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Treatment of hydrocarbon-rich wastewater using oil degrading bacteria and phototrophic microorganisms in rotating biological contactor: Effect of N:P ratio

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Abstract

Treatment of hydrocarbon-rich industrial wastewater in bioreactors using heterotrophic microorganisms is often associated with various operational problems. In this study, a consortium of phototrophic microorganisms and a bacterium is developed on the discs of a rotating biological contactor (RBC) for treatment of wastewater containing diesel oil. The reactor was fed with oil degrading bacterium, *Burkholderia cepacia* and oil tolerant phototrophic microorganisms. After biofilm formation and acclimatization to 0.6% (v/v) diesel, continuous-mode operation was initiated at 21 h hydraulic retention time (HRT). Residual diesel in the effluent was 0.003%. Advantages of this system include good total petroleum hydrocarbon (TPH) removal, no soluble carbon source requirement and good settleability of biosolids. Biofilm observations revealed the predominance of *B. cepacia* and cyanobacteria (*Phormidium*, *Oscillatoria* and *Chroococcus*). The N:P ratio affected the relative dominance of the phototrophic microorganisms and bacterial culture. This ratio was a critical factor in determining the performance efficiency of the reactor. At 21 h HRT and organic loading of 27.33 g TPH/m^2 d, the N:P ratio 28.5:1 and 38:1 both yielded high and almost comparable TPH and COD removal efficiencies. This study presents a feasible technology for the treatment of hydrocarbon-rich wastewater from petrochemical industries and petroleum refineries.

Keywords: Bacteria; Bioreactor; Cyanobacteria; N:P ratio; Total petroleum hydrocarbons

1. Introduction

Petrochemical industries and petroleum refineries generate large amounts of priority pollutants. The major pollutants found in these industries are petroleum hydrocarbons, specifically aliphatic hydrocarbons, arising from storage of crude oil, spills, wash downs and vessel clean-outs from processing operation. Treatment of this wastewater is performed through a series of on-site treatment technologies, such as, American petroleum institute (API) separators, tilted plate interceptor (TPI) separators and dissolved air floatation (DAF) units. These operations are followed by biological treatment in a suspended growth process. However, these processes are typically associated with numerous operational problems, which include: poor settleability of the sludge due to low F/M (food to microorganism) ratio; production of extra-cellular polymers consisting of lipids, proteins and carbohydrates that adversely affect sludge settling; biological inhibition due to toxic compounds, which necessitates very long sludge retention time; long period of acclimation or start-up and production of large amount of biological sludge [1,2]. Some of these problems can be avoided by replacing the suspended growth process by fixed film biological reactors, such as, the trickling filter and the rotating biological contactor (RBC). The inherent advantages of RBC include good COD removal efficiency and capability for handling toxic pollutants [2]. Moreover, the rotating discs facilitate oxygen transfer in the system, thus, eliminating the need for additional aerators.

Hydrocarbon degradation has been widely reported in laboratory-scale batch studies [3–5]. However, studies demonstrating biological treatment of hydrocarbon-rich wastewater in continuous flow bioreactors are limited and need attention. Some researchers have reported the degradation of low concentration of hydrocarbons using bacterial cultures [2,6] and algal cultures in bioreactors [7]. Compared to bacterial systems, bioreactors utilizing a symbiotic association between algae and bacteria have been reported to yield higher treatment efficiency [8–10].

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Moreover, under certain environmental conditions, algae can facilitate spontaneous flocculation of bacteria to improve the quality of the treated effluent.

In the coastal environment of the Arabian Gulf experiencing frequent oil pollution, oil degrading bacterial cultures are found to associate with cyanobacterial (blue-green algae) mats [11–13]. While some cyanobacterial cultures may play a direct role in hydrocarbon degradation, others may facilitate hydrocarbon degradation indirectly by providing surfaces for adherence of oil degrading bacterial cultures [12–16]. As a result of this association, the bacterial cultures are prevented from being washed-out even under turbulent conditions. The removal of high concentration of hydrocarbons by algal-bacterial system has been primarily reported in lab-scale batch studies [11,12,16,17]. However, there are no reports on the degradation of high concentration of complex non-aqueous phase liquids (NAPLs) using phototrophic microorganisms and bacterial cultures in RBC. In the present study, the association between oil-tolerant phototrophic microorganisms (comprising of green algae and cyanobacteria) and an oil-degrading bacterium was utilized for the treatment of diesel oil in a RBC. The effect of N:P ratio on total petroleum hydrocarbons (TPH) and COD removal was also studied. This ratio is likely to affect the relative dominance of phototrophic microorganisms and the bacterium in the reactor.

2. Materials and methods

2.1. Source of chemicals and cultures

All the chemicals used in this research work were of analytical grade and had high purity. Diesel purchased from a petrol station (Bhandup, Mumbai, India) was artificially weathered in a fume hood at room temperature over a period of 48 h and subsequently stored in an air-tight container. The bacterium, *Burkholderia cepacia* (IMTECH, Chandigarh, India, MTCC 5332), enriched and isolated from Arabian Sea sediments [3] was used in this study. The culture was capable of using aliphatic hydrocarbons and diesel oil as the sole source of carbon and energy. The consortium of fresh water phototrophic microorganisms was obtained from the surface of rocks near Powai Lake (IIT Bombay, Mumbai, India). Independent batch experiments revealed that these phototrophic microorganisms could tolerate 0.8% diesel oil (unpublished results).

2.2. Analytical techniques

The performance of the reactor was analyzed by measuring the various parameters [18]. Measurement of COD for diesel oil using the standard protocol resulted in very low COD compared to the theoretical value. These results possibly occurred due to the high influent diesel concentration (0.6%, v/v) and inability in obtaining representative dilutions because of its immiscibility with water. Thus, an attempt was made to modify the procedure for COD measurement by increasing the normality of the oxidizing agent (0.1 M $K_2Cr_2O_7$) and enhancing the digestion period employed in the conventional procedure while maintaining a constant temperature of 150 °C [18]. COD was measured for various concentrations of diesel (0.2–0.6%, v/v). The digestion period was varied from 2 to 12 h. Total biomass and abundance of phototrophic microorganisms was determined by scrapping the biofilm (1 cm × 1 cm) and subsequently determining the volatile suspended solids (VSS) and chlorophyll-*a* by methanol extraction [19].

Concentration of TPH in the effluent samples was analyzed by gas chromatograph (GC) as discussed by Mohanty and Mukherji [20] with minor modifications. The samples were acidified and centrifuged at 10,000 rpm for 15 min with hexane (5:1). After phase separation, the aqueous phase was subjected to two serial hexane extractions (10:1). The combined extract was first analyzed gravimetrically. Subsequently, after adding the internal standard (IS) (5α-andostane, 2 mg/mL), the extracted samples were analyzed using a GC (Agilent Technologies, 6890N) equipped with FID detector and HP5 capillary column. The oven temperature programme used in this study was as follows: initial temperature 50 °C with hold time 1 min; ramping at 10 °C/min up to 150 °C with hold time 1 min; ramping at 5 °C/min up to 175 °C with hold time 1 min; ramping at 40 °C/min up to 200 °C with hold time 25 min. Valley to valley integration was performed between retention time (RT) 4–35 min and the peak areas normalized to area of IS were added to determine the concentration of diesel range resolved peaks (DRRP). Since diesel oil is primarily composed of aliphatic hydrocarbons; DRRP essentially serves as a measure of TPH. Identification of *n*-alkanes was carried out based on relative RT (normalized to IS) using a reference calibration mixture (D2887, AccuStandard) [20].

2.3. Mass cultivation of cultures and reactor start-up

Mass cultivation of phototrophic microorganisms (3 L) was carried out in Haffkin's flasks, containing nutrient medium having the following composition (mg/L): Di sodium EDTA (0.5), citric acid (3), $Na_2CO_3{\cdot}H_2O$ (20), $CaCl_2{\cdot}2H_2O$ (7), $MgSO_4{\cdot}7H_2O$ (370), KCl (500), ferric ammonium citrate (3), $K_2HPO_4{\cdot}3H_2O$ (21.75), KH_2PO_4 (8.5), Na_2HPO_4 (33.4), $NaNO_3$ (750) and trace elements [4]. This medium was designed for optimal growth of *B. cepacia* and phototrophic microorganisms [21]. The flasks were incubated under illumination of 1000–1100 lx (Lutron LX-101, Mumbai) provided with an incandescent bulb and at light:dark (L:D) cycle of 18:6 for 20 days. Simultaneously, *B. cepacia* (1 L) was grown in a rotary shaker set at 28 ± 2 °C and 120 rpm (Trishul Equipments, Mumbai) with 1% (v/v) diesel and 1% (v/v) inoculum containing 10^9 MPN/mL. The mass cultivated bacterium was centrifuged (15,000 rpm, 45 min) and the pellet was re-suspended in 1 L of fresh nutrient medium. Subsequently, the RBC trough was filled with a suspension of these two cultures. Characteristics of synthetic wastewater with 0.6% (v/v) diesel was as follows (mg/L): alkalinity as $CaCO_3$ (58), NH_4^+-N (0.066), NO_3^--N (40), PO_4^{3-}-P (7), TCOD (4512.56) and TPH (4961.4). The pH of the wastewater was 7.5. The design specification of the RBC is shown in Table 1.

Table 1
Reactor design and operating parameters for laboratory scale RBC

Specifications	Values
Number of stages	3
No. of disc/stage	9
Diameter of the disc (mm)	140
Spacing between the disc (mm)	14
Cross-sectional area of tank (m^2)	0.09
Total surface area of discs (m^2)	0.83
Specific surface area of discs (m^{-1})	206.75
Working volume (L)	4.0
Submergence (%)	35
Rotations per minute	10
Peripheral velocity (m/min)	0.7

2.4. Development of biofilm and acclimatization of biofilm to complex NAPL, diesel

For development of the biofilm comprising of *B. cepacia* and phototrophic microorganisms on the RBC discs, the reactor was operated in batch mode with 0.2% (v/v) diesel. Throughout the reactor studies, a constant L:D cycle of 18:6 was maintained with light intensity of 1000–1100 lx at the surface of the trough. The reactor operation was conducted at room temperature ($28 \pm 2\,^{\circ}C$). After development of biofilm on the RBC discs, diesel concentration in the feed was gradually increased by operating the reactor in semi-batch mode with hydraulic retention time (HRT) of 24 h. The reactor contents were drained out completely every day and homogenized by stirring on a magnetic stirrer. An aliquot was filtered and subsequently analyzed for various parameters, such as, pH, alkalinity, NH_4^+-N, NO_3^--N and PO_4^{3-}-P. Another aliquot was allowed to settle for 30 min and the supernatant was analyzed for COD and TSS (total suspended solids). Using an aliquot of the well mixed sample without filtration, TPH was determined. The reactor was operated with 0.2% (v/v), 0.4% (v/v), 0.6% (v/v) and 0.8% (v/v) diesel for a period of 10 days each. Subsequently, the reactor was operated in a flow-through mode at 21 h HRT. The nutrients and diesel were pumped separately using two peristaltic pumps (PP-50V, Electrolab, India and Miniplus 3, Gilson, France).

2.5. Effect of N:P ratio

Performance of the reactor at varying N:P ratio (19:1, 28.5:1, 38:1 and 47.4:1) was studied at a fixed HRT (21 h) by varying the concentration of $NaNO_3$ in the synthetic wastewater, while maintaining a constant concentration of phosphate salts as determined through batch studies [21]. The studies were carried out at a fixed diesel concentration of 0.6% (v/v) and organic loading rate (OLR) of 27.33 g TPH/m^2 d. The corresponding C:N:P ratios (mass basis) were 100:1.82:0.98, 100:2.73:0.98, 100:3.65:0.98 and 100:4.56:0.98, respectively. Effluent samples were analyzed daily for various parameters. Each ratio was studied for a period of 9–10 days under pseudo steady-state condition.

2.6. Microscopic examination of biofilm and characterization of various cultures

For observing the bacterial culture, a loop full of biomass scrapped from the surface of the disc was inoculated in the nutrient medium with 1% (v/v) diesel oil. After five serial transfers, plating, streaking and isolation was performed. The colonies were observed by Gram staining and microscopic examination (Axio Star Plus, Carl Zeiss, Germany). Further, the culture was inoculated with different types of hydrocarbon substrates (v/v), i.e., 1% n-C16, 1% cyclohexane (cycloalkane), 0.1% naphthalene (aromatic hydrocarbon) and 0.1% phthalic acid to observe the substrate preferences for these cultures.

Simultaneously, characterization of phototrophic microorganisms was performed. Cycloheximide was used to examine the type of phototrophic microorganisms, since it is known to restrict the growth of eukaryotes [22]. Morphology of these microorganisms observed under the microscope was used for characterizing them based on unicellular or filamentous structure, presence or absence of heterocysts, presence or absence of spores, trichomes with false or true branching and presence or absence of sheath [23]. The phototrophic microorganisms were also tested for their nitrogen fixing ability by testing for the presence of nitrogenase using acetylene reduction technique [19]. Gas samples were analyzed using GC (Shimandzu GC 15A) equipped with a 10% Carbowax 20 column and FID detector. The oven temperature programme was as follows: initial temperature 40 °C, with hold time 5 min, ramping at 10 °C/min up to 70 °C, with hold time 5 min, with total run time of 14 min. The experimental chromatograms obtained were compared with the controls (devoid of the phototrophic microorganisms) and with pure acetylene and ethylene chromatograms to establish the presence/absence of ethylene. Estimation of water soluble phycobiliprotein pigments [phycoerythrocyanin (PE), allophycocyanin (APC) and C-phycocyanin (PC)] was also carried out to test for the presence of cyanobacteria and rhodophyta in the system [19].

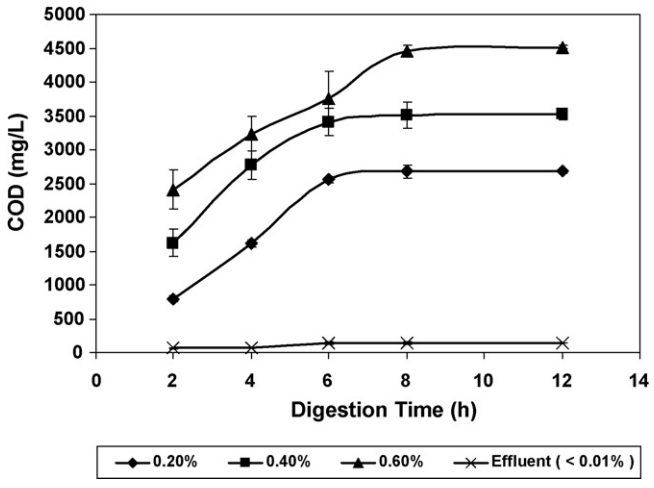

Fig. 1. COD estimation for various diesel concentrations using 0.1 M $K_2Cr_2O_7$: effect of digestion time (2–12 h) for digestion at 150 °C.

2.7. Determination of residual oil in the biofilm

After operating the reactor in flow-through mode for a period exceeding 1 year, flow was stopped and the reactor contents were drained. Biomass covering the discs was collected by scrapping and was autoclaved and dried. Subsequently, soxhlet extraction of biomass was performed to determine the mass of diesel sorbed per unit mass of biomass. Since soxhlet extraction may potentially extract out some portions of the biomass, autoclaved samples of *B. cepacia* and phototrophic microorganisms, grown in batch culture in the absence of diesel oil, were used as controls. Residual oil in the extract was determined gravimetrically and was also characterized by GC analysis.

3. Results and discussion

3.1. Standardization of COD determination for diesel and characterization of diesel

During standardization of the COD determination procedure for water containing 0.2–0.4% (v/v) diesel, it was observed that with increase in digestion period from 2 to 6 h, the COD values increased and subsequently stabilized after 6–8 h (Fig. 1). For 0.6% (v/v) diesel, the COD values stabilized at 4512.56 mg/L after 8–12 h of digestion. This indicates the requirement for higher digestion time for high diesel concentration. For the effluent samples containing very low concentration of diesel (<0.01%), it was observed that increase in the digestion time and normality of $K_2Cr_2O_7$ had no significant impact on the COD values. Thus, for the reactor studies, influent COD was estimated using 0.1 M $K_2Cr_2O_7$ and 12 h digestion, while effluent COD was estimated using the conventional procedure. Fig. 2 illustrates the abundance of various *n*-alkane components in DRRP based on GC analysis for 0.6% (v/v) diesel. It was observed that in this diesel sample, concentration of *n*-alkanes, C14 and C15 were highest.

3.2. Development of biofilm and acclimatization of biofilm to diesel

Visual observations revealed the immobilization of phototrophic microorganisms and bacterium on the surface of the discs after 17 days of inoculation. Table 2 represents percentage TPH and TCOD removal during the acclimatization period. The TCOD removal efficiency was found to increase with increase in diesel concentration, whereas the TPH removal efficiency was found to be almost constant at 98% up to 0.6% (v/v) diesel. When concentration of diesel was increased from 0.6 to 0.8% (v/v), the average TPH and TCOD concentration in the effluent increased to 201 and 1158.75 mg/L, respectively. Simultaneously, sloughing of the biofilm was observed and TSS concentration in the effluent increased from 20 to 200 mg/L. Thus, the influent oil concentration was subsequently reduced to 0.6% (v/v).

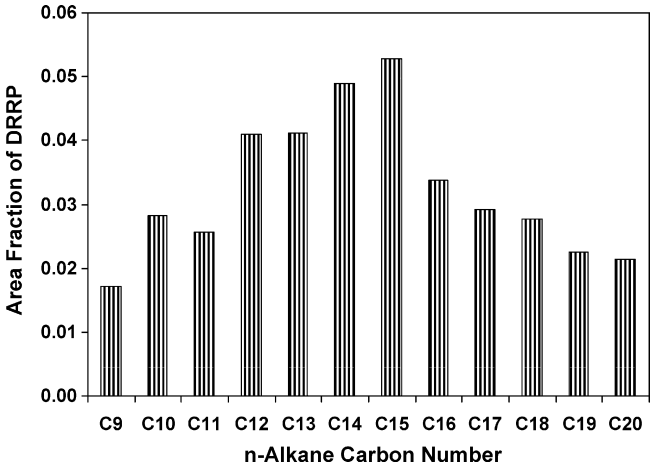

Fig. 2. Abundance of *n*-alkane components present in DRRP based on gas chromatographic analysis of 0.6% (v/v) diesel.

3.3. Reactor performance

Table 3 depicts the average concentration of various parameters in the effluent under pseudo-steady state condition at various N:P ratios. Removal of various *n*-alkanes present in the diesel was estimated based on GC analysis. It was found that at N:P ratio of 28.5:1 and 38:1, almost complete removal of *n*-alkanes from C9 to C20 were obtained (Fig. 3), and chromatograms of the effluent primarily consisted of the unresolved complex mixture (UCM) hump. At low N:P ratio of 19:1, almost complete removal of *n*-alkanes from C9 to C16 was obtained and removal efficiencies were similar to that at N:P ratio of 28.5

Table 2
Reduction in TPH and TCOD in RBC reactor during the acclimatization phase

Run time (days)	% Diesel in the influent (v/v)	TPH concentration[a] (mg/L)			Total COD concentration[b] (mg/L)		
		Influent (mg/L)	Effluent[c] (mg/L)	% Removal[c]	Influent[d] (mg/L)	Effluent[c] (mg/L)	% Removal[c]
0–9	0.2	1653.8	26.73 (±6.57)	98.99	2677.30 (±14.08)	201.4 (±6.76)	92.48
10–19	0.4	3307.6	41.18 (±6.79)	98.76	3523.61 (±53.23)	114.60 (±9.37)	96.74
20–29	0.6	4961.4	93.78 (±8.41)	98.12	4512.56 (±14.75)	126.55 (±12.48)	97.19
29–34	0.8	6615.2	201.38 (±44.12)	95.08	5406.38 (±15.52)	1158.75 (±64.36)	78.56

Values in the parentheses demonstrate standard error.
[a] Determined by gravimetric analysis.
[b] Total COD is reported instead of soluble COD since residual TPH would tend to associate strongly to solids.
[c] Effluent and % Removal are both computed after achieving pseudo-steady state.
[d] COD determination procedure required 0.1 M $K_2Cr_2O_7$ and 12 h digestion time since representative dilutions cannot be prepared for NAPLs dispersed in aqueous phase.

Table 3
Summary of reactor performance at 21 h HRT at various N:P ratios

Parameters	Influent	Effluent at pseudo-steady state			
		19:1[a] 26.5[b] 9[c]	28.5:1[a] 39.7[b] 10[c]	38:1[a] 53.04[b] 10[c]	47.4:1[a] 66.3[b] 9[c]
pH	7.5 (±0.05)	7.88 (±0.028)	7.96 (±0.070)	8.21 (±0.043)	8.31 (±0.041)
Dissolved oxygen	–	4.19 (±0.01)	5.86 (±0.25)	5.06 (±0.17)	4.06 (±0.18)
Total suspended solids	–	119.33 (±6.24)	8.22 (±1.90)	21.14 (±7.69)	73.00 (±14.25)
Alkalinity (as $CaCO_3$)	58.0 (±1)	457.77 (±3.64)	513.75 (±10.23)	477.14 (±9.17)	811.25 (±21.93)
TPH by gravimetric analysis	4961.4[d]	74.83 (±3.32)	16.5 (±5.46)	27.71 (±1.50)	49.75 (±1.75)
TPH by GC analysis	4961.4[d]	68.95 (±3.27)	28.07 (±0.57)	28.34 (±1.10)	32.09 (±1.13)
TCOD[e]	4512.56 (±14.75)	692.20 (±19.99)	97.35 (±5.71)	134.52 (±13.25)	198.18 (±12.38)
NH^{4+}-N	0.066 (±0.007)	0.383 (±0.002)	0.398 (±0.006)	0.294 (±0.010)	0.322 (±0.066)
NO_3^--N	Varying	0.48 (±0.02)	3.89 (±0.66)	24.47 (±1.00)	1.67 (±0.08)
PO_4^{3-}-P	6.2 (±0.5)	3.11 (±0.007)	3.73 (±0.854)	4.45 (±0.164)	2.36 (±0.419)

Note: All parameters are expressed in mg/L except pH, values in the parentheses demonstrate standard error; SS: Pseudo-steady state.
[a] N:P Ratio.
[b] NO_3^--N in influent.
[c] SS duration (days).
[d] Influent TPH reported are not measured values, these values were obtained based on the volumetric concentration of diesel (% v/v) and its density.
[e] TCOD includes COD due to TPH, non-biodegradable soluble organics and suspended cells.

and 38:1. However, low removal efficiencies were obtained for *n*-alkanes from C17 to C20, with a minimum removal efficiency of 96% for C17. At a high N:P ratio of 47.4:1, almost complete removal of *n*-alkanes from C9 to C20 was obtained, except for C17. Thus, C17 was found to be the most persistent *n*-alkane at non-optimal N:P ratios. For diesel degradation studies in batch cultures inoculated with *B. cepacia* and *Exiguobacterium aurantiacum*, Mohanty and Mukherji [20] demonstrated that biodegradation caused a loss of symmetry in the distribution of *n*-alkanes in the residual diesel while the un-degraded diesel yielded a symmetric *n*-alkane distribution (C9–C26) with peak at C14–C15. After a 15 day biodegradation study, the intermediate carbon number compounds (C16–C17) were found to accumulate due to their high abundance, although the maximum decay rate for these compounds was comparatively higher. The abundance of a component in residual diesel was attributed to factors, such as, maximum decay rate, duration of active phase, initial abundance of the component and the rate and extent of decay of other components in the complex mixture.

Successful degradation of *n*-alkanes by cyanobacteria and algal-bacterial association in batch systems is reported by various researchers. The macroalgae, *Padina*, in association with oil degrading bacterial cultures, i.e., *Acinetobacter* and nocardioforms was reported to cause 98% degradation of n-C18 [16]. Maximum degradation of n-C16 and n-C19 by non-axenic cyanobacterial culture, *Phormidium corium*, from oil contaminated sediments was found to be 60 and 22.2%, respectively. In contrast, maximum consumption of n-C16 and n-C19 by non-axenic cyanobacterial culture, *Microcoleus chthanoplasts*, was only 20.6 and 4.1%, respectively [12]. Moreover, pure strains of *Aphanocapsa* sp., *Plectonema tere-*

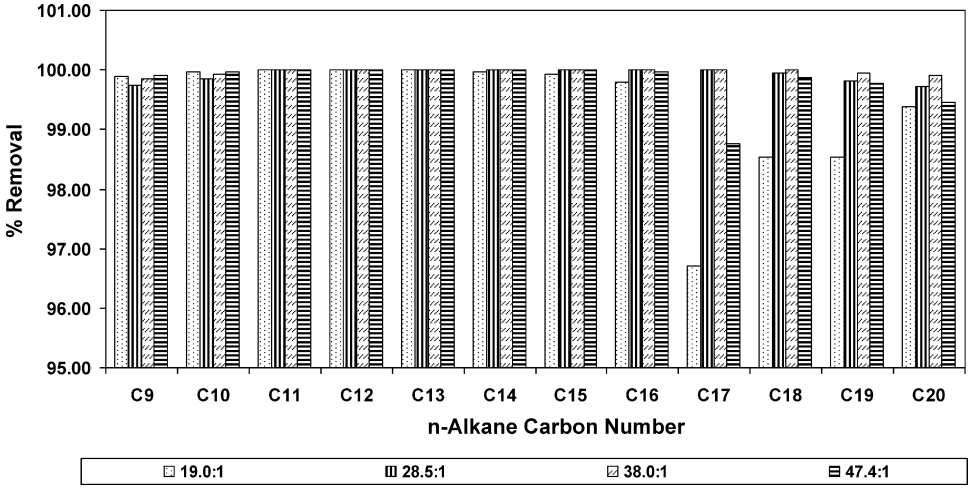

Fig. 3. Removal of the *n*-alkane components in diesel at varying N:P ratios for the reactor operated at 21 h HRT and 27.33 g TPH/m^2 d under pseudo steady-state condition.

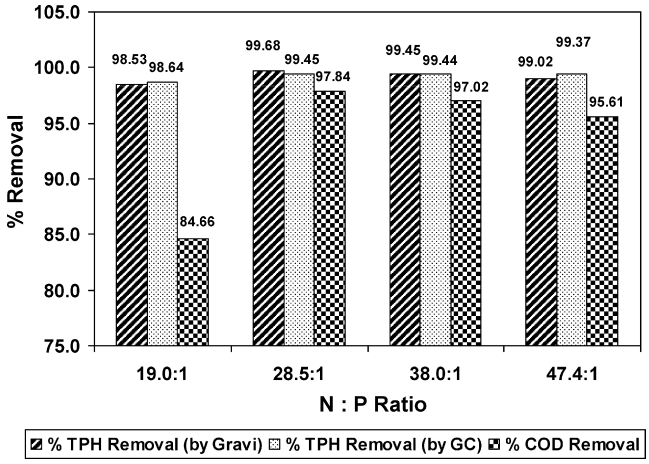

Fig. 4. TPH and COD removal efficiencies for the reactor operated at varying N:P ratios.

brans and *Oscillatoria salina* were found to degrade 65.2%, 60.1% and 48.4% of n-C16 (0.1%) in natural sea water within 10 days [14].

In this study, at N:P ratio of 19:1, 28.5:1, 38:1 and 47.4:1, the TPH removal efficiencies were 98.6, 99.4, 99.4 and 99.3%, respectively, and the corresponding COD removal efficiencies were 84.6, 97.8, 97.0 and 95.6%, respectively (Fig. 4). Thus, there is good correspondence between TPH and COD removal efficiencies at N:P ratio of 28.5:1 and 38:1. The lower N:P ratio of 28.5:1 requiring lower nitrogen source addition may be considered as the optimum N:P ratio for performance of the reactor.

Treatment of oil and oily wastewater by heterotrophic cultures has been carried out in batch systems and in different types of bioreactors, including RBC. In batch cultures with 1% diesel oil, a pure strain of *B. cepacia* demonstrated 51% loss of diesel over 15 days with greater than 70% degradation of *n*-alkanes. The rate and extent of degradation of diesel by this culture was significantly enhanced (66% degradation over 5 days) in the presence of the emulsifying chemical surfactant, Triton X-100 [3,20]. RBC with polyurethane foam lined discs used for the treatment of refinery wastewater (influent COD: 234–925 mg/L; oil: 26–124 mg/L) could achieve a maximum of 85.7% COD removal for OLR of 0.26–4.96 g TPH/m^2 d [2]. RBC with a biofilm of the achlorophyllous algae, *Prototheca zopfii* fed with a model mixture of aliphatic hydrocarbons (n-C_{14}, n-C_{15}, n-C_{16}) operated in batch mode could achieve 60% hydrocarbon removal in 30 days [7].

In batch studies for hydrocarbon degradation using algal-bacterial system, the hydrocarbon concentration employed was found to range from 400 to 10,000 mg/L and the degradation efficiency of *n*-alkanes was in the range of 22–98% [12,16,17,24]. The highest oil degradation in suspended growth system was reported for association of algae obtained from Oredege river (AS-45 and AS-47) with *Rhodococcus* sp. *7HX* [24]. When algal-bacterial cultures were immobilized on glass plates for removal of crude oil, the degradation was found to be 83% [15]. However, in this study, the RBC reactor with algal-bacterial biofilm was found to be successful in achieving TCOD and TPH removal of 97.8% and 99.4%, respectively, at high influent TCOD (4512.5 mg/L) and TPH (4961.4 mg/L) concentration.

The effluent pH (7.8–8.3) and alkalinity (400–850 mg/L) was always found to be higher than influent and was found to increase with the increase in N:P ratio (Table 3). This observation is related to the consumption of CO_2 by phototrophic microorganisms. A good correlation was observed between chlorophyll-*a* (representing phototrophic microorganisms on the discs) and alkalinity in the system ($r^2 = 0.95$). It has been reported that, in open systems, phototrophic microorganisms can reduce the free CO_2 concentration below its equilibrium concentration with air and consequently cause an increase in pH. These microorganisms can continue to extract CO_2 from water until an inhibitory pH is reached (pH 10–11) [25]. It has also been reported that assimilation of nitrate ions by actively growing phototrophic microorganisms also tends to raise the pH [19].

Dissolved oxygen (DO) in the effluent was always found to be in the range of 4.0–5.8 mg/L under the ambient temperature conditions (26–30 °C). The high level of DO in the system in-spite of the high organic loading is also related to the photosynthetic activity of the phototrophic microorganisms. Similar observation was reported in an algal-bacterial RBC for treatment of low concentration of TCE [9]. The loading of ammonical nitrogen (NH_4^+-N) in the system was 0.00036 g/m^2 d. NH_4^+-N was found to be higher in the effluent (0.2–0.4 mg/L) compared to the influent (0.066 mg/L). During assimilation of nitrogen by phototrophic microorganisms and heterotrophs, nitrate nitrogen is first reduced to NH_4^+-N through four reducing steps [19]. It was found that NH_4^+-N loading in this study was much lower than in the algal-bacterial system studied by Sahu [9] (0.2–0.8 g NH_4^+-N/m^2 d). Moreover, in their study, the NH_4^+-N decreased in the effluent (42–0.84 mg/L).

At N:P ratio of 19:1, 28.5:1, 38:1 and 47.4:1 (mole basis), the nitrate (NO_3^--N) removal efficiencies were 95.77%, 92.36%, 52.0% and 97.48%, respectively. At N:P ratio of 38:1, NO_3^--N removal was much lower than at the other N:P ratios. Correspondingly, the density of phototrophic microorganisms on the disc (chlorophyll-*a*, mg/cm^2) and the effluent alkalinity was much lower than the expected trend. The total algal growth on the discs was found to be similar to that at the low N:P ratio of 19:1. Thus, similar NO_3^--N utilization in these two scenarios is expected. Since, the influent NO_3^--N concentration is higher at N:P of 38:1, the NO_3^--N removal efficiency is lower (Table 3). Thus, the N:P ratio is a critical factor determining the relative abundance of phototrophic microorganisms and bacterium in the system and is an important factor determining the performance of the reactor.

Variation in the total biomass (phototrophic microorganisms and bacterial biomass) on the RBC discs in different stages of the reactor at varying N:P ratio is illustrated in Fig. 5 as VSS (mg/cm^2). At the low N:P ratio of 19:1, total biomass was found to be comparatively lower in all the stages of the reactor. At all the N:P ratios, biomass of phototrophic microorganisms was found to be highest in the IIIrd stage of the reactor (Fig. 6). Growth of these microorganisms in the IInd and IIIrd stage of the reactor

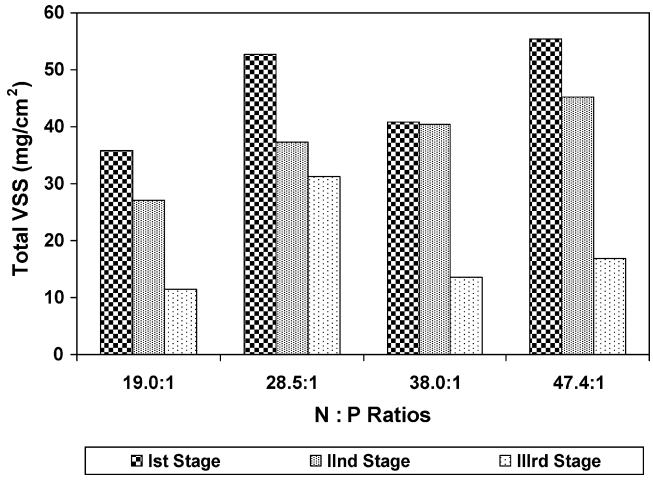

Fig. 5. Total attached biomass of phototrophic microorganisms and bacteria on RBC discs (VSS, mg/cm^2) in the Ist, IInd and IIIrd stage for the reactor operated at varying N:P ratios.

was found to be lower at N:P ratios of 19:1 and 38:1 compared to the other N:P ratios. From Table 3, it can be observed that alkalinity in the effluent increased for all N:P ratios. Maximum increase in alkalinity was observed at the highest N:P ratio of 47.4:1 which also corresponded with the highest chlorophyll-*a* concentration on RBC discs.

Minimum total suspended solids (8–20 mg/L) were observed in the effluent at N:P ratio of 28.5:1 and 38:1. At the lowest N:P ratio of 19:1 and at the highest N:P ratio of 47.4:1, the average effluent TSS concentration was found to be 120 and 73 mg/L, respectively. At N:P ratio of 19:1, sloughing was observed in the IInd and IIIrd stage of the reactor at the end of the 9th day. At N:P ratio of 47.4:1, high growth of microorganisms was observed in suspension in the Ist and IInd stage of the reactor. These studies also demonstrate that the optimum N:P ratio for degradation of diesel in this system is 28.5:1.

The optimal N:P ratio for the degradation of diesel oil obtained in this study was found to be same as that reported for

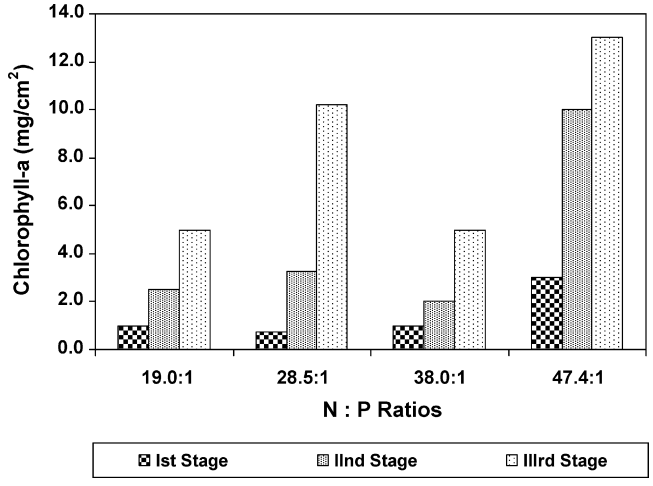

Fig. 6. Phototrophic microorganisms attached on RBC discs (chlorophyll-*a*, mg/cm^2) in the Ist, IInd and IIIrd stage for reactor operation at varying N:P ratios.

batch studies with *B. cepacia* and a consortium of marine algal culture [21]. For the degradation of crude oil (C10-C40) in algal-bacterial suspended growth batch systems, some researchers have reported degradation at a low N:P ratio of 0.98:1 (mole basis) [11,13,16], whereas, other researchers have reported degradation at a relatively higher N:P ratio of 23.9:1 (mole basis) [17]. For the degradation of aromatic pollutants in algal-bacterial suspended growth batch studies, N:P ratio of 1.01:1 (mole basis) was utilized [10]. When treatment of 1% crude oil was studied by immobilizing oil degrading bacterial cultures and cyanobacterial cultures on glass plates and gravel particles [15], a ratio of 4.04:1 was found to result in high degradation of crude oil. For the treatment of palm-oil mill wastewater in a suspended growth reactor, N:P ratio of 4.41:1 (mole basis) was used for facilitating the growth of *Chlorella pyrenoidosa* and various other microorganisms [26]. During the treatment of crude oil in a packed column reactor filled with perlite, N:P ratio of 3.26:1 (mole basis) was used for growing cyanobacterial mats from Ebro Delta [27]. The optimum N:P ratio for the degradation of nitrobenzene and TCE [8,9] was found to be 0.9:1 (mole basis) for RBC reactors with algal-bacterial biofilm. In this study, although the N:P ratio is relatively higher, the concentration of nitrogen and phosphorus sources in the influent is much lower compared to most other studies reported in the literature.

This treatment process was found to provide significant advantages over traditionally used oily wastewater treatment technologies. The specific advantages include: no soluble substrate/additional carbon source requirement; low requirement for nitrogen and phosphorus sources; capability of handling relatively high concentration of oil; good effluent quality with very low residual concentration of oil and COD; lower sludge generation rate; good settleability of sludge; high DO levels maintained by rotation of the discs and photosynthetic activity of the phototrophic microorganisms; and alkalinity generation due to phototrophic microorganisms. High alkalinity can counter the tendency for pH drop caused by accumulation of acidic intermediates during degradation of hydrocarbons by bacterial cultures. The lower sludge generation rate reduces the problems associated with sludge treatment and disposal. In treatment units employing oil degrading cultures, sludge settleability problems are commonly encountered, hence, enhanced settleability due to presence of phototrophic microorganisms is a distinct benefit. Due to its ability to handle relatively high concentration of oil, this biological treatment process may be employed as a single step process in industries generating oily wastewater, after recycling the recoverable oil.

3.4. Biofilm observations

Two types of colonies were observed after streaking the heterotrophic culture. The transparent colonies consisting of Gram negative and rod shaped cells characterized *B. cepacia* that was spiked into the reactor (Fig. 7a). Another distinct colony type comprising of white opaque colonies, larger in size than those of *B. cepacia* was also observed. Microscopic examination without staining revealed that the white opaque colony was

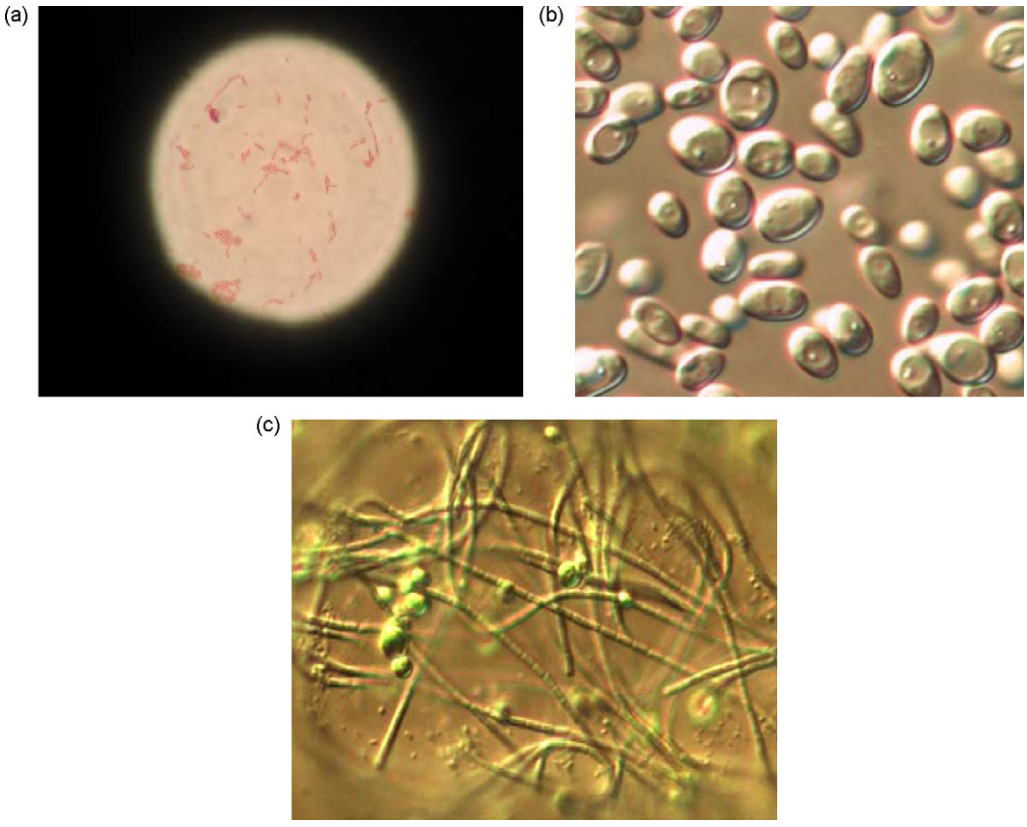

Fig. 7. Microscopic examination of cultures present in the biofilm of the reactor, (a) *B. cepacia*, (b) yeast culture and (c) cyanobacterial cultures.

a unicellular fungus and appeared to reproduce by budding, which is typical of yeast (Fig. 7b). Moreover, when the preference to the different type of substrates was studied, it was observed that both the cultures could only use n-hexadecane (present in aliphatic fraction of diesel) as the sole source of carbon and energy. These studies show that both the cultures could not grow on the other hydrocarbon fractions in the complex NAPL.

Based on the cycloheximide test, it was concluded that the phototrophic culture contained both green algae and cyanobacteria. Microscopic examination of the cyanobacterial culture (Fig. 7c) revealed the predominance of *Phormidium*. *Phormidium* was characterized by its distinct filaments, trichomes with false branching, without heterocysts, absence of spores and filaments forming a thallus with more or less confluent sheath. The other species of cyanobacteria present included *Oscillatoria* and *Chroococcus*. *Oscillatoria* was characterized by its distinct filaments, trichomes with false branching, without heterocysts, absence of spores, clearly visible cells in the trichome, more or less straight trichomes not spirally coiled and absence of sheath. *Chroococcus* was characterized by its unicellular/united colonies with colorless individual sheath and by the non-vesicular spherical cells [23]. The cyanobacterial cultures were unable to reduce acetylene to ethylene in the acetylene reduction test, thus indicating the absence of nitrogenase activity and absence of heterocysts. Estimation of phycobiliprotein pigments for the cyanobacterial consortia yielded the concentration of PE, APC and PC as 35.41, 76.14 and 22.56 mg/mL, respectively. These values were found to be greater than those present in a pure strain of *Oscillatoria BDU* 100731 (obtained from NFMC, Tiruchirappalli, India), which contained 20, 46 and 14 mg/mL of PE, APC and PC, respectively.

3.5. Residual oil in the biofilm

Upon termination of reactor operation after continuously operating the reactor for more than a year, 82.11 g of dry biomass and sorbed oil was collected from the RBC discs. The mass of oil associated with 1 g of biomass was 0.582 g over and above the controls (biomass assumed to have equal abundance of *B. cepacia* and phototrophic microorganisms). Gravimetric analysis revealed that negligible mass of cellular components was extracted out from *B. cepacia* and phototrophic microorganisms during soxhlet extraction, i.e., 0.086 and 0.026 g/g, respectively. The GC chromatogram of the extracted oil did not contain any of the resolved *n*-alkane peaks typically present in diesel (Fig. 8a). The extracted oil primarily consisted of the unresolved complex mixture (UCM) hump representing the un-degraded aromatic fraction of diesel (Fig. 8b). The extract of *B. cepacia* and phototrophic microorganisms grown in the absence of oil, showed very few peaks in the GC chromatograms. These results confirm that bulk of the oil representing the aliphatic fraction is biodegraded by the biofilm on the RBC discs while the aromatic fraction of oil accumulates on the biofilm. Moreover, TPH in the effluent is also primarily composed of the aromatic fraction.

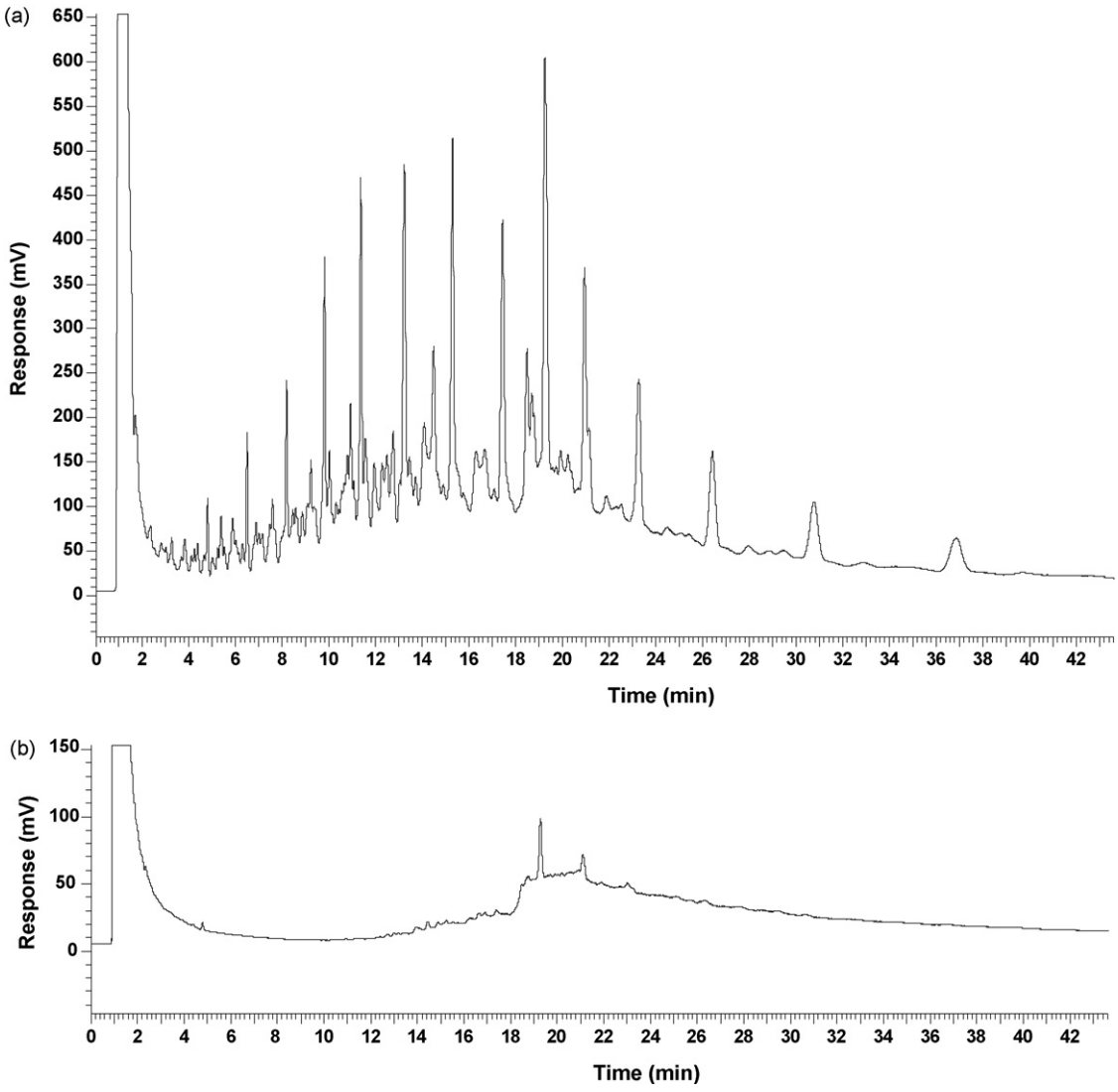

Fig. 8. GC chromatogram of (a) diesel and (b) oil associated with the biofilm recovered from RBC discs.

These results are expected since none of the microorganisms constituting the biofilm were capable of degrading the aromatic hydrocarbons in diesel.

4. Conclusions

The present study indicates that association of phototrophic microorganisms and bacterium can result in better TPH removal efficiency as compared to systems employing only heterotrophic cultures in bioreactors. At N:P ratio of 19:1, 28.5:1, 38:1 and 47.4:1, the TPH removal efficiencies were found to be 98.6%, 99.4%, 99.4% and 99.3%, respectively and TCOD removal efficiencies were found to be 84.6%, 97.8%, 97.0% and 95.6%, respectively, at a HRT of 21 h and OLR of 27.33 g TPH/m^2 d. N:P ratios 28.5:1 and 38:1 were found to yield the best effluent quality marked by low TPH, TCOD and TSS concentration. This association of *B. cepacia* and phototrophic microorganisms at a reactor scale could overcome various operational problems commonly associated with hydrocarbon degradation. The major advantages of this system apart from high TPH removal efficiency include good settleability of sludge, low phosphorus requirement, no soluble carbon source requirement and pH stability. Thus, although RBCs are well known for treatment of low-strength wastewater using bacterial cultures, RBCs utilizing the association of phototrophic microorganisms and bacteria can effectively treat wastewater with high organic loading. This study presents a feasible technology for the treatment of wastewater generated from petrochemical industries and petroleum refineries using algal-bacterial biofilm in RBC.

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