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Efficient degradation of lube oil by a mixed bacterial consortium

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Abstract

A laboratory study was performed to assess the biodegradation of lube oil in bio-reactor with 304# stainless steel as a biofilm carrier. Among 164 oil degrading bacterial cultures isolated from oil contaminated soil samples, *Commaonas acidovorans* Px1, *Bacillus* sp. Px2, *Pseudomonas* sp. Px3 were selected to prepare a mixed consortium for the study based on the efficiency of lube oil utilization. The percentage of oil degraded by the mixed bacterial consortium decreased slightly from 99% to 97.2% as the concentration of lube oil was increased from 2000 to 10,000 mg/L. The degradation of TDOC (total dissolved organic carbon) showed a similar tendency compared with lube oil removal, which indicated that the intermediates in degradation process hardly accumulated. Selected mixed bacterial consortium showed their edge compared to activated sludge. Scanning electron microscopy (SEM) photos showed that biofilms on stainless steel were robust and with a dimensional framework constructed by EPS (extracellular polymeric substances), which could promote the biodegradation of hydrocarbons. The increase of biofilm followed first-order kinetics with rate of 0.216 μg glucose/(cm^2·day) in logarithm phase. With analysis of Fourier transform infrared spectroscopy (FT-IR) and gas chromatography-mass spectrometry (GC-MS) combined with removal of lube oil and TDOC, mixed bacterial consortium could degrade benzene and its derivatives, aromatic ring organic matters with a percentage over 97%.

Key words: lube oil; mixed bacterial consortium; biofilm reactor; stainless steel
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Introduction

Crude oil remains a major source of energy and chemical raw material nowadays. Large-scale production, transport, use and disposal of petroleum globally have made it a major contaminant in both prevalence and quantity in the environment. One of the major derivatives (accounting for 60%) from petroleum hydrocarbon is lube oil, which is practiced world-wide and served as lubricates in multi-industries (Casas-Liza and Pinto, 2005). The spreading application of lube oil could induce serious environmental problems (Rahman et al., 2002). The spills due to leakage and pollution may be small but continuous and prolonged. The persistency of residual oil increases the chances of surface water and groundwater contamination (Van and Mukherji, 2008).

In recent years, researchers are working hard to find an effective and efficient way to remove the oil contaminants from the environment (Zeng et al., 2007; Urum et al., 2006; Bate and Lehrle, 1999; Carmona et al., 2006; Baeta-Hall et al., 2005; Wei et al., 2005; Jian et al., 1999). However, biodegradation with selected bacterial isolates is strongly recommended for treating wastewater containing lube oil since it is low-cost and highly-efficient on converting contaminants to harmless end products (Muthukumar et al., 2003; Yamaguchi et al., 1999). Biodegradation can be described as the conversion of chemical compounds by living organisms, into energy, cell mass, carbon dioxide and biological waste products. Conventional activated sludge treatment has some drawbacks, such as low resistant to loading rate, sludge expansion, sensitivity to low temperatures and toxic compounds, loss of active biomass and more equipment needed for accumulating sludge, instability due to loading shock and fluctuation, and further treatment of excess sludge (Chen et al., 2008). High concentrations of hydrocarbons (aromatic structures, benzene derivatives) in lube oil associated with heavy, big oil slicks in water can inhibit the biodegradation process by nutrient or oxygen limitation. The toxic substances in lube oil, such as volatile hydrocarbons, benzene and its derivatives could deteriorate the biological process.

Biofilm reactors are increasingly used because of their resistance to short-term toxic loads, heavy metals and high organic loads, the ability of the attached biomass to survive at low influent substrate concentrations (oligotrophic traditions), performances of high volumetric biomass (Hsien and Lin, 2005; Sandasi et al., 2008). In addition, sludge expansion hardly happens in biofilm reactors.

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Some isolates have been verified their abilities to degrade oil, including *Pseudomonas* sp., *Bacillus* sp., *Micrococcus* sp., *Corynebacterium* sp., *Flavobacterium* sp., *Brucella* sp., *Gallionella* sp., *Prototheca zopfii*, and so on (Rahman et al., 2002; Muthukumar et al., 2003). By mixed bacterial consortium, oil pollutants could be effectively degraded. The degradation of lube oil with mixed was paid attention by few researchers.

This study investigated the degradation of lube oil by mixed bacterial consortium selected from oil polluted soil. A continuous biological reactor constructed with 304# stainless steel as biofilm carrier was used for the treatment of water contaminated with lube oil and compared with traditional activated sludge treatment. The morphology of biofilm formation with mixed bacterial consortium was characterized by scanning electron microscope (SEM). The degradation kinetics of lube oil was studied at different lube oil concentrations. Combined with Fourier transform infrared spectroscopy (FT-IR) and gas chromatography-mass spectrometry (GC-MS) analysis, degradation process of aromatic components was also discussed.

1 Methods and materials

1.1 Isolation and identification of bacterial cultures

Soil samples were collected from an oil refinery field in Shanghai, where oil refinery products had been continuously released into environment for over 30 years, in pre-sterilized glass bottles and transported to the laboratory for analysis. Enrichment and isolation of oil degradation bacterial cultures were operated using medium composed of (g/L): beef extract 15, NaCl 5, peptone 10, yeast extract 5, agar 20, and pH 7.2. Selective medium was composed of (g/L): $NaNO_3$ 0.1, KH_2PO_4 0.1, $CaCl_2$ 0.03, NH_4NO_3 0.15, $MgSO_4$ 0.1, lube oil 2.0 (emulsification with ultrasonic). Selective medium also contained trace elements as following (mg/L): $FeSO_4$ 5, $ZnSO_4$ 2, $MnSO_4$ 2, KI 1.

The isolated bacterial cultures were analyzed by their morphological and biochemical characteristics. All these chemicals and reagents were purchased from National Chemical Group, China.

1.2 Preparation of inoculum

The bacterial cultures (24 hr old) from enrichment medium were inoculated in above selected mineral salts medium with lube oil as sole carbon source. They were kept in a shaker at 190 r/min and 30°C for 48 hr. The growth rate was monitored by measuring absorption spectrophotometrically at 600 nm (data not given). All isolates were stored at –20°C as the liquid cultures containing 20% glycerol (*V/V*). The isolates for degradation of lube oil with highest growth on lube oil were selected and characterized by Gram staining, oxydase reaction, biochemical test, SEM photos, 16S rDNA gene sequence analysis (implemented by Shanghai Shennengbocai Biochemical Company, China). *Commaonas acidovorans* Px1, *Bacillus* sp. Px2, *Pseudomonas* sp. Px3 were selected to prepare a mixed consortium for the study based on the efficiency of lube oil utilization and cell density. Parameters of initial pH, shaking speed (190 r/min), temperature were optimized (data not given). Initial pH was controlled by buffer solutions at 7.2.

In this study, activated sludge (comparison sample) from a local beverage company was fetched due to the plenty kinds of microorganisms in the sludge. The wastewater in the beverage company was composed of high content of sugars, which could be beneficial for survive of various microorganisms. In order to compare the ability of bacterial selected from two sources, the paralleled experiment was exerted. Activated sludge was cultivated in lube oil solutions for 40 days and gradually increased the lube oil concentration from 100 to 2000 mg/L.

1.3 Biodegradation of lube oil

The mixed bacterial consortia at the log phase were kept in a conical flask (1000 mL) in selective medium at 30°C. Each shaker contained 600 mL of sterile selective medium with 2000 mg/L lube oil. The inoculation of mixed bacterial consortium was 0.1 wet biomass (75% water content)/liquid (*W/V*). The flasks were incubated and cultivation in a shaker at 190 r/min and 30°C for 32 days. With 2-day interval, the degradation of lube oil and TDOC (total dissolved organic carbon) was tested to sustain stable degradation process for mixed bacterial consortium. Five concentrations of lube oil from 2000 to 10,000 mg/L were chosen. The lube oil is mainly composed of benzene and its derivatives, cycloalkanes, hydrocarbons, asphalt, alkanes and so on.

1.4 Biological reactor with 304# stainless steel as biofilm carriers

The schematic diagram of the experimental set-up is in Fig. 1. The reactor consisted of a polyethylene column of 0.20 m internal diameter with total volume of 6 L, the solution volume of which is 4 L. The magnetic stirrer used

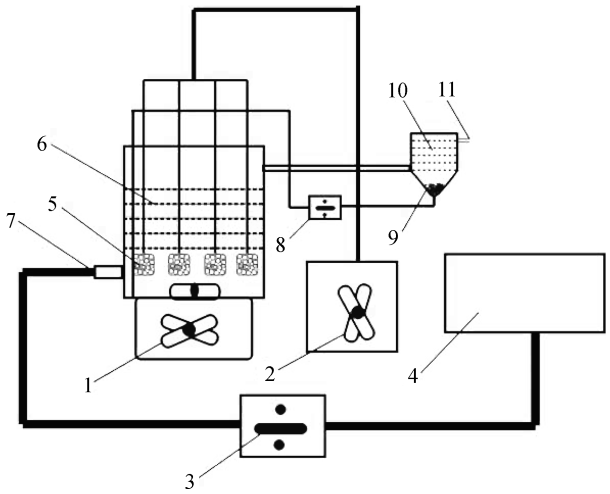

Fig. 1 Schematic of experimental set-up. (1) magnetic stirrer; (2) aeration pump; (3) circulating pump; (4) nutrient storage tank; (5) aeration distributors; (6) stainless steel biofilm carriers; (7) liquid influent; (8) sludge recirculation pump; (9) sludge flocs; (10) water levels; (11) liquid effluent.

to promote the oxygen transfer in biofilm reactor. Twelve aeration distributors (12 W) were used to supply enough oxygen for bacteria growth, which was designed to assure bubble size up to 1 mm and uniform dispersion in the reactor. A peristaltic pump (Grunfos, Denmark) was used to control the flow rate with hydraulic retention time of 48 hours. The flow rate was about 0.08 L/hr. The superficial gas velocity was between 0.003 and 0.015 m/sec. 304# stainless steel was distributed horizontally with thickness of 0.75 cm. Stainless steel pieces (ten pieces with holes of 0.4 cm diameter) were first grinded gradually with abrasive paper from 600 to 1200 mesh and then washed as previous assay (Cerca et al., 2005). pH value was sustained at 7.2 with NaOH (0.5 mol/L) or HCl (0.5 mol/L) during cultivation period. KH_2PO_4 was used as P source supply with ratio of 1/100 lube oil concentration (*W/W*). Degradation experiment was implemented in temperature of 30°C in thermostatic chamber. The inoculation biomass of mixed bacterial consortium was 0.1% (wet biomass (*m*)/solution (*V*)) in stimulated solutions. According to the COD_{Cr} load (3.6–18 g COD/(L·day)) was higher than these in previous reports, but not beyond the limitation of microorganisms (Munoz et al., 2009; Renato et al., 2009). The components of 304# stainless steel were mainly composed of Ni (8%–9%), Cr (18%–19%).

1.5 Physicochemical determination

Routine parameters of samples were measured according to standard methodology (APHA and AWWA, 1998). The concentrations of lube oil in the treatments were determined by ultraviolet spectrum as described by (Rahman et al., 2002). Hydrocarbons could be precisely detected spectrophotometrically. The maximum absorption wavelength was at 257.6 nm, using lube oil as standard substances (R^2 = 0.9999). Aromatic matters were the inhibitory components in lube oil. The absorbance at 257.6 nm could represent aromatic characteristics of organic matters (Zhang et al., 2009). SEM photos were taken in scanning electronic microscopy (S-4800, Hitachi, Japan). Polysaccharide was measured using the modified classical methods described previously with glucose as standard matters (Dubois and Gilles, 1956).

Fe^{2+} can be oxidized to Fe^{3+} with oxygen in the solution, forming different hydroxyl irons, such as $Fe(OH)^{2+}$, $Fe(OH)_2{}^{+}$, $Fe(OH)_2$, $Fe(OH)_3$, $Fe(OH)_4{}^{-}$, $Fe(H_2O)_6{}^{3+}$, $Fe(H_2O)_5(OH)^{2+}$, $Fe(H_2O)_4(OH)_2{}^{+}$, $Fe_2(H_2O)_8(OH)_2{}^{4+}$ and $Fe_2(H_2O)_6(OH)_4{}^{4+}$, when the ferrous dissolved from stainless steel. Total irons were detected by ICP-OES (Optima 2100DV, USA) with high-purity iron powder (99.99%) as standard substance.

TDOC was an important parameter to represent the dissolved carbon content in solutions (Zhao et al., 2006). The accumulation of organics in degradation process would be harmful. In this study, different concentrations of lube oil were biodegraded with TDOC measurements, which could show the ability of degradation by mixed bacterial consortium. TDOC was first filtered by 0.45 μm fiber film, then detected by TOC analyzer (TOC-VCPN, Japan).

Biomass formed on stainless steel were washed by sterile distilled water dramatically for twice to remove tight organics on surface of biofilm, followed by washing in ultrasonic cleaner (50 Hz) for 10 min to remove the biofilm from substrates and disperse in sterile solution.

Each value represents the mean of three repeats with a standard deviation less than 4%. Statistical calculations were based on confidence level equal or higher than 95%.

1.6 FT-IR and gas chromatography mass spectrometry analysis

After degradation process, the degraded samples were separated for analytical purpose using a separation funnel. The extraction solvent is *n*-hexane, which guarantees the solution without any water content. The sample was characterized by Fourier transform infrared spectroscopy (FT-IR, 570, Nicolet, USA). The FT-IR study spectrum was taken in the mid IR region of 400–4000 cm^{-1} with 16-scan speed. The samples were mixed with spectroscopically pure KBr in the ratio of about 1:100, pellets were fixed in the sample holder, and the analyses were carried out.

The initial and residual lube oil were extracted twice with 10-times volume, modified from previous assay (Adebusoye et al., 2007) of *n*-hexane extract (2.0 μL) was subsequently analyzed with a QP2010S gas chromatograph mass spectrometry (Schimadu, Japan) equipped with capillary column (30 m × 0.25 mm × 0.25 μm, HP-5). The carrier gas was nitrogen. The injector and detector temperature were maintained at 220 and 280°C, respectively. The column was programmed at an initial temperature of 60°C, held for 2 min. Then, the temperature ramped at 15°C/min to 180°C, held for 2 min. The sequent temperature was ramped at 40°C/min to 280°C, and held for 10 min. Benzene, its derivatives and non-polar ring compounds could be precisely detected (Chung et al., 2008).

1.7 Data analysis

The data were analyzed using the statistical program Statistical Package for Social Sciences (SPSS), version 13.0 and Origin software, version 7.5.

2 Results and discussion

2.1 Biofilm formation on 304# stainless steel

Stainless steel has been widely used as the support for biofilm formation due to its high stability and anticorrosion. It can be noted in Fig. 2 that the growth rate of biofilm biomass on day 0–1 was obviously slower than day 2–6 due to the lag phase of microbial growth after incubating. In addition, the biomass needed to establish a balance between attachment and detachment on the substrate surface to ensure a readily formation of biofilm. After that, polysaccharide experienced a rapid growth, which was called logarithm increase phase. According to Fig. 2, biomass growth from day 1 to day 4 is well fitted for the Eq. (1), in which the coefficient of fastest growth μ_{max} (μg glucose/(cm^2·day)) of polysaccharide is 0.216

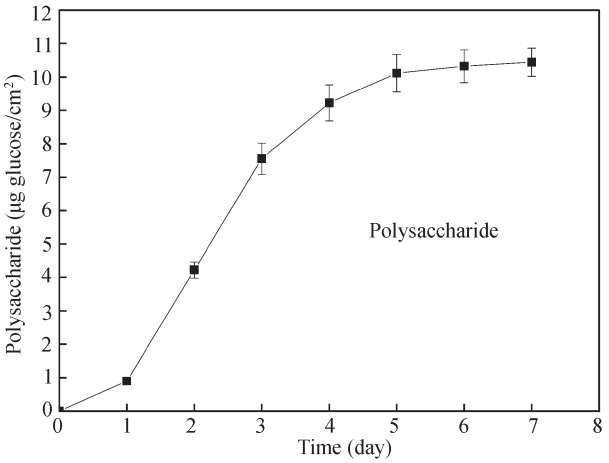

Fig. 2 Biomass formation on 304# stainless steel carriers (n = 3, lube oil: 2000 mg/L).

(R^2 = 0.96).

$$\frac{dx}{dt} = \mu_{max} x \quad (1)$$

where, x (μg glucose/cm^2) is the biomass on stainless steel; t (day) is time.

Until day 7, biofilm formed on stainless steel became mature and the biomass tended to be in a stable state. However, 304# stainless steel also showed excellent biofilm formation ability in this experiment.

2.2 Removal of lube oil and TDOC in biofilm reactor

According to Fig. 3, lube oil was greatly removed by mixed bacterial consortium in biofilm reactor with stainless steel carriers. Except for the first 8 hr, called lag phase (Fernandez et al., 2008) in degradation process, lube oil could be greatly biodegraded in 24 hr. In the first 8 hr, mixed bacterial consortium normally excreted biosurfactants (Pornsunthorntawee et al., 2009; Yin et al., 2009) to emulsify lube oil. As a result, lube oil dispersed evenly in the solution and did not attach. The experimental data showed that the surface tension of solution was reduced significantly from 68.5 to 35.1 mN/m in the first 8 hr. After the emulsification period, mixed bacterial consortium would be in the state of increased logarithmic phase and began to degrade lube oil. Within the first 24 hr, the oil degradation was obvious and over 95% of lube oil was removed. Within 48 hr, the degradation rate was slightly decreased from 99% to 97.2%, which demonstrated that most aromatic matters were greatly reduced (Janhom et al., 2009). Mixed bacterial consortium also showed their edge of bio-degradation in aromatic structure decomposition in a short period. The increase of lube oil concentration would not greatly influence the biological treatment efficiency.

Figure 4 shows the residual TDOC in biological bioreactor was greatly reduced. The removal efficiency of TDOC was slightly reduced from 95.35% to 93.82% (from 2000 to 10,000 mg/L). The decrease curve of TDOC showed similar tendency with that of lube oil, which demonstrated that the contaminants were thoroughly destroyed. Although the decrease of TDOC was lower than that of lube oil by mixed bacterial consortium, the residual concentration of TDOC was very low compared to original content. Through the biological reactor with stainless steel as biofilm carriers, the fast degradation process (from 8 to 24 hr) avoided accumulation of harmful substances to inhibit the biodegradation process. Compared with previous studies (Hii et al., 2009), the degradation time was greatly shortened and the organic load was higher. Once the degradation process was in short period, the inhibitory compounds produced in the degradation process would not induce numerously. Owing to the reason that fast degradation process would avoid the accumulation of other contaminants, the elimination of pollutants was fairly high by mixed bacterial consortium. Combination with removal of lube oil and TDOC, not only aromatic matters could be greatly reduced, but also dissolved organic carbons were decomposed thoroughly.

2.3 Comparison between biofilm reactor with activated sludge reactor

It was obviously observed from Fig. 5 that mixed bacterial consortium showed high efficiency in oil removal.

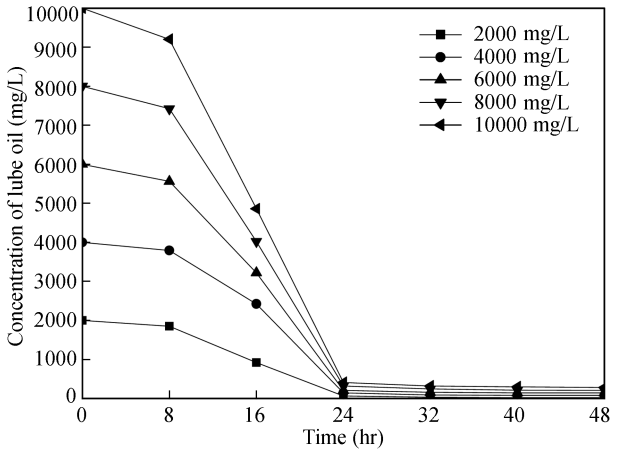

Fig. 3 Removal of lube oil by biofilm reactor at different concentrations.

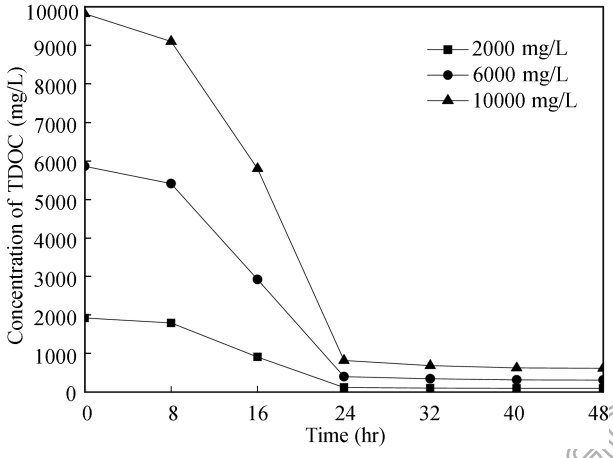

Fig. 4 Concentrations of residual TDOC (total dissolved organic carbon) after treatment by bioreactor.

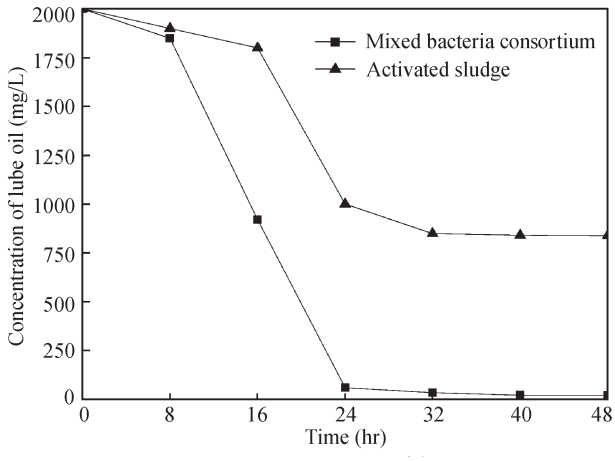

Fig. 5 Lube oil degradation by the mixed bacterial consortium and activated sludge.

Within 8 hr, 2000 mg/L lube oil was totally emulsified by selected mixed bacterial consortium and there were no lube oil drops attached. Compared with activated sludge inoculated from the beverage company without previous emulsification treatment, it will take at least 24 hr for microorganisms to make lube oil dissolved in the solution. The removal efficiency was just 55% compared with 99% by selected mixed bacterial consortium because the suspended oil may hinder the oxygen transfer efficiency by the formation of lipid coats (Lobos-Moysa et al., 2009). Although the degradation was longer than 48 hr, the removal efficiency was not obviously improved (data not given). In comparison, the activated sludge from the beverage company did not destroy lube oil with high efficiency.

2.4 Mechanisms of the degradation in lube oil

2.4.1 Dynamics of lube oil degradation

If the substrate was not performed as inhibitory substances and the degradation accumulation phenomenon would not function, the biodegradation process of hydrocarbons should obey the first-order decay equation (Rahman et al., 2002). Table 1 shows the simulation with Origin software in first-order decay equation.

According to Table 1, the degradation model was obviously matched with first-order decay equation. The fitting equation showed that different concentrations of lube oil did not inhibit the degradation efficiency of mixed bacterial consortium, even the concentration of lube oil was 10,000 mg/L. The contaminants accumulated in degradation process also seemed to hardly influence the ability of selected mixed bacterial consortium. In short cut, mixed bacterial consortium could efficiently uptake lube oil, which ensured little harmful intermediate matters produced.

Table 1 Kinetics of lube oil degradation process

$y = A_1 \times \exp(-x/x_1)+y_0$[a]	2000 mg oil/L	6000 mg oil/L	10,000 mg oil/L
A_1 (mg/L)	–662	–1107	–2857
x_1	–17	–13	–16
y_0 (mg/L)	2738	7273	13,189
R^2	0.98	0.99	0.98

[a] Where, y (mg/L) is residual lube oil concentration and x (mg/L) is original lube oil concentration. In addition, $1/x_1$ is decay constant and y_0 is theoretical concentration of lube oil controlled by decay constant. A_1 is maximum decay rate coefficient.

2.4.2 SEM photos of biofilm formed on 304# stainless steel

From morphology analysis of biofilm formed on stainless steel, mixed bacterial consortium was constructing dimensional structures. Figure 6 shows that strength of biofilm formed on stainless steel at day 6 (Fig. 6a) was not greatly changed after 24-hr treatment in buffer solutions (pH 7.2, Fig. 6b), which was matched with previous report (Neria-Gonzalez et al., 2006). Biofilm was very thick, surrounded tightly by EPS (extracellular polymeric substances), which make biofilms matrix to anti-resistant different organic loads (Yang et al., 2000). EPS were also substances of polysaccharides, phospholipids, proteins, which could serve as bio-surfactants to increase the emulsification process and greatly degraded organics in liquid-solid interface (Mehdi and Giti, 2008). The suspended and attached mixed bacterial consortium could digest emulsified lube oil with high efficiency.

2.4.3 Dissolution of ferrous ions from stainless steel

It is reported that the cast stainless steel can form a primary micro battery in the electrolyte solutions. The

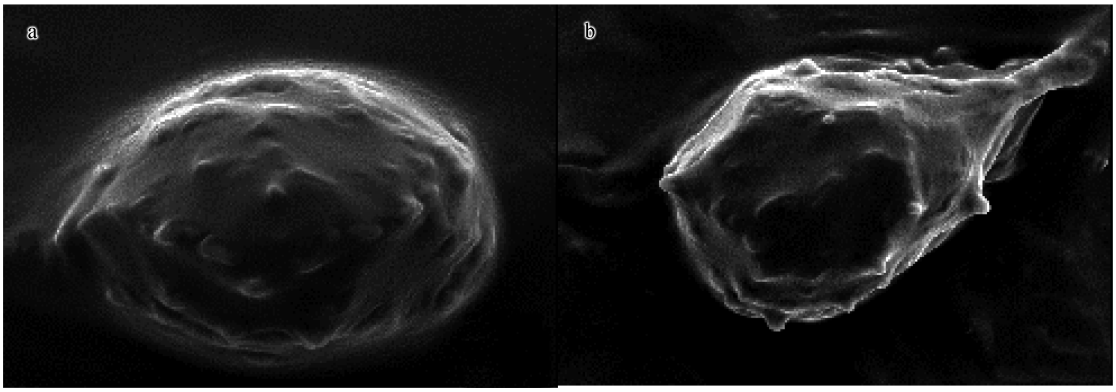

Fig. 6 Morphology of biofilm on day 6 (a) and dipped in buffer sample for 24 hours (b) (scale bar = 5 μm).

basic reactions in the micro battery are as Eq. (2):

$$\text{Anode reaction:} \quad Fe - 2e = Fe^{2+} \tag{2}$$

The nascent Fe^{2+} can be produced in the micro-electrolysis (5–6 mg/L in average). These hydroxyl irons have strong flocculation and absorption ability (Li et al., 2009). Besides the flocculation ability, mixed bacterial consortium could oxidize ferrous ions into ferric oxide in the presence of oxygen. Ferric oxide could precipitate on surface of stainless steel as catalyst medium (Shi et al., 2002). Mixed bacterial consortium utilizes energy from carbon and hydrogen during oil degradation, which could break aromatic rings firstly and degrade into small molecular matters.

2.4.4 FT-IR analysis of lube oil degradation

The IR spectroscopy of pure lube oil shows the characteristics bonds at 2930 cm^{-1} (C–H aliphatic stretch); 1450 cm^{-1} (C=C stretch in aromatic nuclei); 672 (meta disubstituted benzene) (Muthukumar et al., 2003). After adding the mixed bacterial consortium, the aromatic nuclei peaks disappeared because of degradation.

2.4.5 Lube oil degradation by gas chromatography mass spectrum analysis

Commaonas acidovorans Px1, *Bacillus* sp. Px2, and *Pseudomonas* sp. Px3 have been verified their high removal efficiency toward PAH (persistent organic carbons) in oil in previous reports (Fuchedzhieva et al., 2008; Loo and Sudesh, 2007; Das et al., 2008; Zeinali et al., 2008). According to persistent organic pollutants such as aromatic structures, mixed bacterial consortium could open the benzene ring at first stage. From Fig. 7a, with *n*-hexane extraction, lube oil contains non-polar organics mainly including benzene and its derivatives. After the treatment of mixed bacterial consortium, all the peaks were dramatically reduced due to the biomass production and mineralization process (Obayori et al., 2009), especially for that the sharp peaks in Fig. 7a generally represented benzene while the tiny short peaks are shown in Fig. 7b. The degradation result showed that nearly all the benzene was degraded by mixed bacterial consortium. In addition, Fig. 7 also shows that *n*-alkanes benzene derivatives were better removed by mixed bacterial consortium than branched alkane-benzene derivatives, in which the point was also supported by Genov et al. (2008).

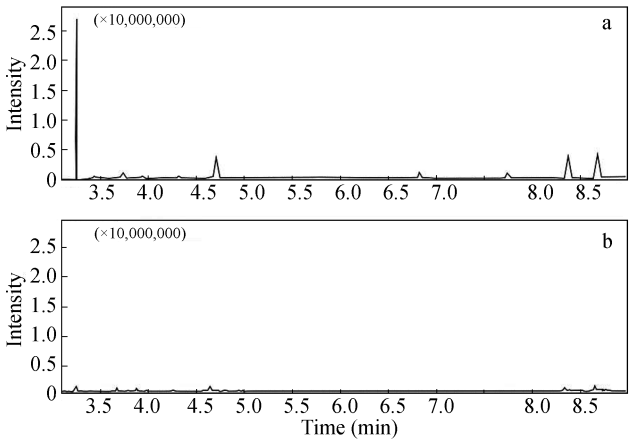

Fig. 7 Gas chromatography mass spectrum of original lube oil (a) and residual oil (b) recovered from biological reactor treated with mixed bacterial consortium. The lube oil substrate was supplied at a concentration of 5000 mg/L. Sample was diluted by *n*-hexane for 5000 times.

3 Conclusions

Mixed bacterial consortium was selected among 164 isolates from oil polluted soil samples with lube oil as sole carbon source. Three isolates (*Commaonas acidovorans* Px1, *Bacillus* sp. Px2, *Pseudomonas* sp. Px3) were selected and analyzed to identify their characterizations. Mixed bacterial consortium could thoroughly degrade lube oil from 0.2% to 1% within 48 hours in biofilm reactor with stainless steel as biofilm carriers (first sponsored). The TDOC degradation curve showed that the contaminants produced in degradation process would not greatly accumulate. Compared to isolates inoculated from the beverage company, mixed bacterial consortium exhibited better performance in lube oil degradation. The degradation of lube oil followed the first-order decay model, which showed that in study ranges, lube oil would not influence performance of mixed bacterial consortium. SEM photos displayed that biofilm formed on stainless steel had morphology of dimensional structures and surrounded by EPS, which could endure starvation of organic carbons, increased organic load and promote the lube oil to be decomposed. Through the analysis of FT-IR, total irons measurement and gas chromatograph mass spectrum, combined with removal of lube oil and TDOC, benzene derivatives and ring aromatic matters, as typical pollutants could be effectively reduced. Treatment of lube oil with mixed bacterial consortium by biofilm reactor was a promising method. The further degradation mechanism should be discussed in sequent works.

Acknowledgments

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