Ann Microbiol (2014) 64:1769–1777
DOI 10.1007/s13213-014-0821-3

ORIGINAL ARTICLE

Enhanced crude oil hydrocarbon degradation by self-immobilized bacterial consortium culture on sawdust and oil palm empty fruit bunch

Mohammad Hazaimeh · Sahilah Abd Mutalib · Palsan Sannasi Abdullah · Wong Kok Kee · Salmijah Surif

Received: 27 June 2013 / Accepted: 17 January 2014 / Published online: 6 February 2014

Abstract This study reports enhanced degradation of crude oil hydrocarbons by a bacterial consortium culture (CC) immobilized onto sawdust (SD) and oil palms empty fruit bunch (OPEFB). The bacterial surface hydrophobic property of the bacterial CC was 60.3±2.20 %, which suggested high bacterial cell attachment onto the carrier materials. The free bacterial CC exhibited the ability to produce exopolysaccharide (EPS) in minimal salt medium (MSM). The highest value of EPS produced by free bacteria CC was 61.3±2.10 %. Due to their surface hydrophobic properties and ability to produce EPS, the bacterial CC was self-immobilized onto OPEFB and SD. The immobilized bacterial CC was then used to degrade crude oil hydrocarbons. The results showed that bacterial CC immobilized onto OPEFB and SD increased degradation of crude oil by 17.52 % and, 15.85 % respectively, at week 6 of incubation, and shortened the time to complete degradation by 25 % (from 8 to 6 weeks) compared to free bacterial CC. Immobilized bacterial CC enhanced biosurfactant production, as indicated by the emulsification index (E24%). Thus, the present study demonstrated that bacterial CC immobilized onto carrier materials increases crude oil degradation by increasing production of biosurfactants.

Keyword Consortium culture · Immobilized cells · Crude oil hydrocarbon degradation · Oil palm empty fruit bunch · Sawdust · Exopolysaccharide

M. Hazaimeh (✉) · S. Abd Mutalib · S. Surif
Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM, Bangi, Selangor, Malaysia
e-mail: moh_hazaimeh@yahoo.com

P. S. Abdullah · W. Kok Kee
Faculty of Health & Life Sciences, INTI International University, 71800, Nilai, Negeri Sembilan, Malaysia

Introduction

The increasing extraction and usage of crude oil resources and oil products has caused major environmental problems, particularly from crude oil spills and contamination (Si-Zhong et al. 2009). The British petroleum (BP) crude oil spillage in the Gulf of Mexico in 2010 (Zeller 2010) and the Exxon Valdez spill in Alaska in 1989 (Bourne 2010) for example spewed about 4.4 million and 262,000 barrels of crude oil into the sea, respectively, leading to costly and massive cleanup operations.

Recently, the use of biological materials as a cleanup technique for hydrocarbons contamination in the environment has gained much attention. Microorganisms, for instance, are useful as bioremediation agents for degradation of petroleum hydrocarbons and their utilization as carbon/energy sources (Ivshina 2001). Bioremediation techniques using microorganisms are cheaper and more environmentally friendly than physical and chemical methods (Si-Zhong et al. 2009). It is reported that the consortium cultures are better degraders compared to individual strain cultures (Chhatre et al. 1996; Sugiura et al. 1996; Rahman et al. 2002; Sathishkumar et al. 2008) because each member strains of the consortium acts in concert to effect the break down of all the different hydrocarbon chains. A consortium culture also provides the necessary micro-environment, such as providing nutrients and being at the correct pH, thus stabilizing the culture for each of the members.

One of the challenges in the environmental application of bacteria as bioremediation agents is that the introduced bacteria have to compete with indigenous microorganisms or that they are less adapted to the ambient environmental conditions (Li et al. 2007). Introduced bacteria are washed out and diluted in open water systems, leading to a lowering of their concentration and inefficient use of the bioremediation technique (Rahman et al. 2006). Thus, to avoid this, immobilizing bacteria onto a carrier matrix is the alternative of choice (Gentili et al. 2006).

In order to immobilize bacterial cells onto carrier(s), cells must possess physico-chemical characteristics such as cell surface hydrophobicity (CSH) and the ability to produce exopolysaccharide (EPS) (Obuekwe and Al-Muttawa 2001) to form biofilm. The formation of biofilm begins initially by cells attaching to the carrier surface through hydrophobic forces found on the surfaces of bacteria. As hydrophobic molecules on the bacterial cell, fimbriae, flagella and proteins play important roles in the attachment of bacterial cells, overcoming repulsive forces such as the electrostatic force on the carrier surface and between cells (Bendinger et al. 1993). The positive correlations between bacterial hydrophobicity and the ability to attach onto a glass surface of *Pseudomonas aeruginosa*, *Pseudomonas putida* and *Escherichia coli* showed that the bacterium with the highest hydrophobic surface showed the highest attachment (Wang et al. 2011). The second step of biofilm formation is EPS production by bacterial cells to enable them to cement to the surface. The EPS is responsible for the cohesion of bacterial cells and the particulate nutrients surroundings them, and binds all of these masses onto carrier surfaces to form a biofilm layer (Sutherland 2001).

Biofilms are useful in protecting bacterial cells from harsh environmental conditions such as desiccation or variations in humidity (Sutherland 2001), and they persist in enabling degradation without decreasing bacterial cell activity (Wang et al. 2007). Biofilms also help to increase cell density by easing diffusion of nutrients and gaseous exchange, thereby increasing degradation activities (Baillie and Douglas 2000; Mah and O'Toole 2001; Simões and Vieira 2009).

Various materials, both natural and synthetic, have been used as carriers to support immobilization of bacterial cells. Ideally, such carriers should be made of readily available and cheap materials that are nontoxic and resistant to chemical and mechanical damage (Kuyukina et al. 2009). Oil palm empty fruit bunch fibers (OPEFB) are plentiful in Malaysia. It is reported that 50.0 million tons of this agro-waste is produced annually in Malaysia (Rahman et al. 2007). OPEFB is nontoxic and rich in organic content such as fatty acids that support bacterial growth, and is potentially an ideal carrier (Mumtaz et al. 2008). Another potential agro-waste is sawdust (SD), which has been used successfully to immobilize bacterial cells by other researchers (Obuekwe and Al-Muttawa 2001; Podorozhko et al. 2008). In this paper we report the biodegradation efficiency of crude oil hydrocarbons by a bacterial consortium culture (CC) immobilized onto OPEFB and SD carriers.

Materials and methods

Preparation of inoculums

Isolation and bacterial CC from industrial wastewater was described previously by Sannasi et al. (2009). The bacteria CC consists of six Gram-negative strains [*Pseudomonas* sp. (7 s4), *Serratia* sp. (11 s2), *Flavobacterium* sp. (28 s4), *Chryseomonas* sp. (5 s2), *Xanthomonas* sp. (9 s5), and *Agrobacterium* sp. (10 s4)] and three Gram-positive strains [*Arthrobacter* sp. (9 s4), *Bacillus* sp. (1 s10), and *Micrococcus* sp. (2 s9)]. These bacteria are able to absorb heavy metals (Sannasi et al. 2009) and possess the ability to degrade petroleum hydrocarbon compounds such as benzene, toluene, ethyl benzene and xylene (BTEX) (Fellie et al. 2012). The bacterial CC from glycerol stock was grown in nutrient broth and incubated on an orbital shaker (200 rpm) at 29±1 °C for 24 h. The bacterial CC was then harvested by centrifugation at 3,000 rpm for 15 min and washed twice with 10 mM phosphate buffer (pH 7) using similar conditions. The pellet was re-suspended in phosphate buffer to give approximately 10^7 CFU/mL (Hamzah et al. 2013). This suspension served as the starting inoculum for all subsequent experiments.

Cell surface hydrophobicity test

Cell surface hydrophobicity (CSH) of bacterial CC was measured using microbial adhesion to hydrocarbon test (MATH) (Chae et al. 2006). One milliliter of bacterial CC inoculum (Preparation of inoculum) was suspended in 10 mM phosphate buffer (pH 7) (OD_{600nm}≈0.3) and added to 0.15 mL crude oil in a test tube. The tube containing the mixture was vortexed for 1 min and left to stand for 15 min to allow separation into two phases. The aqueous phase containing the cells was read at OD_{600nm} in a UV-visible spectrophotometer (Hitachi U-1900, Tokyo, Japan) to determine cell density.

The CSH was calculated as follows:

$$CSH = 100 \text{ x } [1-(A/A0)], \text{ where } A0 \text{ is the } OD_{600nm} \text{of cell suspension before mixing and} A \text{is the } OD_{600nm} \text{after mixing.}$$

Measurement of EPS production by bacterial CC

Production of EPS was measured using Alcian Blue dye, which stains the EPS secreted by bacterial cells (Vandevivere and Kirchman 1993). One milliliter of bacterial CC inoculum was inoculated into 4 mL minimal salt medium (MSM) (per liter of deionized water: 1.0 g K_2HPO_4, 1.0 g KH_2PO_4, 1.0 g NaCl; 1.0 g KNO_3; 0.2 g $MgSO_4$ and 0.02 g $CaCl_2$) (Podorozhko et al.

2008) and incubated on an orbital shaker (200 rpm) at 29±1 °C for 5 days. At every 24 h interval, 4 mL bacterial CC was pipetted out and added to 50 μL Alcian Blue dye and left to stand for 5 min. The mixture was centrifuged at 10,000 rpm to precipitate the bacterial cells. The absorbance of the dye that was not bound to bacterial CC in the supernatant was then read at OD_{606nm} (Obuekwe and Al-Muttawa 2001). The amount of EPS produced was calculated from the reduction (%) in the absorbance of dye at OD_{606nm} in the supernatant relative to the control. The control for bacterial CC was prepared using MSM without bacterial cells.

Immobilization of bacterial CC onto carriers

The OPEFB was supplied by Malaysian Palm Oil Board (MPOB), and SD was from Sawmill Kajang. These carriers were washed several times with ethanol (70 %), followed by distilled water to remove surface impurities, and dried at room temperature. The carriers were then sterilized by autoclaving at 121 °C for 20 min and cooled down prior to use. One milliliter of bacterial CC inoculum was pipetted into 20 mL vials that contained 1 g sterilized carrier and incubated at room temperature for 5 days. The progression of the immobilization process of bacterial CC was analyzed every 24 h by measuring EPS production. At the end of the incubation, 10 mL fresh MSM was added to the culture, sonicated (BRANSON 1210) for 2 min, followed by vortexing (VELP Scientifica, Usmate, Italy) for 10 min to dislodge the bacterial CC from the carriers. Four milliliters of the bacterial CC suspended in MSM, which had been dislodged from carrier surface, was then pipetted into a vial. It was then stained with 50 μL Alcian Blue dye and incubated for 5 min at room temperature and the concentration of dye that was not bound to EPS was read at OD_{606nm}, as described in "Measurement of EPS production by bacterial CC".

Progression of the immobilization was also observed through the formation of biofilm under scanning electron microscopy (SEM). At 24-h intervals, 0.01 g bacterial CC-carriers incubation mixture was removed and washed with 0.85 % saline to remove unattached cells. The carrier with attached bacterial CC was then fixed with 4 % (w/v) glutaraldehyde solution for 24 h at 4 °C and washed three times with 0.1 M sodium cacodylate buffer. Samples were fixed using 1 % (w/v) osmium tetroxide for 2 h at 4 °C and then washed with 0.1 M sodium cacodylate buffer. The samples were then dehydrated using a series of acetone (35–100 %), 10 min times each, and dried using the CO_2 critical point drying technique for 30 min. The samples were then sputtercoated with gold and viewed under SEM (SUPRA 55VP, Zeiss, Jena, Germany) (Díaz et al. 2002).

Biodegradation of crude oil hydrocarbon by free and immobilized bacterial CC

Biodegradation of crude oil hydrocarbon was conducted by mixing 1.0 mL bacterial CC inoculum in a conical flask containing 98.0 mL MSM medium (pH 7) and 1.0 mL crude oil. This mixture was for free bacterial CC. For immobilized bacterial CC, a separate experiment was conducted by adding 1.0 g immobilized bacterial CC on OPEFB (see Immobilization of bacterial CC onto carriers) to 98.0 mL MSM medium and 1.0 mL crude oil. The same experiment was done using 1.0 g SD immobilized bacterial CC (Immobilization of bacterial CC onto carriers) in 98.0 mL MSM medium and 1.0 mL crude oil. A control for free bacterial CC was made by adding 99.0 mL MSM medium to 1.0 mL crude oil. A control for immobilized bacterial CC was prepared by adding 1.0 g sterile OPEFB or SD without immobilized CC. All flasks were incubated on an orbital shaker (200 rpm) at 29±1 °C for 8 weeks. The residual crude oil was analyzed every 2 weeks as described in the section on "Residual crude oil analysis". Each experiment was carried out in triplicate.

Residual crude oil analysis

Residual crude oil was extracted by two successive liquid–liquid extraction methods (Gentili et al. 2006). All the mixtures mentioned above (Biodegradation of crude oil hydrocarbon by free and immobilized bacterial CC) in each extraction were poured into a separation funnel and added to 5 mL *n*-hexane. The mixture was shaken vigorously for 1 min and left to stand for 10 min to separate the organic phase from the aqueous phase. The lower layer (aqueous phase) was used in the bacterial CC emulsification test (see Emulsification activity). The upper layer (organic phase) was collected into a round bottom flask. This solution was then evaporated on a rotary evaporator (EYELA N-1000, Tokyo Rikakikai, Tokyo, Japan) with the water bath at 50 °C. The dried crude oil residue was resuspended in 1 mL *n*-hexane and immediately analyzed using gas chromatography with flame ionization detector (GC-FID) (Clarus 500 GC, Shelton, CT).

The capillary column gas of the GC-FID used was a 320 μm×30 m silica column (J&W Scientific, Folsom, CA). One microliter of sample was injected by split injection using helium as carrier gas at a constant flow rate of 1.2 mL/min and the oven temperature programmed as follows: 5 min at 60 °C followed by 5 °C/min to 320 °C and a final bake out at 320 °C for 10 min. The percentage of crude oil degraded was determined by comparing the total area of the chromatogram of the tested samples with that of the control. The GC fraction was analyzed for the carbon 17 (C17)/pristane (pri) and carbon 18 (C18)/phytane (phy) ratios. The ratios of C17/pri and C18/phy were used to estimate crude oil biodegradation. If the level

ratios of C17/pri and C18/phy decreased compared to the control, this indicated biodegradation.

Stability of the immobilized bacterial CC

The stability of bacterial CC immobilized on OPEFB and SD (Biodegradation of crude oil hydrocarbon by free and immobilized bacterial CC) was studied as described by Obuekwe and Al-Muttawa (2001). The viable bacterial CC on the surfaces of the respective carriers was determined every 2 weeks. The carriers in each experiment were removed from the flasks into vials using sterile forceps and washed twice with 10 mL phosphate buffer (pH 7) by inverting the vial several times to remove free bacteria cells. The immobilized bacteria CC on carriers were then sonicated for 2 min (BRANSON 2210) and vortexed for 10 min to dislodge the bacterial CC. One hundred microliters of the dislodged bacterial CC was then plated onto nutrient agar and incubated at 29±1 °C overnight. The numbers of viable bacterial cells grown on the agar were calculated as colony forming unit per milliliter (CFU/mL).

Emulsification activity

Emulsification activity during crude oil degradation was evaluated using the emulsification index E24% as described by Cooper and Goldenberg (1987). The lower aqueous phase from Residual crude oil analysis was transferred into 100 mL tubes and centrifuged at 10,000 rpm for 15 min. Three milliliters of aqueous phase from the above centrifugation was transferred into a glass test tube, and 3.0 mL crude oil was then added to the tube and vortexed for 2 min. The mixture was left to stand for 24 h at room temperature. Emulsification index E24% was calculated as follow:

$$E24\% = [\text{Height of the emulsified layer} / \text{Total height of the liquid column}] \times 100.$$

Statistical analyses

Data were analyzed by means of one way ANOVA with 95 % confidence level using SPSS PASW Statistic 17 software (SSPS, Chicago, IL). Results are reported as mean ± standard deviation (n=3).

Results and discussion

Cell surface hydrophobicity and exopolysaccharide production

The bacterial consortium used in this study contains nine species of bacteria isolated from waste industrial water. It showed a high ability to survive in extreme conditions and to absorb heavy metals at the laboratory scale (Sannasi et al. 2009). Due to these abilities, an attempt has been made to explore its ability in degrading crude oil hydrocarbon and to immobilize it onto a carrier surface such as OPEFB and SD.

One of the important aspects in their capacity to degrade crude oil is the CSH value of a consortium. The CSH is important in initiating the attachment of bacterial cells onto the carrier surface. In this work, the CSH was examined prior to immobilization and the value of free bacterial CC was shown to be 60.3±2.20 %. This result was significantly higher than the values reported by Wang et al. (2011), who studied the attachment of each single isolate of three species bacteria, namely *P. aeruginosa* (36.7±4.5 %), *P. putida* (10.8±2.8 %) and *E. coli* (5.1±4.1 %). In this work, we applied a bacterial consortium rather than a single isolate; this may explain why our CSH value was much higher than that of Wang et al. (2011).

Since biofilm formation is reported to be correlated with the production of EPS, we evaluated EPS production by semi-quantitative determination using Alcian Blue dye (Obuekwe and Al-Muttawa 2001). This technique was chosen because we are interested in EPS bound onto a bacterial surface. The reduction in OD reading relative to the control indicates EPS production due to Alcian Blue dye bound with these molecules. In our study, EPS production was calculated as a percentage relative to the control and the value was 61.3±2.10 % after 3 days incubation. This result is slightly higher (8.6 %) as compared to findings reported by Obuekwe and Al-Muttawa (2001), which was 56 % after 4 days incubation using a single isolate of *Arthrobacter* sp. The EPS production of the bacterial consortium also showed 1 day earlier compared to single isolate bacteria, which may indicate better survival and activity of bacterial cells. However, this semi-quantitative method is able to measure only the EPS bound to the bacterial cells, but not soluble EPS that may present in the supernatant. The actual amount of EPS being produced may be slightly higher, if soluble EPS was measured.

Development of immobilized CC

Determination of CSH and EPS showed the potential of our bacterial consortium in biofilm formation. Two carrier materials for immobilization were studied: OPEFB and SD. The bacterial CC (Immobilization of bacterial CC onto carriers) was immobilized on the carrier surface for 5 days. Results indicated that, after 12 h of incubation, the bacterial CC was

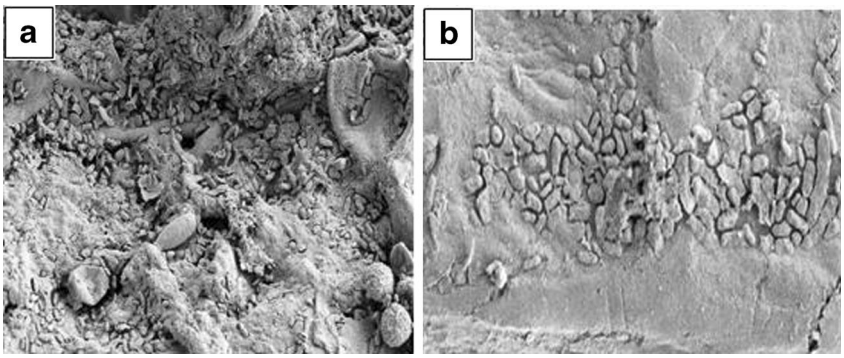

Fig. 1 Micrographs of bacterial consortium culture (CC) on **a** oil palm empty fruit bunch (OPEFB) (4,000×) and **b** sawdust (SD) (5,000×) at 12 h of incubation

attached firmly to the surfaces of OPEFB and SD as shown in Fig. 1. Donlan (2002) suggested that the initial adhesion of bacterial CC onto a carrier surface relied on hydrophobic forces since EPS was not synthesized. He reported that the hydrophobic properties of bacterial fimbriae, flagella and protein cell walls overcame repulsive forces such as the electrostatic force between cells and carrier surfaces.

Our finding is in agreement with that of Wang et al. (2011), who reported a positive correlation between CSH values and attachment of cells onto surfaces, where CSH indicated the capability to bind onto the carrier surface. When the incubation was prolonged to 24 h, EPS production was observed. EPS was measured using the reduction in the percentage of Alcian Blue dye at optical density 606 nm. Incubation of bacterial CC with OPEFB as a carrier gave a value of 56.7± 3.20 %, with 55.3±4.01 % for SD. The bacterial CC synthesized EPS fibers that allow individual cells to latch onto the carrier surface and penetrate between cells. SEM results after 3 days of incubation showed that the bacterial CC produced a thicker biofilm layer than after 24 h of incubation (Fig 2). The value of EPS at day 3 was 67±4.0 for OPEFB and 67.3±3.50 for SD. There was no further significant EPS production after 3 days of incubation ($P>0.05$). The increased value of EPS would increase the aggregation of bacterial CC onto both carriers.

Figure 2 shows micrographs of OPEFB and SD surfaces with excessive EPS production functioning as a bridge to bind bacterial CC to form a biofilm. Sutherland (2001) reported a varied structure and composition of EPS but the majority was either polyanionic uronic acids or ketal-linked pyruvate. The charged surfaces of EPS provide various interaction sites for binding to solid surfaces via interactive forces such as hydrogen or electrostatic bonding (Mayer et al. 1999). To this extent, the bacterial CC used in this study was able to attach onto OPEFB and SD, and produced excessive EPS thus forming a stable biofilm.

Crude oil hydrocarbon degradation

The ability of free and immobilized bacterial CC to degrade crude oil hydrocarbon was evaluated and compared. Figure 3 shows crude oil degradation by free and immobilized bacterial CC from week 2 to week 8 (> 97 % degradation). Bacterial CC immobilized on OPEFB degraded 81.1±4.36 %, 91.7± 1.03 % and 97.0±0.96 % of crude oil at week-2, week-4 and week-6; and SD degraded 70.3±4.12 %, 89.5±2.80 % and 95.9±1.67 % of crude oil at week-2, week-4 and week-6, respectively.

The efficiency of immobilized bacterial CC in degrading crude oil hydrocarbon was compared to that of bacterial free cells. As shown in Fig. 3, the immobilization of bacterial CC on OPEFB enhanced crude oil degradation by 24.21 %, 19.15 % and 17.52 % at week-2, week-4 and week-6, respectively, compared to free cells bacterial CC. A similar observation was also made with crude oil degradation on SD, which increased values of degrading crude oil hydrocarbon by 12.56 %, 16.30 % and 15.85 % at week-2, week-4 and week-6, respectively (Fig. 3). The ability of immobilized CC to degrade crude oil hydrocarbon was further evaluated by the

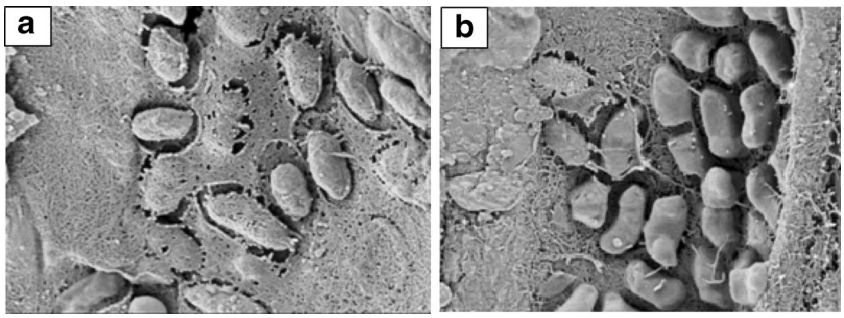

Fig. 2 Formation of biofilm by bacterial CC on **a** OPEFB and **b** SD surfaces (50,000×) at day 3 of incubation

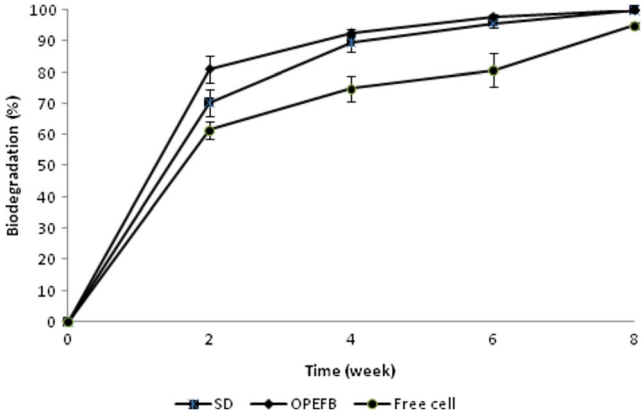

Fig. 3 Crude oil degradation using free and immobilized cells of bacterial CC on OPEFB and SD from week 2 to week 8 of incubation. Values are the mean of three separate experiments ± standard deviation

decrease of *n*- C17/pri and *n*-C18/phy ratios extrapolated from GC profiles (Table 1).

In biodegradation processes, the value of the aliphatic fraction of hydrocarbons is estimated by the *n*- C17/pri and *n*-C18/phy ratios. The value of this ratio would decrease due to degradation activity by microbes (Antic et al. 2006); without degradation the ratios of *n*-C17/pri and *n*-C18/phy remain constant (Wang et al. 1998). The degradation of alkanes (C17 and C18) and isoprenoids (pristane and phytane) compounds by bacterial CC caused a decrease in *n*-C17/pri and *n*-C18/phy ratios. The bacterial CC immobilized onto OPEFB reduced the percentage of *n*-C17/pri and *n*- C18/phy to 99 % and 95 %, respectively, at week 6 of incubation, while immobilization of bacterial CC onto SD reduced the percentage of *n*-C17/pri to 99 % and reduce 100 % of *n*-C18/phy (Table 1, Fig. 4). None of these compounds were detected after 6 weeks of incubation.

In the present study, bacterial CC was capable of degrading both alkanes and isoprenoids. The degradation of alkanes has been shown to be faster compared to isoprenoid compounds. NAS (1985) described that recalcitrant isoprenoids were less degraded compared to alkanes because only a few microbes are able to break down the beta oxidation blockage of methyl branching in isoprenoid structure. Immobilizing bacterial cells enabled increased contact between bacterial cells and hydrophobic hydrocarbon compounds, thus increasing the degradation rate of hydrocarbon compounds (alkanes and isoprenoids), as shown by the decrease in *n*-C17/pri and *n*-C18/phy ratios (Mehdi and Giti 2008). Our results were in agreement with those findings.

Stability of immobilized bacterial CC

The stability of immobilized bacterial CC during crude oil degradation was studied by measuring the number of viable cells attached to OPEFB and SD surfaces. Figure 5 shows the changes in the number of viable bacterial CC cells (log10 CFU/mL) attached to OPEFB and SD surfaces during biodegradation of crude oil hydrocarbons. At day-0, the number of bacterial CC attached to OPEFB and SD was 3.01×10^7 and 2.86×10^7, respectively. At week-2, the number of viable cells immobilized on OPEFB and SD showed a significant increase ($P<0.05$) of 18.9 % and 23.7 %, respectively. Thereafter, the number of viable cells started to decline steadily. At week-8, the CC declined to 4.43×10^6 in OPEFB and to 3.87×10^6 in SD; however, the decline was not significant in the case of OPEFB. This observation suggested that OPEFB gave better support to bacterial CC in maintaining survivability of cells compared to SD. The solid biomass of OPEFB is made up of 15 % fiber (Wu et al. 2010), and the fibrous component acts as a water sorbent material, entrapping water molecules within its structure.

Although the number of viable cells was observed to decrease, crude oil degradation continued to increase significantly ($P<0.05$) from week-2 to week-8 as shown in Fig. 5. This suggested that bacterial CC immobilized on OPEFB and SD remained metabolically active to degrade crude oil hydrocarbons throughout 8 weeks of incubation.

Emulsification activity

Emulsification of crude oil occurs when the surface tension due to repulsive forces between crude oil and aqueous media is reduced, this allowing the two phases to mix easily (Soberón-Chávez and Maier 2011). Formation of small oil droplets increases the surface area of hydrocarbon and this

Table 1 Ratio of *n*-C17/pristane (pri) and *n*-C18/phytane (phy) in crude oil hydrocarbon samples incubated for 8 weeks using free cells and immobilized CC on sawdust (SD) and oil palm empty fruit bunch (OPEFB). Values are the mean of three separate experiments ± standard deviation

Time (week)	2		4		6		8	
	n-C17/pri	*n*-C18/phy	*n*-C17/pri	*n*-C18/phy	*n*-C17/pri	*n*-C18/phy	*n*-C17/pri	*n*-C18/phy
Free cell	3.4±1.30	4.0±0.50	0.9±0.06	1.7±0.48	0.1±0.02	0.4±0.04	ND[a]	ND
SD	0.4±0.07	0.5±0.08	0.2±0.04	ND	0.05±0.03	ND	ND	ND
OPEFB	0.4±0.02	1.2±0.42	0.1±0.05	1.2±0.30	0.06±0.01	0.3±0.03	ND	ND

[a] Not detected

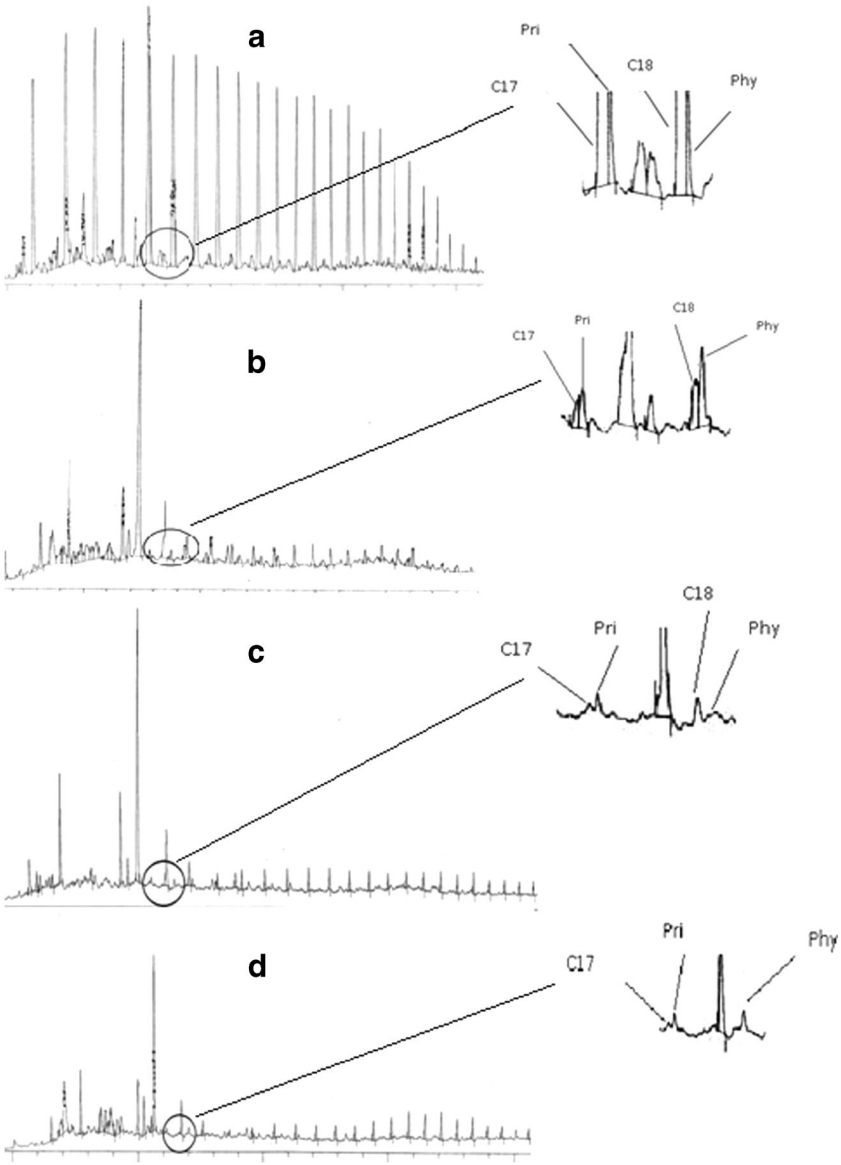

Fig. 4a–d Gas chromatography with flame ionization detector (GC-FID) analyses of the saturated fraction. **a** Initial crude oil and biodegraded samples using **b** free and immobilized bacterial CC cells onto **c** OPEFB and **d** SD at 6 weeks of incubation

increases the contact between emulsified hydrocarbons and membrane-bound enzymes on bacteria (Zhang and Miller 1992).

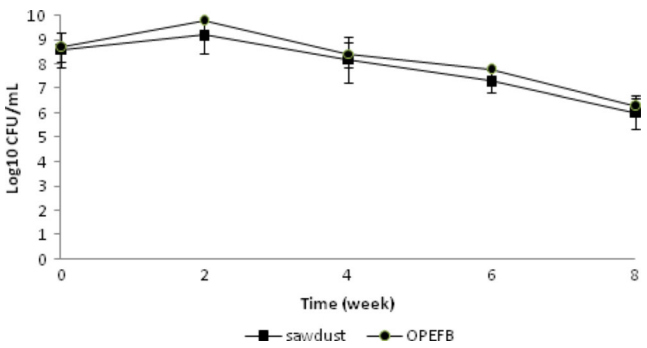

Fig. 5 Changes in viable bacterial CC cell number, immobilized on OPEFB and SD during crude oil biodegradation. Values are the mean of three separate experiments ± standard deviation

In the present study, the emulsification activity of bacterial CC immobilized onto OPEFB increased by 8.5 %, 20.4 % and

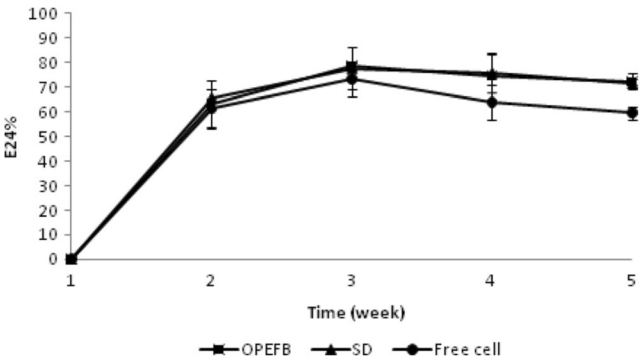

Fig. 6 Emulsification index (E24 %) of crude oil hydrocarbon using supernatant obtained from free and immobilized bacterial CC cells on OPEFB and SD. Values are the mean of three separate experiments ± standard deviation

26.1 % at weeks-4, week-6 and week-8, respectively, compared to bacterial CC free cells (Fig. 6), whereas immobilized CC onto SD increased the emulsification activities by 6.8 %, 22.5 % and 24.2 % at weeks-4, week-6 and week-8, respectively. Those observations show that bacterial CC immobilized onto carrier materials could enhance biosurfactant activity. Similar findings were also observed by Zhang and Miller (1992), who reported that biosurfactant activity increased the emulsification of crude oil in the aqueous phase. Thus, bacterial CC immobilized on carrier materials enhances emulsification activity and this led to increased degradation of crude oil hydrocarbon.

Conclusion

Bacterial self-potential in degradation of crude oil hydrocarbon was studied prior to immobilization. Cell surface characteristics such as CSH and EPS production were evaluated to ensure the free bacterial CC was able to form biofilm. This bacteria CC was then immobilized onto two carrier materials—OPEFB and SD—to improve crude oil hydrocarbon bioremediation on the laboratory scale. The bacterial CC used in the present study showed high emulsification activity when immobilized compared to free bacterial CC, and thus increased the degradation of crude oil hydrocarbon.

Acknowledgments The work described in this paper was supported by Exxon-Mobil STGL-008-2006 and the Malaysian Ministry of Science, Technology and Innovation (MOSTI), Escience Fund 06-01-02-SF0469.

References

Antic MP, Jovancicevic BS, Ilic M, Vrvic M, Schwarzbauer J (2006) Petroleum pollutant degradation by surface water microorganisms. Environ Sci Pollut R 13:320–327

Baillie GS, Douglas LJ (2000) Matrix polymers of *Candida* biofilms and their possible role in biofilm resistance to antifungal agents. J Antimicrob Chemother 46:397–403

Bendinger B, Rijnaarts HHM, Altendorf K, Zehnder AJB (1993) Physicochemical cell surface and adhesive properties of *Coryneform* bacteria related to the presence and chain length of mycolic acids. Appl Environ Microbiol 59:3973–3977

Bourne JK Jr (2010) The deep dilemma. National Geographic. http://connection.ebscohost.com/c/articles/53874173/deep-dilemma. Accessed 16 October 2010

Chae MS, Schraft H, Hansen LT, Mackereth R (2006) Effects of physicochemical surface characteristics of *Listeria monocytogenes* strains on attachment to glass. Food Microbiol 23:250–259

Chhatre S, Purohit HJ, Shanker R, Khanna P (1996) Bacterial consortia for crude oil spill remediation. Water Sci Technol 34:187–193

Cooper DG, Goldenberg BG (1987) Surface-active agents from two *Bacilllus* species. Appl Environ Microbiol 53:224–229

Díaz MP, Boyd KG, Grigson SJW, Burgess JG (2002) Biodegradation of crude oil across a wide range of salinities by an extremely halotolerant bacterial consortium MPD-M, immobilized onto polypropylene fibers. Biotechnol Bioeng 79:145–153

Donlan RM (2002) Biofilms: microbial life on surfaces. Emerg Infect Dis 8:881–890

Fellie EA, Sannasi P, Wong KK, Salmijah S, Kader J (2012) Tolerance and biodegradation of benzene, toluene, ethylbenzene and xylene (BTEX) by a metal acclimatized bacterial consortium culture. Res J Biotechnol 7:52–58

Gentili AR, Cubitto MA, Ferrero M, Rodriguèz MS (2006) Bioremediation of crude oil polluted seawater by a hydrocarbon degrading bacterial strain immobilized on chitin and chitosan flakes. Int Biodeter Biodegrad 57:222–228

Hamzah A, Phan CW, Abu Bakar NF, Wong KK (2013) Biodegradation of crude oil by constructed bacterial consortia and the constituent single bacteria isolated from Malaysia. Bioremed J 17:1–10

Ivshina IB (2001) Operation and establishment of the Russian biological resource centre. WFCC Newsletter 33:8–14

Kuyukina MS, Ivshina IB, Serebrennikova MK, Krivorutchko AB, Podorozhko EA, Ivanov RV (2009) Petroleum-contaminated water treatment in a fluidized-bed bioreactor with immobilized *Rhodococcus* cells. Int Biodeter Biodegrad 63:427–432

Li H, Zhang Y, Kravchenko I, Xu H, Zhang C-G (2007) Dynamic changes in microbial activity and community structure during biodegradation of petroleum compounds: a laboratory experiment. J Environ Sci 19:1003–1013

Mah TF, O'Toole GA (2001) Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol 9:34–39

Mayer C, Moritz R, Kirschner C, Borchard W, Maibaum R, Wingender J, Flemming HC (1999) The role of intermolecular interactions: studies on model systems for bacterial biofilms. Int J Biol Macromol 26: 3–16

Mehdi H, Giti E (2008) Investigation of alkane biodegradation using the microtiter plate method and correlation between biofilm formation, biosurfactant production and crude oil biodegradation. Int Biodeter Biodegrad 62:170–178

Mumtaz T, Abd-Aziz S, Abdul Rahman NA, Yee PL, Shirai Y, Hassan MA (2008) Pilot-scale of recovery of low molecular weight organic acids from anaerobically treated palm oil mill effluent (POME) with energy integrated system. Afr J Biotechnol 21:3900–3905

National Academy of Sciences (NAS) (1985) Oil in the sea: inputs, fates and effects. National Academy Press, Washington, DC

Obuekwe CO, Al-Muttawa EM (2001) Self-immobilized bacterial cultures with potential for application as ready-to-use seeds for petroleum bioremediation. Biotechnol Lett 23:1025–1032

Podorozhko EA, Lozinsky VI, Ivshina IB, Kuyukina MS, Krivorutchko AB, Phillip JC, Cunningham CJ (2008) Hydrophobised sawdust as a carrier for immobilisation of the hydrocarbon-oxidizing bacterium *Rhodococcus ruber*. Bioresour Technol 99:2001–2008

Rahman KSM, Thahira-Rahman J, Lakshmanaperumalsamy P, Banat IM (2002) Towards efficient crude oil degradation by a mixed bacterial consortium. Bioresour Technol 85:257–261

Rahman RNZA, Ghazali FM, Salleh A, Basri M (2006) Biodegradation of hydrocarbon contamination by immobilized bacterial cells. J Microbiol 44:354–359

Rahman SHA, Choudhury JP, Ahmad AL, Kamaruddin AH (2007) Optimization studies on acid hydrolysis of oil palm empty fruit bunch fiber for production of xylose. Bioresour Technol 98:554–559

Sannasi P, Kader J, Othman O, Salmijah S (2009) Physical growth and biomass characterization of bacterial cells exposed to Cd(II), Cr(VI), Ni(II) and Pb(II). J Environ Res Dev 4:8–18

Sathishkumar M, Binupriya AR, Baik SH, Yun SE (2008) Biodegradation of crude Oil by individual bacterial strains and a mixed bacterial consortium isolated from hydrocarbon contaminated areas. Clean 36:92–96

Simões M, Vieira MJ (2009) Persister cells in *Pseudomonas fluorescens* biofilms treated with a biocide. In: Proceedings of the international

conference processes in biofilms: fundamentals to applications, Davis, CA, pp 58–62

Si-Zhong J, Hui-Jun J, Wei Z, Rui-Xia H, Yan-Jun JI, Xiu-Mei LI, Shao-Peng YU (2009) Bioremediation of oil spills in cold environments. Pedosphere 19:371–381

Soberón-Chávez G, Maier RM (2011) Biosurfactants: a general overview. In: Soberón-Chávez G (ed) Biosurfactants. Springer, Berlin, pp 1–11

Sugiura K, Ishihara M, Shimauchi T, Harayama S (1996) Physicochemical properties and biodegradability of crude oil. Environ Sci Technol 31:45–51

Sutherland IW (2001) Biofilm exopolysaccharides: a strong and sticky framework. Microbiology 147:3–9

Vandevivere P, Kirchman DL (1993) Attachment stimulates exopolysaccharide synthesis by a bacterium. Appl Environ Microbiol 59:3280–3286

Wang Z, Fingas M, Blenkinsopp S, Sergy G, Landriault M, Sigouin L, Foght J, Semple K, Westlake DWS (1998) Comparison of oil composition changes due to biodegradation and physical weathering in different oils. J Chromatogr A 809:89–107

Wang Y, Ye T, Bin H, Hua-bing Z, Jian-nan B, Bao-li C (2007) Biodegradation of phenol by free and immobilized *Acinetobacter* sp.strain PD12. J Environ Sci 19:222–225

Wang HM, Sodagari Y, Chen X, He BM, Newby Z, Ju LK (2011) Initial bacterial attachment in slow flowing systems: effects of cell and substrate surface properties. Colloid Surface B 87:415–422

Wu TY, Mohammad AW, Jahim JM, Anuar N (2010) Pollution control technologies for the treatment of palm oil mill effluent (POME) through end-of-pipe processes. J Environ Manage 91:1467–1490

Zeller T Jr (2010) Estimates suggest spill is biggest in US History. http://www.nytimes.com/2010/05/28/us/28flow.html. Accessed 27 May 2010

Zhang YM, Miller RM (1992) Enhanced octadecane dispersion and biodegradation by a *Pseudomonas* rhamnolipid surfactant (biosurfactant). Appl Environ Microbiol 58:3276–3828