Int. J. Environ. Sci. Technol. (2015) 12:2253–2262
DOI 10.1007/s13762-014-0619-7

ORIGINAL PAPER

Self-immobilised bacterial consortium culture as ready-to-use seed for crude oil bioremediation under various saline conditions and seawater

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Received: 1 August 2013 / Revised: 11 April 2014 / Accepted: 10 May 2014 / Published online: 4 June 2014
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Abstract Biodegradation of crude oil hydrocarbon by microorganisms in seawater is generally slow because of the harsh environmental condition due to high salinity. The aim of this study was to compare sawdust (SD) and oil palm empty fruit bunch wastes as suitable carrier material to immobilise hydrocarbon-degrading bacterial consortium culture to accelerate and improve crude oil degradation in seawater. The consortium culture was found able to tolerate salinity up to 3 %, where the degradation of crude oil was not inhibited ($p > 0.05$). In artificial seawater, suspension of bacterial consortium culture was able to degrade 83.3 ± 3.00 % of crude oil within 8 weeks, which indicated the possibility of using consortium culture in seawater. When tested in seawater, suspension of consortium culture managed to degrade 47.7 ± 1.53 % of crude oil in 8 weeks. In order to improve the performance of consortium culture, immobilisation of consortium culture onto SD and oil palm empty fruit bunch was successfully undertaken when formation of biofilm layers was observed under scanning electron microscope. Immobilising consortium culture onto oil palm empty fruit bunch and SD was shown to increase crude oil biodegradation to 68.7 ± 4.04 and 62.3 ± 5.51 % in 8 weeks, respectively. This study demonstrated immobilisation of consortium culture onto SD and oil palm empty fruit bunch can be utilised as ready-to-use seeds to improve and accelerate crude oil biodegradation in seawater.

Keywords Bacteria · Biodegradation · Biofilm · Carrier materials · Hydrocarbon · Petroleum wastes

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Introduction

Seawater pollution by crude oil hydrocarbon is a serious international concern for it causes ecological damage to marine shorelines and having an adverse impact on fishery activities and public health (Vila et al. 2010). The main sources of seawater hydrocarbon pollution are crude oil offshore exploration and the use and transportation of crude oil products. Physicochemical methods used to remove hydrocarbon pollution are costly and limited in effectiveness (Ławniczak et al. 2010). Therefore, there is an increase tension to develop environmental-friendly and cost-effective method (Bao et al. 2012). At present, bioremediation using microbial cells has gained popularity because microbes can synthesize a variety of enzymes to degrade an extensive range of hydrocarbons (Mahjoubi et al. 2013), easily regenerated, can be optimised and incorporated into other treatment plants and can be applied in situ or ex situ (ITOPF 2011).

Numerous investigators have studied the use of hydrocarbon-degrading consortia isolated and enriched from polluted marine sediments (Mille et al. 1991) or from hypersaline environments such as salt marshes (Bertrand et al. 1993). In the present study, consortium culture (CC) with metal-resistant characteristic (Sannasi et al. 2009) will be tested under different salinity, artificial seawater and seawater to degrade crude oil hydrocarbon since crude oil contamination sites are frequently co-contaminated by

heavy metals as well (Mielke et al. 2004). Of the nine isolates identified using Biolog system, *Pseudomonas*, *Flavobacterium* (Ko and Lebeault 1999), *Serratia* (Fellie et al. 2012) and *Brevundimonas* have been reported to successfully degrade hydrocarbon compounds such as benzene, toluene, naphthalene, decalin and phenol (Tumaĭkina et al. 2008), and *Alcaligenes* (Wentzel et al. 2007) was shown to degrade hydrocarbons. Sathishkumar et al. (2008) further reported on *Corynebacterium* and *Pseudomonas*, two dominant strains commonly found in soil with the capability of degrading 54 and 67 % of crude oil, respectively. Ławniczak et al. (2010) reported the ability of *Stenotrophomonas maltophilia* to degrade diesel oil. The remaining two isolates in this study, *Gemella palaticanis* and *Brevibacterium otitdis*, have not yet been reported to degrade hydrocarbons. However, *Brevibacterium otitdis* was known to produce extracellular polysaccharide (EPS) which can function as biosurfactant (Asker and Shawky 2010) that facilitates emulsification and degradation of crude oil hydrocarbon.

Despite large number of enriched microbes possessing hydrocarbon degradative abilities has been isolated from pollution sites, however, most were unable to adapt to field environment, rendering their application useless (Yakimov et al. 2007). One of the ways to increase survival of bacteria in field application is by immobilising these bacterial cells onto suitable carrier. Carrier surfaces can provide protection and nutrition, which improves the activity and survivability of bacterial cells when introduced to harsh environment (Gentili et al. 2006). In this study, immobilisation of CC onto sawdust (SD) and oil palm empty fruit bunch (OPEFB) as suitable carriers to improve and accelerate crude oil biodegradation in seawater was further investigated for the purpose of practical application as ready-to-use seeds for bioremediation. Selection of OPEFB is based on reported waste generated amounting to 50.0 million tons in Malaysia annually (Rahman et al. 2007) and rich in organic content such as fatty acids, which can support bacterial growth (Mumtaz et al. 2008). SD was selected based on existing reports whereby it has been used successfully for immobilisation of bacterial cells (Podorozhko et al. 2008; Obuekwe and Al-Muttawa 2001). Both OPEFB and SD are suitable potential carriers because they are aplenty locally in Malaysia, cheaply available, biodegradable and non-toxic to both bacterial cells and the environment. Furthermore, both OPEFB and SD offer plenty of rough surfaces with macropores to provide wide area for attachments to bacteria.

The research was conducted entirely in the laboratory of Universiti Kebangsaan Malaysia, Malaysia from June 2009 to April 2012. Seawater was collected from Port Dickson-Malaysia (latitude N 02°32′31.9′, longitude E 101°47′56.0′) in July 2010.

Materials and methods

Bacterial sources

The bacterial CC used in this study was previously isolated from industrial waste water (Sannasi et al. 2009) and was shown able to degrade benzene, toluene, xylene and ethylbenzene, monoaromatic compounds commonly found in crude oil (Fellie et al. 2012; Kok-Kee et al. 2013). From the glycerol stock, the CC was re-grown in fresh nutrient broth and incubated at 29 ± 1 °C on a rotary shaker at 200 rpm. At the end of 24 h, the bacterial CC cells were harvested by centrifugation at 3,000 rpm for 15 min and the pellet obtained was then rinsed twice using saline (0.85 % NaCl). The rinsed pellet was then resuspended using fresh saline to obtain a cell density of 4.71×10^7 CFU/mL as enumerated using pour plate method (Hamzah et al. 2013) and served as starting inoculums.

Biodegradation of crude oil under different salinity

The ability of CC to degrade crude oil under different salinity was tested. Minimal salt media (MSM) with salinity of 1, 2, 3, 4 and 5 % was prepared by dissolving NaCl salt in MSM (1.0 g of K_2HPO_4, 1.0 of KH_2PO_4, 1.0 g of NaCl; 1.0 g of KNO_3; 0.2 g of $MgSO_4$ and 0.02 g of $CaCl_2$ in 1 L of deionised distiled water), autoclaved and the pH adjusted to 7.0 ± 0.2. Biodegradation experiments were conducted in Erlenmeyer flask containing MSM (98.0 mL) with different salinity, inoculated with 1.0 mL of CC starting inoculums and then added with 1.0 mL of crude oil. The flask was then incubated on a rotary shaker at 200 rpm and 29.0 ± 1 °C. Crude oil degradation was measured after 2 weeks. At the end of the incubation period, 1.0 mL of the culture was analysed for growth of CC using pour plate method (Hamzah et al. 2013) and the rest were extracted using *n*-hexane for analysis of crude oil residues using GC-FID ("Extraction and gas chromatographic (GC-FID) analysis of residual crude oil" section).

Biodegradation in artificial seawater

The potential of bacterial CC to degrade crude oil under seawater condition was first studied using artificial seawater. Artificial seawater (ASW) was prepared according to Austin (1993) by mixing 1 g/L NH_4Cl; 1.47 g/L $CaCl_2{\cdot}2H_2O$; 0.026 g/L H_3BO_3; 0.68 g/L KCl; 10 g/L $MgCl_2$; 0.196 g/L $NaHCO_3$; 4 g/L Na_2SO_4; 0.5 g/L K_2HPO_4; and 30 g/L NaCl to give 3 % salinity to the ASW

and autoclaved at 121 °C for 15 min. Biodegradation experiments were conducted in Erlenmeyer flask containing 98.0 mL of ASW, inoculated with 1 mL of CC starting inoculums and 1 mL of crude oil hydrocarbon as sole carbon source. The inoculated media were then incubated on a shaker at 200 rpm and 29.0 ± 1 °C. At every 2 weeks, 1.0 mL of the culture was analysed for growth using pour plate method and the rest were extracted using *n*-hexane for analysis of crude oil residues using GC-FID ("Extraction and gas chromatographic (GC-FID) analysis of residual crude oil" section).

Sampling and analyses of seawater

Seawater was collected from Port Dickson-Malaysia (latitude N 02°32′31.9′, longitude E 101°47′56.0′), in July 2010 and kept in sterile glass bottles, transported to the laboratory to be analysed for total petroleum hydrocarbon contents and indigenous microbial population within 24 h.

The total petroleum hydrocarbons (TPH) were measured by extracting 1 L of seawater with 30 mL chloroform, and the extract was then evaporated in a vacuum evaporator (EYELA N-1000, Japan) with the water bath temperature set at 50 °C. The TPH residue after evaporation was re-dissolved in 1 mL of *n*-hexane and analysed using GC-FID as described in "Extraction and gas chromatographic (GC-FID) analysis of residual crude oil" section. The number of indigenous bacteria in the seawater samples was determined using spread plate method on nutrient agar (Oxoid, United Kingdom) and on solid minimal salt media (MSM) supplemented with 1 % of crude oil. The MSM agar was prepared by adding 1.5 % of agar into MSM media and autoclaved at 121 °C for 15 min (Sorkhoh et al. 1990). After cooling down to 60 °C, 1 mL (v/v) of crude oil was added into the MSM and poured into petri dishes and left to solidify. In total, 100 µL of seawater was spread onto nutrient agar and incubated for 24 h to assess the population of microbes in seawater. In total, 100 µL of seawater was also spread on MSM agar supplemented with crude oil and incubated for 7 days to determine the population of hydrocarbon degrader. Both plates were incubated at 29 ± 1 °C, and at the end of each incubation period, the bacterial colonies were calculated and expressed as colony-forming units per mL (CFU/mL).

Biodegradation of crude oil in seawater

Degradation of crude oil in seawater was carried out in Erlenmeyer flasks that contained 98 mL of non-sterile seawater added with 1 mL of filter-sterilised crude oil. In triplicates, flasks were then inoculated with (1) 1.0 g of OPEFB immobilised with bacterial CC, (2) 1.0 g of SD immobilised with bacterial CC, (3) 1 mL of free suspension CC (10^7 CFU/mL) and (4) without inoculation of CC to assess the activity of the indigenous microbial community (positive control). Another three flasks contained autoclaved seawater and filter-sterilised crude oil were prepared to assess abiotic loss of crude oil (negative control). All the flasks were incubated at 29 ± 1 °C on an orbital shaker set at 200 rpm. Samples were taken on day 14, 28, 42 and 56 and subjected to GC-FID analysis to calculate percentage of crude oil degraded.

For immobilising bacterial CC, two organic carrier, the OPEFB (Malaysian Palm Oil Board Center) and SD from sawmill were used. The OPEFB was shredded to obtain particles with a uniform size approximately 15–20 cm in length. SD and OPEFB particles collected were then rinsed with boiling water followed by 70 % alcohol and distiled water in order to remove debris and impurity. Both carriers were then autoclaved at 121 °C for 15 min and air-dried. Immobilisation of CC onto carriers was carried out by inoculating 1 mL (10^7 CFU/mL) of CC from starting inoculums into 20 mL vials containing 1 g of sterilised carrier and incubated at room temperature for 5 days. In order to determine the number of CC attached to carriers after 24 h incubation, the carriers were removed and resuspended in 10 mL of phosphate buffer, sonicated for 2 min (BRANSON 2210), followed by vortexing for 10 min to detach CC. In total, 100 µL of the detached CC was then plated onto nutrient agar and incubated at 29 ± 1 °C overnight. The numbers of viable bacterial cells on the agar were reported as colony-forming unit (CFU/mL). The progression of immobilisation of CC was analysed using 0.01 g of immobilised CC and fixed with 4 % (w/v) glutaraldehyde solution in 0.1 M sodium cocodylate buffer for 24 h at 4 °C and washed thrice with 0.1 M sodium cocodylate buffer. Samples were fixed using 1 % osmium tetroxide for 2 h at 4 °C and then washed with 0.1 M sodium cocodylate buffer. The samples were then dehydrated using a series of acetone (35–100 %), 10 min each times and dried using the CO_2 critical point drying technique for 30 min. The samples were then sputter coated with gold and viewed under scanning electron microscope (SUPRA 55VP-Germany) (Díaz et al. 2002).

Extraction and gas chromatographic (GC-FID) analysis of residual crude oil

Residual crude oil was extracted from ASW media, seawater and culture sample (" Biodegradation of

crude oil under different salinity, Biodegradation in artificial seawater, Sampling and analyses of seawater and Biodegradation of crude oil in seawater" section) according to the International Standard Method ISO 9377-2 (2000) with minor modification that determines long-chain or branched aliphatic, acyclic, aromatic and alkyl-substituted aromatic hydrocarbons. Briefly, samples mixed with 5 mL *n*-hexane were shaken vigorously and poured into a separation funnel. The contents were rested for 10 min to allow separation of the organic and aqueous phases. The organic *n*-hexane phase was then collected into a round bottom flask. The extraction process was repeated twice. The extracted crude oil in *n*-hexane was then evaporated on a rotary evaporator (EYELA N-1000, Japan) with the water bath temperature set at 50 °C. The crude oil residue after evaporation was resuspended in 1 mL *n*-hexane and quantified chromatographically using gas chromatography GC-FID.

One µL of residual crude oil hydrocarbon in *n*-hexane was analysed on a capillary gas chromatograph (Clarus 500 GC, J&W Scientific, USA) equipped with a 320 µm × 30 m silica capillary column (J&W Scientific, Folsom, CA, USA) and flame ionisation detector (FID). The samples were injected by split injection using helium as carrier gas at a constant flow rate of 1.2 mL/min, and the oven temperature was 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. Percentage of crude oil degraded was determined by comparing total area of chromatogram of the test samples with that of the control.

$$(\text{Total peak area of crude oil sample after incubation} / \text{Total peak area of crude oil of the control}) \times 100.$$

Results and discussion

Effect of salinity on the growth of CC and crude oil degradation

For practical application of bioremediation in contaminated seawater, bacterial CC has to overcome osmotic pressure of seawater with higher salinity and maintain their biodegradation activities. Thus, the survivability and degradative ability of CC were tested in the presence of different salt concentrations. Figure 1 shows that increase in NaCl concentration caused a decrease in both CC population and percentage of crude oil hydrocarbon degraded. The population of CC was observed to decrease up to 75.68 % compared with control when the salinity was increased 5-folds from 1 to 5 %. Similarly, the percentages of crude oil hydrocarbon degraded were observed to reduce by 7.59, 13.00, 24.17, 56.73 and 62.85 %, respectively, at 1, 2, 3, 4 and 5 % salinity when compared to control.

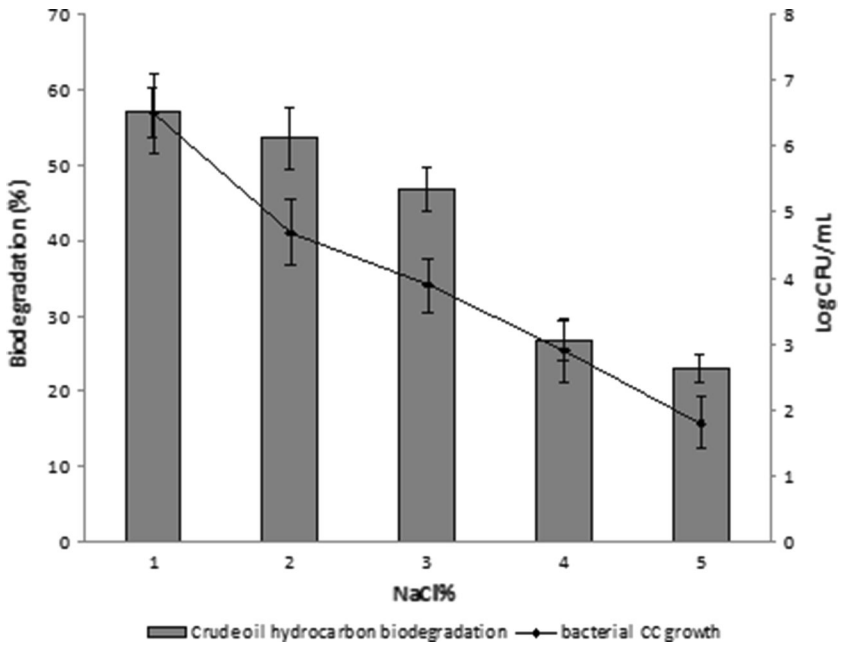

Fig. 1 Bacterial CC population growth and crude oil degradation under different salinity

However, the percentage of crude oil degraded at increasing salinity from 1 to 3 % was not significantly different ($p > 0.05$) compared with control. This result suggested degradation of crude oil hydrocarbon by CC was not affected by increasing salinity up to 3 %. However, inhibition of crude oil hydrocarbon degradation >50 % was observed when the salinity exceeded 3 %.

Minai-Tehrani et al. (2006) similarly reported a decrease in crude oil degradation from 41 to 12 % when the salinity was increased from 0 to 5 %. The study attributed the decrease in crude oil biodegradation to inhibition of microbial growth. Increase in salinity can retard microbial growth by the increasing osmolarity, which causes hyper osmotic shock to microbial cells (Abdulkarim et al. 2009). Salinity at 5 % was reported to inhibit the synthesis of macromolecules (Csonka 1989), which suggests degradative enzymes that play a major role in degrading crude oil hydrocarbons are also inhibited. Furthermore, Ward and Brock (1978) reported that an increase in salinity will decrease oxygen uptake by bacterial cells, resulting decrease in crude oil oxidisation. However, bacterial CC in this study was able to tolerate and maintain its crude oil degradative ability up to 3 % salinity; a salinity similar to seawater (Liu et al. 2009) and thus potentially useful to degrade crude oil in seawater.

Biodegradation of crude oil in artificial seawater by CC

Figure 2a shows that under artificial seawater (ASW) condition, bacterial CC was able to degrade up to 83.3 ± 3.00 % of crude oil within 8 weeks. The CC population was seen to increase by 79 % within the first 2 weeks of incubation (Fig. 2b) that coincided with the rapid degradation of crude oil of 51.0 ± 4.36 % in the same period. This suggests the crude oil hydrocarbon was rapidly degraded by CC to obtain carbon source to support growth. The CC population gradually declined after week-2 (Fig. 2b) along with the crude oil hydrocarbons degradation, as the carbon source was increasingly depleted.

Consortium culture ability to maintain crude oil hydrocarbon degradative activities despite being exposed to increasing salinity up to 3 % suggested that strains within the CC were able to activate certain mechanism to preserve the functionality of cells under such harsh condition. Vreeland (1987) reported strains could adapt to high salinity by concentrating compatible solutes to create an osmotic balance between the cytoplasm and the external environment or by altering membrane permeability to control the movement of water and allowing the cell to exist with an ionically dilute cytoplasm. Some bacterial strains can lower its cytoplasmic ionic content compared with the outside medium by activating a Na^+: H^+ antiporter to maintain a large Na^+ gradient in the cytoplasm (Vreeland 1987). Within the CC, three of the strains, *Pseudomonas fluorescens* (Viggor et al. 2013), *Brevundimonas* and *Stenotrophomonas* (Mahjoubi et al. 2013) may possess such mechanism since they were reported in other studies to survive in the sea and marine environments. This further strengthens CC usefulness as bioremediation tool to degrade crude oil hydrocarbon in seawater.

Biodegradation of crude oil in seawater by CC

The seawater samples collected from station near a petroleum refinery in Port Dickson, Malaysia showed

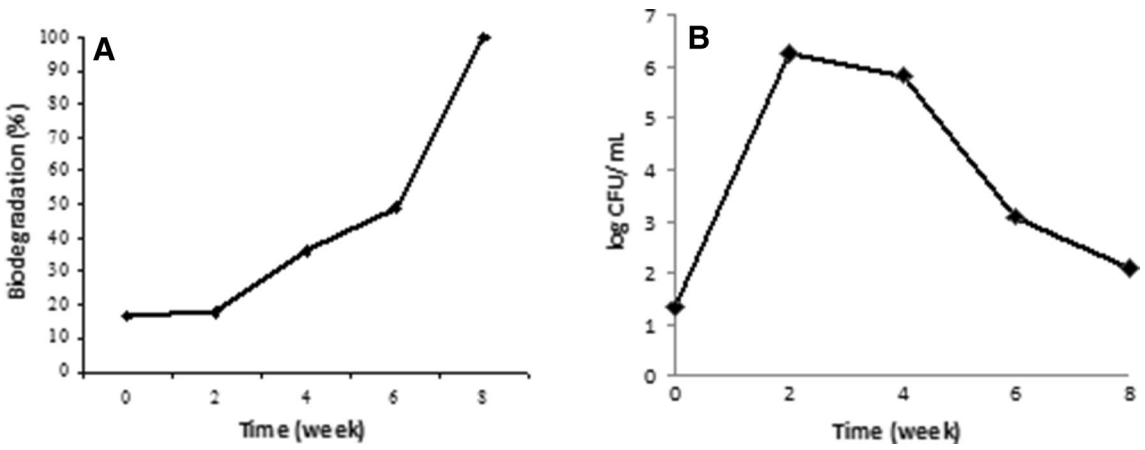

Fig. 2 Crude oil hydrocarbon degradation (**a**) and bacterial CC growth (**b**) during 8 weeks in artificial seawater

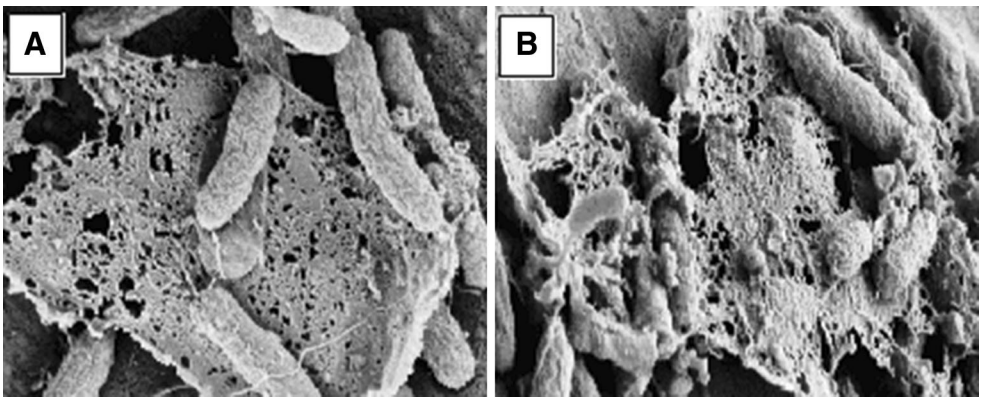

Fig. 3 Biofilm formation of CC on (**a**) SD and (**b**) OPEFB after 3 days of incubation

the following parameters: temperature 29.6 °C, dissolved oxygen 6.12 mg/L, salinity 3.05 %, PO_4^{3-} 5.24 mg/L, NH_3 0.9 mg/L and pH 7.63. The concentration of TPH in the sample was 46.21 μ/L, which was safely below the contamination level of 100 μ/L (Bishop 1983). Approximately 34 % of the total indigenous microbial population found in the seawater samples were crude oil hydrocarbon degraders. The percentage of hydrocarbon degrader found are thrice higher than reported by Atlas (1981) who reported that in pristine environment, microbial hydrocarbon degraders are less than 1 % and in contaminated area exceeding 10 % of the total microbial population. In pristine environment, the microbial hydrocarbon degrader generally degrades hydrocarbon compounds produced naturally by algae and plants (Atlas 1995). Although the seawater sample obtained was found not to be contaminated, the sampling location near to an oil refinery allowed gradual exposure to residual crude oil, which may encourage the indigenous bacteria to start using crude oil hydrocarbon as carbon source.

In order to test whether immobilisation can protect CC from the harsh condition of seawater and hence enhanced degradation of crude oil hydrocarbon, CC was immobilised onto SD and OPEFB in phosphate buffer. After 3 days of incubation, the surfaces of SD and OPEFB were covered with CC as observed under scanning electron microscope (Fig. 3). After sonication and washing with saline solution, the number of viable CC dislodged from SD and OPEFB was 60.9 and 64.0 % of the starting inoculums (10^7 CFU/mL), respectively. The number of viable CC immobilised was relatively high and suggested promising performance

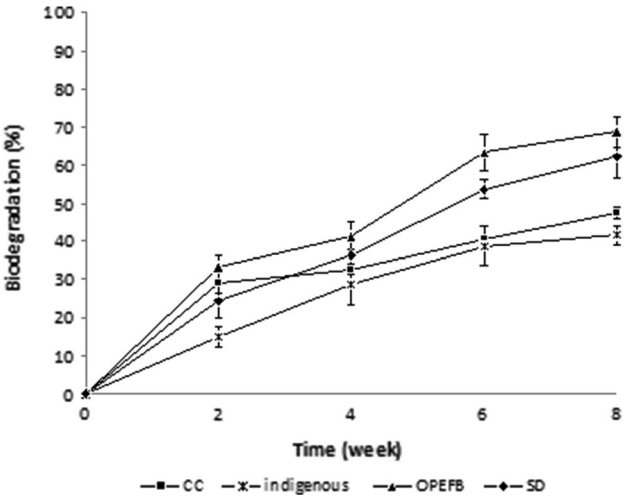

Fig. 4 Percentage of crude oil hydrocarbon biodegradations in fresh water using indigenous microbial cells, free CC and bacterial CC immobilised onto OPEFB and SD

to degrade crude oil. In comparison, Zhen-Yu et al. (2012) who showed immobilisation of 10^4 CFU of bacterial cells onto sodium alginate-diatomite reported a 46.3 % of crude oil degradation. Whereas, both SD and OPEFB ability to retain 2.86×10^7 and 3.01×10^7 CFU, respectively, indicated stable survivability and good potential as ready-to-use seed in crude oil hydrocarbon degradation application.

Figure 4 shows the highest biodegradation of crude oil was obtained when using bacterial CC immobilised onto SD and OPEFB as carrier compared with free

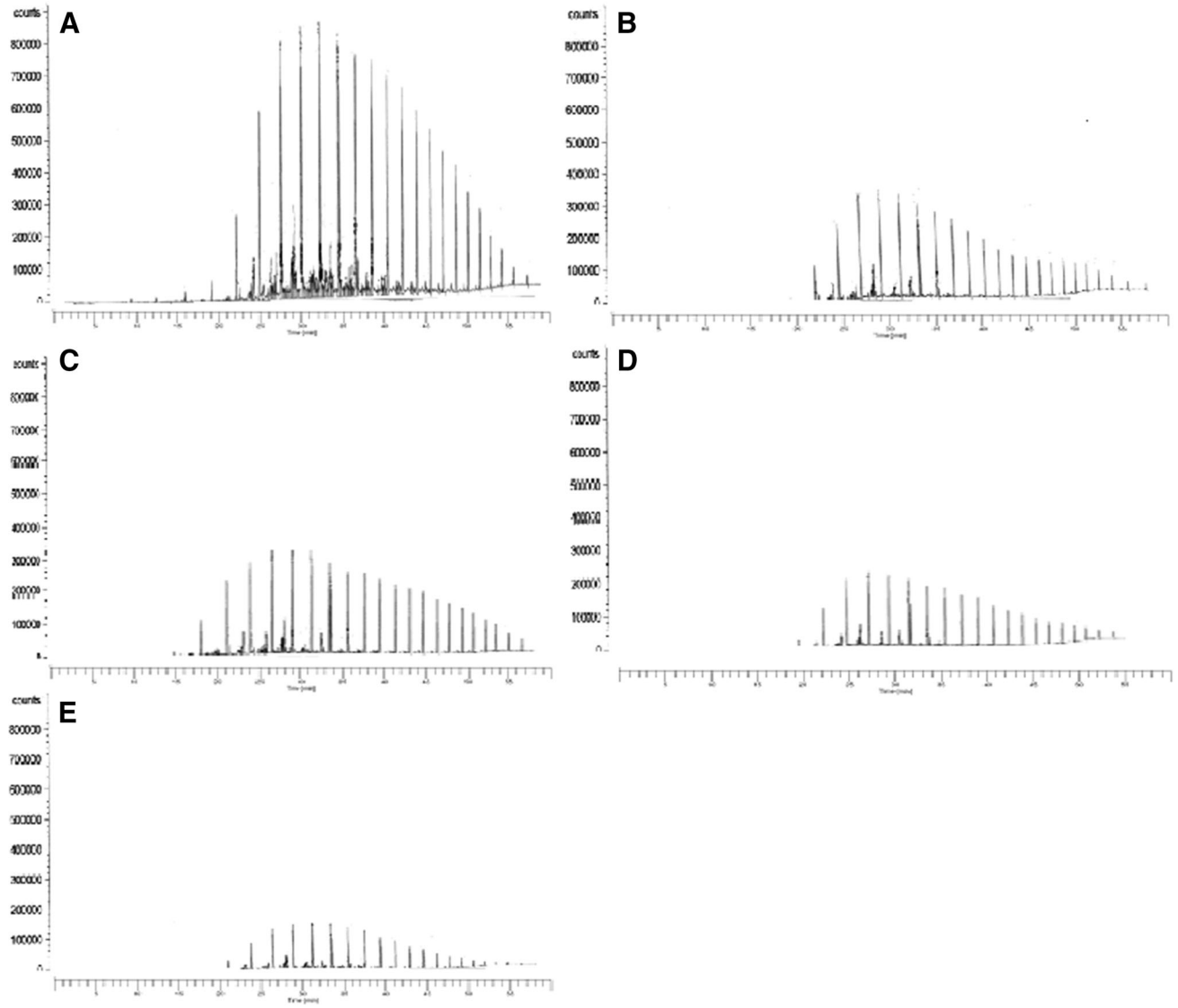

Fig. 5 Gas chromatogram of crude oil at (**a**) day-0 and after week-8 of incubation using (**b**) indigenous microbial cells, (**c**) free CC, (**d**) immobilised CC onto SD, (**e**) immobilised CC onto OPEFB

suspension CC. Compared with positive control, inoculation of seawater using free suspension CC increased degradation from 41.7 ± 2.52 to 47.7 ± 1.53 %. Immobilising CC onto SD and OPEFB further enhanced the degradation by 33.7 and 39.3 %, respectively, compared with control.

Figure 5 further shows in detail that all the initial peaks detected at day-0 were significantly reduced after week-8 using the various treatment. This observation further supported the ability of CC and the process of immobilising CC on SD and OPEFB can further enhance degradation. After week-8, all treatments showed rather similar gas chromatogram whereby it suggests that CC degraded the various light and heavy chains hydrocarbon fractions simultaneously and not selectively, the light chain hydrocarbon first before utilising the heavy chains after the

former is depleted. This is interesting because microorganisms are widely known to attack short chain hydrocarbons first (Etoumi 2007; Lliròs et al. 2008) as hydrocarbons with lower number of carbon atoms are less hydrophobic and easily available to degradation. One possible explanation is that members within CC can actively secret biosurfactant to increase the solubility and thus the bioavailability of heavy chain hydrocarbon fractions (C29–C43) within the crude oil for degradation. This is an advantage because heavy chain hydrocarbons are usually rendered non-biodegradable due to their low bioavailability for attachment onto bacterial surfaces with membrane-bound enzymes (Harayama et al. 1999; Kok-Kee et al. 2013).

The ability of free suspension CC to increase crude oil degradation also suggested that CC was able to survive and compete with the indigenous microbes in the seawater. Immobilising bacterial CC on OPEFB and SD increased the stability of bacterial CC, and thus, enhanced crude oil degradative activities. The surfaces of SD and OPEFB covered with "pores" and "crevices" encouraged the colonisation of CC, which later developed into an extensive network of biofilm (Fig. 3). In our previous report, we demonstrated the role of exopolysaccharide (EPS) secretion by CC accelerating the biofilm formation on OPEFB and SD (Hazaimeh et al. 2014). Obuekwe and Al-Muttawa (2001) reported lower secretion of EPS by *Arthrobacter* sp. (56 %) and another bacillus-like species (43 %) when immobilised on sand. Although at present, we do not know the exact role of the carriers' organic content in relation to biofilm formation; however, circumstantial evidence above suggests the higher organic content in OPEFB and SD, compared with sand, enhanced the secretion of EPS and subsequently influencing the development of biofilm. This interesting aspect of how the organic content of the chosen carriers might influence the outcome of biofilm formation by bacterial cells worth further investigation. Thus, the development of biofilm is an important key strategy by CC to ensure survivability in hostile marine environment, as in seawater with high salinity. The thick layer of exopolysaccharide surrounding the biofilm prevented desiccation and gives protection against the variation in humidity and against bacteriophage activity (Sutherland 2001), which lends a high survivability percentage of CC after inoculated into seawater. The immobilised CC can be contained into perforated cassette, tubes or similar apparatus and tied to boomers during the initial oil spill clean up operations. This will prevent the immobilised CC from being washed away, and at the same time, degrading the oil spill contained by the boomers. These results suggested CC immobilised onto SD and OPEFB can be utilised as ready-to-use seeds for in situ application to improve and accelerate crude oil biodegradation in seawater with high salinity.

Conclusion

Bacterial CC isolated from industrial wastewater was shown first to degrade crude oil under different salinity; then, in artificial seawater and finally in seawater. Immobilising bacterial CC cells onto SD and palm oil empty bunch fruit increased the survivability of CC through formation of biofilm, leading to enhance biodegradation of crude oil. This study demonstrated immobilisation of CC onto easily available and cheap material such as SD and OPEFB can be utilised as ready-to-use seeds to improve and accelerate crude oil biodegradation in seawater.

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.

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