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ORIGINAL ARTICLE**Degradation of Crude Oil in the Presence of Lead (Pb) and Cadmium (Cd) by a Metal-Adapted Consortium Culture****¹Kok-Kee Wong, ²Brid Quilty, ¹Salmijah Surif**¹*Faculty of Science & Technology, Universiti Kebangsaan Malaysia, 43650 UKM, Bangi, Selangor Malaysia*¹*School of Biotechnology, Dublin City University, Dublin 9, Ireland*

Kok-Kee Wong, Brid Quilty, Salmijah Surif: Degradation of Crude Oil in the Presence of Lead (Pb) and Cadmium (Cd) by a Metal-Adapted Consortium Culture

ABSTRACT

A consortium culture (CC) which was adapted to heavy metals was able to degrade 1% (v/v) of crude oil and after 8 weeks of incubation, no detectable peaks were found when analysed using GC-FID. There were significant decrease ($p < 0.05$) in the surface tension (dynes/cm) of the culture filtrate measured starting from week-2 to week-8 compared to starting week-0, suggesting that biosurfactant was rapidly produced by CC within the first two weeks as a strategy to increase solubility of hydrophobic components of crude oil. In the presence of 0.1 and 1.0 mg/L of lead (Pb), degradation of crude oil was observed at 96% and 94%, respectively. In the case of cadmium (Cd), concentration at 0.01 mg/L resulted in 96% of crude oil degradation. However, at higher concentration of 0.1 mg/L Cd, only 73% of degradation was achieved. The CC was also shown able to remove Pb and Cd up to 90% from the culture media. The bacterial CC can adsorb metals onto the cell surfaces as shown using transmission electron microscopy (TEM). These results showed that the CC could be effective for bioremediation of crude oil waste contaminated with heavy metals.

Key words: consortium culture, biodegradation, biosurfactant, crude oil, heavy metals.**Introduction**

Crude oil, source of petroleum hydrocarbons is widely used as the principle source of energy and raw material in industries [21]. It can contaminate the environment via releases of crude oil from tankers, offshore platforms, drilling rigs and wells, spills of refined petroleum products (such as gasoline, diesel) and their by-products, and heavier fuels used by large ships such as bunker fuel constitute a major source of contamination [7]. An effective way to mineralise crude oil completely to carbon dioxide is by biodegradation process using microbes [9]. However, complete biodegradation of complex petroleum or crude oil can be limited by biotic factors such as paucity of bacterial population with hydrocarbon degrading capabilities [11] or the inability to increase the bioavailability of hydrophobic hydrocarbons through reduction of the media surface tension [27].

Degradation of crude oil components can also be further inhibited by heavy metals [12]. Crude oil contamination sites are frequently found to be co-contaminated by heavy metals [15]. Heavy metals are known to exert toxicity on the growth and metabolism of bacterial cells, which altogether reduces the effectiveness of using them to degrade crude oil waste [18]. Heavy metals can inhibit

enzyme function through their binding to thiol and other groups on enzyme molecules. They can also compete with the metals acting in the enzymes by replacing them and thus inhibiting the enzyme actions, severely affecting metabolism and growth of the microbes [4] and biodegradation capacity [25]. Lead (Pb) and cadmium (Cd) among the most toxic metals predominantly found in wastewater of various industries including hydrocarbon processing, iron/steel manufacturing, electronics manufacturing and smelting [28]. This paper reports the ability of a metal adapted consortium culture degrading crude oil in the presence of Pb and Cd, up to 1.0 mg/L and 0.1 mg/L, respectively.

Methods:**Chemicals and Media:**

Crude oil used in this study was obtained from Kuala Terengganu Crude Oil Terminal, Terengganu, Malaysia. Freshly prepared Difco™ Bushnell-Haas (BH) media that contained (g/L): MgSO₄, 0.2; CaCl₂, 0.02; KH₂PO₄, 1.0; (NH₄)₂HPO₄, 1.0; KNO₃, 1.0; FeCl₂, 0.05 were used for all biodegradation studies. The pH of the media was adjusted to 7.0±0.2 and autoclaved at 121°C for 15 mins. Stock solutions for

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lead (Pb) and cadmium (Cd) were made by dissolving the metal salts individually in distilled water to obtain a final concentration of 100 mg/L.

Consortium Culture:

The consortium culture (CC) comprises of *Agrobacterium* sp., *Chryseomonas* sp., *Flavobacterium* sp., *Pseudomonas* sp., *Serratia* sp., *Xanthomonas* sp., *Arthobacter* sp., *Bacillus* sp. and *Micrococcus* sp. was developed from environmental isolates obtained from ten wastewater metal-based industrial premises (Sannasi et al. 2006) and the stock culture has been maintained in 10 ppm lead (Pb). Each strain was individually grown overnight to reach the exponential phase in nutrient broth. The bacterial cells were then collected by centrifugation and the pellet obtained was washed twice and resuspended with 0.85% NaCl to give an OD_{600nm} of ≥ 0.5 . The starting consortium culture was obtained by adding each of the bacteria in the ratio of 1:1.

Time Course Biodegradation of Crude Oil by CC:

Five mL CC (5% v/v) inoculum was inoculated into conical flasks, each containing 1 mL crude oil (1% v/v) in Bushnell-Haas media (100 mL). The flasks were incubated in a shaker at 200 rpm at 30°C for 8 weeks. At 2 weeks interval, a pair of flasks was taken for enumeration of microbial population using pour plate technique and the residual crude oil in each flasks extracted and analysed using GC-FID (Clarus 500 GC, J & W Scientific, USA). Control flasks were set up to contain crude oil without inoculation.

Surface Tension Measurement:

At day-0 and each subsequent 2 weeks, the flasks after crude oil extraction were then filtered through 0.45 μm Milipore nitrocellulose membrane. The surface tension of the filtrate was then measured using a du Nouy ring-type tensiometer (Fisher Scientific, USA). Deionized water was used as the blank. The surface tension activity was expressed as the percentage of reduction of surface tension compared to day-0 [19]. Surface tension measurements shows the bioavailability of crude oil to be degraded by CC [23].

Effects of Lead and Cadmium on Biodegradation of Crude Oil:

5% (v/v) CC was incubated with 0.4 mL of KTCOT crude oil and (i) 0.1 mg/L Pb (ii) 1.0 mg/L Pb (iii) 0.01 mg/L Cd (iv) 0.1 mg/L Cd. The concentration used (Pb-0.1 & 1.0 mg/L; Cd- 0.01 & 0.1 mg/L) in this present study was chosen based on the permissible level suggested by Environmental Quality Act 1974 Malaysia (Sewage and Industrial

Effluents), and ten times higher to reflect conditions of contamination. Crude oil at 0.4% (v/v) was based on data on field sample collected. Control contains all components except the CC. At the end of day 4 and 8, the residual crude oil in the culture was extracted with *n*-hexane and analysed using GC-FID. For metals analysis, 5 mL culture samples were filtered on 0.45 μm pore size nitrocellulose membrane and the concentrations of the respective Pb and Cd in the culture were analysed using ICP-MS (Perkin Elmer ELAN 9000, USA).

Transmission Electron Microscopy:

In the microscopy analysis, 1.0 mL of the CC was collected by centrifugation (10 000 rpm; 5 mins). The resulting pellet was washed twice using NaCl solution (0.85%) to remove all the residual media before resuspending the pellet in 1.0 mL of autoclaved de-ionised water. 100 μL of the dissolved pellet was transferred to a copper grid (mesh: 200). Samples unexposed to metals (control) were stained with methylamine tungstate (2%) for 1 min so that the cells can easily be observed under microscope. Samples exposed to Pb and Cd on the other hand, can be easily observed without any staining [3], under transmission electron microscope (Hitachi H-1700 EM) operating at accelerating voltage of 75kV.

Extraction and Analysis of Crude Oil using GC-FID:

The residual crude oil in the culture was extracted twice, using 20 mL *n*-hexane. The extracted crude oil in *n*-hexane was then collected in a 250 mL round bottom flask and evaporated using a rotary evaporator (EYELA N-1000, Japan) and a water bath set at 50°C (MOOPAM 1999) to reduce the volume to 1.0 mL. The extracted residual crude oil in *n*-hexane was then analysed on a capillary gas chromatograph (Clarus 500 GC, J & W Scientific, USA) equipped with a 320 μm x 30 m silica capillary column (J&W Scientific, Folsom, California, USA) and flame ionization detector (FID). The samples were injected by split injection with helium as carrier gas, and the oven temperature was programmed as follows: 2 min at 45°C followed by 10°C/min to 280°C. The major hydrocarbon compounds of the crude oil were identified on the basis of their retention time and by comparing them to those of analytical standards. The reduction of the crude oil was calculated as below:

$$\left[\frac{\text{Total peak area of crude oil (before incubation P0)} - \text{Total peak area of crude oil (after incubation Pt)}}{\text{Total peak area of crude oil (before incubation P0)}} \right] \times 100\%$$

Statistical Analysis:

Data are reported as mean values \pm standard deviation of the number of replicates. Statistical

analysis was performed using SPSS PASW Statistic 17 software (SPSS Inc., USA) for Windows Vista™ and Student's "t" test with levels of confidence at 95% ($\alpha = 0.05$).

Results and Discussion

Biodegradation of Crude Oil and Surface Tension Reduction:

Figure 1B shows that crude oil was progressively degraded by CC starting from the first week and completed by the eighth week. Within the first two weeks, 53.2% of the crude oil was degraded which was 6-folds higher than control. After 4 weeks, 70.1% crude oil was degraded, 4-folds higher compared to control. After 6 weeks 96.3% of crude oil have been degraded by CC, 3-folds higher than control. By 8 weeks, all the crude oil was degraded. In comparison, the control CC only showed 32.0% of crude oil being degraded after 8 weeks and the degradation activity can be attributed to the existence of indigenous population found within the crude oil. The rapid crude oil degradation by CC corresponded with the CC population (\log_{10} CFU/mL) as shown in Figure 1A. Starting from day-0, growth of CC increased to 13.1% at week-2. The CC population further increase to 42.0% and to 62.2% at week-4 and week-6, respectively. In contrast, the indigenous population in the control showed a lag phase of two weeks before showing an increase ($p < 0.05$) of 15.2% in the population at week-4. The bacterial population remained constant until end of the incubation period at week-8. The population of CC was 5-fold higher than the indigenous population at the end of the week-8 incubation period. This suggests that CC was able to degrade the wide range of hydrocarbons within the complex crude oil to support growth. There was no lag phase indicating the fitness of CC to grow in an oil-contaminated environment. The CC showing robust growth in crude oil is important because survival of microbes in crude oil medium after inoculation is the first pivotal step towards successful biodegradation [22].

The ability of CC to biodegrade crude oil, which has hydrophobic components, suggests that these hydrocarbons were readily bioavailable to the CC. One of the ways to achieve this was by secreting biosurfactant to emulsify non-water soluble fractions of the crude oil. After two weeks of incubation, the

two distinct water-oil layers in the culture becoming less evident and more turbid, as the oil slick was transformed into many small oil droplets (miscelles). Formation of miscelles are attributed to the reduction of the surface tension between water-oil interface [23]. When the surface tension of the culture was measured, there were significant decrease ($p < 0.05$) in the surface tension (dynes/cm) of the culture filtrate at week-2 to week-8 when compared to starting week-0 (Figure 2). The first two weeks was also the most active for emulsification of crude oil into droplets and this coincided to the phase when the crude oil was most actively degraded (48.2%) (Figure 2). However, after week-2, the surface tension measured were not further reduced and it was reflected by the slowing down of crude oil degraded from week-2 to 4, and from week-4 to 8, with increment approximately 20% each (Figure 2). This suggests that biosurfactant was rapidly produced by CC in the first two weeks to emulsify crude oil, and the formation of oil-water emulsion was an important first step in hydrocarbon biodegradation. Non-water soluble hydrocarbons such as the branched/long chain *n*-alkanes and high molecular weights PAHs need to be bound to the surface of bacterial cells before biodegradation could occur [27]. Kirschner et al. [10] described the binding process as hydrocarbon adhesion to the fatty acid moieties of cell membrane. This suggested that CC in this study adopted similar strategy to increase contact with the crude oil by emulsifying the crude oil. Rapid secretion of biosurfactant by CC was instrumental to breakdown crude oil physically, which then facilitates the adhesion of hydrocarbons onto bacterial cells. Emulsified hydrocarbons allowed greater contact between hydrophobic hydrocarbons and bacterial membrane-bound enzymes, and this action will accelerate the biodegradation process [30].

Figure 3 shows the degradation of crude oil given as number of carbons analysed from the chromatogram of GC-FID. There was a high percentage of heavy chain compounds (C27 to C37) along with recalcitrant pristane and phytane initially present in the crude oil. As the incubation time progress, there was a shift towards an increase in the short chain hydrocarbons (C14 to C27) at the expense of heavy chain hydrocarbons. Even recalcitrant pristane and phytane that were detected at the initial stage were degraded to an undetectable level within two and six week time, respectively.

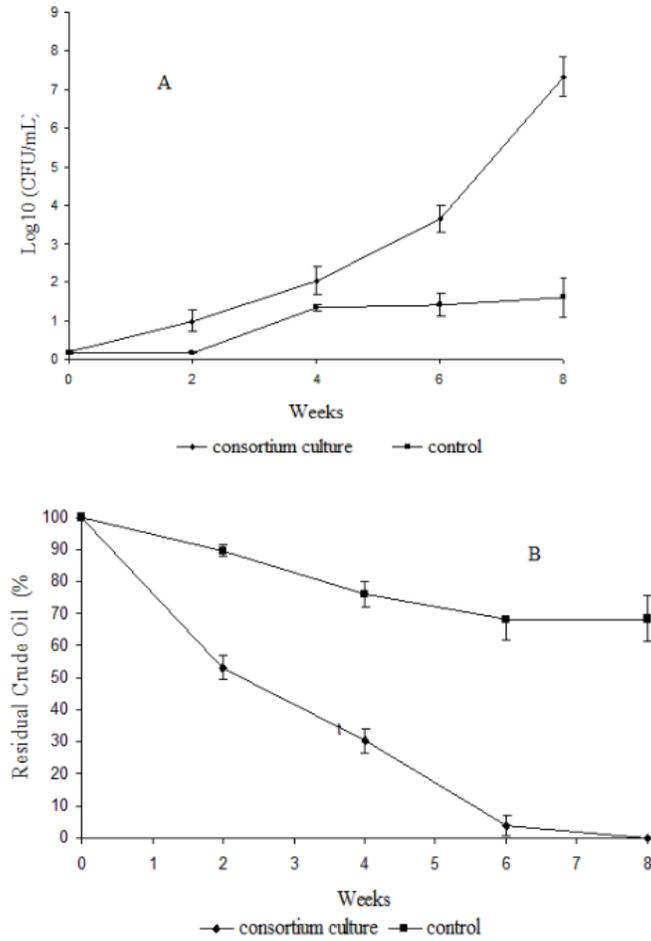


Fig. 1: Growth of CC (a) and percentage of crude oil residues (b) after 8 weeks.

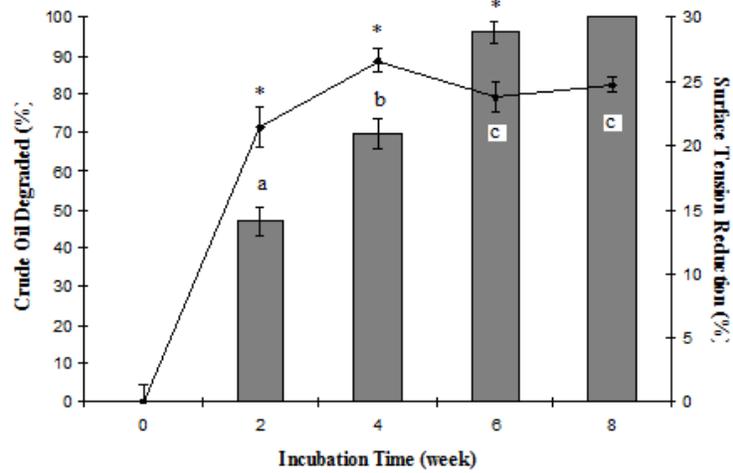


Fig. 2: Percentage of KTCOT crude oil degraded (-■-) and surface tension reduction (-♦-) measured in the CC culture. (*) indicates significant differences of the surface tension ($p < 0.05$) compared to week-0. Different alphabets indicates significant differences of crude oil degraded ($p < 0.05$) compared to week-0 and between samples.

The progressive build up of short chain hydrocarbon especially C16 and C18 seen in week-2 and week-4 is interesting because it was expected to disappear at early stages of incubation, since microorganisms are widely known to attack short chain hydrocarbons first [6]. It is possible that CC rapidly exhausted most of the initial short chain hydrocarbons present in the media but were quickly replenished through fragmentizing pieces of longer chain hydrocarbons. This suggests, as the incubation time progressed, CC was forced to metabolise longer chain hydrocarbons when the ready supply of short-chain hydrocarbons become scarce. Experiment on *Pseudomonas* sp. and *Actinomyces* sp. incubated with Sarir crude oil similarly showed decreases in heavy hydrocarbon fractions (>C22) corresponding to increases in the C15-C20 fractions [6].

The ability of CC to degrade crude oil efficiently can be attributed to the fact that the consortia comprises of *Agrobacterium* sp., *Chryseomonas* sp., *Flavobacterium* sp., *Pseudomonas* sp., *Serratia* sp., *Xanthomonas* sp., *Arthobacter* sp., *Bacillus* sp. and *Micrococcus* sp. Several of the species found within the CC have been reported to degrade hydrocarbons. *Pseudomonas* sp. and *Bacillus* sp. are frequently reported to degrade hydrocarbons, for example degrading 70% of oil in contaminated soil [14] and approximately 78% of Bombay High crude oil [21]. Other species in CC such as *Agrobacterium* sp. was reported to degrade benzo(a)pyrene, a 5-ring component of crude oil [1], demonstrating possible

involvement of the species in the long sequential degradation process of polycyclic aromatic hydrocarbons found within the crude oil in this study. *Serratia plymuthica* was reported to exhibit benzene-ring cleaving enzymatic activity and capable of biodegrading phenol [20]. This also suggests the possible role and contribution of *Serratia* sp. in the CC to cleave various aromatic-ring structures in the crude oil, thus further facilitating the biodegradation process. The presence of a diverse species allows synergistic effect to occur, whereby different bacteria within the CC can deplete distinct hydrocarbons in the crude oil during biodegradation.

Effects of Heavy Metals on Biodegradation of Crude Oil and Metal Removal:

Figure 4A shows that 5% CC degraded up to 60% of crude oil, which is 3-fold higher compared to indigenous population at day-4. CC at day-8 showed 27-30% increased in crude oil degradation ($p < 0.05$) compared to day-4. The exception was with culture incubated with 0.1 mg/L Cd that showed only 8.5% of increment. Cd at the higher dosage of 0.1 mg/L rendered the degradation of crude oil at day-8 to be less than 90% compared to other treatments (Figure 4A). The indigenous population in the control flasks showed crude oil degradation to be less than 20% in Pb and Cd at both concentrations throughout day-4 and day-8. Crude oil degradation was not observed to increase from day-4 to day-8 in the control flasks.

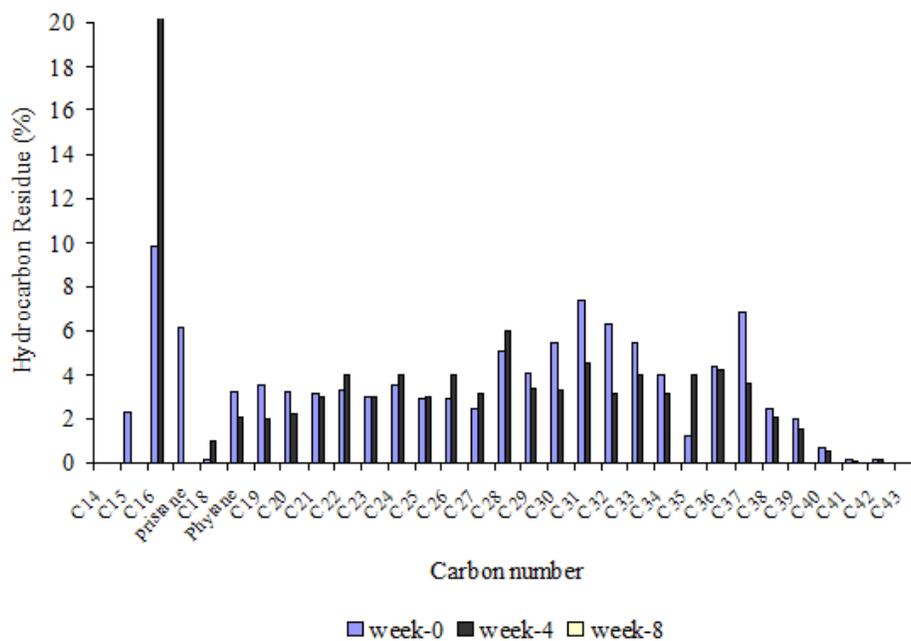


Fig. 3: Percentage of hydrocarbon residues detected according to carbon numbers analysed from samples at week-0, week-4 and week-8. Each value is a SEM of duplicates

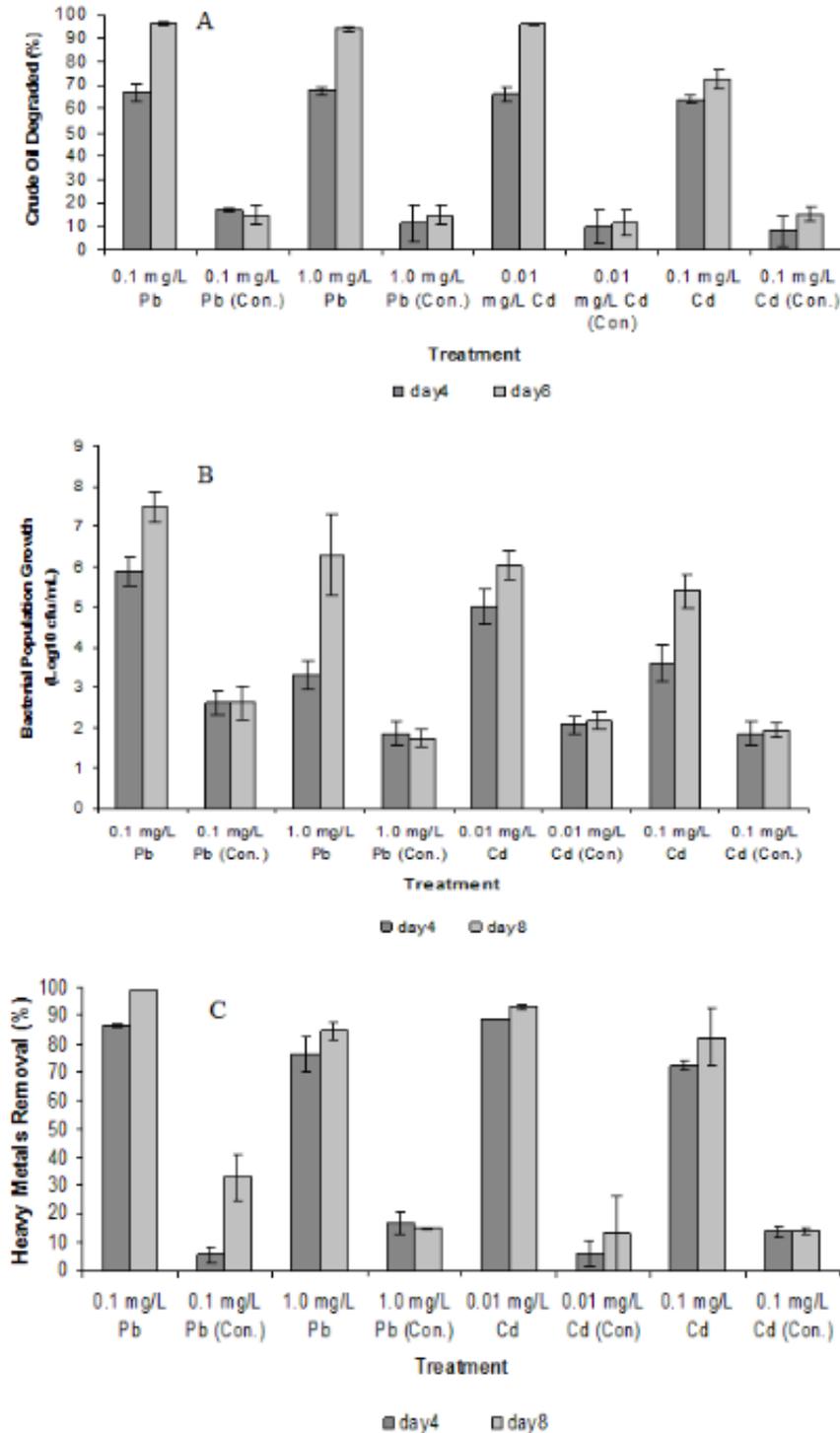


Fig. 4: Effects of lead and cadmium on (A) crude oil degradation, (B) growth of bacterial population and (C) heavy metals removal by mixed culture

The CC was also able to remove both Pb and Cd efficiently from the culture media (Figure 4C). At lower metal concentration (0.1 mg/L Pb; 0.01 mg/L Cd), the CC was able to remove up to 86% and 88% Pb and Cd ($p < 0.05$), respectively compared to higher

metal concentrations (1.0 mg/L Pb; 0.1 mg/L Cd) of Pb (76%) and Cd (72%) at day-4. At day-8, the removal of Pb and Cd were increased ($p < 0.05$) to 98% and 93%, respectively in flasks with 0.1 mg/L Pb and 0.01 mg/L Cd. The removal of Pb (84%) and

Cd (82%) with 1.0 mg/L Pb and 0.1 mg/L Cd however did not increase at day-8. No significant differences in metal removal were observed between day-4 and day-8 in the indigenous population. Only control flask with 0.1 mg/L Pb showed removal ($p < 0.05$) of Pb up to 33% at the end of day-8 compared to day-4.

Results of crude oil degradation were reflected by the bacterial population growth (Figure 4B). The increase in degradation ($p < 0.05$) in all treatments coincided with the increased in growth ($p < 0.05$) observed between day-4 to day-8. There was also a significant increase in Pb and Cd removal in flasks containing Pb-0.1 mg/L and Cd-0.01 mg/L that coincided with significant growth in bacterial populations from day-4 to day-8. However, flasks with Pb-1.0 mg/L and Cd-0.1 mg/L did not show significant increase in metal removal from day-4 to day-8. This indicated higher concentrations of Pb and Cd adversely affected the removal of both metals from the culture media. The increasing toxicity of higher concentrations of Pb and Cd on CC can also be observed whereby the initial bacterial population at day-4 were significantly lower in flasks with 1.0 mg/L Pb and 0.1 mg/L Cd compared to Pb at 0.1

mg/L and Cd at 0.01 mg/L. Although no differences in population was observed in all treatments at the end of day-8, this could be due to the decreasing toxic effect of Pb and Cd after CC started to adapt to the higher metals concentrations and the chelating of metals by biosurfactant secreted by CC to reduce the bioavailability of Pb and Cd, rendering them less toxic.

It is possible that biosurfactant secreted by CC acted as metal chelating agents, reducing the bioavailability of Pb and Cd and rendering these metals to be less toxic. Das et al. [5] reported that a biosurfactant produced by *Bacillus circulans* was able to chelate positively charged lead and cadmium to the outer hydrophilic surface of biosurfactant, which consisted of anionic peptide head groups.

Figure 5 shows the dense area around the bacterial CC from culture exposed to Pb and Cd observed under TEM compared to control. The dense area showed metal adsorption on the surface of bacterial cells, possibly coated with biosurfactant, suggesting this to be the mechanism employed by CC to remove metals from the culture solution to reduce metals toxicity.

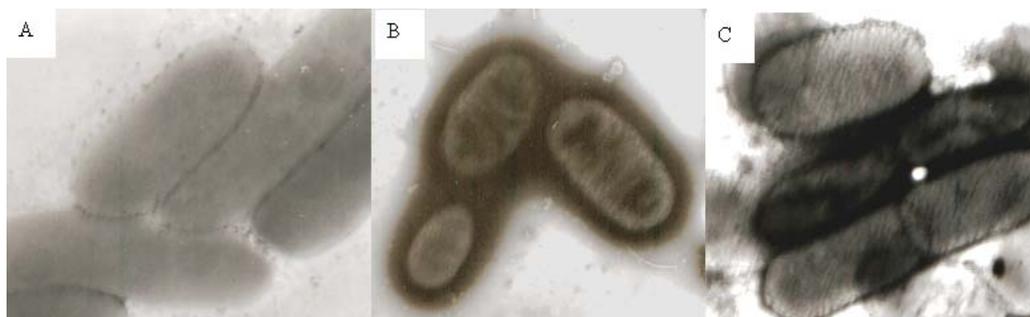


Fig. 5: Bioadsorption of (B) Pb and (C) Cd on bacterial cells as seen under TEM compared to (A) control without metals

Toxicity from the combination of metals and hydrocarbon compounds has been reported to cause structural and functional damages to cells. The combined effects of fluoranthrene and copper resulted in ultra-structural impairments including cytoplasmic vacuolization, organelle changes and anomaly on the multilayered cell walls of marine diatom *Phaeodactylum tricoratum* [29]. Amor et al. [2] further reported on growth inhibition in *Bacillus* sp. by alkylbenzene and cadmium (0.33mM). In this study, only Cd at higher concentrations of 0.1 mg/L adversely affected the crude oil degradation by displaying a lower total percentage of crude oil removal and inhibition on the growth of bacterial population. This can be due to reactive oxygen species (ROS) generated by metal stress, causing damages to cells membrane, chloroplasts and mitochondria resulting in the inhibition of growth and physiological activities [16]. Growth of the

indigenous population remained low and constant up to day-8, showing poor adaptability towards crude oil and poor tolerance towards Pb and Cd. The ability of the CC to show greater tolerance towards increased Pb concentrations was most probably due to the fact that CC used in this study was adapted to 10 ppm of Pb. The present study demonstrated CC is adaptable and robust with tolerance towards toxicity posed by crude oil and heavy metals (Pb and Cd), and thus potentially useful to bioremediate wastewater of mixed pollutant.

Conclusion:

The consortium culture was able to biodegrade crude oil completely within eight weeks. During the degradation process, CC secreted biosurfactant to reduce the surface tension of the media up to 50.2 (dynes/cm), thus increasing the solubility and

bioavailability of hydrophobic crude oil for biodegradation. The ability of CC to degrade crude oil was not inhibited by the presence of heavy metals. In fact, CC was shown to remove Pb and Cd from the media. Hence, the CC can be exploited to enhance degradation of crude oil in the presence of Pb and Cd.

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