Biodegradation process of crude oil and bioemulsifier activity by a bacterial consortium culture

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Abstract. This study reports the analysis of biosurfactant production by a bacterial consortium culture (CC) during biodegradation of crude oil. The ability of CC (5% v/v), grown in Minimal Medium (MM) added with 1% (v/v) Tapis crude oil, to produce biosurfactant under different incubation times (0, 2, 4, 6 and 8 week) was investigated. Biosurfactant activity was estimated by water surface tension changes and emulsification index ($E_{24}$). Maximum biosurfactant production was observed at 2 weeks incubation period, after which the yield gradually decreased. The biosurfactant produced by the CC significantly decreased the surface tension of water from 72 dynes/cm to 52 dynes/cm ($p < 0.05$) at week 4. Emulsification test indicated that this biosurfactant effectively emulsified the crude oil with an emulsification index ($E_{24}$) of 85% ($p < 0.05$). This is significantly better ($p < 0.05$) when compared to other chemical surfactants tested namely SDS (53.3%), Tween-20 (50.8%) and Triton X-100 (55%). These results indicate the capability of CC to produce biosurfactant during crude oil degradation and this can be exploited for enhanced bioremediation in oil contaminated areas.

1. Introduction
Many microorganisms are able to produce a wide range of amphipathic compounds, those with both hydrophilic (heads) and hydrophobic (tails) moieties. These compounds called biosurfactants or bioemulsifiers can diffuse into water and adsorb at interfaces between air and water or at the interface between oil and water. They form suspensions or micelles and reduce surface and interfacial tension [1].

Biosurfactants are categorized according to chemical composition, molecular weight, mode of action and bacterial origin, and general physico-chemical properties, unlike their chemically synthesized counterpart such as Triton-X 100, sodium dodecyl sulphate (SDS) which is classified based on their dissociation in water. Based on molecular weight, biosurfactant are divided either into the low-molecular-weight biosurfactants which include lipopeptides, glycolipids and phospholipid, or the high molecular weight for those containing amphipathic polysaccharides, lipopolysaccharides, proteins or lipoproteins or complex mixtures of these polymers [2].

The ability of biosurfactants to reduce surface and interfacial tensions both in aqueous solutions and hydrocarbons mixtures, results in increased aqueous concentration of poorly soluble compounds, such as hydrocarbons which lead to improved accessibility and bioavailability of these substrates for degradation by the microorganisms [3]. Biosurfactants also stabilizes hydrocarbons water emulsions or vice versa, by increasing the surface area available for interaction with the aqueous phase and at the same time providing the necessary aqueous interphase for the bacteria to carry out enzymatic
degradations of petroleum hydrocarbon [4; 5]. These emulsification properties have been demonstrated to enhance hydrocarbons degradation in the environment, making them potentially useful to aid oil spill pollution control [6]. Biosurfactants are known to be synthesised intercellularly and extracellularly. Extracellularly secreted biosurfactants aid emulsification of the hydrocarbons while those produced within the cell wall facilitate the penetration of hydrocarbons into the periplasmic space [7]. These two sources of biosurfactants act in tandem during the biodegradation of hydrocarbons. The aim of this research is to investigate biosurfactant production and emulsification activity during crude oil biodegradation.

2. Materials and Methods

2.1. Source of microorganisms

The microorganisms used are collections of biology department laboratory Faculty of Mathematics and Natural Sciences State University of Medan (UNIMED). It consists of three Gram-negative (Pseudomonas sp., Serratia sp., and Agrobacterium sp.) and one Gram-positive (Bacillus sp.).

2.2. Media and inoculum preparation

Inoculum of the CC was prepared in nutrient broth, the stock culture growth medium. An incubation time of between 8-12 hours was needed for the CC to reach OD$_{600}$ of 0.5 ($2.5 \times 10^8$ cfu/mL), which is the optimal time to transfer the CC (5% v/v) to the minimal medium. The minimal medium (MM) was used to the production biosurfactant during crude oil biodegradation (Abu-Ruwaida et al., 1991). The composition of MM prepared in distilled water (g/L) was as follows: Na$_2$HPO$_4$ (2.2), KH$_2$PO$_4$ (1.4), Mg SO$_4$·7H$_2$O (0.6), FeSO$_4$·7H$_2$O (0.01), NaCl (0.05), CaCl$_2$ (0.02), yeast extract (0.02) and 0.1 mL of trace element solution containing (g/L): ZnSO$_4$·7H$_2$O (2.32), MnSO$_4$·4H$_2$O (1.78), H$_3$BO$_3$ (0.56), CuSO$_4$·5H$_2$O (1.0), Na$_2$MoO$_4$·2H$_2$O (0.39), CoCl$_2$·6H$_2$O (0.42), EDTA (1.0), NiCl$_2$·6H$_2$O (0.004) and KI (0.66). pH of the medium was adjusted to 7.0 ± 0.2 and autoclaved at 121ºC for 15 min.

2.3. Growth of CC in crude oil

The 5% (v/v) CC from an overnight culture was transferred into 250 mL conical flasks containing 100 mL of sterile MM medium [8; 9] with 1% (v/v) crude oil, as carbon source for biodegradation test and biosurfactant analysis. The set of flasks were then incubated on a shaker (200 rpm) at 29 ± 2ºC (to mimic ambient temperature) for eight weeks. At every 2 weeks interval, 2.0 mL of culture was removed from the sets of flasks for CC growth analysis by the spread plate technique. Cell growth was reported by the number of viable colonies as colony forming unit per milliliter (cfu/mL).

2.4. Surface tension measurement

Surface tension changes of the crude oil were determined every two weeks with a tensiometer (Fisher Scientific, USA) using ring method [10] at room temperature. The platinum wire ring was rinsed three times with water, three times with acetone, and then left to dry [10]; [11]. The tensiometer was calibrated with deionized water by dipping the ring into water and the surface tension measured and recorded. The platinum ring was then washed with acetone and air dried before dipping it into the CC cell culture (25.0 mL) which was placed in a clean 50 mL glass beaker and the reading of the surface tension (dynes per cm) recorded. All readings were taken in triplicates.

2.5. Determination of emulsification index (E$_{24}$)

Every two weeks for 8 weeks, 2.0 mL Tapis crude oil was added into a screw cap test tube containing 2.0 mL of culture filtrate, in triplicates. The mixture was vortexed for 2 minutes and left to stand for 24 hours. The percentage of height of the emulsified layer (mm) from total height of the liquid layer (mm), the E$_{24}$ index [10]; [12] was then calculated.
2.6. Extraction of residual crude oil for gc-fid analysis
At every two weeks 50 mL sample was taken from each set of flasks to extract the residual crude oil. Sample was poured into a separatory funnel, 30 mL of n-hexane was added [13] and the residual crude oil in sample extracted by shaking the funnel vigorously for 2 minutes. It was then left to stand for a minimum of 10 minutes to allow separation of the organic phase from the aqueous phase. The aqueous layer (lower layer) was drained into the sample flask and the organic layer separated into another flask. The extraction process was repeated twice, each with 30 mL n-hexane to ensure complete extraction of the crude oil residue. The extract was then collected in a round bottom flask and evaporated by using a rotary evaporator (EYERLA N-1000, Japan) in a water bath at 60°C. The residue was then reconstituted with 1.0 mL n-hexane being analyzed by GC-FID.

One microliter of the extractable crude oil was analyzed on a 30 m polydimethyl siloxane capillary column (HP 1) in a capillary gas chromatography (Agilent-Auto System) equipped with a flame ionization detector (FID) using the HP ChemStation software. The oven temperature was initially set to 30°C and was increased at a rate of 4°C min⁻¹ to 300°C [14]. Degradation was estimated as the difference between the initial and final concentrations of total hydrocarbons in the sample.

2.7. Statistical data analyses
Data were analyzed by one-way analysis of variance (ANOVA) with Tukey-HSD multiple comparison test.

3. Results and Discussions

3.1. Crude oil degradation by consortium culture

![Figure 1](image)

Figure 1. The growth of consortium culture and percentage of crude oil degradation during biodegradation process in the MSM medium at optimal pH 7.0 and 29 ± 2°C for 0 to 8 weeks. a: significant difference (p < 0.05) with week 0; significant difference between with week 0 and 2, and non-significant difference between week 4, 6 and 8.

Figure 1 shows that the CC was able to grow in mineral salt medium with crude oil as the sole carbon source. CC population increased gradually within the first two to four weeks, but showed a decline after six to eight weeks. The results showed the ability of CC to utilize crude oil as the carbon source and energy for biomass proliferation. No lag phase was observed indicating the robustness of CC to
grow in crude oil and its ability to adapt to using the hydrocarbon as sole carbon source very quickly. Figure 1 also shows significant (p < 0.05) increases in total viable colonies, from \(3 \times 10^5\) (cfu/mL) at day 0 to \(4 \times 10^7\) (cfu/mL) at week 2 and reaching its maximum at \(7 \times 10^9\) (cfu/mL) in week 4. The population count however declined to \(5 \times 10^6\) (cfu/mL) after week 6 and decreased further by week 8.

Generally, biodegradation of the crude oil correlated well with growth of the CC (Figure 1). By Week 6, up to 97% of crude oil was already degraded, and degradation was complete (100%) after 8 weeks (Figure 1). Thus the decline in the growth of CC after week 6 to week 8 is likely due to the depletion of crude oil as the sole carbon source.

The ability of CC to grow and completely degrade crude oil within eight weeks suggests that the CC was robust and able to survive in the crude oil medium after their inoculation. pH 7 was used in these investigation as departure from the optimal pH can impair ability of microbial populations to degrade hydrocarbons [15]; [16]; [17]. Survival of microorganisms in medium containing crude oil after their inoculation has been shown to be the key deciding factor in ascertaining the rate of biodegradation of hydrocarbons either in soil or in liquid phase [18]. The composition of a microbial consortium is an important factor, as they are critical in ensuring the synergistic enhancement of catabolic activities. The microbial consortium culture exhibited higher biodegradation activity than an axenic culture of Acinetobacter for the degradation of light and heavy crude oils [19]. Salleh et al [20] reported that bacterial consortium comprising of hydrocarbon degrading Pseudomonas aeruginosa (1 strain) and Bacillus sp (2 different strains), was shown to be effective in biodegrading crude oil petroleum in liquid cultures as well as in polluted soil and sand. Rahman et al [9] and Kalme et al [21] reported better efficiency of crude oil biodegradation by mixed bacterial cultures as opposed to single cultures. The presence of different bacteria in mixed bacterial cultures increase competition in utilizing new sole carbon sources, forcing mixed bacteria to adapt quickly to the environment.

3.2. The changes in surface tension during biodegradation process

![Figure 2](image-url)

**Figure 2.** The change in surface tension and percentage of crude oil biodegradation over a period of 8 weeks. a: significant difference (p < 0.05) with week 0; b: significant difference between week 0 and 2, and non-significant difference between week 4, 6 and 8.

Overall, a gradual decrease in the surface tension of the culture medium was observed during the biodegradation of crude oil by CC. Figure 2 shows the presence of CC reduced surface tension of the sample during the biodegradation process. These results reveal the ability of the CC to produced biosurfactant, which improves the solubility of hydrophobic pollutants and thus, their biodegradability (Banat 1995). The surface tension of the culture medium showed significant decreases (p < 0.05) when
compared at week 2 to week 8 but not at weeks 4, 6 and 8. Das et al [22] reported the ability of Micrococcus sp. grown on n-octadecane to reduce surface tension to 58.3 dynes/cm. Kaczorek et al [23] also observed the gradual decrease in surface tension of culture media during the process of biodegradation and emulsification of hydrocarbon.

3.3. The emulsification of crude oil by consortium culture
The emulsification activity of CC as measured by the emulsification index showed significant increases (p < 0.05) at week 2 (85%) and week 4 (58%) compared to week 0. There was however no significant difference between weeks 4, 6 and 8 (Figure 3). Emulsification activity increased with increasing cell growth and reaching their optimum at week 2 and gradually decreasing towards week 8. These results indicate that biosurfactant biosynthesis from CC occurred predominantly during the exponential growth phase at week 2. Sepahi et al [24] also observed the release of biosurfactant into the culture medium during the exponential phase and suggested that the biosurfactant was produced as a primary metabolite accompanying cellular biomass formation.

![Figure 3. The emulsifying index of CC as indicated by E<sub>24</sub> during biodegradation of crude oil.](image)
a: significant difference (p < 0.05) with week 0; b: significant difference between with week 0 and 2, and non-significant difference between week 4, 6 and 8.

Emulsification activity of crude oil occurs when the surface tension is reduced due to the repulsive forces between crude oil and media, allowing the two phases to mix more easily [25]. During this phase the formation of small oil droplet increases the surface area of hydrocarbon thus facilitating the enhanced attachment of the bacterial cell to crude oil and helps to increase the degradation process [26].

Another function of biosurfactants or microbial surface active molecules with interesting biotechnological potential is the ability to form stable emulsions. The two most important properties evaluated for biosurfactant are surface tension reduction and, emulsion forming and stabilizing capacities [27]. The emulsion stabilizing property is the ability to maintain at least 50% of the original emulsion volume 24 h after formation [28] and be sustainable in the long term for months and years [29].

3.4. The evaluation of emulsifying activity
Emulsification activity as measured by Emulsification Index (E<sub>24</sub>) from CC was calculated to be 85%. This is relatively higher than the values reported previously by Elouzil et al [30] and Abouseod et al [31], at 70% and 66%, respectively. Figure 4 shows the emulsifying activity of CC on crude oil
compared to several commercial chemical surfactants. The capability of CC to emulsify was found to be higher (85%) compared to 1% SDS (53.3%), 2% Tween 20 (50.8%), and 2% Triton X-100 (55%).

![Image of emulsion production](image)

**Figure 4.** The sample of emulsion produced by CC compared with the commercial surfactants. a. 2% Triton X-100, b. 2% Tween 20, c. CC and d. 1% SDS.

## 4. Conclusion

This paper demonstrates a consortium culture which can grow and utilize crude oil as the sole carbon and energy source. This was aided by the production of bioemulsifier that aid the biodegradation process. With these ability, the CC is potentially useful for the bioremediation of crude oil contaminated environment.

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