Characterization of biosurfactant produced by petrofilic bacteria isolated from hydrocarbon impacted soil and its potential application in bioremediation

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Abstract. The study adopts a multi-level perspective in technology transition to analyse how the transition process in the development of geothermal energy in Indonesia is able to compete against the incumbent fossil-fuelled energy sources. Three levels of multi-level perspective are socio-technical landscape (ST-landscape), socio-technical regime (ST-regime) and niche innovations in Indonesia geothermal development. The identification, mapping and analysis of the dynamic relationship between each level are the important pillars of the multi-level perspective framework. The analysis considers the set of rules, actors and controversies that may arise in the technological transition process. The identified geothermal resource risks are the basis of the emerging geothermal technological innovations in Indonesian geothermal. The analysis of this study reveals the transition pathway, which yields a forecast for the Indonesian geothermal technology transition in the form of scenarios and probable impacts.

Keywords: biosurfactants, emulsification index, fatty acids, surface tension

1. Introduction

Bioremediation is a process by which biological agents such as bacteria, fungi, or green plants are used to remove or neutralize contaminants in soil or water. Bioremediation typically involves augmentation of soil or other media, contaminated with pollutants act as nutrient for microorganisms, to improve process for biodegradation of the contaminants. Biodegradation rate of contaminant in soil depends on its bioavailability to the metabolizing organisms, which is influenced by factors such as desorption, diffusion, and dissolution [1]. Addition of biosurfactants as surface tension reduction agents became a promising method to improve bioremediation effectiveness of hydrocarbon-contaminated environments.

Most of persistent contaminants exhibit low water solubility, and hence the addition of emulsifier can increase bioavailability of the contaminants for the metabolizing microorganisms. By reducing surface and interfacial tension among liquids, solids, and gases, biosurfactants are allowed to disperse readily as emulsions so that the intake process of contaminants as a nutrient for microorganism can be improved [2]. Researcher [3] classified biosurfactants into five categories: (i) glycolipids, carbohydrate with the combination of aliphatic acids and long chained hydroxyl aliphatic acids, group of low molecular weight biosurfactants, (ii) phospholipids and fatty acids, containing one or more phosphate
groups with phospholipid and glycolipid membranes, (iii) lipopeptide, classified as biosurfactants with high molecular weight, (iv) polymeric surfactants, biopolymer that can be used as thickener or emulsion stabilizer, and (v) particulate biosurfactants, as membrane or extracellular products act in uptake process of hydrocarbon as the substrate. This studies focusing on isolating bacteria that capable to produce biosurfactants, its properties and characteristics of biosurfactants, also its application for biodegradation of petroleum hydrocarbon.

2. Research Method

2.1. Preparation of Bacteria Isolates
In the beginning, screening process is carried out to obtain bacteria that potentially capable of producing biosurfactants. Mixed culture from Laboratory of Environmental Biotechnology, Institut Teknologi Bandung was incubated at room temperature 25°C with agitation speed of 110 rpm. In the mixed culture, 0.1% (v/v) of crude oil was added to create a suitable condition and environment for biosurfactants producing bacteria to grow. Furthermore, the culture is inoculated in nutrient agar on petri dish to acquire bacteria colonies that pass the screening process.

2.2. Emulsification Index
In emulsification test, 5 ml of supernatant is added with crude oil (1:1 v/v) and mixed in vortex with maximum speed for two minutes, and left for 24 hours. E24 is height measurement of the crude oil emulsion formed, divided with the total of column height after 24 hours, in percentage.

2.3. Surface Tension Measurement
In surface tension measurement, supernatants are tested for its surface tension at room temperature with Fisher® Tensiometer model 21, with Du-Nuoy platinum-iridium ring method. 30 ml of sample was put into a clean glass vessel and placed on tensiometer platform. The ring is pulled slowly until it breaks the surface of sample through the liquid-air interface. Between each measurement, the platinum wire ring was rinsed with water and allowed to dry.

2.4. Measurement of Microorganism Growth
The growth of biomass is measured through spectrophotometry analysis, with the measurement of optical density (O.D.) and Mixed liquor suspended solids (MLSS). O.D. are measured by Jenway® spectrophotometer model 6305 with 420nm wavelength. MLSS measurement is carried out by measuring its total suspended solid (TSS).

2.5. 16S rRNA Gene Sequencing Analysis
Polymerase Chain Reaction (PCR) was carried out by Macrogen Inc., South Korea. Taxonomic assignments of sequences were performed using Ribosomal Database Project. The sequences in GenBank were retrieved using Basic Local Alignment Search Tool (BLAST) from NCBI.

2.6. Isolation of Biosurfactants
In this research, the measurement of biosurfactants quantity is including fatty acids fraction and exopolysaccharide fraction (EPS). The cell free broth, previously incubated with the same condition mentioned above is precipitated by two different methods. To obtain exopolysaccharide fraction, cold acetone is added to precipitate emulsifiers. Then mixture is allowed to stand overnight at 4°C to get the precipitate exopolysaccharide. Furthermore, the mixture sample is filtered through number 42 Whatman paper, and heated in oven at temperature 105°C for one hour, the measurement is performed by gravimetric method. Cold acetone method also chosen as biosurfactants extraction with organic solvents by [4]. To measure fatty acids fraction, culture supernatant is acid hydrolysed with HCl 2N until it reaches at pH 2, because in this condition, biosurfactants becomes insoluble in aqueous [5]. It then allows to stand for overnight at 4°C to precipitate lipid and protein fraction from biosurfactants. After that, extraction is performed by adding chloroform to the sample to extract fatty acids, because it will be soluble in chloroform. The organic later was transferred to a round-bottom flask and heated in water
bath at temperature 70°C, allowing the chloroform to evaporate, so then fatty acids will be left for further gravimetric method of measurement [6].

2.7. CMC Values
Determination of the critical micelle concentration (CMC) of rhamnolipids was performed by measuring the surface tension of aqueous solutions. The measurements were carried out with a tensiometer.

2.8. Biodegradation Assay
To determine the performance of biosurfactant on enhancing petrophilic bacteria in degrading petroleum hydrocarbon, a biodegradation assay developed and set up as follows:
- Blank reactor; no petrophilic bacteria were added.
- Control reactor; with addition of petrophilic bacteria but without biosurfactant.
- Reactor with the addition of petrophilic bacteria and biosurfactant.
- Reactor with the addition of petrophilic bacteria and chemical surfactant as comparison

  5% (v/v) of biosurfactant was added to 500 ml of Basal Salts Medium (BSM) containing 5% crude oil. The flasks were incubated at room temperature on a rotary shaker (150 rpm). Total petroleum hydrocarbon (TPH) concentration are observed each day for 10-days period.

2.9. Total Petroleum Hydrocarbon
Measurement of TPH was conducted by gravimetric method. Sample was extracted with n-hexane, the organic layer was pooled and dried at 70°C temperature by evaporation of solvents. After evaporation, the amount of residual TPH recovered was weighted [7].

3. Results and Discussion

3.1. Biosurfactant production
From screening process, there are four samples of isolates that undergo selection process while its biosurfactants are tested for its E24 and surface tension value. Table 1 is showing the results of biosurfactants quality from each isolate. The chosen isolates are the one with highest E24 and the lowest surface tension, due to consideration that it will describes the best quality of biosurfactants. From the measurement, isolate A is chosen as isolate that will be inoculum for the next biosurfactants production process.

| No. | Isolate | E24 (%) | Surface Tension (dyne/cm) |
|-----|---------|---------|--------------------------|
| 1   | A       | 84      | 43.5                     |
| 2   | B       | 52      | 52.5                     |
| 3   | C       | 61      | 49.5                     |

It was then revealed by gene sequencing analysis of 16S rRNA that isolate A is identified as Burkholderia sp. For further reference, the isolates will be referred as as Burkholderia sp. PAU02. Growth and biosurfactant production from Burkholderia sp. PAU02 with 2% glucose as sole carbon source is described in Figure 1. It is indicating that biosurfactant production kinetics is categorized as “Growth Associated” [8]. The maximum biosurfactant produced by Burkholderia sp. PAU02 is obtained after 42-hour incubation amount to 5.18 g/l.
Figure 1. Time course study of biosurfactant production with glucose as a carbon source.

Figure 2. The quality of biosurfactant produced by *Burkholderia* sp. PAU02.

The quality of biosurfactants is measured each day for consecutive four days. Figure 2 shows the data related to quality of the biosurfactant of the respective isolate. *Burkholderia* sp. PAU02 produced optimum biosurfactant at 72-hour incubation period, reducing surface tension to the point of 47.0 dyne/cm and emulsification index of 84%. Based on comparison study of biosurfactant produced by *B. thailandensis* by [9], different method of extraction and purification of biosurfactant can alter the property of biosurfactant itself, including its surface tension. For this research, crude biosurfactant was measured without further purification process. The quantity of biosurfactants produced by each isolate was measured and can be seen in Figure 3. For *Burkholderia* sp. PAU02, the maximum biosurfactant production is achieved at 42-hour. Majority of biosurfactants is a secondary metabolite and some have an essential role in the defence mechanism, either through nutrient transport mechanisms, interactions between the microbial hosts or as biocides. By releasing biosurfactants into the growth medium, microorganisms can survive by using non-polar substrates as a carbon source, to sustain its growth and survival.
In contrast to primary metabolites, secondary metabolite is a product of metabolism produced due to change of the environment and inducible. Definition of secondary metabolites is not because the product is produced after growth, but because those products are not involved in the growth of the culture itself [10]. The biosurfactant kinetic production patterns is categorized as type of kinetics with a pattern of "simultaneous formation of an external product with growth" indicating that biosurfactant production rate proportional to the rate of growth. Measurement of CMC value of biosurfactant yielding 279 mg/L. CMC is important for biodegradation mechanism. After reaching CMC, solubilisation process is occurred thus forming a micelle and increasing oil solubility.

3.2. Biodegradation Assay
Laboratory scale of biosurfactant enhanced biodegradation of crude oil was conducted. Effect of addition of biosurfactant and surfactant in the biodegradation process is shown in Table 2.

Table 2. TPH removal efficiency of petroleum hydrocarbon after 10-days by petrophilic bacteria in batch reactor with addition of various surfactants.

| Sample                        | Initial TPH (%) | Final TPH (%) | Efficiency (%) |
|-------------------------------|-----------------|---------------|----------------|
| Blank*                        | 5               | 4.479         | 10.43          |
| Control** (C)                 | 5               | 2.626         | 47.48          |
| C + Biosurfactants *Burkholderia sp.* PAU02 0.34 ×CMC | 5 | 1.728 | 65.44 |
| C + Tween 80 (3.53 ×CMC)      | 5               | 1.687         | 66.26          |
| C + Tergitol NP-10 (1.18 ×CMC)| 5               | 2.836         | 43.29          |

*Blank: Medium + crude oil 5%; **Control: Medium + crude oil 5% + petrophilic bacteria

The efficiency of chemical surfactant Tween 80 is as effective as biosurfactant, due to its low toxicity and biodegradability. However, Tergitol NP-10 is showing even lower efficiency than control parameter. Compared to other non-ionic surfactants, Tergitol NP-10 have non-biodegradable characteristic with higher toxicity. Due to this reason, addition of Tergitol NP-10 is not as efficient compared to other kinds of surfactant. Based on GC/MS results (data not shown), it is known that biosurfactant addition to the system can enhanced the hydrocarbon removal process.
4. Conclusion

Biosurfactants produced by *Burkholderia* sp. PAU02 have a great potential for bioremediation application, due to its emulsifying effects and ability in reducing surface tension. From biodegradation assay studies, it is known that with addition of biosurfactant can enhance the rate of biodegradation efficiency on removing petroleum hydrocarbon up to 65%.

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