Preliminary study : optimization of pH and salinity for biosurfactant production from *Pseudomonas aeruginosa* in diesel fuel and crude oil medium

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Abstract. Biosurfactant is secondary metabolite surface active compound produced by microorganisms which is nontoxic and eco-friendly. Microorganism producing biosurfactant that is quite potential to use in many applications is from *Pseudomonas aeruginosa* strains. Good quality of biosurfactant production from microbes is supported by the suitable nutrients and environmental factors. The aim of this research was to obtain preliminary o data upon the optimum pH and salinity for the production of biosurfactant from *Pseudomonas aeruginosa* ATCC 15442 in diesel fuel and crude oil medium. *P. aeruginosa* ATCC 15442 cultured in diesel fuel and crude oil as carbon source showed biosurfactant activity. *P.aeruginosa*-derived biosurfactant was capable to form stable emulsion for 24 hours (EI24) in hydrocarbons n-hexane solutions. The particular biosurfactant showed EI_{24} highest value at pH 7 (31.02%) and 1% NaCl (24.00%) when *P. aeruginosa* was grown in 10% diesel fuel medium in mineral salt solution. As for the media crude oil, the highest EI_{24} value was at pH 6 (52.16%) and 1% NaCl (33.30%).

1. Introduction

Surfactants are compounds that can reduce the surface tension of a liquid, interfacial tension between two liquids or between a liquid and a solid. Surfactant is characterized as an organic compound consisting of a hydrophobic group (tail) and hydrophilic (head). This indicates that the surfactant molecules are not water soluble (oil-soluble components) as well as water-soluble components [1]. Meanwhile, biosurfactants is a surface-active compounds produced by microorganisms. Metabolites of these microorganisms are nontoxic and environmentally friendly. Characteristic of microbial surfactants is associated with surface activity, tolerance to pH, temperature and ionic strength, biodegradability, low toxicity, emulsion and de-emulsion ability, as well as antimicrobial activity. Those advantages make it a potential object of research for multiple applications such as in industrial cosmetics, pharmaceutical, food preservatives, detergents, petroleum, mining, metallurgy and many others [2].

Biosurfactants produced by various microorganisms, mainly bacteria, fungi, and yeast are vary in terms of their chemical compositions, as it depends on its natural environment, the type of

microorganisms and nutrients availability. Environmental and nutrient conditions that may limit biosurfactant production including pH, temperature, agitation, aeration, dilution rate, metal ion concentration, as well as carbon and nitrogen sources [1]. Environmental factors such as pH is very important in the acquisition and characterization of biosurfactants. Quality of biosurfactant is determined by its emulsification activity. For example, a high emulsification activity of biosurfactant was produced by *P. aeruginosa* PBSC1 at pH 7 [3]. Other environmental factors that is quite important for some applications is salinity. Strain *Virgibacillus salarius* KSA-T was known to produce high concentration of biosurfactant at salinity of 4% (w/v) [4]. Meanwhile, production of surfactant by *P. aeruginosa* RS29 was decreased after the addition of NaCl> 0.8% (w/v), indicating that the particular strain in sensitive toward high salinity [5].

In this study, biosurfactant production by *P. aeruginosa* ATCC 15442 was optimized based on pH and salinity conditions. It was reported that *P. aeruginosa*-derived biosurfactant production is applicable to many purposes, including for the microbe-enhanced oil recovery (MEOR) and bioremediation. Biosurfactants produced from these bacteria is a type of glycolipid, particularly rhamnolipid, a secondary metabolite that is produced at the beginning of the stationary phase of the microbial growth [6].

This research aimed to optimize biosurfactant production from *P. aeruginosa* ATCC 15442 in laboratory scale. *P. aeruginosa* ATCC 15442 was grown in two types of fermentation medium, namely diesel oil and crude oil-enriched saline mineral (mineral salt solution (MSS)). The parameters used in this optimization were pH and salinity of the media. Biosurfactant obtained from each treatment were tested for emulsification index. The study was conducted in May 2016 to July 2016 at the Laboratory of Biochemistry, Department of Biochemistry, Faculty of Mathematics and Natural Sciences IPB.

1.1. Materials

The bacteria that used in this study was *P. aeruginosa* ATCC 15442 from Bogor Agricultural Institute Culture Collection (IPBCC) which routinely grown in nutrient broth (NB) or triptic soy agar (TSA) for starter culture. Media used in the growth of the bacteria producing biosurfactant was diesel oil and crude oil enriched with mineral salt solution (MSS). Mineral salts solution was composed by MSS composition used was 2.0 g/L KH_2PO_4, 5.0 g/L K_2HPO_4, 3.0 g/L $(NH_4)_2SO_4$, 0.1 g/L NaCl, 0,01 g/L $FeSO_4.7H_2O$, 0.2 g/L $MgSO_4.7H_2O$, 0.01 g/L $CaCl_2.2H_2O$, and 0.002 g/L $MnSO_4.H_2O$, with the addition glucose (0.03%) and yeast extract (0.03%). pH of the medium was adjusted by 4 N HCl and 3 M NaOH. The material used for biosurfactant activity response to test emulsification index (EI) is n-hexane. Other materials used are for refreshment bacterial liquid medium as well as starter medium and for a refresher on solid media.

1.2. Tools

Equipment used in for routine handling of bacteria including Petri dish, ose, Bunsen, Erlenmeyer flasks, beakers, laminar flow cabinets, autoclave, pH meter, incubator temperature, rotary shaker, and water bath shaker. Equipment used for biosurfactant analysisng via EI test including refrigerated centrifuge, test tubes, vortex mixer and a ruler. Other equipment used in this study was micropipette and 0.2 μm millipore filter, and other chemical glassware.

1.3. Procedures data analysis

1.3.1. Cultivation biosurfactant producing bacteria (modification Francy et al. 1991 [7]). P. aeruginosa ATCC 15442 was grown in 4% TSA (w/v) and then incubated at 37 °C for 18 hours. The single colonies of *P. aeruginosa* culture was further taken and grown in 5% NB (w/v) and incubated for 14 hours at room temperature using a rotary shaker (110 rpm). That broth culture was grown in 50 mL of a mixture diesel fuel or crude oil with MSS in 1% (v/v). Diesel fuel and cured oil were added to the media for for 10% and 5%, respectively. Culture was then incubated at 30 °C.

1.3.2. Effect of pH in biosurfactant production (modification Yakimov et al. 1995 [8]*).* Medium for the growth of *P. aeruginosa* was adjusted to pH value of 4, 5, 6, 7, 8, and 9. The medium was then incubated in water bath shaker at 110 rpm, 30 °C for an *optimum* time of biosurfactant production based on EI_{24} value. *P. aeruginosa* was incubated for 4 and 7 days in diesel oil and crude oil medium, respectively, which further were used for emulsification index analysis.

1.3.3. Effect of salinity on biosurfactant production (modification Prommachan et al. 2001 [9]*).* Effect of salinity on biosurfactant production was done by adding NaCl at different concentrations to the growth medium. NaCl concentration tested was 1%, 5%, 9%, 13%, and 17% (w/v) while pH was set up to 7. The culture was then incubated on a water bath shaker at 110 rpm, 30 °C for an optimum time of biosurfactant production based on EI_{24} in each medium which then was tested for emulsification index analysis.

1.3.4. Emulsification index analysis test (Pereira et al. 2013 modification [10]*).* A total of 2 mL of n-hexane was added to the 2 mL of the supernatant free of cell in a test tube. Supernatant was then centrifuged at 10000 g, 4 °C for 20 minutes which was followed with vortexing at high-speed for 2 minutes. Solution was then incubated for 24 hours at room temperature. Emulsification index was measured by the percentage of the emulsified layer height (cm) divided by the total height of the liquid in the tube (cm).

2. Result and discussion

P. aeruginosa biosurfactant-producing bacteria can be obtained from various sources, either directly from nature or culture collections that have been registered. *P. aeruginosa* used in this study was the collection of American Type Culture Collection (ATCC) strain number 15442 which is the re-culture from Institut Pertanian Bogor Culture Collection (IPBCC). This bacterial strain was isolated from water bottle animal with optimum growth temperature of 37 °C in TSA (tryptic soy agar) or TSB (tryptic soy broth) media under aerobic condition. Since the purpose of this study was to obtain good quality of biosurfactant, thus the *P. aeruginosa* was cultured in the presence of degradable hydrocarbon resources [12] and minimal nitrogen sources [13]. The carbon source in the media was either diesel oil or crude oil, while the nitrogen source was obtained from some mineral salts in the MSS and yeast extract.

Microbial fermentation process needs to be optimized to obtain a good quality of biosurfactant. In this study, optimization of pH and salinity of each media were conducted with further observation toward the emulsification index for 24 hours (EI_{24}).

2.1. Effect of pH in growth medium to emulsification index

Supernatant from the culture of *P. aeruginosa* used in this study showed biosurfactant activity on diesel fuel and crude oil medium at a slightly different pH conditions. Stable emulsion for 24 hours was seen at both neutral pH (7) and basic pH (8-9) on the diesel fuel medium, while in crude oil medium, it was found stable at acidic pH (5-6). On the other hand, at alkaline pH, the EI_{24} value was fairly low for less than 15%. The highest EI_{24} value in diesel medium and crude oil medium was found at pH 7 (27.35%) and pH 6 (52.16%), repectively (figure 1 and 2).

EI_{24} value optimum at pH 7 of diesel fuel medium in this study was also obtained by Saikia *et al*. (2012) with the same incubation time (4 days), but different carbon source, temperature and agitation speed. Saikia *et al*. (2012) get the value of EI_{24} about 70% at pH 7 and 8 with the incubation temperature was 35 °C and 110 rpm agitation speed—meanwhile in this study use 30 ^{o}C and agitation speed was 150 rpm—using glycerol as carbon source. Glycerol is used by Saikia *et al*. (2012) after previously tried with some other carbon sources. Their research showed that the strain of bacteria they used, namely *P. aeruginosa* RS29 produce maximum biosurfactant on the water-soluble substrate.

The highest EI_{24} using 10% diesel fuel medium in this research only get 27.35% at pH 7. A lot of data already reported mention that EI_{24} value > 50% is potential for biosurfactant production by

microorganisms (Rodríguez-Rodríguez *et al*., 2012). Research of Prabakaran and Sumathi get 76% EI_{24} value on the carbon source media of 1% diesel fuel with pH 7, in the enrichment Bushnell Haas media, at 37 ^{o}C [14]. Furthermore, the enrichment media used in this study is MSS that being modified by Francy *et al*. (1991) which refered from Knetting and Zajic [15]. The difference EI_{24} result is fairly high value that can be caused by several factors, such as the bacterial strain used and the conditions of growth medium.

The enrichment compositions medium in this study and Prabakaran and Sumathi (2014) is almost the same. Both media are commonly used to study the utilization of hydrocarbons by microorganisms. The content of the medium is all important nutrients for bacterial growth except hydrocarbons as an energy source.

Magnesium sulphate ($MgSO_4$), calcium chloride ($CaCl_2$), and ferric chloride ($FeCl_3$) in Bushnell Haas media or iron phosphate ($FeSO_4$) on Knetting-Zajic media provide essential elements for the growth of the organism. Potassium phosphate is a buffer agent and ammonium nitrate (NH_4NO_3) on Bushnell Haas media or ammonium sulphate ($(NH_4)_2SO_4$) on Knetting-Zajic media is a source of nitrogen. Other elements present in the media Knetting-Zajic but none on Bushnell Haas medium are Mn, Na and Cl. In addition, the MSS which refers Francy *et al*. (1991) added 0.03% glucose as the carbon source and 0.03% yeast extract as nitrogen source.

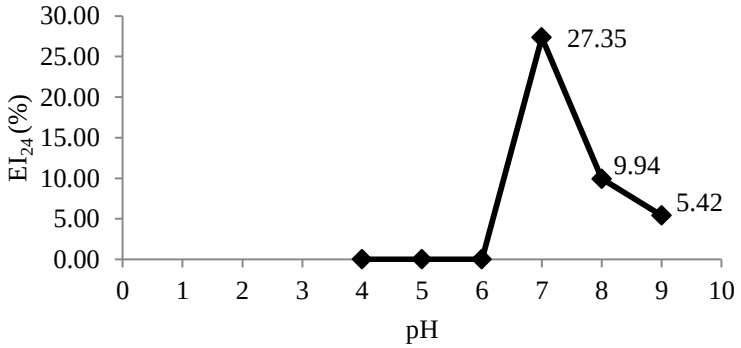

Figure 1. Effect of pH on the emulsification index (EI_{24}) of biosurfactant produced by *Pseudomonas aeruginosa* in diesel fuel medium.

Eraqi *et al*. research used 1% glycerol as a carbon source and 2% $NaNO_3$ as nitrogen source showed that rhamnolipid was produced at its maximum level at pH 6 [16]. Moreover *P. aeruginosa* OCD1 in showed the highest EI_{24} value at pH 6 with 2% n-octadecane as carbon source [17] which is similar to our result. Interestingly, it was reported that high EI_{24} (75.12%) value could be shown by *P. aeruginosa* PBSC1 at pH 7 in 1% crude oil as carbon source. Meanwhile, lower EI_{24} was exhibited by this particular bacterial strain when it was cultivated in pH 6. The bacteria used in their study was *P. aeruginosa* PBSC1 isolated from mangrove ecosystem.

Rhamnolipid which is the main type of bacterial biosurfactant strains derived from *P. aeruginosa* is known to reversibly change their morphology due to pH alterations. Their molecular aggregates changing from vesicles to lamella under acidic conditions and from lipid particles to micelle at slightly acidic conditions, in narrow pH range about 5-7. Rhamnolipid therefore is anionic surfactant at pH 6.8 and almost entirely protonated at pH 5, so that they are nonionic [18]. Rhamnosyl group structure is not affected in this narrow pH range, since their dissociation occurs at higher pH values is more than 11 [19].

In this study, the emulsion of biosurfactant in two different biosurfactant producing media at pH 4 was not detected. Similar results was shown by previous study with surfactant rhamnolipid [16]. This

can be explained by rhamnolipid under acidic conditions which is strong enough to be in the protonated form, so that the water solubility decreases [20] [21].

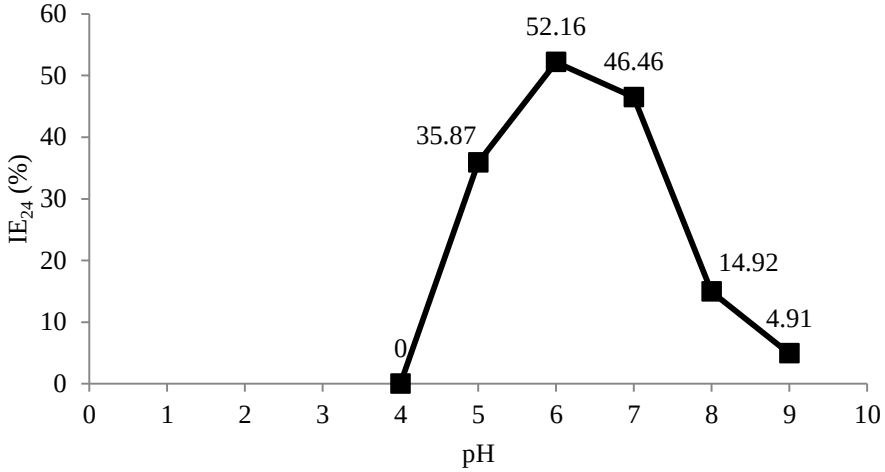

Figure 2. Effect of pH on the emulsification index (EI_{24}) of biosurfactant produced by *Pseudomonas aeruginosa* in crude oil medium.

2.2. Effect of salinity in growth medium on emulsification index

The concentration of salt in certain media can influence the production of biosurfactant from microorganisms. Indeed, in our study, the supplementation of salt for more than 1% did not induce any emulsion, both in diesel fuel and crude oil medium. The emulsion was formed only in 1% NaCl addition both in diesel fuel and crude oil medium for about 24.00% and 26.39%, repectively (figure 3 and 4). Previous study showed that 1.5% NaCl resulted an optimum production of biosurfactant [22]. *P. aeruginosa* used in this study was isolated from fishing harbor area. Other studies reported that *P. aeruginosa* DHT2 isolated from oil contaminated soil was tolerance to salinity for up to 10% due to biosurfactant production. Intrestingly, this *P. aeruginosa* DHT2 strain which were grown in diesel fuel medium as carbon source could yield 60% EI_{24} at 30 ^{o}C [23]. NaCl is known to activate the biosurfactant performance of many strains isolated from sea water or oil reservoir [8].

Previous study showed that EI_{24} value of biosurfactant produced by P. aeruginosa RS29 was decreased in NaCl supplementation for more than 0.8%, however, treatment of 1% NaCl could still maintain its EI_{24} for around 70%, significant reduction for about 40% was detected in 5% NaCl treatment. This indicated that *P. aeruginosa* strain RS29 was not quite tolerant toward salinity.In our study, activity of biosurfactant was not affected (EI_{24} value < 50%) by various concentrations of NaCl tested.

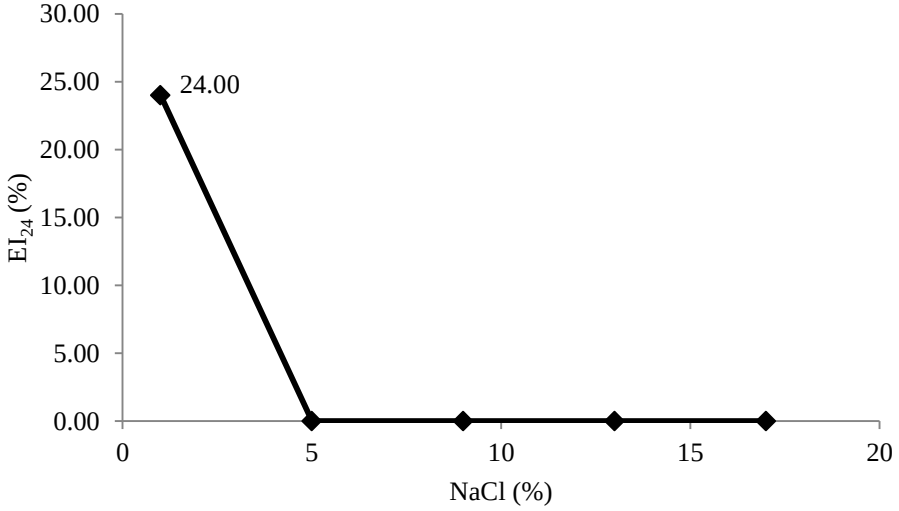

Figure 3. Effect of salt (NaCl) on the emulsification index (EI_{24}) of biosurfactant produced by *Pseudomonas aeruginosa* in diesel fuel medium.

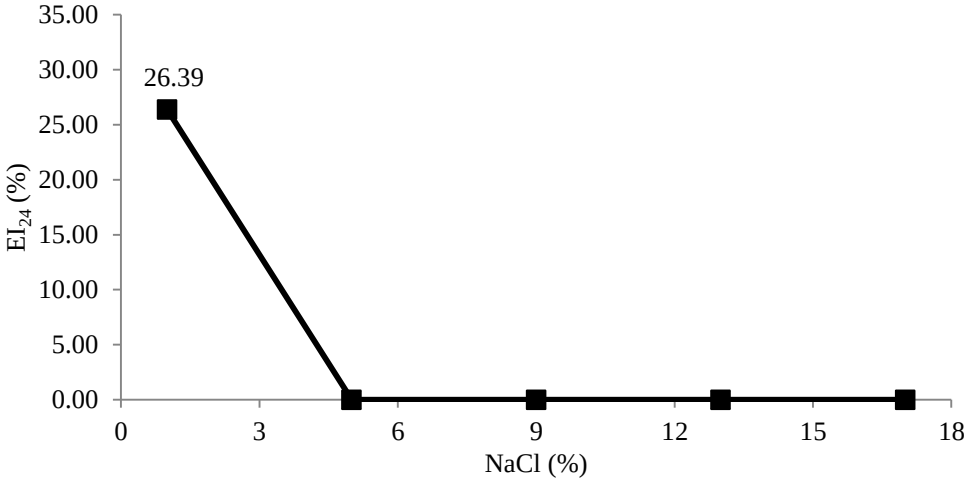

Figure 4. Effect of salt (NaCl) on the emulsification index (EI_{24}) of biosurfactant produced by *Pseudomonas aeruginosa* in crude oil medium.

3. Conclusion

Pseudomonas aeruginosa ATCC 15442, cultured in diesel fuel and crude oil as carbon source, showed biosurfactant activity. Biosurfactant activity is clear from stable emulsions for 24 hours (EI_{24}) in the emulsification test performed. Preliminary optimization showed EI_{24} highest value at pH 7 (27.35%) and 1% NaCl (24.00%) on 10% diesel fuel in mineral salt solution (MSS) medium. As for the crude oil media, the highest EI_{24} value was resulted at pH 6 (52.16%) and 1% NaCl (26.39%).

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