Optimization of rhamnolipid production from bioconversion of palm oil mill effluent (POME) waste by *Pseudomonas stutzeri* BK-AB12 using response surface methodology

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Abstract. Rhamnolipid is one of the most widely used biomaterials in the industry due to it has various applications, such as antimicrobial, anti-corrosion, emulsification, bioremediation, and surfactant for enhanced oil recovery. Rhamnolipid is produced by several bacteria that grow in a medium containing high concentration of lipid as a major carbon source. The production of rhamnolipid is still relatively high cost, therefore, this study is aimed to decrease the production cost of rhamnolipid by utilizing palm oil mill effluent (POME) waste as a major carbon source. *Pseudomonas stutzeri* BK-AB12 has been identified in our previous study as one of the potential rhamnolipid producing bacteria. In order to obtain the best conditions in rhamnolipid production by bacteria, response surface methodology (RSM) was applied in this study. The study begins by observing bacterial growth in a medium salt mineral (MSM) containing POME as a carbon source within concentration 10% to 30% (v/v). The RSM results showed that *P.stutzeri* BK-AB12 optimally produced rhamnolipid after the fermentation was running for 90 hours in the medium containing POME 20% (v/v) and 0.175% (w/v) urea. The produced rhamnolipid was then extracted and characterized. The critical micelle concentration (CMC) of biosurfactant was about 390 mg·L^{-1} with a decrease in surface tension of water about 16 dyne·cm^{-1}, which can be categorized as the potential surfactant for various applications.

1. Introduction

Biosurfactant is one of the secondary metabolites synthesized by natural microorganisms such as bacteria[1]. Biosurfactants are classified into glycolipids, lipopeptides, lipoproteins, fatty acids, neutral lipids, phospholipids, polymeric surfactants and particulate biosurfactants[2]. Rhamnolipid is a biosurfactant of glycolipid group synthesized by natural bacteria, such as *Bukholredia pseudomallei*[3], *Pseudomonas aeruginosa*[4,5], and *Pseudomonas stuzteri*[6,7]. Rhamnolipid has various physiological and industrial applications[8,9]. Rhamnolipid is a biosurfactant of the glycolipid group having a hydrophile group in the form of a sugar ring and a hydrophobic group in the form of a fatty acid tail[10]. The rhamnolipid itself consists of various types and structures depending on the number of sugar groups present and the number of carbon atoms present in the fatty acid chain[2].

Several studies suggested that *P.stutzeri* belongs to a group of bacteria that are unable to produce rhamnolipid[11,12] although other studies suggest vice versa[6,7]. By using blue agar test[13] it is known that *P.stutzeri* BK-AB12 is one of the strains that can produce rhamnolipid. This bacterium was isolated from a salty mud crater at Bledug Kuwu village, Central Java, Indonesia, which is a unique terrestrial

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halophilic habitat that periodically spill out brine to the surface in spite of the fact that it is far from the sea. It is likely made *P.stutzeri* BK-AB12 different from the other typical *P.stutzeri.*

Indonesia is one of the countries that has a large amount of palm oil industry. However, the utilization of waste from the palm oil industry is still rarely used[14]. One of the waste that can be utilized is palm oil mill effluent (POME). Based on the previous research it has been known that *P.stutzeri* BK-AB12 is able to convert POME into rhamnolipid and to get the best condition in rhamnolipid production, optimization step should be done. Therefore, this study aims to optimize the production of rhamnolipid by *P.stutzeri* BK-AB12 by applying response surface methodology.

2. Materials and Methods

2.1. Chemicals and standards

Palm oil mill effluent (POME) was provided by Darmex Argo company, tryptone, yeast extract, sodium chloride, potassium dihydrogen phosphate, potassium hydrogen phosphate, magnesium sulphate heptahydrate and calcium chloride dehydrate were purchased from Sigma-Aldrich.

2.2. Determination of POME composition

POME composition was determined using gas chromatography-mass spectrometry (GC-MS). The POME was first converted into fatty acid methyl esters (FAME) through an esterification reaction[14]. 1 µL of the sample was injected for analysis into GC-MS injector port with the GC injection port temperature was 280 ˚C.

2.3. Biosurfactant identification

Biosurfactant identification was carried out using blue agar plate method[13]. Productive colonies of bacteria that produced rhamnolipid were surrounded by dark blue halos. In this research the supernatant of free cells were tested also on blue agar plate to determined presence of rhamnolipid.

2.4. Biosurfactant production

2.4.1. Microorganism. The microorganism used in this work was *P.stutzeri* BK-AB12 which was obtained from the collection of Biochemistry Research Division, Bandung Institute of Technology.

2.4.2. Media and culture condition. P.stutzeri BK-AB12 was maintained at 8 ˚C on Luria–Bertani (LB) agar plates with 5% (w/v) NaCl added. One colony of cells taken from the LB agar plate was grown in an LB liquid medium at 37 ˚C with an aeration rate of 150 rpm for 24 hours. A total of 2% (v/v) of the LB liquid medium was added to fermentation medium containing 0.1% K_2HPO_4, 0.05% KH_2PO_4, 0.03% $MgSO_4 \cdot 7H_2O$, 0.001% (w/v) $CaCl_2 \cdot 2H_2O$, 5% (w/v) NaCl, urea (w/v) as a nitrogen source and POME waste (v/v) as a carbon source.

2.4.3. Growth measurement. Optical density was used to measure cell growth during fermentation. Optical density was determined by monitoring the absorption at 600 nm using a Spectrophotometer UV-VIS (Shimadzu, USA) of 1 ml of the sample medium. Fermentation medium at 0 hours was used as a control.

2.4.4. Optimization POME and urea composition. Experiments were conducted to determine the optimal concentration of the carbon and nitrogen sources for biosurfactant production. POME content ranged from 10% to 30% (v/v) of the media. Urea optimization was carried out from concentrations of 0.1% to 0.3% (w/v). Biosurfactant production was monitored by the oil spreading test.

2.4.5. Design of experiment. Central composite design (CCD) is one of the most widely used statistical methods based on a multivariate nonlinear model for optimization some variables and also used for determine the equation of the regression model and the optimum conditions in an experiment[15]. This also useful in studying the interactions among variables that may influence the experiment[16]. The response surface methodology was applied to study the optimum condition for bacterial growth and produced rhamnolipid. Analysis of the experimental design data and calculation of the predicted response were carried out using the RSM on Minitab Statistical Software (version 18). The factorial was designed with 20 runs and 1 replicates at the central point (listed in Table 1).

Table 1. Factorial design box to evaluate the effects of POME and urea concentration also effect of incubation time for rhamnolipid production

Run Order	% POME (v/v)	% Urea (w/v)	Incubation (h)
1	14	0.100	72
2	20	0.175	90
3	20	0.175	120
4	20	0.050	90
5	14	0.250	108
6	20	0.175	90
7	20	0.300	90
8	26	0.250	108
9	20	0.175	90
10	20	0.175	90
11	26	0.250	72
12	30	0.175	90
13	14	0.100	108
14	10	0.175	90
15	26	0.100	72
16	14	0.250	72
17	20	0.175	90
18	20	0.175	90
19	20	0.175	60
20	26	0.100	108

2.4.6. Biosurfactant extraction. The rhamnolipid extraction technique was used in combination with base precipitation and organic solvent extraction using chloroform: methanol (2:1). The mixture was then centrifuged at 8000 rpm for 30 min to remove bacterial cells. After that 6 M NaOH was added to the supernatant until reaching pH 12. The precipitate was collected by centrifugation at 8000 rpm for 30 min and then it was diluted with deionized water until pH was about 7. The suspension was transferred to a separating funnel and mixed with chloroform: methanol (2:1) and shaken vigorously. If the yellow faded solution is developed, indicating that the rhamnolipid was successfully extracted. This extraction was carried out three times until no further color persisted in the organic solutions. The sample was filtered and evaporated using a rotary evaporator to yield a yellow-brown biosurfactant[17].

2.5. Biosurfactant characterization

Testing of reduction of water surface tension and critical micelle concentration (CMC) is an important character for biosurfactants[18]. The reduction in surface tension was measured using a tensiometer and Du-Nouy ring. The CMC point was determined by drawing a straight line between points indicating the decrease in surface tension and points when the addition of the surfactant no longer lowers the surface

tension. Extrapolation of both lines and the point where the two lines cross are plotted perpendicular to the biosurfactant concentration to obtain CMC values.

3. Results and Discussions

3.1. POME composition

POME is liquid waste of palm oil industry process. The GC-MS analysis and composition of POME are shown in Table 1. POME is a complex and variable mixture of fatty acids, glycerol and some organic molecules; mainly glycerol 49.00% followed by others component like shown in Table 2.

Table 2. Composition of POME detected by GC-MS.

Name	Formula	Percentage (%)
Glycerol	$C_3H_8O_3$	49.00
Acetone	C_3H_6O	11.60
3-hidroxy-3methoxycarbonil-pentadioic acid	$C_7H_{10}O_7$	10.89
Palmitic acid	$C_{16}H_{32}O_2$	7.46
Butyric acid	$C_4H_{18}O_2$	6.62
Methyl oleic acid	$C_{19}H_{37}O_2$	4.06
1,2-O-Isopropiliden glycerol	$C_6H_{12}O_3$	3.79
5-methyloktena-1	C_9H_{18}	1.60
2-hidroxy-cyclopentadekanon	$C_{15}H_{28}O_2$	1.10
Oktadecanoic acid	$C_{18}H_{34}O_2$	1.03
Toluene	C_7H_8	0.72
Hidantoic acid	$C_3H_6N_2O_3$	0.68
Methyl cyclohexene	C_7H_{14}	0.62
Methyl linoleic acid	$C_{19}H_{34}O_2$	0.45
Methyl myristic acid	$C_{15}H_{30}O_2$	0.21
Methyl 6,9-octadecanoic acid	$C_{19}H_{32}O_2$	0.17

3.2. Optimization of rhamnolipid production

The result of RSM approach in determining optimum condition of rhamnolipid production by varying incubation time, POME and urea concentration is presented in **Table 3**. If bacteria have produced rhamnolipid, it can be checked by performing oil spreading test (OST)[19]. The relationship between OST as dependent variable in each experimental condition was established by a second order polynomial equation (**Eq. 1**) that includes linear, quadratic, and interaction term correspond to CCD.

$$Y = -6.55 + 0.360(X_1) - 3.2(X_2) + 0.0836(X_3) - 0.00951(X_1)^2 - 19.3(X_2)^2 - 0.000668(X_3)^2 - 0.226(X_1X_2) + 0.00141(X_1X_3) + 0.1697(X_2X_3) \quad (1)$$

Y = OST (cm) as dependent variable. The independent variables were X_1 = % (v/v) POME, X_2 = % (w/v) urea, X_3 = incubation time (h). The regression coefficient: -6.55 is a constant coefficient; 0.360,

3.2, 0.0836 are the linear coefficients; 0.00951, 19.3, 0.000668 are the quadratic coefficients, and 0.226, 0.00141, 0.1697 are the interaction coefficient between variable X_1, X_2, X_3.

Table 3. Experimental design and data for response surface analysis

Run order	% POME (v/v)	% Urea (w/v)	Incubation (h)	OST (cm)
1	14	0.100	72	0.8
2	20	0.175	90	2.5
3	20	0.175	120	2.2
4	20	0.050	90	2.0
5	14	0.250	108	1.8
6	20	0.175	90	2.2
7	20	0.300	90	1.8
8	26	0.250	108	3.0
9	20	0.175	90	2.3
10	20	0.175	90	2.5
11	26	0.250	72	1.5
12	30	0.175	90	1.5
13	14	0.100	108	0.8
14	10	0.175	90	1.0
15	26	0.100	72	1.8
16	14	0.250	72	0.8
17	20	0.175	90	2.0
18	20	0.175	90	2.0
19	20	0.175	60	1.0
20	26	0.100	108	2.5

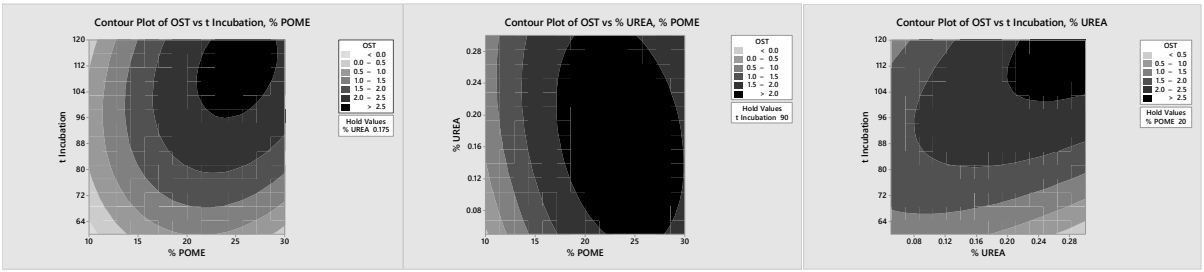
Analysis of variance (ANOVA) was performed to validate the quadratic model. The result provided correlation coefficients (R^2) and P-values of 87.15% and 0.000 (<0.05) respectively, which means the model is significantly fit to the variation observed. Therefore, this regression model could be accepted and satisfactorily useful to obtain the best OD value. This model shows the best OD at POME 26% (v/v), urea 0.25% (w/v) and incubation for 90 hours. To validate this optimum condition, five replicates experiments were performed, which resulting in OST of 2.8 cm in average, less than the model prediction value.

In order to examine the correlation significance of each independent factor, the Minitab program was adopted and the result is presented in **Table 4**. Probability (p) values were used to check the significance correlation between %POME, %urea and incubation time. Among the three factors tested, POME concentration and incubation time appeared to have the highest impact on OST production as it had highest linear coded coefficient and its p-value is less than 0.05. The negative square coefficient found for % POME, % urea, and incubation time indicated that these independent variables were already in its optimum range. Urea concentration does not have a significant effect because the p value is above 0.05[20]. Urea concentration does not have a significant effect because the p-value is above 0.05. This is because the urea variations performed in this experiment are included in the limited nitrogen concentration (under 0.3% (w/v)), rhamnolipid production should be performed in nitrogen limited concentration[21]. In term of interaction between the three tested independent variables, the obtained p-values are all > 0.05 indicating that there was no significant interaction between concentration POME, urea and incubation time on OST.

Table 4. Coded coefficient and significance level (p) of model determined OST

Source	Coded Coefficient	p-Value
Linear		
% POME (v/v)	0.3984	0.001
% urea (w/v)	0.0632	0.484
t incubation (h)	0.3821	0.001
Square		
% POME (v/v) x % POME (v/v)	-0.3362	0.003
% urea (w/v) x % urea (w/v)	-0.1064	0.238
t incubation (h) x t incubation (h)	-0.2125	0.031
Interaction		
% POME (v/v) x % urea (w/v)	-0.100	0.400
% POME (v/v) x t incubation (h)	0.150	0.217
% urea (w/v) x t incubation (h)	0.225	0.076

Further validation on independent variables interaction was performed by visualizing two-dimensional (2D) contour on its response to OST production. Contour showing effects of concentration POME, urea and incubation time was presented in **Figure 1**. Darker region on the contour indicates wider OST performed.



(a) (b) (c)

Figure 1. Contour plots of OST as function of % POME and t incubation (a), % POME and % urea (b), % urea and t incubation (c).

3.3. Rhamnolipid characterization

One of the surfactant characteristics is the ability to lower surface water tension. The critical micelle concentration (CMC) value is determined by drawing a straight line between the drop points of surface tension and the points when the addition of the surfactant no longer lowers the surface tension. The second meeting of the straight line is a CMC title. **Figure 2** shows the water surface tension drop curve.

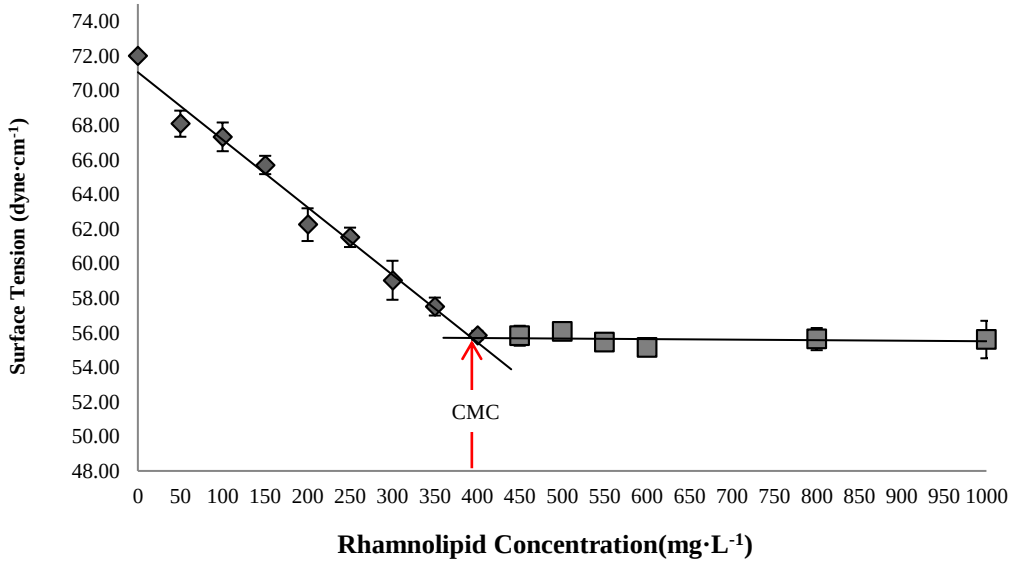


Figure 2. Reduction of surface tension of water and CMC value

Based on **Figure 2**. it can be concluded that the water surface tension reduction is 16 dyne·cm^{-1} and the value of CMC is 390 mg·L^{-1}. The value of CMC is useful for knowing the proper use of biosurfactants in a particular field. For example, in the soap industry small values of CMC are required otherwise in the process of corrosion inhibition of large CMC values are more valuable[20].

4. Conclusions

This study has shown that optimization of factors affecting rhamnolipid production needs to be done to obtain the best formula. Based on the optimization results using response surface methodology, rhamnolipid was optimally produced after fermentation for 90 hours of fermentation in POME concentration of 20% (v/w) and urea 0.175% (w/v), as carbon and nitrogen sources, respectively. The biosurfactant can reduce the surface tension of water by 16 dyne·cm^{-1} with a CMC value of 390 mg·ml^{-1}.

5. Acknowledgment

This research funded and supported by Program Magister dan Doktor untuk Sarjana Unggulan (PMDSU) from Ministry of Research and Higher Education, Indonesia.

References

[1] Lan G, Fan Q, Liu Y, Chen C, Li G, Liu Y, and Yin X 2015 *Biochemistry Engineering Journal* **101** 44 – 45
[2] Desai J D and Banat I M 1997 *Microbiology and Molecular Biology Reviews* **61** 47 – 64
[3] Häuβler S, Rohde M, von Neuhoff N, Nimtz M, Steinmetz I 2003 *Infection and Immunity* **71(5)** 2970 – 2975
[4] Dobler L, de Carvalho B R, Alves Wd S, Neves B C, Freire D M G, Almeida R V 2017 Enhanced rhamnolipid production by *Pseudomonas aeruginosa* overexpressing *estA* in a simple medium. PLoS ONE 12(8): e0183857
[5] Lang S and Wullbrandt D 1999 *Applied Microbiology and Biotechnology* **51(1)** 22 – 32
[6] Celik G Y, Aslim B, and Beyatli Y 2008 *Journal of Environmental Biotechnology* **29** 867 – 870
[7] Onbasli D and Aslim B 2009 *Journal of Enviromental Biotechnology* **30** 161 – 163

[8] Abdel-Mawgoud A M, Lepine F, and Deziel E 2010 *Journal of Applied Microbiology Biotechnology* **86** 1323 – 1336
[9] Maier R M, Soberon-Chavez G 2000 *Applied Microbiology and Biotechnology* **54** 625 – 633
[10] Henkel M, Müller M M, and Kügler J H 2012 *Processing Biochemistry* **47** 1207 – 1219
[11] Chong L and Li Q 2017 *Microbial Cell Factories*,**16**:137
[12] Zhao F, Mandlaa M, Hao J, Liang X, Shi R, Han S and Zhang Y 2014 *Lett Appl Microbiol* **59** 231–7
[13] Siegmund I, and Wagner F 1991 *Biotechnology Technique* **5** 265 – 268
[14] Radzuan M N, Banat I M, and Winterburn J 2017 *Journal of Bioresource Technology* **225** 99 – 105
[15] Sadhukhan B, Mondal N K and Chattoraj S 2016 *Karbala International Journal of Modern Science* **2** 145 – 155
[16] Chattoraj S, Mondal N K, Das B, Roy P and Sadhukhan B 2014 *Journal of Environmental Chemistry and Engineering* **2** 1920 – 1928
[17] Geys R, Soetaert W, and Van Bogaert I 2014 *Current Opinion in Biotechnology* **30** 66 – 72
[18] Pereira A G, Pacheco G J, Taveres L F, Neves B C, Kronemberger F D A, Reis R S, and Freire D M G 2013 *Processes Biochemistry* **48** 1271 – 1278
[19] Satpute S K, Banpurkar A G, Dhakephalkar P K, Banat I M and Chopade B A 2010 *Critical Review of Biotechnology* **30** 1 – 18
[20] Saha P, Chowdhury S, Gupta S, Kumar I and Kumar R 2010 *Clean Soil Air Water* **38** 437 – 445
[21] Gudiña E J, Rodrigues A I, Alves E, Domingues M R, Teixeira J A, and Rodrigues L R 2015 *Bioresource Technology* **177** 87 – 93