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Characterization and Production of Rhamnolipid Biosurfactant in Recombinant *Escherichia coli* Using Autoinduction Medium and Palm Oil Mill Effluent

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HIGHLIGHTS

- Recombinant *E. coli* can produce mono-rhamnolipid and di-rhamnolipid biosurfactants.
- Rhamnolipid congeners were identified using HRMS.
- Di-rhamnolipid has lower surface tension and CMC than mono-rhamnolipid.
- Rhamnolipid production have been optimized to enhance rhamnolipid yield.

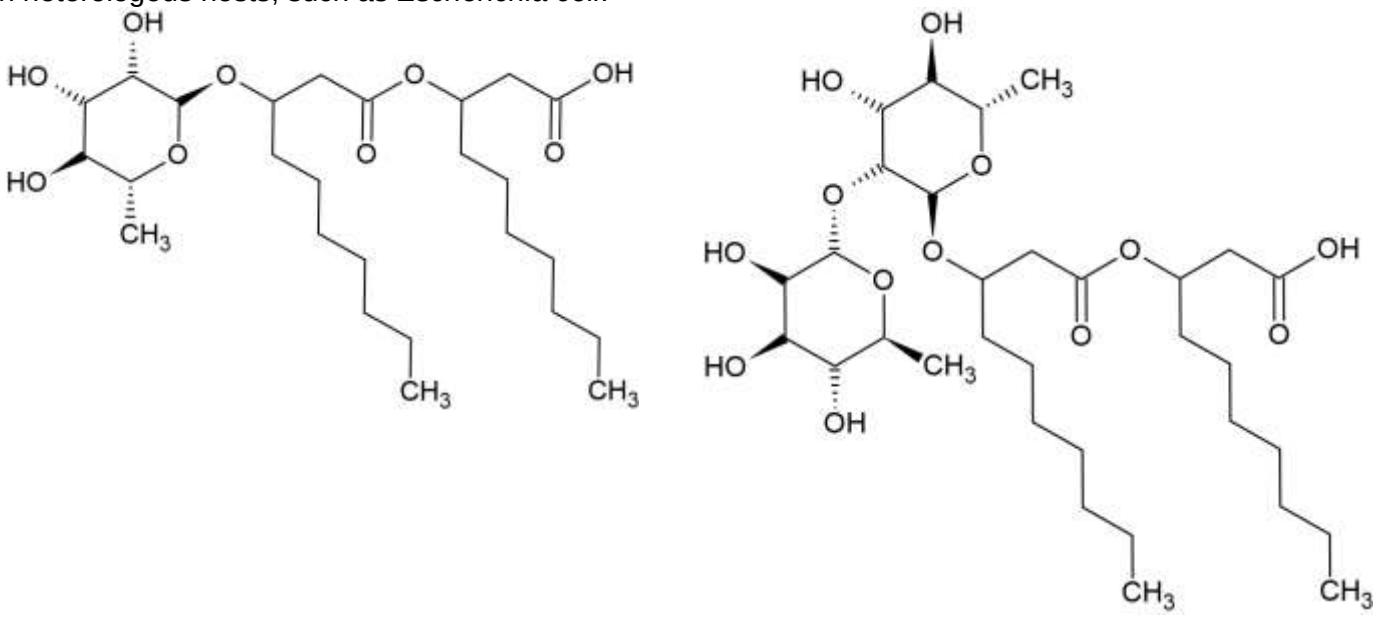
Abstract: Rhamnolipid is a potent biodegradable surfactant, which frequently used in pharmaceutical and environmental industries, such as enhanced oil recovery and bioremediation. This study aims to engineer *Escherichia coli* for the heterologous host production of rhamnolipid, to characterize the rhamnolipid product, and to optimize the production using autoinduction medium and POME (palm oil mill effluent). The construction of genes involved in rhamnolipid biosynthesis was designed in two plasmids, pPM RHLAB (mono-rhamnolipid production plasmid) and pPM RHLABC (di-rhamnolipid production plasmid). The characterization of rhamnolipid congeners and activity using high-resolution mass spectrometry (HRMS) and critical micelle concentration (CMC). In order to estimate rhamnolipid yield, an oil spreading test was performed. HRMS and CMC result show *E. coli* pPM RHLAB mainly produced mono-rhamnolipid (Rha-$C_{14:2}$) with 900 mg/L and 35.4 mN/m of CMC and surface tension value, whereas *E. coli* pPM RHLABC mainly produced di-rhamnolipid (Rha-Rha-C_{10}) with 300 mg/L and 34.3 mN/m of CMC and surface tension value, respectively. The optimum condition to produce rhamnolipid was at 20 h cultivation time, 37 ^{o}C, and pH 7. In this condition, the maximum rhamnolipid yield of 1245.68 mg/L using autoinduction medium and 318.42 mg/L using 20% (v/v) of POME. In conclusion, the characteristics of the rhamnolipid by recombinant *E. coli* is very promising to be used in industries as the most economical way of producing rhamnolipid.

Keywords: rhamnolipid; *Escherichia coli*; biosurfactant; autoinduction medium; POME.

INTRODUCTION

Rhamnolipid is a glycolipid biosurfactant that is naturally produced by *Pseudomonas aeruginosa* [1]. Many rhamnolipid congeners have been identified as having one or two rhamnose residues and β-hydroxy fatty acid chain (C_8-C_{16}) to form mono-rhamnolipid and di-rhamnolipid congeners [2]. As a biosurfactant, rhamnolipid has highest potential prospects, such as biodegradability and non-toxicity compared to synthetic surfactants, low levels of critical micelle concentration (CMC), high emulsion capacity and good solubility that could potentially be used in the pharmaceutical, agricultural industries, and enhanced oil recovery technology [3].

Rhamnolipid biosynthesis is linked to three genes, *rhlA, rhlB,* and *rhlC*. *rhlA* encodes the RhlA enzyme that catalyzes the synthesis of the fatty acid dimer of rhamnolipids and free (3-(3-hydroxyalkanoxyloxy) alkanoic acids (HAA). *rhlB* encodes rhamnosyltransferases I (RhlB), which catalyzes the transfer of activated L-rhamnose to HAA and generates mono-rhamnolipid. *rhlC* encodes rhamnosyltransferases II (RhlC), which catalyzes the transfer of activated L-rhamnose to mono-rhamnolipid and generates di-rhamnolipid (Figure 1) [4,5]. Rhamnolipid synthesized by *P. aeruginosa* are the most frequently studied of biosurfactant. However, *P. aeruginosa* is an opportunistic pathogenic bacterium, therefore limited to industrial-scale production [6]. The pathogenicity of *P. aeruginosa* for rhamnolipid production can be overcome through genetic engineering in heterologous hosts, such as *Escherichia coli*.



(a) (b)

Figure 1. Molecule structure of mono-rhamnolipid (a) and di-rhamnolipid (b).

In this study, we report the expression of rhamnolipid in recombinant *E. coli* BL21(DE3) and isolated novel rhamnolipid congeners. A previous study [6–8] has succeeded in producing rhamnolipid in recombinant *E. coli,* but still low production yield (0.07 - 0.64 g/L) with high-cost material production that using IPTG for T7 promoter induction. So we modified media production by using lactose as an inducer (autoinduction medium) [9] and an agricultural-waste namely palm oil mill effluent (POME)-based medium, which is abundant waste and high environmental impact problems in the palm oil industry in Indonesia [10]. POME has high glycerol and fatty acid contents [11], therefore, it may be used as a carbon source to convert low-value POME to high-value rhamnolipid.

This study aimed to characterize rhamnolipid expression in recombinant *E. coli* by determining rhamnolipid congeners and activity using high-resolution mass spectrometry (HRMS) and critical micelle concentration (CMC). We also optimize rhamnolipid production using the autoinduction medium and observe the addition of POME in recombinant *E. coli*.

MATERIAL AND METHODS

Bacterial strains, plasmids, and culture conditions

Escherichia coli TOP10 (NEB, USA) and *E. coli* BL21 (DE3) (NEB, USA) was used as the heterologous host strain for the plasmids. The plasmid was derived from pUC57 plasmid (Genescript, USA) and inserted *rhlAB* (pPM RHLAB) and *rhlABC* sequences (pPM RHLABC). The strains were maintained on LB (Luria Bertani) agar medium at 37 ^{o}C for 16 h. Experiment were carried out in 250 mL Erlenmeyer flask with 50 mL of production media with following media: autoinduction medium (tryptone, 12 g/L; yeast extract, 24 g/L; $(NH_4)_2SO_4$, 3.3 g/L; KH_2PO_4, 6.8 g/L; Na_2HPO_4, 7.1 g/L; glucose, 0.5 g/L; lactose, 2 g/L, $MgSO_4$, 0.15 g/L; trace elements, 0.03 g/L), M9 medium (M9 salt (Na_2HPO_4, KH_2PO_4, NaCl, NH_4Cl), 5.25 g/L; 1 M $MgSO_4$, 1 M $CaCl_2$), LB (Luria Bertani) medium (tryptone, 10 g/L; yeast extract, 5 g/L; NaCl, 10 g/L) and palm oil mill effluent/POME (Dermex Agro, Indonesia) mixed with M9 medium. The time and pH of the culture were optimized, and when appropriate, 0.5 mM isopropyl-β-D-thiogalactoside (IPTG) and 1% (w/v) lactose were added to induce the *rhlABC* gene. The antibiotic concentration used was ampicillin (10^5 ppm).

Plasmids construction design

The *rhlAB* gene was designed by positioning *rhlA* (Acc. No: AAG06867), and *rhlB* (Acc. No: AAG06866) genes adjacent to each other with a 65 bp nucleotide separating them. The tandem sequence was then ordered to be synthesized at Genescript, USA. The synthesized *rhlAB* gene was inserted into pUC57 as the backbone plasmid, containing the T7*lac* promoter, RBS, CDS (*rhlAB*), rrnb T1 terminator, and T7 terminator, hereinafter referred to as pPM RHLAB. This plasmid constructed (pPM RHLAB) was a donation by the iGEM_ITB2015 team. The *rhlC* gene sequence (Acc. No: AAG04519) was ordered to be synthesized at Macrogen Inc., South Korea, and codon optimization was performed using the GeneArt® GeneOptimizer tool for *Escherichia coli*. The synthetic *rhlC* gene was equipped with the T7*lac* promoter, RBS, CDS (*rhlC*), and T7 terminator. This cassette was then sub-cloned into pPM RHLAB to create pPM RHLABC, which was facilitated by *Sph*1 and *Hind*III digestion.

PCR confirmation was carried out using GoTaq® Green Master Mix (Promega, USA). PCR confirmation was conducted with the specific primer of the *rhlAB* sequence as follows: forward primer (5'-GTTTCTTCGAATTCGCGGCCGCTTCTAG-3') and reverse primer (5'-GTTTCTTCCTGCAGCGGCCGCTACTAGTA-3') in 30 cycles of denaturation at 95 ^{o}C, annealing at 54 ^{o}C, and extension at 72 ^{o}C, while the specific primer of the *rhlC* sequence as follows: forward primer (5'-CTGCATGCGAAGCCAATACGAC-3') and reverse primer (5'-TACGCCAAGCTTTATAAACGCAG-3') in 30 cycles of denaturation at 95 ^{o}C, annealing at 57 ^{o}C, and extension at 72 ^{o}C. The success of this cloning was confirmed by sequencing at Macrogen Inc., South Korea, and analyzed using SnapGeneTM v.4.3.10. The pPM RHLAB and pPM RHLABC plasmids were transformed into competent of *E. coli* TOP10 for cloning purposes and competent of *E. coli* strain BL21(DE3) for expression.

Expression of rhamnolipid biosurfactant in recombinant E. coli

A positive *E. coli* colony containing pPM RHLAB and pPM RHLABC was inoculated into a liquid culture of 5 mL LB medium. Five percent (v/v) of the overnight culture was inoculated into 50 mL LB at 37 ^{o}C, with a shake at 250 rpm for 2 - 4 h. After OD_{600} reached to obtain OD_{600} of 0.5 - 0.7, five mL of this culture was separated as non-induced culture, and then the other 45 mL was induced by 0.5 mM IPTG (final concentration). After 20 h, the culture was taken to extract rhamnolipid and characterized.

Extraction of rhamnolipid from recombinant E. coli

Extraction of mono-rhamnolipid (pPM RHLAB) and di-rhamnolipid (pPM RHLABC) by liquid extraction [12]. The culture was centrifuged for 15 min at 7000 g and 4 ^{o}C. The supernatant was mixed with chloroform:ethanol (2:1), stirred for 5 min for equilibrium, and repeated for extraction twice. The organic phase was collected and evaporated the solvent by oven-drying and leaving a brown paste (rhamnolipid).

Rhamnolipid congener diversity by HRMS and CMC determination

Rhamnolipid extraction from pPM RHLAB and pPM RHLABC strains was characterized by high-resolution mass spectrometry (HRMS), and the rhamnolipid activity was determined by critical micelle concentration (CMC) as well as surface tension. To determine of rhamnolipid congener diversity from *E. coli* BL21(DE3) pPM RHLAB and pPM RHLABC, each 10 mg of rhamnolipid were dissolved into deuterated

chloroform, and 100 µL solution was injected for TOF-MS analysis in negative ion mode (LCT Premier XE Micromass, USA). The scanning mass spectrum ranged from 300 m/z to 800 m/z. HRMS peaks were identified and analyzed based on $[M-H]^-$ m/z [2] to determine rhamnolipid congeners from *E. coli* pPM RHLAB and *E. coli* pPM RHLABC.

Each of the surfactant (sodium dodecyl sulfate/SDS as the positive control, mono-rhamnolipid, and di-rhamnolipid) at 50 mg/L - 2500 mg/L concentration was made to determine CMC value. The surface tension of each surfactant was measured until no reduction in surface tension for an added higher concentration of surfactants. The determination of CMC was measured using a Du-Nouy ring tensiometer (Surface Tensiomat® FISHER Model 21, USA). All of the experiments were conducted in triplicate, and the mean values ± standard deviation were reported. The best rhamnolipid activity from recombinant strains (pPM RHLAB or pPM RHLABC) was selected and optimized the production by using autoinduction and POME media.

Determination of the rhamnolipid production curve in recombinant *E. coli*

The standard growth curve was first created as a reference to estimate the number of living colonies (colony form unit/cfu) on each OD value to calculate the cell number of recombinant *E. coli.* In this study, the method was used for creating the standard curve throughout OD 0.1 - 0.9 and maintained on LB medium agar containing ampicillin antibiotics (10^5 ppm) at 37 ºC for 16 hours. Colonies growing on each Petri dish were counted, and a regression curve was made.

The rhamnolipid production curve was carried out by sampling every 3 h of cultivation culture. A 5% (v/v) pre-culture of recombinant *E. coli* was inoculated into 50 mL of the autoinduction medium and grown at 37 ºC at 250 rpm. The oil spreading test (OST) was performed to estimate the highest production time and rhamnolipid yield. The experiment was performed in triplicate, and the mean values ± standard deviation were reported.

Analysis of rhamnolipid concentration using oil spreading test (OST)

The oil spreading test (OST) technique was used to analyze the surfactant activity qualitatively. The OST technique was carried out by adding 30 mL of water to a Petri dish (Area: 78.5 cm^2) and adding 100 µL of crude oil (from PT Pertamina, Mangunjaya refinery, South Sumatera). A supernatant containing the surfactant was dripped as much as 100 µL at the midpoint of a Petri dish; as a result, an oil-spreading zone (OSZ) appeared [13]. The area of the OSZ was performed using the NIH ImageJ software [14] with the following formula:

$$Area_{OSZ} = Area_{DISH} (Pixel_{OSZ}/Pixel_{DISH}) \quad (1)$$

$Area_{OSZ}$ and $Area_{DISH}$ are the areas of the circle of OSZ and the Petri dish (78.5 cm^2), while $Pixel_{OSZ}$ and $Pixel_{DISH}$ are pixel measurements from the NIH ImageJ software, respectively. Determination of rhamnolipid concentration from recombinant *E. coli* fermentation culture was carried out using an oil spreading test technique on rhamnolipid standards. The rhamnolipid standard (AGAE Tech, USA) was used from 100 mg/L to 700 mg/L. The $Area_{OSZ}$ was obtained from the OST at each standard rhamnolipid standard concentration and formed a linear regression curve. The rhamnolipid standard curve was used to estimate the rhamnolipid concentration from recombinant *E. coli* cultures [15].

Optimization of pH and temperature of rhamnolipid production in autoinduction medium and POME

Optimization of rhamnolipid production from recombinant *E. coli* was carried out at variations of pH 4, 7 and 9 and temperatures of 30 ºC, 37 ºC and 40 ºC. Cultures were grown for optimal cultivation time, and subsequently, the supernatant was obtained and measured the concentration of rhamnolipid to show of $Area_{OSZ}$, and its value was interpolated by rhamnolipid standard regression. The experiment was performed in triplicate.

The optimal concentration of the POME-based medium was determined by the added variation of POME concentration (10%, 15%, 20%, and 25% (v/v)) in M9 medium. Induction was carried out with 1% (w/v) lactose when the culture OD reached 0.6. Cultures were grown at the optimal cultivation time, temperature, and pH of rhamnolipid production. The supernatant was obtained and measured the concentration of rhamnolipid to show of $Area_{OSZ}$, and the value was interpolated by rhamnolipid standard regression. The experiment was performed in triplicate.

RESULTS

Plasmid construction design of rhamnolipid expression

The size of the *rhlAB* part was 2551 bp, and the *rhlC* part was 1129 bp (Figure 2). This size was also confirmed by PCR confirmation with the specific primers of *rhlAB* and *rhlC* parts (Figure 3).

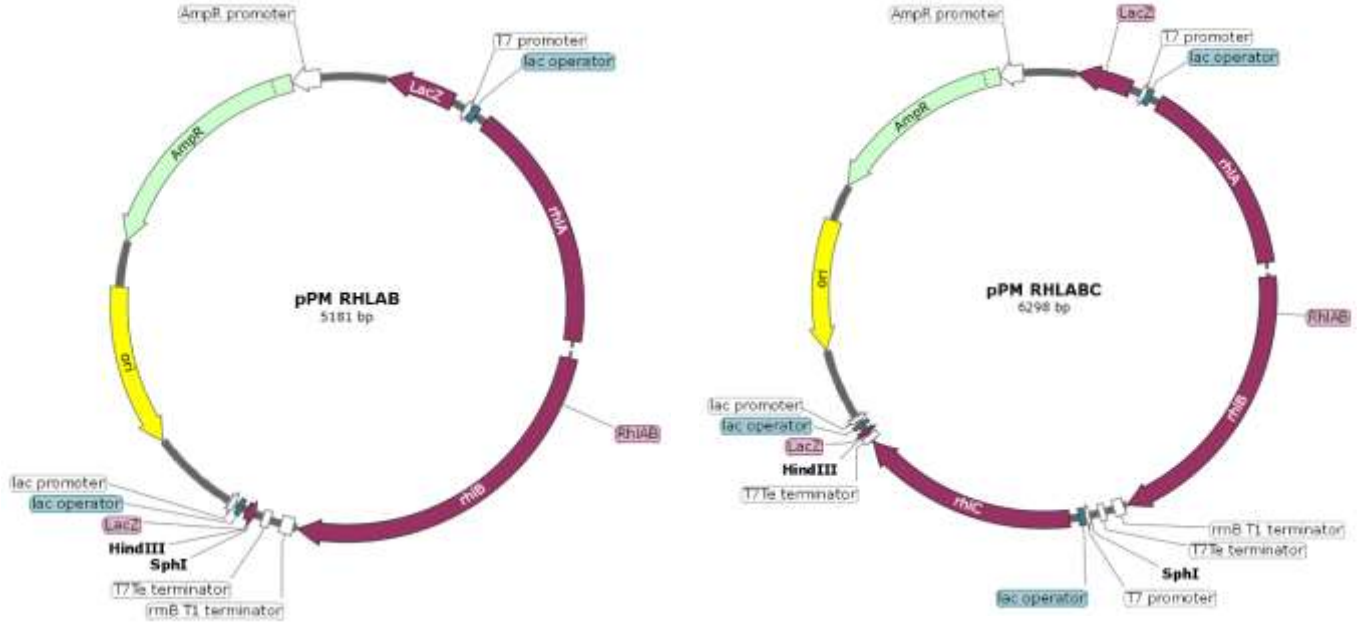


Figure 2. pPM RHLAB (a) and pPM RHLABC (b) construction map.

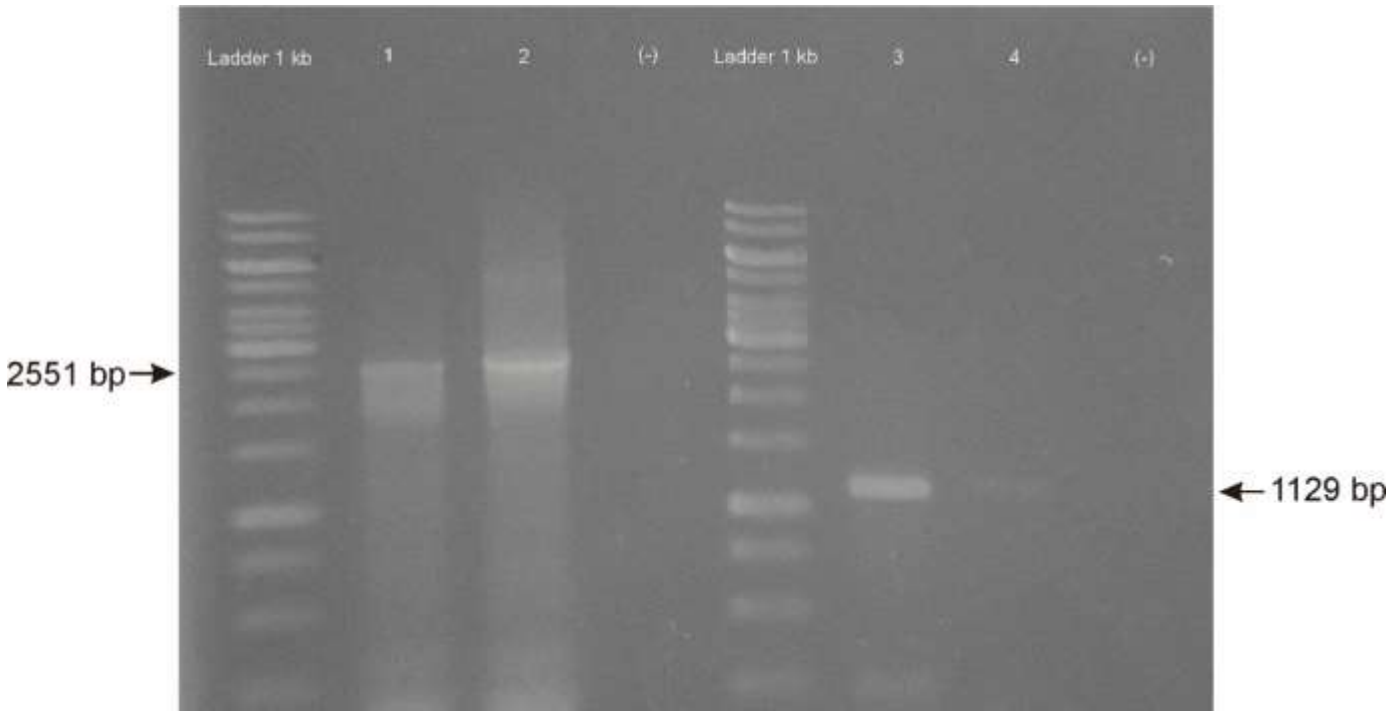


Figure 3. PCR confirmation of pPM RHLAB (1) and pPM RHLABC (2) using specific primers for *rhlA* and *rhlB* genes and PCR confirmation of RHLABC (3 and 4) with the specific primer *rhlC* gene. (-) is the negative control.

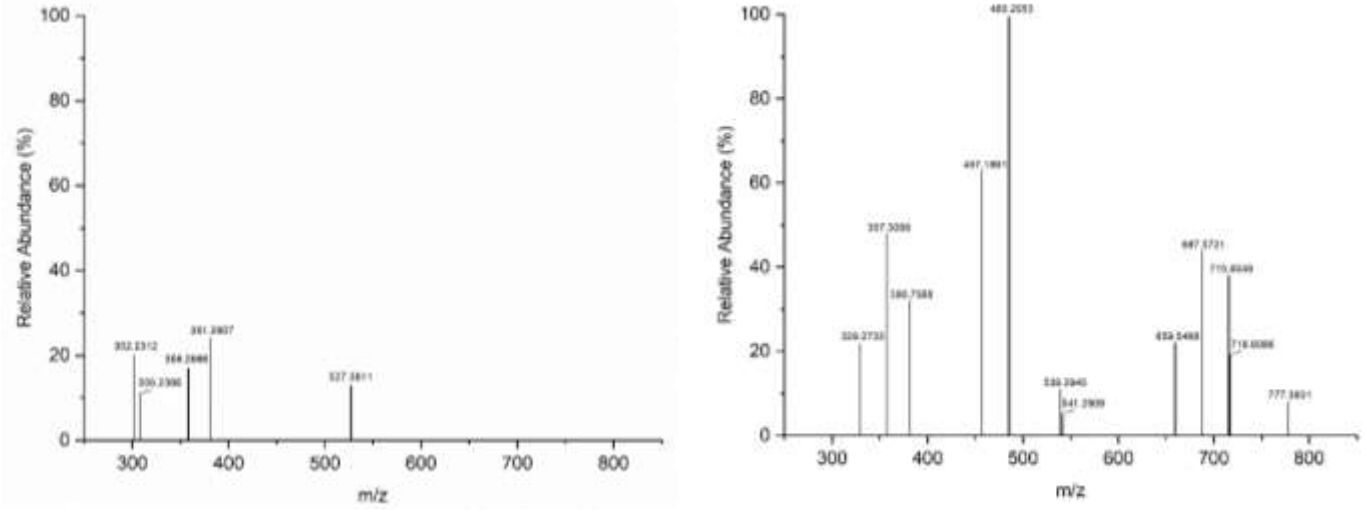
Figure 2 shows that both parts have the same promoter and terminator, namely T7*lac* promoter, and T7 terminator. However, pPM RHLAB has an additional terminator, which is the rrnb T1 terminator. The *rhlC* gene was driven by a separate promoter from the *rhlAB* gene. PCR confirmation of pPM RHLAB and pPM RHLABC (Figure 3) confirmed that both recombinant strains consisted of desired genes (*rhlAB* and *rhlC*).

Therefore, it can be concluded that both plasmids were successfully transformed into *E. coli* BL21(DE3) and continued for rhamnolipid expression and characterization.

Characterization of rhamnolipid expression by HRMS and determination of CMC

Characterization of rhamnolipid expression by HRMS

HRMS analysis aims to confirm rhamnolipid congeners expressed by *E. coli* pPM RHLAB and pPM RHLABC. Peaks of HRMS (Figure 4) from both rhamnolipid products were analyzed and compared to the rhamnolipid diversity database, which has been reported by Abdel-mawgoud [2]. Table 1 and Table 2 show rhamnolipid congeners from rhamnolipid of *E. coli* pPM RHLAB and pPM RHLABC.



(a) (b)

Figure 4. HRMS spectra of the rhamnolipids produced by *E. coli* pPM RHLAB (a) and *E. coli* pPM RHLABC. The *X*- and *Y-axe*s indicate m/z (mass-to-charge ratio) and intensity/relative abundance (%), respectively.

Table 1. Relative abundance of main rhamnolipid congeners produced by recombinant *E. coli* pPM RHLAB by HRMS.

Rhamnolipid congeners	M. Form	$[M-H]^-$ m/z	Relative abundance (%)
Mono-rhamnolipid			
Rha-$C_{8:2}$	$C_{14}H_{22}O_7$	302.2312	20
Rha-C_8	$C_{14}H_{36}O_7$	308.2386	11
Rha-$C_{12:2}$	$C_{18}H_{30}O_7$	358.2986	17
Rha-$C_{14:2}$	$C_{20}H_{34}O_7$	381.2907	24
Rha-C_{10}-$C_{12:1}$	$C_{28}H_{46}O_9$	527.3611	13

Table 2. Relative abundance of main rhamnolipid congeners produced by recombinant *E. coli* pPM RHLABC by HRMS.

Rhamnolipid congeners	M. Form	$[M-H]^-$ m/z	Relative abundance (%)
Mono-rhamnolipid			
[1]Rha-$C_{10:2}$	$C_{16}H_{26}O_7$	329.2733	21.7
Rha-$C_{12:2}$	$C_{18}H_{30}O_7$	357.3086	48
Rha-$C_{14:2}$	$C_{20}H_{34}O_7$	380.7588	32
[1]Rha-C_{10}-$C_{13:2}$	$C_{29}H_{52}O_9$	539.2943	11
[1]Rha-C_{10}-$C_{13:1}$	$C_{29}H_{54}O_9$	541.2909	5
Di-rhamnolipid			
Rha-Rha-C_8	$C_{20}H_{36}O_7$	457.1891	63
Rha-Rha-C_{10}	$C_{22}H_{40}O_{11}$	485.2053	99.5
[1]Rha-Rha-C_{10}-$C_{11:2}$	$C_{33}H_{52}O_{13}$	659.5488	22
[1]Rha-Rha-C_{12}-$C_{11:2}$	$C_{35}H_{60}O_{13}$	687.5721	44
[1]Rha-Rha-C_{12}-$C_{13:2}$	$C_{38}H_{64}O_{13}$	715.6049	38
[1]Rha-Rha-$C_{13:2}$-C_{12}	$C_{38}H_{64}O_{13}$	716.6086	19
[1]Rha-Rha-C_{14}-C_{15}	$C_{41}H_{78}O_{13}$	777.3831	8

[1] Novel rhamnolipid congeners produced by *E. coli* pPM RHLABC

High-resolution mass spectrometry (HRMS) results show that rhamnolipid from recombinant *E. coli* pPM RHLAB consist of five rhamnolipid congeners and rhamnolipid from *E. coli* pPM RHLABC consists of 12 rhamnolipid congeners. Furthermore, *E. coli* pPM RHLAB produced mono-rhamnolipid congeners, namely Rha-$C_{14:2}$ with higher relative abundance (24%) and Rha-C_8 with lower relative abundance (11%). In comparison, the *E. coli* pPM RHLABC produced di-rhamno mono-lipid (Rha-Rha-C_{10}) with higher relative abundance (99.5%) and mono-rhamno di-lipid (Rha-C_{10}-$C_{13:1}$) with lower relative abundance (5%). Therefore, it can be concluded that *E. coli* pPM RHLAB only produces mono-rhamnolipid (by expression of *rhlA,* and *rhlB* genes) and *E. coli* pPM RHLABC can produce both mono-rhamnolipid and di-rhamnolipid (by expression of *rhlA, rhlB,* and *rhlC* genes).

In this stimulated high-resolution mass spectrometry, the ion of Rha-$C_{14:2}$ has the highest relative abundance and peak among the other of mono-rhamnolipid congeners, so Rha-$C_{14:2}$ was the base peak of rhamnolipid produced by *E. coli* pPM RHLAB. However, in the di-rhamnolipid congeners, Rha-Rha-C_{10} has the highest relative abundance and peak among the other of rhamnolipid congeners (mono- or di- types), so Rha-Rha-C_{10} was the base peak of rhamnolipid produced by *E. coli* pPM RHLABC. In the interpreting of HRMS result, for instance, Rha-Rha-C_8 has a relative abundance of 63%, meaning the mass spectrum contains 63% as many ions of *m/z* Rha-Rha-C_{10} (the base peaks). Note that this does not mean 99.5% of the ions produced have *m/z* = Rha-Rha-C_{10} and another 63% have *m/z* = Rha-Rha-$C_{8,}$ because it was not possible to produce more than 100%.

Tables 1 and 2 show that recombinant *E. coli* pPM RHLABC produces novel rhamnolipid congeners that different congeners have been reported, but it was not from *E. coli* pPM RHLAB [2]. The novel rhamnolipid congeners from *E. coli* pPM RHLABC consist of mono-rhamno mono-lipid (Rha-$C_{10:2}$), mono-rhamno di-lipid (Rha-C_{10}-$C_{13:2}$ and Rha-C_{10}-$C_{13:1}$), and di-rhamno di-lipid (Rha-Rha-C_{10}-$C_{11:2}$, Rha-Rha-C_{12}-$C_{11:2}$, Rha-Rha-C_{12}-$C_{13:2}$, Rha-Rha-$C_{13:2}$-C_{12} and Rha-Rha-C_{14}-C_{15}).

Determination of rhamnolipid CMC from recombinant E. coli

The best biosurfactant activity has a lower surface tension (ST) value and critical micelle concentration (CMC). The critical micelle concentration (CMC) is the minimum concentration required to form micelles and is defined by the surface tension of various surfactant concentrations. Rhamnolipid from *E. coli* pPM RHLAB and pPM RHLABC showed different values of CMC (Figure 5).

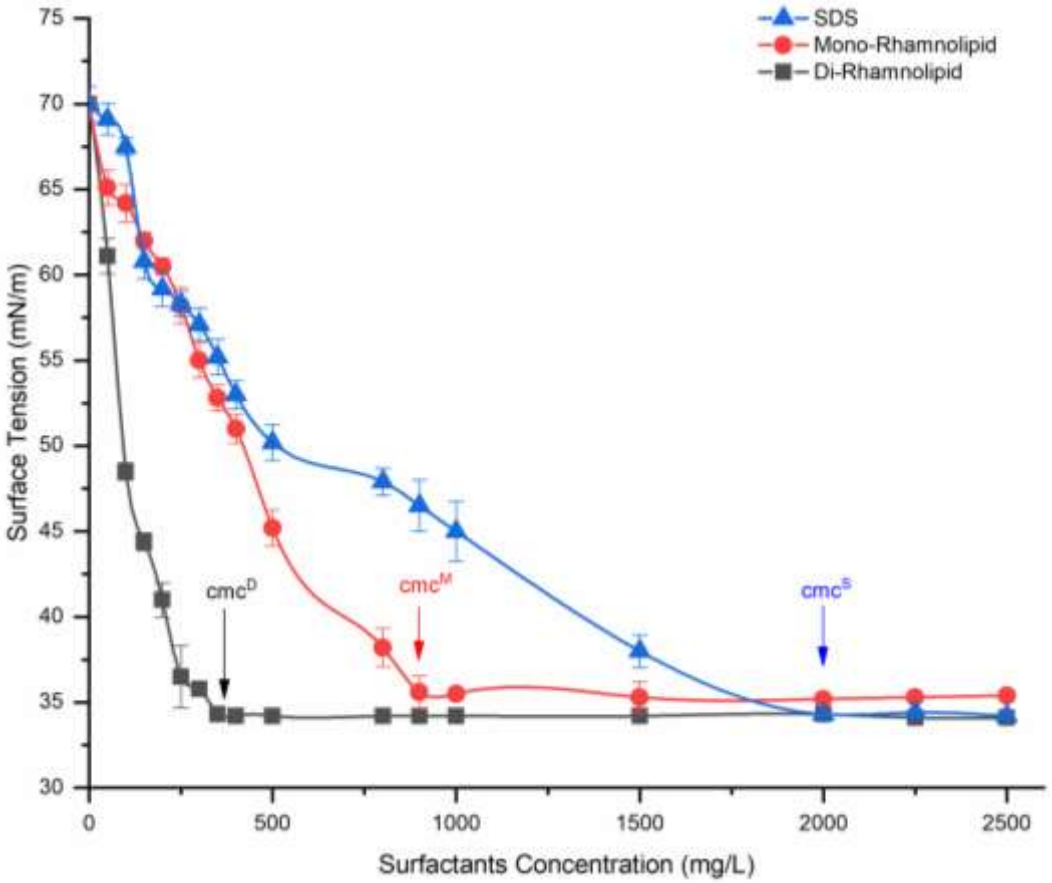
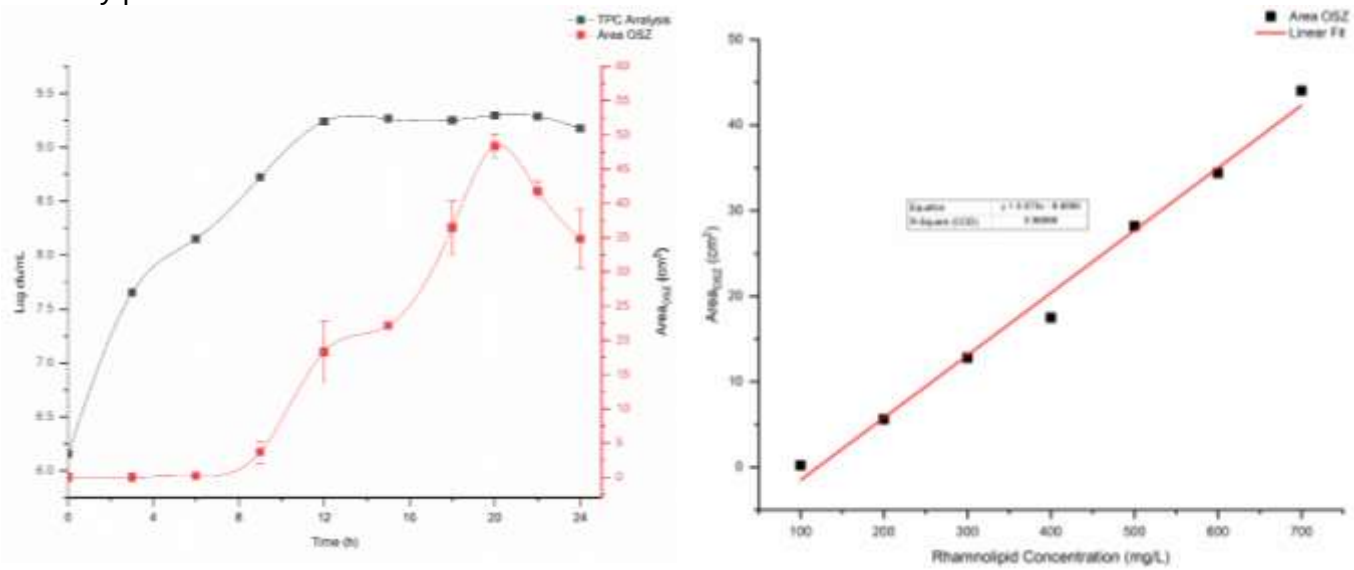


Figure 5. Critical micelle concentration (CMC) of SDS (sodium dodecyl sulfate), mono-rhamnolipid, and di-rhamnolipid. cmc^S, cmc^M, and cmc^D are the values of CMC for SDS, mono-rhamnolipid, and di-rhamnolipid, respectively.

Figure 5 shows that di-rhamnolipid has the lowest CMC value (300 mg/L) with a surface tension value of 34.3 mN/m than mono-rhamnolipid (900 mg/L CMC value and 35.4 mN/m surface tension value). Whereas, sodium dodecyl sulfate had the highest CMC value (2000 mg/L) with a surface tension value of 34.2 mN/m than both biosurfactants. Because di-rhamnolipid from *E. coli* pPM RHLABC has lower surface tension and CMC, *E. coli* pPM RHLABC was continued to optimize production by using autoinduction medium and POME-based medium.

Optimization of rhamnolipid production from recombinant *E. coli* in autoinduction medium

The culture duration and physiological conditions for biosurfactant production were essential to optimize for the determination of optimal conditions. Figure 6a shows the rhamnolipid production curve in recombinant *E. coli* using the autoinduction medium. Optimal production of rhamnolipid can be considered on the production curve at 20 h culture time with $Area_{OSZ}$ of 48.3 cm^2. The optimal culture time of 20 h lies in the stationary phase of recombinant *E. coli*.



(a) (b)

Figure 6. Rhamnolipid production from recombinant *E. coli* in autoinduction medium. TPC analysis is the total plate count is counted in cfu/mL, and area OSZ is the area from the oil spreading test.

Based on Figure 6a, rhamnolipid was early expressed at 9 h of cultivation time with an $Area_{OSZ}$ of 3.8 cm^2, but still with a deficient concentration. Production of rhamnolipid begins to be fully expressed at the end of the log phase, and the optimal time was 20 h. However, at the end of the stationary phase (exceed the optimal time), rhamnolipid expression decreased due to enter the cell death phase and the accumulation of toxic substances. On the other sides, the precursor of RhlB (L-rhamnose) may be declined after the optimal time and mainly produced HAA (the expression of *rhlA)*, which lower of surface tension and $Area_{OSZ}$ than rhamnolipid.

We determined rhamnolipid concentration in culture supernatant from recombinant *E. coli* culture using oil spreading test and obtained the rhamnolipid standard curve equation; $y = 0.073x - 8.8094$; $R^2 = 0.99$, which x = rhamnolipid concentration and y = $Area_{OSZ}$ (Figure 6b). Figure 6b shows that there was a positive correlation between $Area_{OSZ}$ and rhamnolipid concentration; therefore, this method can be applied to estimated rhamnolipid concentrations more accurately than using the staining method, such as anthrone or orcinol reagent, with a spectrophotometer due to overestimating result of rhamnolipid yield measured [15].

Rhamnolipid production in the wild type, e.g., *Pseudomonas aeruginosa*, it was dependent on pH and temperature. Hence, we considered observing pH and temperature effects of rhamnolipid production in recombinant *E. coli*. Based on Figure 7 shows that the optimal rhamnolipid production lies at 37 ^oC with rhamnolipid concentrations reaching 1245 ± 142.49 mg/L compared to temperatures of 30 ^oC (516 ± 66.12 mg/L), and 40 ^oC (130 ± 7.50 mg/L). The optimum pH was found at pH 7, with a similar quantity produced at 37 ^oC. However, changing the pH value affects of rhamnolipid yield with at pH 5 and pH 9 was 499 ± 113.20 mg/L, and 389 ± 68.10 mg/L, respectively.

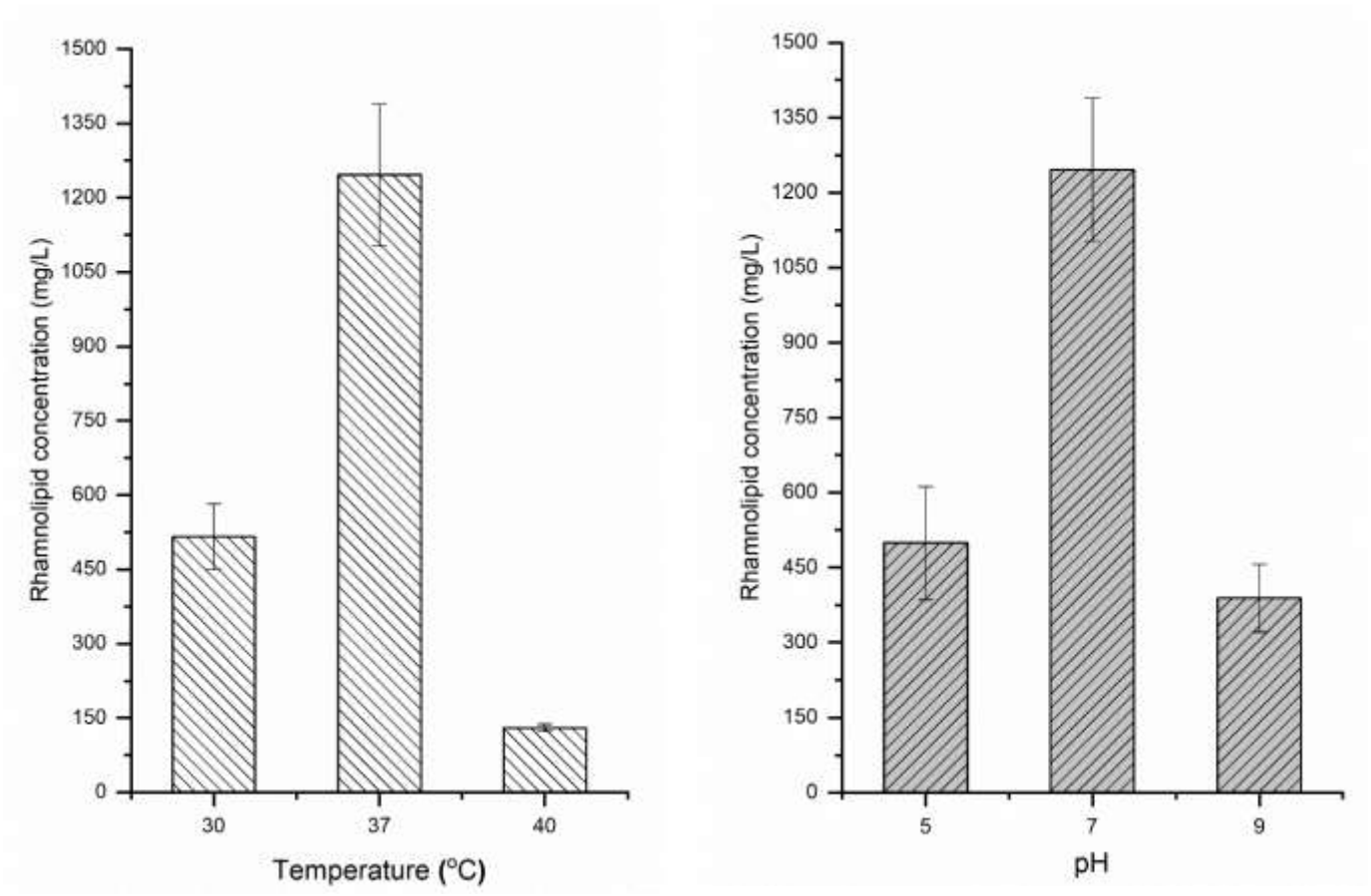


Figure 7.Optimization of rhamnolipid production from recombinant *E. coli* at variations of temperatures and pH. The values are mean ± S.D of triplicate analyses

Optimization of rhamnolipid production in palm oil mill effluent (POME) medium

In this study, we optimized the POME concentration as an economical medium in recombinant *E. coli* to produce rhamnolipid. POME was mixed with M9 medium as a source of pH buffer and nitrogen sources. The culture in this medium was induced by 1% (w/v) lactose, which optimal lactose concentration to induce T7*lac* promoter in recombinant *E. coli* [17]. Based on Figure 8, the highest concentration of POME medium was obtained at a concentration of 20% (v/v) (318.42 ± 35.12 mg/L), while the lowest concentration was obtained at a concentration of 25% (v/v) (180.40 ± 32.31 mg/L). In comparison, at a concentration of 10% (v/v) and 15% (v/v) was 211.58 ± 24.5 mg/L and 305.59 ± 11.29 mg/L, respectively. However, we found that the optimal range of POME concentration was 15% - 20% (v/v).

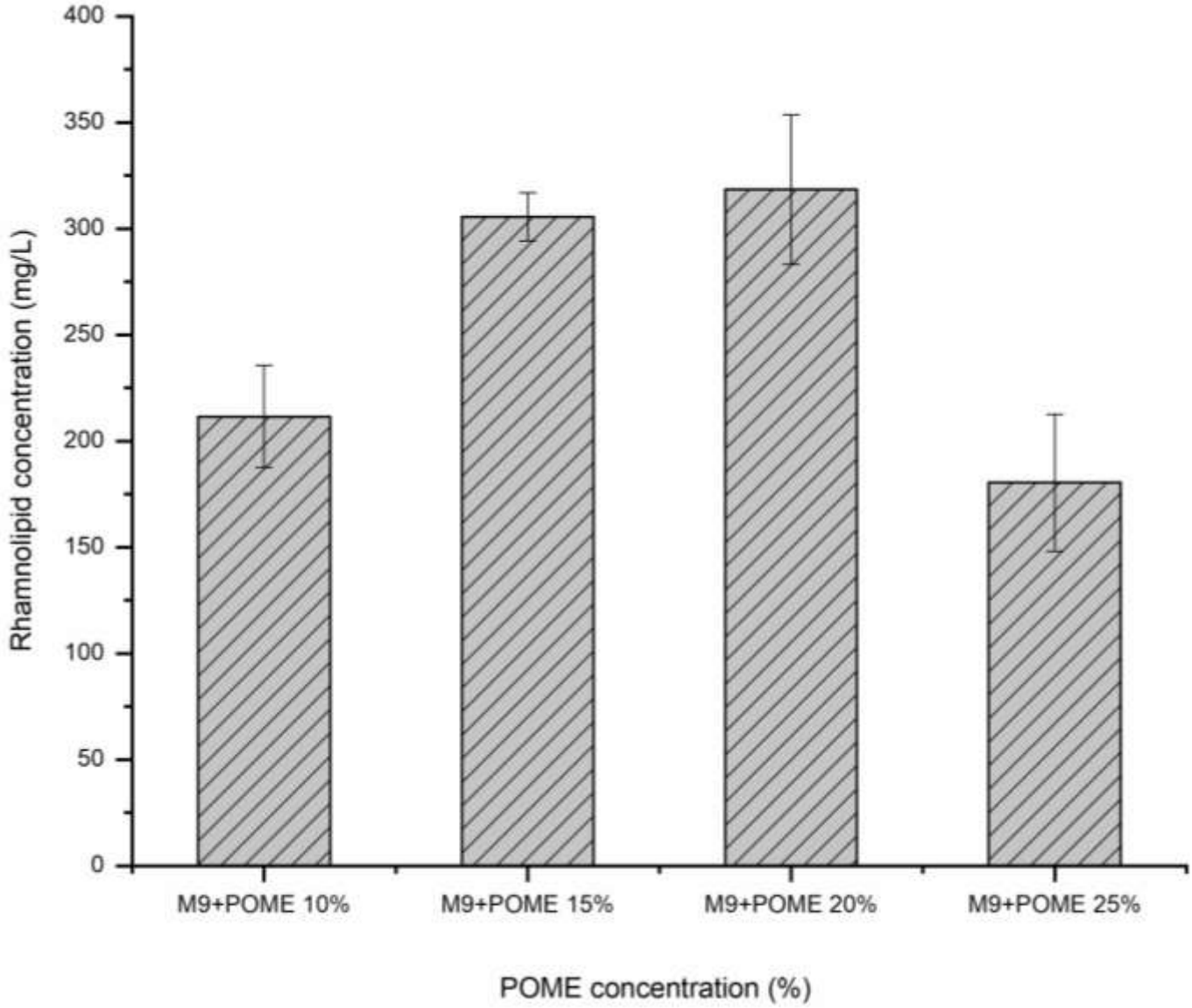


Figure 8. Optimization of rhamnolipid production from recombinant *E. coli* in POME-based medium. The values are mean ± S.D of triplicate analyses.

DISCUSSION

Rhamnolipid is a biodegradable surfactant, which is extensively used in industrial and enviromental application such as the pharmaceutical, food industries, and enhances oil recovery [18]. In this study, we transformed the rhamnolipid gene by synthetic approach from *Pseudomonas aeruginosa,* which is pathogenic bacterium, into a GRAS (generally recognized as safe) strain, such as *Escherichia coli*. The *rhlABC* gene was driven by the T7*lac* promoter to increase synthesis of rhamnolipid. However, the *rhlC* gene was driven by a separate T7*lac* promoter from the *rhlAB* gene. They consist of two separate operons that mimic the two natural operons in *Pseudomonas aeruginosa* [16]. In *P. aeruginosa*, the *rhlA* and *rhlB* genes are arranged in an operon adjacent to *rhlRI*, a regulator that plays a role in quorum sensing whereas the *rhlC* gene was located at a distance far away from the *rhlAB* operon (PA1131 locus), which has no promoter part in the upstream section [19].

To investigate the composition of rhamnolipid congeners by *E. coli* pPM RHLAB and pPM RHLABC whether both plasmids encode mono-rhamnolipid or di-rhamnolipid, each of the rhamnolipids extractions were subjected to HRMS analysis. We found that *E. coli* pPM RHLAB only encodes mono-rhamnolipid, and *E. coli* pPM RHLABC may encode mono-rhamnolipid and di-rhamnolipid. However, rhamnolipid congeners by *P. aeruginosa* mainly consist of Rha-C_{10} and Rha-Rha-C_{10}-C_{10} [13], but our recombinant strain mainly produces Rha-$C_{12:2}$ (mono-rhamnolipid) and Rha-Rha-C_{10} (di-rhamnolipid). The difference in rhamnolipid congeners may be caused by various factors such as strains, carbon sources, and culture conditions [20]. Additionally, it influences rhamnolipid activity, which di-rhamnolipid facilitates a decrease in surface tension

values [21]. Furthermore, in the analysis of critical micelle concentration (CMC), we found that di-rhamnolipid have lower CMC values and surface tension value compared to mono-rhamnolipid and SDS (sodium dodecyl sulfate) as synthetic/chemical surfactants. Mono-rhamnolipid is less soluble and adsorbed to surfaces more strongly, requiring higher CMC for the solubilization of hydrocarbons than di-rhamnolipid [22]. Because di-rhamnolipid has lower CMC, di-rhamnolipid has more activities and efficiency than mono-rhamnolipid [19,23]. Further, we optimized di-rhamnolipid production from *E. coli* pPM RHLABC using autoinduction and POME media.

The rhamnolipid production in recombinant *E. coli* using autoinduction medium was inspired by the research of Studier [9], where the production of rhamnolipid itself was not induced by IPTG, but by lactose. This is because IPTG is expensive and not suitable for later to continue on an industrial application. Using the autoinduction medium allows more optimal production of rhamnolipid without knowing specific OD values in the induction process (as was carried out in expressions with the IPTG inducer). Growth of recombinant *E. coli* using autoinduction medium may use glucose as a primary carbon source until glucose depletes in the mid to late-log phase, and lactose will be used as a second carbon source while inducing T7*lac* promoter [9], so the expression of the *rhlABC* gene can occur. The glucose and lactose content in autoinduction medium may also increase rhamnolipid yield production. This is because glucose as a source in the formation of rhamnose in rhamnolipid biosynthesis [24]. This autoinduction medium itself has been previously used in protein expression but has never been conducted in rhamnolipid expression from wild-type or recombinant strains. Previous research on rhamnolipid production from recombinant *E. coli* still uses LB medium and was induced with IPTG [6,8,25]. The use of autoinduction medium is a novelty for producing high yield rhamnolipid in recombinant *E. coli*.

In *P. aeruginosa*, the wild-type rhamnolipid production strain, the best rhamnolipid production was in the range of 25 - 37 ^{o}C, and the best pH was in the range of 6 - 6.8, whereas above 42 ^{o}C and pH 7 decreased rhamnolipid production [26]. Similarly, we found that rhamnolipid production in recombinant *E. coli* was optimum at 37 ^{o}C and pH 7 using autoinduction medium. *P. aeruginosa* and *E. coli* may live inside the human body therefore the human body temperature of 37 ^{o}C and pH 7 will be comfortable for both microorganisms. Interestingly, for 24 h of cultivation time, our rhamnolipid yield in recombinant *E. coli*, which harboring pPM RHLABC, by using autoinduction medium was higher (1245 mg/L) than from *P. aeruginosa* (206 mg/L) [7], and other recombinant strains that have been reported, such as *E. coli* HB101 (pINC94-*rhAB)*, *E. coli* BL21(DE3) (ETRABC-*rhlABC)*, and *E. coli* BL21(DE3) (DJ208-*rhlABC)* were 70.5 mg/L, 120 mg/L, and 640 mg/L, respectively [6–8]. As a result, it is very promising for further bioprocessing of our recombinant strain to enhance large-scale production.

To investigate the addition of POME in fermentation culture, variations of POME concentration were observed. We found that the optimal concentration was 20% (v/v) in POME addition. POME is an agricultural waste with high BOD and COD content, so the addition of POME exceeding 20% (v/v) may cause nonoptimal bacterial growth and decreased rhamnolipid production. Analysis of POME by GC-MS shows that it contains complex compounds, including glycerol, fatty acids, and other organic compounds with a glycerol content of 49% [11] therefore, it can be used by recombinant *E. coli* as a substrate to produce rhamnolipid. Glycerol can be converted to lipid precursor for rhamnolipid production as a carbon source by the respiratory metabolism pathway of glycerol in *E. coli.* In this way, glycerol may be converted to acetyl-CoA, which entered to FAS II (fatty acid synthase type-II) pathway and produce HAA as the precursor of RhlA [2]. Production of rhamnolipid using POME medium has been carried out previously in *Pseudomonas aeruginosa* [27] and *P. stutzeri* [11] but has never been carried out in recombinant *E. coli*. Using POME medium is expected to be used for further economic rhamnolipid production. However, the rhamnolipid yield using POME was lower than using autoinduction medium. It may be considered for further by using POME in the M9 medium with the addition of glucose as a rhamnose substrate to enhance rhamnolipid yield production.

CONCLUSION

Escherichia coli BL21(DE3) recombinant was able produced mono-rhamnolipid (pPM RHLAB) and di-rhamnolipid (pPM RHLABC). The size of *rhlAB* part was 2551 bp and *rhlC* part was 1129 bp, which was regulated by T7*lac* promoters and T7 terminators. The properties of biosurfactants show that di-rhamnolipid have the lowest surface tension and CMC than mono-rhamnolipid and synthetic surfactant (SDS). HRMS analysis of rhamnolipid extraction shows that recombinant *E. coli* pPM RHLAB mainly produced mono-rhamnolipid (Rha-$C_{14:2}$) while *E. coli* pPM RHLABC strain mainly produced di-rhamnolipid (Rha-Rha-C_{10}). In this study, rhamnolipid production using the autoinduction medium was relatively higher than other systems known today in recombinant *Escherichia coli*. The best rhamnolipid production was found at 20 h cultivation

time, temperature 37 ^{o}C, pH 7 with $Area_{OSZ}$ of 48.3 cm^2. The POME medium successfully produced rhamnolipid with an optimal addition of POME of 20% (v/v) in the medium.

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