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Enhanced di-rhamnolipid production with an indigenous isolate *Pseudomonas aeruginosa* J16

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ABSTRACT

A newly isolated indigenous strain *Pseudomonas aeruginosa* J16 was able to produce a glycolipid biosurfactant (i.e., rhamnolipid). Analyses with ^{13}C NMR and mass spectrometry show that the biosurfactant consisted mainly of mono-rhamnolipid (RL1) and di-rhamnolipid (RL2). Since RL2 possesses higher surface activity than RLl and is of better applicability and commercial benefits, the present work attempted to enhance the production of RL2 over RL1 in *P. aeruginosa* J16 from the perspective of medium improvement. The results show that the abundance and composition of RL1 and RL2 varied with the type of carbon and nitrogen sources used. When glycerol and NH_4Cl were used as the carbon and nitrogen sources, respectively, an RL2 production of 448.3 mg/L was obtained with a volumetric productivity of 4.67 mg/(L h) and a RL2/RL1 ratio of 4.22 (by mass). Replacing NH_4Cl by $(NH_4)_2SO_4$, the RL2 concentration and volumetric productivity were improved to 2121 mg/L and 22.1 mg/(L h), respectively, with the RL2/RL1 ratio of 3.0. To further enhance RL2 production, statistical experimental design methodology was applied to optimize the culture medium composition favoring RL2 synthesis. Three key parameters (glycerol, $(NH_4)_2SO_4$, and $MgSO_4{\cdot}7H_2O$) were selected by two-level factorial design. Response surface methodology was then used to identify the optimal composition of the three key parameters, giving an optimal concentration of 0.38 M, 33.3 mM, and 577 μM for glycerol, $(NH_4)_2SO_4$, and $MgSO_4{\cdot}7H_2O$, respectively. With this optimal medium, the RL2 production could be markedly elevated to a maximum concentration of 3190 mg/L and a volumetric productivity of 44.3 mg/(L h), while the RL2/RL1 ratio maintained nearly constant at a value of 3.0.

1. Introduction

A variety of microorganisms, such as bacteria, yeasts and fungi, are able to produce biosurfactants [1]. Compared to synthetic surfactants, biosurfactants have higher surface-activity, lower toxicity, higher biodegradability and better environmental compatibility [2]. Therefore, they have been widely applied in environmental remediation, pharmaceutical, cosmetics, and food industries. Biosurfactants can be categorized as five groups in terms of their chemical composition, being glycolipids, lipopeptides, phospholipids, fatty acids, and polymeric biosurfactant [3]. Among them, rhamnolipid, a glycolipid-type biosurfactant primarily produced by *Pseudomonas aeruginosa* strains, has prominent surface-activity as it can reduce surface tension of water from 72 to 30–32 mN/m with a critical micelle concentration of 5–65 mg/L and displays a high emulsifying activity of 10.4–15.5 U/mL filtrate [4]. Given these features, rhamnolipid is well suited to apply in the petrochemical industry for enhanced oil recovery, hydrocarbon remediation, removal of heavy metals from soils, and decontamination of soil from oil [3,5–7].

Recent findings show that while growing on different substrates, *P. aeruginosa* could produce different rhamnolipid homologues, which may differ in the chain length of fatty acids or in the number of rhamnose units [8]. It was mentioned that rhamnolipids with a longer fatty acid chain length would possess stronger hydrophobicity, while they are more hydrophilic if the sugar component is a rhamnose dimer [9]. Hence, the difference in chemical structure and component can influence the surface-activity and stability of rhamnolipids in the aqueous phase. Literature also shows that the two most common structures in rhamnolipid family are mono-rhamnolipid (rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate; RL1) and di-rhamnolipid (rhamnosyl-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate; RL2), which are composed of a monomer or dimmer of L-rhamnose molecule connecting with β-hydroxydecanoyl-β-hydroxydecanoate, respectively [10]. The synthesis of RL1 is catalyzed by rhamnosyltransferase 1 (encoded by *rhlAB*) combining dTDP-L-rhamnose and β-hydroxydecanoyl-β-hydroxydecanoate to form RL1, while RL2 is synthesized form RL1

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and dTDP-L-rhamnose with the aid of rhamnosyltransferase 2 (encoded by *rhlC*) [11,12]. Some researchers [4,8] reported that di-rhamnolipids were the predominant component of a rhamnolipid surfactants mixture from their *P. aeruginosa* cultures and pointed out that di-rhamnolipid has lower critical micelle concentration and better bioavailability than mono-rhamnolipid. Zhang et al. [13] applied rhamnolipids in phenanthrene remediation and discovered that phenanthrene was more bioavailable in di-rhamnolipid solutions than in mono-rhamnolipid solution; thus di-rhamnolipid seems to be more useful for remediation of poly-aromatic hydrocarbon (PAH)-contaminated sites. Moreover, di-rhamnolipid is also known to be useful in dermatology and other pharmaceutical applications [14,15]. Unfortunately, there is little information regarding fermentation strategies leading to improvement of RL2 production from *P. aeruginosa* species.

In light of the favorable features of di-rhamnolipid, this study aimed to enhance the production of di-rhamnolipid from indigenous *P. aeruginosa* J16 strain isolated form oil-contaminated wastewater in the southern Taiwan. Medium modification and statistical experimental design were applied to obtain optimal medium composition leading to the highest RL2 production.

2. Materials and methods

2.1. Bacterial strain and culture medium

P. aeruginosa J16 isolated from wastewater of a petrochemical factory located in the southern Taiwan was used in the present work for biosurfactant production. The bacterial strain was pre-cultured on LB medium for 12 h at 37 °C and 200 rpm before being inoculated into a designated medium (5% inoculum) for batch fermentation to produce the biosurfactant. The basal medium used for batch fermentation consisted of $NaHPO_4$, 4.28 mM; KH_2PO_4, 30 mM; $CaCl_2{\cdot}2H_2O$, 7 μM; $MgSO_4{\cdot}7H_2O$, 257.35 μM; $C_{10}H_{16}N_2O_8$ (ethylene diaminetetraacetic acid, EDTA), 4 μM; $FeSO_4{\cdot}7H_2O$, 1.14 μM [16]. The foregoing basal medium was supplemented with a variety of carbon sources (namely, glycerol, methanol, ethanol, mannitol, glucose, sucrose, starch, soybean oil, sunflower oil) and nitrogen sources (namely, NH_4Cl, NH_4NO_3, $(NH_4)_2SO_4$, urea, $NaNO_3$). The batch culture was incubated at 37 °C and 200 rpm with a typical cultivation time of 72 h. Moreover, for statistical experimental design, the composition of the starting medium was that of the aforementioned basal medium supplemented with 20 g glycerol/L and 25 mM $(NH_4)_2SO_4$.

2.2. Purification of rhamnolipids

Cells of fermentation broth were separated by centrifugation (9000 × *g*, 30 min, 4 °C). The pH of the resulting supernatant was adjusted to pH 2.0 with 1N HCl to precipitate rhamnolipids. The precipitate was harvested by centrifugation and extracted by ethyl acetate. The organic phase was collected and the solvent was removed in a rotary evaporator (Buchi, Rotovapor R-200, Germany) to obtain crude rhamnolipid, which was subsequently dissolved in 50% ethanol and filtered with ultrafiltration membrane (30 kDa molecular weight cut-off). The resulting filtrate was again precipitated with acid, re-dissolved in deionized water, and then filtered through 1 kDa membrane. The filtrate was precipitated with acid again and then the final precipitate was lyophilized to obtain the purified rhamnolipid product.

2.3. NMR and mass spectrometry analyses

The purified rhamnolipid was dissolved in methanol and analyzed with nuclear magnetic resonance (NMR) and mass spectrometry. The ^{13}C NMR spectra were obtained with an NMR machine by Bruker Instrument (Bruker Advance 600, 600 MHz, Germany). The mass spectrometry analysis was performed using fast atom bombardment mass spectrometry (FAB/MS, JMS-700 HRMS, JEOL, Tokyo, Japan).

2.4. HPLC analysis

Quantitative analysis of rhamnolipid was also conducted using high performance liquid chromatography (HPLC; Hitachi Model L2130, Tokyo, Japan) equipped with a UV detector. For derivatization of rhamnolipid, an appropriate amount of purified rhamnolipid was dissolved in 1 mL of CH_3CN solution containing 2-bromoacetophenone and triethylamine to reach a molar ratio (glycolipid: 2-bromoacetophenone: triethylamine) of approximately 1:4:2. The reaction was conducted at 80 °C for 1 h and left at room temperature (i.e., 28 °C) for 10 min. After filtration with a 0.22 μm filter membrane (Millipore), the sample was loaded on to HPLC for analysis. The column used to analyze derivatized rhamnolipid phenacyl esters was a reverse-phase LiChrospher 100 C18 column (RP18, 5 μm). The mobile phase was CH_3CN–3.3 mM H_3PO_4 using a gradient of 50:50 (v/v) for 3 min; from 50:50 to 100:0 (v/v) for 19 min; 100:0 (v/v) for 5 min; from 100:0 (v/v) to 50:50 (v/v) for 3 min; and 50:50 (v/v) for 10 min. The flow rate was controlled at 1 mL/min. The detecting wavelength of the UV detector was set at 244 nm [4,17].

2.5. Experimental design

The eight core components in the fermentation medium (glycerol, $(NH_4)_2SO_4$, $NaHPO_4$, KH_2PO_4, $CaCl_2{\cdot}2H_2O$, $MgSO_4{\cdot}7H_2O$, EDTA, $FeSO_4{\cdot}7H_2O$) were selected as the target parameters. Two-level factorial design based on Plackett–Burman folded method [18] was used to screen the most important parameters affecting the production of di-rhamnolipid. Next, the method of the path of the steepest ascent was used to determine the range of key parameters and then response surface methodology (RSM) was applied for experimental design based on Box–Behnken design algorithm with the aid of JMP software (version 3.2.2, SAS Institute Inc., Cary, NC, USA). According to the experimental results, stepwise regression analysis was carried out by generating the second order polynomial equation describing the response surface. The optimal values of key parameters leading to the best response (i.e., the maximum concentration of RL2) were then identified [19–21].

3. Results and discussion

3.1. Qualitative and quantitative analyses of rhamnolipids

The purified rhamnolipid products originating from *P. aeruginosa* J16 was dissolved in methanol and analyzed with FAB/MS. The mass spectrum (Fig. 1a) indicates two significant peaks at 527 *m*/*z* and 673 *m*/*z*, representing the molecular weight of sodium mono-

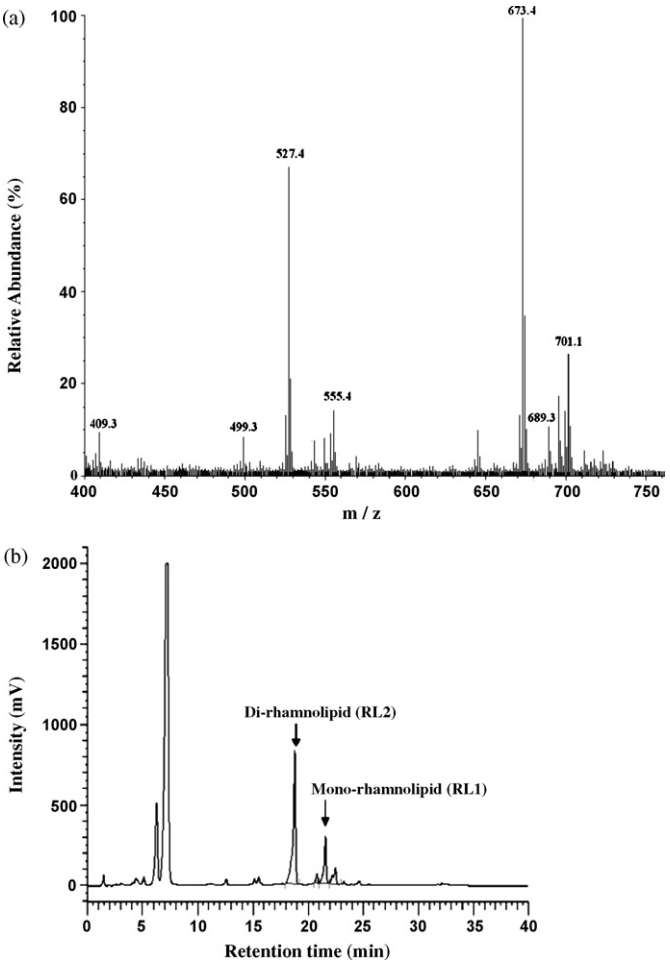

Fig. 1. (a) FAB/MS analysis of purified rhamnolipid sample and (b) HPLC analysis of purified rhamnolipid after derivatization of rhamnolipid (the peak at the retention time of 18.8 and 21.5 min represents di-rhamnolipid and mono-rhamnolipid, respectively).