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Rhamnolipid production with indigenous *Pseudomonas aeruginosa* EM1 isolated from oil-contaminated site

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Abstract

Rhamnolipid is one of the most effective and commonly used biosurfactant with wide industrial applications. Systematic strategies were applied to improve rhamnolipid (RL) production with a newly isolated indigenous strain *Pseudomonas aeruginosa* EM1 originating from an oil-contaminated site located in southern Taiwan. Seven carbon substrates and four nitrogen sources were examined for their effects on RL production. In addition, the effect of carbon to nitrogen (C/N) ratio on RL production was also studied. Single-factor experiments show that the most favorable carbon sources for RL production were glucose and glycerol (both at 40 g/L), giving a RL yield of 7.5 and 4.9 g/L, respectively. Meanwhile, sodium nitrate appeared to be the preferable nitrogen source, resulting in a RL production of 8.6 g/L. Using $NaNO_3$ as the nitrogen source, an optimal C/N ratio of 26 and 52 was obtained for glucose- and glycerol-based culture, respectively. To further optimize the composition of fermentation medium, twenty experiments were designed by response surface methodology (RSM) to explore the favorable concentration of three critical components in the medium (i.e., glucose, glycerol, and $NaNO_3$). The RSM analysis gave an optimal concentration of 30.5, 18.1, and 4.9 g/L for glucose, glycerol, and $NaNO_3$, respectively, predicting a maximum RL yield of 12.6 g/L, which is 47% higher than the best yield (8.6 g/L) obtained from preliminary selection tests and single factor experiments (glucose and $NaNO_3$ as the carbon and nitrogen source). The NMR and mass spectrometry analysis show that the purified RL product contained L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate (RL1) and L-rhamnosyl L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate (RL2). Meanwhile, HPLC analysis indicates that the molar ratio of RL1 and RL2 in the purified rhamnolipid product was ca. 1:1.

Keywords: Biosurfactant; *Pseudomonas aeruginosa*; Response surface methodology; Rhamnolipid

1. Introduction

Oil-contamination in soil and underground water has been a major threat to the environment and human health in Taiwan. Bioremediation has been used to clean up oil polluted sites, but the efficiency of biodegradation of oil pollutants is often limited by their poor water solubility (Banat et al., 2000; Desai and Banat, 1997; Dyke et al., 1993; Janssen et al., 2002; Mulligan and Eftekhari, 2003; Rahman et al., 2002). One of the approaches to enhance biodegradation of oils is to use biosurfactants (Benincasa et al., 2002; Finnerty and Singer, 1983; Rahman et al., 2003; Santos et al., 1984, 2002), which could increase solubility of oils in water to enhance the bioavailability of the hydrophobic substrates, leading to higher oil degradation rates. Biosurfactants are natural surface active products from a variety of microorganisms (Banat et al., 2000; Desai and Banat, 1997) and can be categorized as glycolipids,

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lipopeptides, fatty acids, polysaccharide-protein complexes, peptides, phospholipids, and neutral lipids (Benincasa et al., 2002). In addition to environmental applications, biosurfactants also find their applications in pharmaceutical, cosmetic, fine chemical, and food industries (Banat et al., 2000; Thanomsub et al., 2007).

Rhamnolipid is the most often used biosurfactant in bioremediation (Banat et al., 2000; Dyke et al., 1993; Reiling et al., 1986; Zhang and Miller, 1992). Rhamnolipid is a glycolipid-type biosurfactant primarily produced by *Pseudomonas aeruginosa* (Hauser and Karnovsky, 1957; Maier and Chavez, 2000). It is known that *P. aeruginosa* strains are able to produce six types of rhamnolipids, which possess similar chemical structure and surface activity and have an average molecular weight of 577 (Torrens et al., 1998). Rhamnolipid can reduce surface tension of water from 72 mN/m to 30 mN/m (Abalos et al., 2001; Arino et al., 1996; Ron and Rosenberg, 2001) with a critical micelle concentration of 50–65 mg/L (Sandoval et al., 1999). Although rhamnolipid is an effective biosurfactant and is well suited for applications in bioremediation of oil pollutants (Mulligan, 2005), the major hurdle for commercial application of the biosurfactant has been the low yield and high production cost (Wei et al., 2005). Therefore, there is an urgent demand to develop an efficient biosurfactant producer and a cost-effective bioprocess for the production of rhamnolipid.

In light of this, this work started with an attempt to isolate an indigenous bacterial strain (identified as *P. aeruginosa* EM1) capable of producing rhamnolipid. Next, systematic assessment on the isolated strain was performed to evaluate its potential for commercial production of rhamnolipid. The EM1 strain was grown on different carbon and nitrogen sources to determine the favorable culture medium for rhamnolipid production. Different carbon sources (e.g., glucose, glycerol, vegetable oil, fatty acid, and hydrocarbon) and nitrogen sources (e.g., ammonium salt, nitrate, and urea) were used to explore their effects on the yield of rhamnolipid. Moreover, the effect of carbon to nitrogen ratio on rhamnolipid production was also investigated. Statistical experimental design methodology was utilized to determine the optimal medium composition. The structure and surface activity of the rhamnolipid product produced by *P. aeruginosa* EM1 was also identified. This work was undertaken to assess the commercialization potential and feasibility of this indigenous bacterium in rhamnolipid production.

2. Methods

2.1. Bacterial strain and preparation of seed culture

The rhamnolipid-producing strain used in this study was *P. aeruginosa* EM1, which is an indigenous isolate obtained from an oil-contaminated site near a petrochemical plant in southern Taiwan. For pre-culture, the strain from frozen stock was streaked on LB agar plate, which was then incubated at 37 °C for 14–16 h. After that, a single colony was taken from the plate and transferred into 50 ml LB liquid medium to prepare the seed culture. The cultivation condition for the seed culture was 37 °C, 200 rpm, and 14–16 h of incubation time.

2.2. Fermentation medium and condition

For liquid fermentation, the seed culture (5% inoculum) was inoculated into a 500-ml flask containing 150 ml mineral salts (MS) medium consisting of (g/L): NH_4NO_3, 4; KH_2PO_4, 4.08; Na_2HPO_4, 5.68; $CaCl_2$, 7.77×10^{-4}; $MgSO_4\cdot7H_2O$, 0.2; sodium EDTA, 1.49×10^{-3}; $FeSO_4\cdot7H_2O$, 5.56×10^{-4} (Wei and Chu, 1998). In general, the MS medium was amended with 40 g/L glucose as a sole carbon substrate (designated as GMS medium) (Wei and Chu, 1998; Wei et al., 2005). The culture temperature and agitation rate was 37 °C and 200 rpm, respectively. The pH of the medium was initially adjusted to 6.8 by 1.0 M HCl.

For the experiments exploring the effect of carbon substrates on rhamnolipid production, the carbon source in GMS medium (i.e., glucose) was replaced by glycerol (40 g/L), sucrose (40 g/L), hexane (40 g/L), olive oil (80 g/L), oleic acid (80 g/L), and soybean oil (80 g/L). In addition, the nitrogen source in GMS medium (i.e. NH_4NO_3) was also replaced by NH_4Cl (50 mM), $NaNO_3$ (50 mM), urea (50 mM), and yeast extract (10 g/L) to investigate the effect of nitrogen source on rhamnolipid production. The concentration of carbon and nitrogen source was identical to GMS medium, while that of oil and yeast extract was determined according to the information from literature and preliminary tests. For the experiments examining the effect of carbon to nitrogen (C/N) ratio, the concentration of carbon source (glucose or glycerol) was fixed at 40 g/L, while the concentration of nitrogen source ($NaNO_3$) was adjusted to 0.85, 2.125, 4.25, 8.5, and 17 g/L, resulting in a C/N ratio of 130, 52, 26, 13, and 6.5, respectively. During the course of batch fermentation, samples were taken from the liquid culture to monitor the cell growth, rhamnolipid production, surface tension, and emulsification index.

2.3. Experimental design

Literature indicated that carbon and nitrogen sources usually play a critical role in the performance of rhamnolipid production by *P. aeruginosa* strains (Santos et al., 2002). To investigate the effects of glucose, glycerol, and $NaNO_3$ on rhamnolipid production, surface response methodology was used for the experimental design. The concentration range of the three factors, indicated in Table 1, was estimated according to the data from our preliminary tests as well as the results indicated in the literature (Santos et al., 1984, 2002; Wei et al., 2005). With the aid of JMP software (version 3.2.2, SAS Institute Inc., Cary, NC, USA), 20 batch experiments (Table 2) were designed