ABSTRACT

Pseudomonas aeruginosa produce biosurfactant with biotechnological importance through fermentation. The main factors affecting biosurfactant production are the type of organism used and the components of the fermentation medium (pH, temperature, oxygen, carbon and nitrogen sources, various salts). Increasing the production of rhamnolipid produced in low amounts has been the subject of many studies. In this study, toluene was used to increase rhamnolipid production. The addition of 0.2% toluene at the 48th h resulted in the highest rhamnolipid formation (3.0 g/L), which is a significant 30% increase over the control (2.3 g/L). While rhamnolipid production increased with the addition of toluene, bacterial biomass decreased. This study revealed that adding toluene to the fermentation medium with a new strategy significantly increases rhamnolipid production. Addition of toluene is an easy and effective way to increase rhamnolipid production in P. aeruginosa fermentation processes. The present research is the first to demonstrate that P. aeruginosa improves rhamnolipid synthesis when toluene is added.

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Graphical abstract
INTRODUCTION

*Pseudomonas aeruginosa* is a Gram-negative bacterium and is one of the most commercially and biotechnologically valuable microorganisms. This bacterium is particularly noteworthy due to its high biosurfactant (rhamnolipid), pigment (pyocyanin) and enzymes (protease, lipase, reductase, oxygenase, elastase) production [1-3]. Among these, studies on the production and applications of biosurfactants have gained importance in the last two decades. Surfactants can be obtained by chemical and biological methods [4]. Biosurfactants are low toxicity, biodegradable and environmentally compatible compared to chemical surfactants. This is why there has been a rise in interest in biosurfactants recently. They are also active at high temperature, pH and salinity [1, 5].

Although the production of biosurfactants primarily depends on the producing microorganism, environmental conditions such as carbon and nitrogen source, ion concentration, pH, oxygen and temperature affect their production. Process optimization is required by using low-cost renewable raw materials to reduce costs [1, 6].

Surfactants are in great demand worldwide. The global market for surfactants was $30.64 billion in 2016 and is estimated to be around $40 billion by 2021. Biosurfactants constitute a significant share of the surfactant market. By 2027, it is anticipated that the market for biosurfactants would have grown to $1.9 billion from its estimated $1.2 billion value in 2022 [7]. However, biosurfactants are not as competitive as synthetic surfactants [8]. The market value of synthetic surfactants is around $2/kg. The variety and purity level of biosurfactant have an impact on its current market price. Surfactin 318£/10 mg obtained from *Bacillus subtilis* biosurfactant have an impact on its current market price. The variety and purity level of biosurfactant have an impact on its current market price.

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Anticancer, immune modulator and antioxidant activities of rhamnolipid are also known [17]. The low efficiency and high cost of production of rhamnolipid limit their use. Therefore, it is important to add new promoters that increase the efficiency of rhamnolipid production. The aim of this study is to increase the rhamnolipid production of *P. aeruginosa* by adding toluene as an easy and effective method.

MATERIALS AND METHODS

Chemicals

All of the substances utilized in the research were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Difco (Detroit, MI, USA).

Microorganism Used in the Study

*Pseudomonas aeruginosa* OG1, known to produce rhamnolipid, was used in the present study. This isolate, which possesses the potential to break down pesticides, was isolated from cockroaches that were living in pesticide-contaminated environments [18].

Growth Conditions

*P. aeruginosa* OG1 was grown on OG1 Nutrient Agar medium at 30 °C for 24 hours. Then, bacteria was inoculated into Nutrient Broth (NB) medium and incubated at 150 rpm for 24 hours at 30 °C. Then, the prepared bacterial suspension (OD600 1) was inoculated into rhamnolipid production medium.

Rhamnolipid Production

By adding 4% glycerol to the NB (50 mL medium in 250 mL flasks), the media were sterilized in autoclave at 121 °C for 15 minutes. For the rhamnolipid production, 4% (v/v) of the inoculum (OD600 1) was added to sterilized medium and incubated at 30 °C for 216 hours at 200 rpm.

Analytical Methods

For biomass prediction, fermentation broth was centrifuged (10,000 rpm for 10 min at room temperature), washed several times with sterile distilled water and dried at 70 °C until constant weight. The amount of rhamnolipid was determined using the phenol-sulfuric acid method [19].

Statistical Analysis

All of the experiments were performed in three times. The statistical analyses of the data were carried out one-way analysis of variance (ANOVA) using software package SPSS15.0 (SPSS Inc., Chicago, IL, USA). A p < 0.05 was considered statistically important.
RESULTS AND DISCUSSION

Results

Rhamnolipid synthesis and growth kinetics of *P. aeruginosa*

Rhamnolipid production was carried out in production environment for a period of 216 hours as shown in Figure 1. A stationary growth phase was reached by the cells after 72 hours. At 192 hours, the maximum amount of 2.3 g/L rhamnolipid was produced (Figure 2).

Effect of toluene supplementation on *P. aeruginosa*

At 24, 48, and 72 hours of fermentation, 0.2% toluene was introduced to *P. aeruginosa* OG1 to observe how it affected the formation of rhamnolipids and bacterial biomass (Figure 3). As it is known, when cells enter the stationary growth phase, secondary metabolites (such as rhamnolipids and pigments) are produced more. It was found that adding toluene to the fermentation broth at any time and concentration led to a reduction in biomass, as shown in Figure 3. This indicates that toluene has a negative effect on the growth of *P. aeruginosa*. However, when toluene was added 24 hours or later, there was a significant increase in rhamnolipid production. At the 48th h of fermentation, toluene was added to produce the highest amount of rhamnolipids (3.0 g/L). Similarly, 2.74 g/L rhamnolipid was obtained by adding toluene at the 24th hour of fermentation and this value was 19.1% higher than the control. According to these results, it was determined that toluene addition time is important in rhamnolipid production. Rhamnolipid yield increased after additions of all toluene concentrations in the stationary phase (Figure 4). The findings demonstrated that by adding a suitable toluene concentration in the stationary phase of fermentation, rhamnolipid produce could be increased.

![Figure 1. Bacterial growth in the production environment.](image1)

![Figure 2. Effect of time on the formation of rhamnolipid in the production medium.](image2)

![Figure 3. Effect of toluene addition time on biomass yield and rhamnolipid production of *P. aeruginosa*. Toluene (0.2%, v/v) was added to the production medium at different times (24, 48 and 72 h). An asterisk denotes a value significantly greater than other rhamnolipid values (P<0.05).](image3)

![Figure 4. Effect of different toluene concentrations on biomass yield and rhamnolipid production of *P. aeruginosa*. Toluene was added to the production medium after 48 h. An asterisk denotes a value significantly greater than other rhamnolipid values (P<0.05).](image4)
Discussion

Rhamnolipid, a biosurfactant, is a secondary metabolite produced by *P. aeruginosa* (Figure 1 and 2). Secondary metabolites are mostly produced in the stationary phase of growth [20]. In order to increase the production of rhamnolipid, studies are carried out to optimize media composition and environmental conditions [1, 6, 21].

Several organic solvents (benzene, toluene and xylene) are very toxic when supplemented immediately to the growth medium [22]. At high toluene concentrations, the reduction of rhamnolipid production can be explained by decreased bacterial biomass (Figure 3 and 4). According to these results, it can be said that *P. aeruginosa* is sensitive to toluene. Ozdal [2] showed that toluene changes fatty acids in the cell membrane of *P. aeruginosa*.

Many added organic solvents are toxic to microorganisms even at low concentrations, thus affecting the production of metabolites. Therefore, the concentration, time of addition and type of stimulants must be chosen correctly. The synthesis of enzymes has been increased by some researchers using organic solvents. According to Sumarsih et al. [23], the presence of toluene and hexadecane improved bacterial oxygenase activity. It was also found that *P. aeruginosa* PsEA produces more lipase when exposed to tetradecane, dodecane, isoctane, and heptane [24]. According to Thumar and Singh [25], adding xylene benzene, butanol, and acetone significantly enhanced the production of alkaline protease in *Streptomyces clavuligerus*. Toluene has been reported to dramatically improve the production of pyocyanin by increasing the activity of the protease when it was added to the fermentation medium [2]. According to this study, rhamnolipid synthesis was enhanced by adding toluene to the fermentation medium (Figure 3 and 4).

The interaction between the microorganisms and the chemical to be added can alter the organic solvent addition strategy. It was found that the addition of 0.3% toluene in the production of exopolysaccharide by *Collybia maculata* TG-1 increased the yield more than twice [26]. In another study, it was determined that the addition of 0.2% acetone increased exopolysaccharide production in *Lentinus tigrinus* [27]. It is known that toluene stimulates oxidative stress. Tan et al. [28], it has been reported that *H. H2O2* causes biofilm increase by causing oxidative stress. Rhamnolipid has antioxidant activity [29]. Therefore, considering that rhamnolipid serves as an antioxidant preservative, it seems quite possible to increase rhamnolipid production under oxidative stresses. It is essential to increase the production of biosurfactants due to their many uses [30].

CONCLUSION

As a result, it was determined that the production yield increased with the addition of an appropriate toluene concentration in the stationary phase of rhamnolipid production. Therefore, the adding of toluene may be a simple and effective means for enhancing rhamnolipid production in *P. aeruginosa* fermentation processes. This method can be used to enhance the yield of numerous products, including pigment, biosurfactant, polysaccharide, and enzyme made through fermentation.

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CONFLICT OF INTEREST

The author declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

ETHICS

There are no ethical issues with the publication of this manuscript.

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