Sigma J Eng Nat Sci, Vol. 41, No. 6, pp. 1287–1291, December, 2023

Sigma Journal of Engineering and Natural Sciences
Web page info: https://sigma.yildiz.edu.tr
DOI: 10.14744/sigma.2023.00144

Technical Note

Increasing the yield of rhamnolipid produced by *Pseudomonas aeruginosa* by using toluene

Havva Nur YARLI[1], Murat ÖZDAL[1,*]

[1]*Department of Biology, Ataturk University, Erzurum, 25240, Türkiye*

ARTICLE INFO

Article history
Received: 15 September 2021
Revised: 10 November 2021
Accepted: 11 December 2021

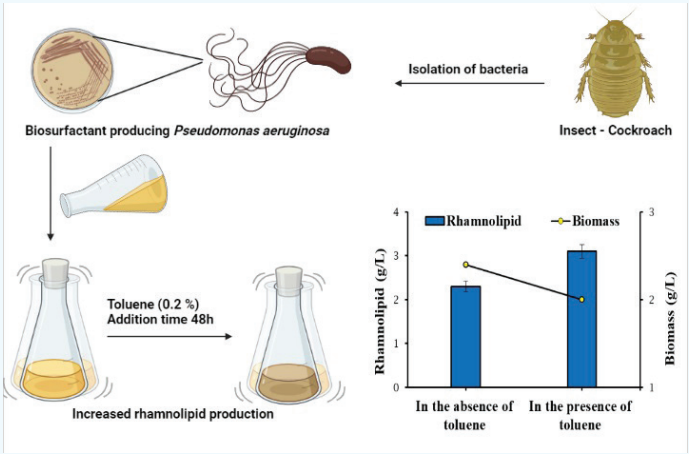
Keywords:
Pseudomonas aeruginosa, rhamnolipid, toluene

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.

Cite this article as: Yarlı HN, Özdal M. Increasing the yield of rhamnolipid produced by *Pseudomonas aeruginosa* by using toluene. Sigma J Eng Nat Sci 2023;41(6):1287–1291.

Graphical abstract



***Corresponding author.**
*E-mail address: murat.ozdal@yahoo.com

This paper was recommended for publication in revised form by Regional Editor Alper Yılmaz

Published by Yıldız Technical University Press, İstanbul, Turkey

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 pricing. Surfactin 318£/10 mg obtained from *Bacillus subtilis* in 98% purity, Iturin A 257£/mg in 95% purity, rhamnolipid obtained in 90% purity from Pseudomonas aeruginosa is 85£/10g, while 95% pure rhamnolipid is 116-430£ /10mg (Sigma-Aldrich Co.). Therefore, efficiency-enhancing and cheap raw materials must be used to make the production process of biosurfactants economical [6].

Biosurfactants are widely used in many industries such as agricultural, textile, mining, food, cosmetic, environmental and pharmaceutical [9, 10]. Pseudomonas, Bacillus, Acinetobacter, Rhodococcus and Candida are the most used genera in the production of biosurfactants with different properties [11, 12]. Rhamnolipid produced by *P. aeruginosa* is the most studied biosurfactant and has many different applications. Rhamnolipid can be used as an insecticide against green peach aphid [13]. Rhamnolipid from Pseudomonas sp S-17 has been reported as a Herpesvirus inhibitor [14]. In general, biosurfactants are more useful than synthetic surfactants in the cosmetic industry due to their low irritant effect and compatibility with the skin. Rhamnolipid has been shown to have anti-wrinkle effects due to their moisturizing properties [9]. Rhamnolipid further improve the washing quality of detergents [15] and are mainly used in the textile industry for degreasing [9]. Due to its antimicrobial properties, rhamnolipid is used in many different cosmetic products such as toothpastes, deodorants, acne creams and hand-nail care products [16]. 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, bacterium was inoculated into Nutrient Broth (NB) medium and incubated at 150 rpm for 24 hours at 30 °C. Then, the prepared bacterial suspension (OD_{600} 1) was inoculated into rhamnolipid production medium.

Ramnolipid 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 DISCUSSIN

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).

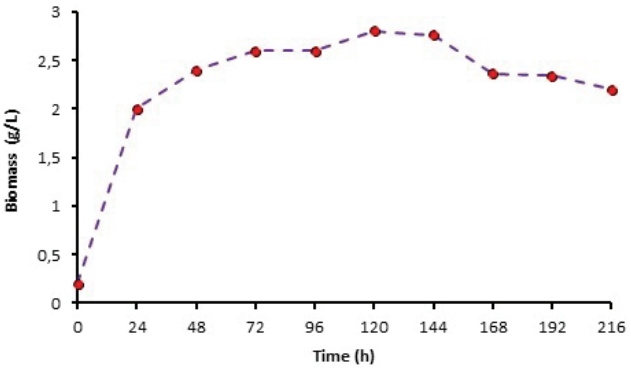


Figure 1. Bacterial growth in the production environment.

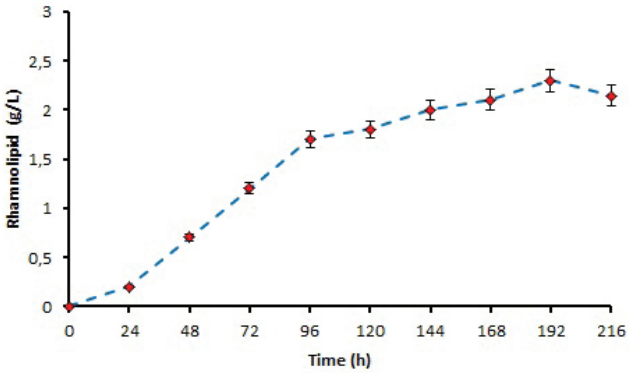


Figure 2. Effect of time on the formation of rhamnolipid in the production medium.

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.

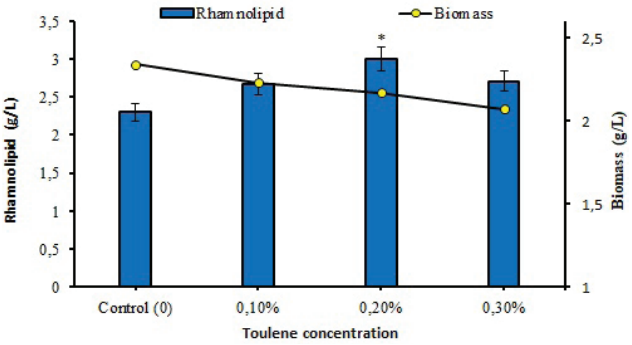


Figure 3. Effect of toluene addition time on biomass yield and rhamnolipid production of *P. aeruginosa*. Toluene (0.2%, v/v) was added to the production medium at different times (24, 48 and 72 h). An asterisk denotes a value significantly greater than other rhamnolipid values ($P < 0.05$).

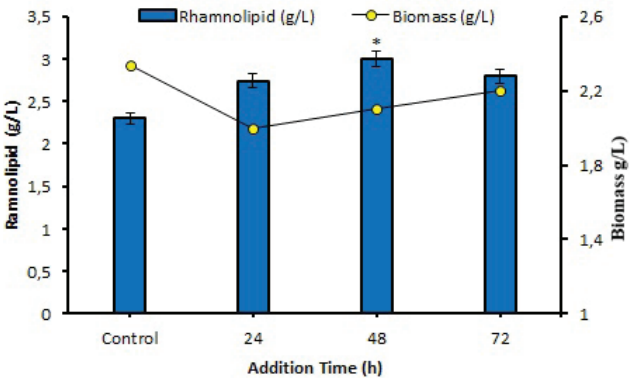


Figure 4. Effect of different toluene concentrations on biomass yield and rhamnolipid production of *P. aeruginosa*. Toluene was added to the production medium after 48 h. An asterisk denotes a value significantly greater than other rhamnolipid values ($P < 0.05$).

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, isooctane, 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_2O_2 causes biofilm increase by causing oxidative stress. Ramnolipid 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.

ACKNOWLEDGEMENTS

Authors thank the TÜBİTAK for the financial support under project no: 1919B011902073.

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.

REFERENCES

[1] Ozdal M, Gurkok S, Ozdal OG. Optimization of rhamnolipid production by Pseudomonas aeruginosa OG1 using waste frying oil and chicken feather peptone. 3 Biotech 2017;7:117. [CrossRef]

[2] Ozdal M. A new strategy for the efficient production of pyocyanin, a versatile pigment, in Pseudomonas aeruginosa OG1 via toluene addition. 3 Biotech 2019;9:1–8. [CrossRef]

[3] Ozdal M, Gurkok S, Ozdal OG, Kurbanoglu EB. Enhancement of pyocyanin production by Pseudomonas aeruginosa via the addition of n-hexane as an oxygen vector. Biocatal Agric Biotechnol 2019;22:101365. [CrossRef]

[4] Sun H, Wang L, Nie H, Diwu Z, Nie M, Zhang B. Optimization and characterization of rhamnolipid production by Pseudomonas aeruginosa NY3 using waste frying oil as the sole carbon. Biotechnol Prog 2021:e3155.

[5] Jahan R, Bodratti AM, Tsianou M, Alexandridis P. Biosurfactants, natural alternatives to synthetic surfactants: physicochemical properties and applications. Adv Colloid Interface Sci 2020;275:102061. [CrossRef]

[6] Gurkok S. Important parameters necessary in the bioreactor for the mass production of biosurfactants. In Green Sustainable Process for Chemical and Environmental Engineering and Science. Elsevier, 2021;347–365. [CrossRef]

[7] Markets and Markets. Available at https://www.marketsandmarkets.com/Market-Reports/biosurfactant-market-163644922.html. Accessed March 24, 2020.

[8] Rivera ÁD, Urbina MÁM, López VEL. Advances on research in the use of agro-industrial waste in biosurfactant production. World J Microbiol Biotechnol 2019;35:155. [CrossRef]

[9] Fenibo EO, Ijoma GN, Selvarajan R, Chikere CB. Microbial Surfactants: The Next Generation Multifunctional Biomolecules for Applications in the Petroleum Industry and Its Associated Environmental Remediation. Microorganisms 2019;7:581. [CrossRef]

[10] Liepins J, Balina K, Soloha R, Berzina I, Lukasa LK, Dace E. Glycolipid Biosurfactant Production from Waste Cooking Oils by Yeast: Review of Substrates, Producers and Products. Fermentation 2021;7:136. [CrossRef]

[11] Singh P, Patil Y, Rale V. Biosurfactant production: emerging trends and promising strategies. J Appl Microbiol 2019;126:2–13. [CrossRef]

[12] Markande AR, Patel D, Varjani S. A review on biosurfactants: properties, applications and current developments. Bioresour Technol 2021;124963. [CrossRef]

[13] Kim SK, Kim YC, Lee S, Kim JC, Yun MY, Kim IS. Insecticidal activity of rhamnolipid isolated from Pseudomonas sp. EP-3 against green peach aphid (Myzus persicae). J Agric Food Chem 2011;59:934–938. [CrossRef]

[14] Remichkova M, Galabova D, Roeva I, Karpenko E, Shulga A, Galabov AS. Anti-herpesvirus activities of Pseudomonas sp. S-17 rhamnolipid and its complex with alginate. Z Naturforsch C 2008;63:75–81. [CrossRef]

[15] Jadhav JV, Anbu P, Yadav S, Pratap AP, Kale SB. Sunflower Acid Oil-Based Production of Rhamnolipid Using Pseudomonas aeruginosa and Its Application in Liquid Detergents. J Surfactants Deterg 2019;22:463–476. [CrossRef]

[16] Elshikh M, Moya-Ramírez I, Moens H, Roelants SLKW, Soetaert W, Marchant R, Banat IM. Rhamnolipids and lactonic sophorolipids: natural antimicrobial surfactants for oral hygiene. J Appl Microbiol 2017;123:1111–1123. [CrossRef]

[17] Kumar R, Das AJ. Application of rhamnolipids in medical sciences. In Rhamnolipid Biosurfactant. Springer, Singapore, 2018;79–87. [CrossRef]

[18] Ozdal M, Ozdal OG, Algur OF. Isolation and characterization of α-endosulfan degrading bacteria from the microflora of cockroaches. Pol J Microbiol 2016;65. [CrossRef]

[19] Dubois M, Gilles KA, Hamilton JK, Rebers PT, Smith F. Colorimetric method for determination of sugars and related substances. Anal Chem 1956;28:350–356. [CrossRef]

[20] Ozdal M, Kurbanoglu EB. Use of chicken feather peptone and sugar beet molasses as low cost substrates for xanthan production by Xanthomonas campestris MO-03. Fermentation 2019;5:9. [CrossRef]

[21] Özdal M, Gürkök S, Özdal ÖG, Kurbanoğlu EB. Rhamnolipid production by Pseudomonas aeruginosa OG1 using waste frying oil and ram horn peptone. In AIP Conference Proceedings. AIP Publishing LLC, 2017;1833:020102. [CrossRef]

[22] Pacífico C, Fernandes P, de Carvalho CCCR. Mycobacterial response to organic solvents and possible implications on crossresistance with antimicrobial agents. Front Microbiol 2018;9:961. [CrossRef]

[23] Sumarsih S, Nimatuzahroh F, Puspitasari M, Rusdiana M. Effect of aliphatic and aromatic hydrocarbons on the oxygenase production from hydrocarbonoclastic bacteria. J Chem Technol Metall 2017;52:1062–1069.

[24] Gaur R, Khare SK. Cellular response mechanisms in Pseudomonas aeruginosa PseA during growth in organic solvents. Lett Appl Microbiol 2009;49:372–377. [CrossRef]

[25] Thumar JT, Singh SP. Organic solvent tolerance of an alkaline protease from salt-tolerant alkaliphilic Streptomyces clavuligerus strain Mit-1. J Ind Microbiol Biotechnol 2009;36:211. [CrossRef]

[26] Lim JM, Yun JW. Enhanced production of exopolysaccharides by supplementation of toluene in submerged culture of an edible mushroom Collybia maculata TG-1. Process Biochem 2006;41:1620–1626. [CrossRef]

[27] He P, Wu S, Pan L, Sun S, Mao D, Xu C. Effect of Tween 80 and acetone on the secretion, structure and antioxidant activities of exopolysaccharides from Lentinus tigrinus. Food Sci Biotechnol 2016;54:290–295. [CrossRef]

[28] Tan Q, Ai Q, Xu Q, Li F, Yu J. Polymorphonuclear leukocytes or hydrogen peroxide enhance biofilm development of mucoid Pseudomonas aeruginosa. Mediators Inflamm 2018;8151362. [CrossRef]

[29] Sanjivkumar M, Deivakumari M, Immanuel G. Investigation on spectral and biomedical characterization of rhamnolipid from a marine associated bacterium Pseudomonas aeruginosa (DKB1). Arch Microbiol 2021;203:2297–2314. [CrossRef]

[30] Ozdal M, Janek T, Satpute SK. Biosurfactants: From renewable resources to innovative applications. Front Bioeng Biotechnol 2022;10:988646. [CrossRef]