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RESEARCH ARTICLE

Production of Rhamnolipid (A Biosurfactant) Using Free and Immobilized Cells of *Pseudomonas* sp.

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Summary

This study presents a method for the production of rhamnolipid, a biosurfactant, by *Pseudomonas* sp. *Pseudomonas* sp. cells that were grown in nutrient agar were inoculated into sterile liquid medium. Following an incubation period of 24 h, 2 ml of cells were inoculated into a different liquid medium and the results were obtained at the end of 26 hours incubation time. In our study, the effects of temperature, pH, and glucose concentration on rhamnolipid production were also investigated. Later, the same procedure was applied to immobilized cells that were kept away from the free microorganisms. The production of rhamnolipid by free cells was found to be much higher than that of immobilized cells. Free cells could be used for rhamnolipid production effectively.

Keywords: *Biosurfactant, Rhamnolipid, Pseudomonas sp., Immobilized cells, Free cells*

Serbest ve Tutuklanmış *Pseudomonas* sp. Hücrelerinden Ramnolipid (Biyosürfektan) Eldesi

Özet

Bu çalışmada, bir büyosürfektan olan ramnolipidin *Pseudomonas* sp.'den üretimi araştırılmıştır. Nutrient agarda üretilen *Pseudomonas* sp. örnekleri sıvı besiyerine aktarılmıştır. 24 saatlik inkübasyon sonrası, 2 ml'lik kültür örnekleri ramnolipid üretimi için, farklı bir sıvı besiyerine aktarılarak 26. saatte sonuçlar gözlenmiştir. Çalışmada, ayrıca ramnolipid üretimine sıcaklığın, pH'ın ve glukoz konsantrasyonunun etkisi de incelenmiştir. İmmobilize (tutuklanmış) hücreler içinde aynı metotlar uygulanmıştır. Ramnolipid üretimi, serbest hücrelerde tutuklanmış hücrelerden daha yüksek bulunmuştur. Serbest hücreler, etkin ramnolipid üretimi için kullanılabilirler.

Anahtar sözcükler: *Biyosürfektan, Ramnolipid, Pseudomonas sp., Tutuklanmış hücre, Serbest hücre*

INTRODUCTION

Many microorganisms are able to synthesize different types of biosurfactants when grown on various carbon sources. These biosurfactants include low molecular weight glycolipids, lipopeptides, and high molecular weight lipid -containing polymers such as lipoproteins, lipopoly-saccharide-protein complexes, and polysaccharide-protein -fatty acid complexes [1,2]. It is well known that several strains of *Pseudomonas* can accumulate surface-active compounds characterized as rhamnolipids when grown on different carbon sources [3]. The rhamnolipids produced by *Pseudomonas aeruginosa* have been widely studied and are reported to be a mixture of the homologous species RL1 (Rha C10C10), RL2 (Rha C10), RL3 ($Rha_2C10C10$) and RL4 (Rha_2C10) [4].

In recent years, the number of investigators studying biosurfactant-producing microorganisms has dramatically increased. Biosurfactants have several advantages over synthetic surfactants for potential industrial applications. The most important advantage of biosurfactants when compared to synthetic surfactants is their ecological acceptance, owing to their low toxicity and biodegradable nature [3,5]. Some of these biosurfactants also have been investigated for their potential to act as biologically active compounds for pharmaceuticals [6].

Microorganisms used for industrial applications are selected to provide the best possible combination of characteristics for the specific process and equipment

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being used. The selected strains should be tolerant of high concentrations of carbohydrates and biosurfactants. *P. aeruginosa* is capable of using different carbon sources to produce rhamnolipids: glycerol, vegetable oils, hydrocarbons and others [3,5]. Biosurfactant production is possible using either free or immobilized cells in culture. Immobilization involves the restriction of cell mobility within a defined space, which provides high cell concentrations and permits the reuse of the cells [7,10]. Both techniques have some advantages and disadvantages, e.g., economical and technical. The use of immobilized whole cells in industrial processes has attracted considerable attention during the past few years due to its advantages over traditional processes, and the development of cell immobilization techniques has gained momentum in recent years due to its potential application in different areas of biotechnology, including biosurfactant production. The aim of this study was to compare the productivity of rhamnolipid production in batch systems in which either free or immobilized *Pseudomonas* sp. cells were grown using glucose as a substrate.

MATERIAL and METHODS

Microorganism

Pseudomonas sp. BKK041 was obtained from the culture collection of the Department of Biology, Faculty of Science and Arts, Mustafa Kemal University (Hatay, Turkey). The stock culture was maintained on a nutrient agar slant at 4°C.

Culture Medium

The stock culture (24 h in nutrient agar) was tranferred into a 250-ml Erlenmayer flask containing 50 ml of liquid medium (1.28 g $NaNO_3$, 0.87 g K_2HPO_4, 0.1 g $MgSO_4{\cdot}7H_2O$, 0.1 g NaCl, 0.2 g KCl, 6.5 g Tris (hydroxymethyl aminomethane), 20.0 g glucose, 5 ml Mineral Salt Solution [0.116g $FeSO_4$ $(NH_4)_2SO_4.6H_2O$, 0.232 g H_3BO_3, 0.41 g $CoCl_2.6H_2O$, 0.008 g $CuSO_4.5H_2O$, 0.008 g $MnSO_4.H_2O$, 0.022 g $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.174 g $ZnSO_4.7H_2O$ in 1 L] in 1 L), then incubated for 24h at 30°C [11]. Culture media were sterilized in an autoclave.

Rhamnolipid Determination

Two ml of the seed culture was inoculated into a 250-ml flask containing 50 ml of liquid culture medium. All samples were incubated at 150 rpm for 26 h. The culture broth was then centrifuged at 5.000 rpm for 12 min. Rhamnolipid production was determined by a colorimetric assay at 480 nm using a Shimadzu UV-VIS spectrophotometer. For this purpose, 2 ml of the cell-free broth (supernantant) was used. Rhamnolipid concentration was estimated by the phenolsulphuric acid method [12].

Glucose Determination

The glucose level was determined according to Miller [13] by the dinitro salicylic acid method at 550 nm using a Shimadzu UV-VIS spectrophotometer.

Cell Immobilization

Immobilization of cells was performed according to Cheetham et al.[8] with a minor modification. For this purpose, 0.6g of sodium alginate was dissolved in 20 ml of distilled water, then mixed with previously centrifuged cells. This mixture was poured into a beherglass that contained 0.5 M $CaCl_2$. All tests were performed in triplicate.

RESULTS

In the present study, we have investigated batch culture system effects on rhamnolipid production in free and immobilized *Pseudomonas* sp. BKK041 cells. The results shown in *Table 1* indicate that there is a substantial influence of temperature on biosurfactant production. The highest biosurfactant concentration (600 mg/L) was obtained at 30°C. Higher and lower temperature levels inhibited rhamnolipid production [14,17]. The glucose utilization curve showed a linear pattern throughout 26 hours of fermentation.

Table 2 shows the pattern of biosurfactant production by the free and immobilized cells of *Pseudomonas* sp. BKK041 in different pH conditions. These results indicate that pH variation had an appreciable effect on rhamnolipid production, as its concentration varied over a wide range

Table 1. *Values of rhamnolipid and final glucose concentration at different temperatures*
Tablo 1. *Farklı sıcaklıklardaki son glukoz konsantrasyonu ve ramnolipid miktarları*

Temperature (°C)	Free Cells		Immobilized Cells	
	Rhamnolipid (mg/L)	Final Glucose Concentration (g/L)	Rhamnolipid (mg/L)	Final Glucose Concentration (g/L)
20	50	0.025	0.2	0.250
25	470	0.375	4	0.300
30	600	3.450	300	3.200
35	10	3.175	3	2.000
40	2	0.250	0.2	0.250

of pHs. An initial pH value of 7 was found to be the most suitable for *Pseudomonas* sp. to produce rhamnolipid [14-18].

The effect of glucose concentration on rhamnolipid production is shown in *Table 3*. The maximum biosurfactant concentration (387.5 mg/L) was obtained at an initial glucose concentration of 2% (w/v). Higher and lower glucose concentrations inhibited rhamnolipid production [17-20].

DISCUSSION

The results shown in *Tables 1-3* represent the average of triplicate trials. When the different batch processes are compared, it can be seen that free cells gave better results than immobilized cells *(Fig. 1-3)*. The reasons for this difference may be explained as a loss of activity during immobilization or the diffusion limitations between alginate gel surfaces and microorganisms. Similar results have been reported by Jamuna et al.[21], Kanwar et al. [22], and Goksungur and Guvenç [23]. However, their studies were on different substrates and used different microorganisms. If the limitations with the rhamnolipid production techniques can be solved, the advantages of free cells utilization would increase, in both batch and continuous culture systems.

It is well known that biosurfactants are surface-active agents produced by bacteria, yeasts, and fungi, and include

Table 2. *Values of rhamnolipid and final glucose concentration at different pH values*
Tablo 2. *Farklı pH değerlerindeki son glukoz konsantrasyonu ve ramnolipid miktarları*

pH	Free Cells		Immobilized Cells	
	Rhamnolipid (mg/L)	Final Glucose Concentration (g/L)	Rhamnolipid (mg/L)	Final Glucose Concentration (g/L)
5.0	2.50	0.250	0.25	0.450
6.0	12.5	0.500	0.10	0.900
7.0	410	4.000	320	3.400
8.0	25	0.400	20	2.100

Table 3. *Values of rhamnolipid and final glucose concentration at different glucose concentrations*
Tablo 3. *Farklı glukoz konsantrasyonlarındaki son glukoz ve ramnolipid miktarları*

Glucose Concentration (%)	Free Cells		Immobilized Cells	
	Rhamnolipid (mg/L)	Final Glucose Concentration (g/L)	Rhamnolipid (mg/L)	Final Glucose Concentration (g/L)
0.5	2.5	0.050	0.25	1.250
1.0	5	0.125	1	2.000
1.5	300	1.375	10	1.500
2.0	387.5	3.200	210	2.200
2.5	200	1.725	100	1.100

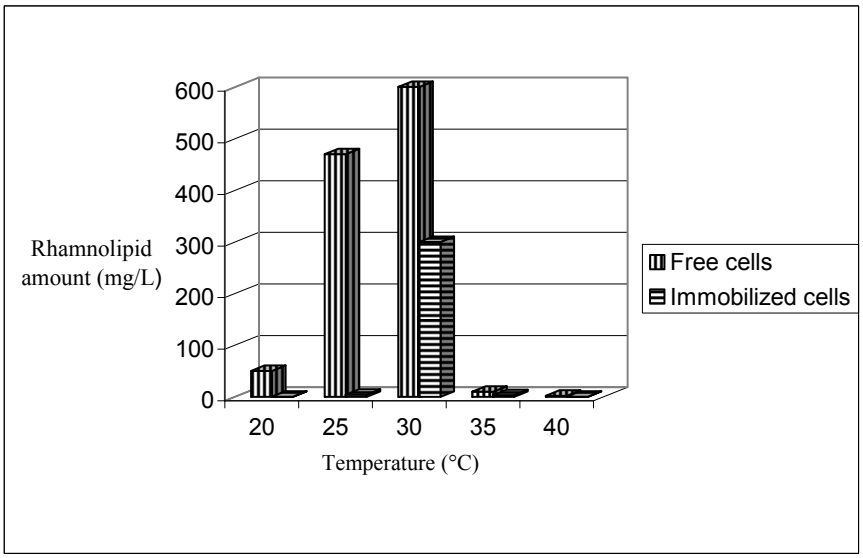

Fig 1. The effect of temperature on rhamnolipid production

Şekil 1. Ramnolipid üretimine sıcaklığın etkisi

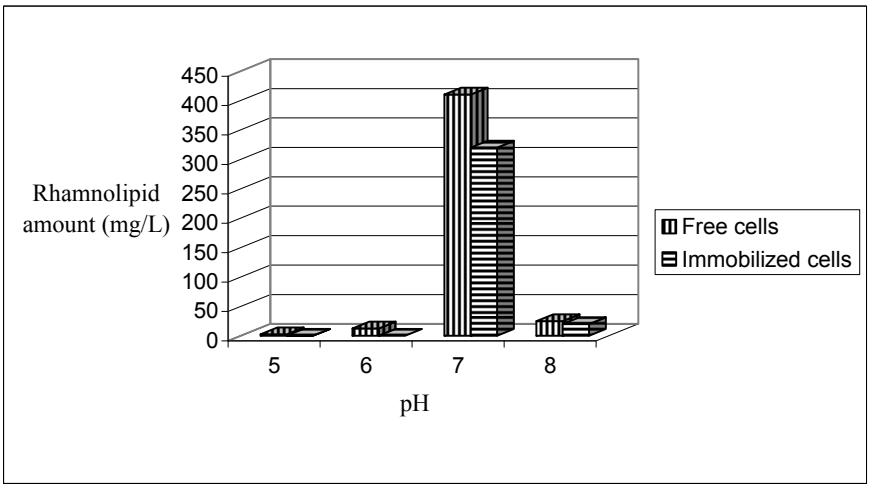

Fig 2. The effect of pH on rhamnolipid production

Şekil 2. Ramnolipid üretimine pH'ın etkisi

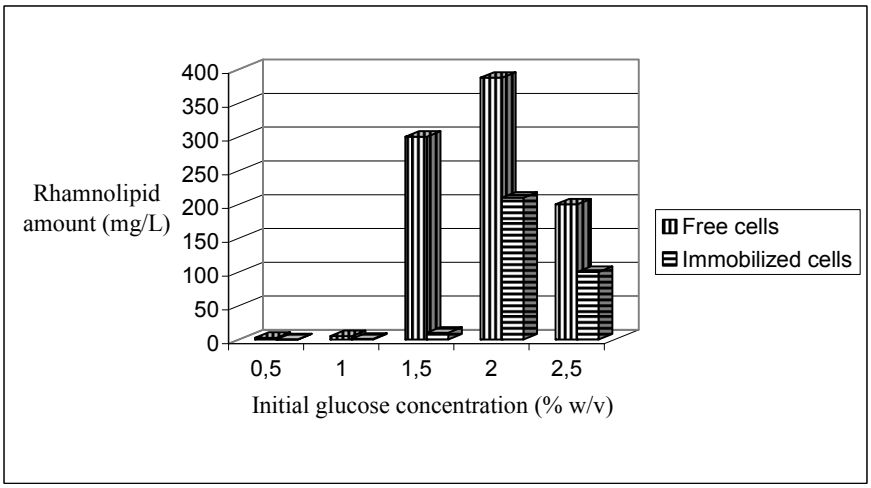

Fig 3. The effect of initial glucose concentration on rhamnolipid production

Şekil 3. Ramnolipid üretimine başlangıç glukoz konsantrasyonunun etkisi

products such as fatty acids, glycerides, phospholipids, lipopeptides, antibiotics, and glycolipids. Thus, in recent years there has been much interest in the production of biosurfactants from microorganisms for use in different applications in many industries. For example, demand for biosurfactants has increased in the oil and cosmetics industries, among others, because biosurfactants are easily degradable by microorganisms, compared with synthetic surfactants, which are highly resistant to biodegradation or are only partially biodegradable [24]. The rhamnolipids from *Pseudomonas aeruginosa* were first described by Jarvis and Johnson [25], and studies on the biosynthesis of these compounds in optimal *in vitro* conditions were carried out by others [26-28], who reported that these glycolipids were secreted into the medium during the stationary phase of growth [29].

For these reasons, many studies have been conducted to improve the production of biosurfactants. Biosurfactant production optimization appears to be one of the main fields of research, owing to the low product yields [30]. However, fermentation technology for biosurfactant production using immobilized cells has not been applied widely yet. In conclusion, this study showed that, the yield of the biosurfactant rhamnolipid was low using the immobilized cell technique. We suggested that it may be possible to improve this method in further studies. In addition, Pseudomonas sp. BKK041 free cells presented good potential for the production of a rhamnolipid-type biosurfactant.

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REFERENCES

1. Koch KA, Kappeli O, Fiechter A, Reiser J: Hydrocarbon assimilation and biosurfactant production in *Pseudomonas aeruginosa* mutants. *J Bacteriol*, 173, 4212- 4219, 1991.

2. Reiser J, Koch AK, Jeny K, Kappeli O: Structure, properties and production of biosurfactants. **In,** Obringer JW, Tillinghast HS (Eds): Advances in Biotechnology for Aerospace Applications. pp. 85- 97, Portfolio, The Woodlands, 1989.

3. Prieto LM, Michelon M, Burkert JFM, Kalil SJ, Burkert CAV: The production of rhamnolipid by a *Pseudomonas aeruginosa* strain isolated from a southern coastal zone in Brazil. *Chemosphere,*71, 1781-1785, 2008.

4. Singer MEV, Finnerty WR: Physiology of biosurfactant synthesis by *Rhodococcus* Species H13-A. *Canadian J Microbiol,* 36, 741-745, 1990.

5. Costa SGAO, Nitschke M, Haddad R, Eberlin, MN, Contiero J: Production of *Pseudomonas aeruginosa* LBI rhamnolipids following

growth on Brazilian native oils. *Process Biochem,* 41, 483-488, 2006.

6. Yilmaz EŞ, Sidal U: Investigation of antimicrobial effects of a Pseudomonas-originated biosurfactant. *Biologia*, 60, 723-725, 2005.

7. Champagne CP, Gaudy C, Poncelet D, Neufeld RJ: *Lactobacillus lactis* Release from calcium alginate beads. *Appl and Environ Microbiol,* 58, 1429-1434, 1992.

8. Cheetham PSJ, Blunk KW, Bucke C: Physical studies on cell immobilization using calcium alginate gels. *Biotech Bioeng*, 21, 2155-2168, 1979.

9. Guoqlang D, Kaul R, Mattiasson B: Evaluation of alginate-immobilized *Lactobacillus casei* for lactate production. *Appl Microbiol Biotechnol*, 36, 309- 314, 1991.

10. Hang YD, Hamamci H, Woodams EE: Production of L (+)- lactic acid by *Rhizopus oryzae* immobilized in calcium alginate gels. *Biotechnology Letters,* 11, 119-120, 1989.

11. Para JL, Guinea J, Manresa MA, Robert M, Mercade ME, Comelles F, Bosch MP: Chemical characterization and physicochemical behavior of biosurfactants. *J Am Oil Chem Society*, 66, 141-145,1989.

12. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F: Colorimetric method of determination of sugar and related substances. *Anal Chem*, 28, 350-356,1956.

13. Miller GL: Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal Chem,* 31, 426- 428, 1959.

14. Mercade ME, Manresa MA: The use of agroindustrial by products for biosurfactant production. *J Amr Oil Chem Society,* 71, 61-64, 1994.

15. Mercade ME, Manresa MA, Robert M: Olive Oil Mill Effluent (OOME) New substrate for biosurfactant production. *Bioresource Technology,* 43, 1- 6, 1993.

16. Ramana KV, Karanth NG: Production of biosurfactants by the resting cells of *Pseudomonas aeruginosa* CFTR- 6. *Biotech Lett*, 11, 437-442, 1989.

17. Sidal U, Kurtonur C, Kolankaya N: *Pseudomonas* sp. ile zeytinyağı fabrikası atığından biyosürfektan eldesi. *Tr J Biology*, 24, 611-625, 2000.

18. Rocha C, San Blas F, San Blas G, Vierma L: Biosurfactant production by two isolates of *Pseudomonas aeruginosa*. *World J Microbiol Biotechn*, 8, 125-128, 1992.

19. Ramana KV, Charyulu NCLN, Karanth NG: A mathematical model for the production of biosurfactants by *Pseudomonas aeruginosa* CFTR-6: Production of Biomass. *J Chem Tech Biotec,* 51, 525-538, 1991.

20. Rosenberg E: Exploiting microbial growth on hydrocarbons-new markets. *Tib Tech October,* 11, 419-424, 1993.

21. Jamuna R, Sai PST, Vora S, Ramakrishna SV: Optimization of critical parameters for immobilization of yeast cells to alginate gel matrix. *J Ferm Bioeng,* 73, 319-322, 1992.

22. Kanwar SS, Chadha BS, Tewari HK, Sharma VK: Continuous production of lactic acid from molasses by free and immobilized *Sporolactobacillus cellulosolvens. World J Microbiol Biotech,* 11, 687- 688, 1995.

23. Goksungur Y, Güvenç U: Batch and continuous production of lactic acid from beet molasses by *Lactobacillus delbrueckii* IFO 3202. *J Chem Tech Biotechnol,* 69, 399-404, 1997.

24. Abu-Ruwaida AS, Banat IM, Hadidirto S, Salem A, Kadri M: Isolation of biosurfactant-producing bacteria, product characterization and evaluation. *ACTA Biotechnologica,* 11, 315-324, 1991.

25. Jarvis FG, Johnson MJ: A glycolipide produced by *Pseudomonas aeruginosa. J Am Chem Soc*, 71, 4124- 4126, 1949.

26. Hauser G, Karnovsky ML: Studies on the production of glycolipide by *Pseudomonas aeruginosa*. *J Bacteriol,* 68, 645- 654, 1954.

27. Hauser G, Karnovsky ML: Rhamnose and rhamnolipide biosynthesis by *Pseudomonas aeruginosa. J Biol Chem*, 224, 91-105, 1957.

28. Hauser G, Karnovsky ML: Studies on the biosynthesis of L-rhamnose. *J Biol Chem,* 233, 287- 291, 1958.

29. Guerra-Santos L, Kappeli O, Fiechter A: *Pseudomonas aeruginosa* biosurfactant production in continuous culture with glucose as carbon source. *Appl Environ Microbiol,* 48, 301-305, 1984.

30. Abouseoud M, Yataghene A, Amrane A, Maachi R: Biosurfactant production by free and alginate entrapped cells of *Pseudomonas fluorescens. J Ind Microbiol Biotechnol,* 35, 1303-1308, 2008.