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Short Communication

Biosurfactant production in sugar beet molasses by some *Pseudomonas* spp.

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Abstract: *In this study, rhamnolipid biosurfactant production was investigated in eighteen strains of Pseudomonas spp.. Rhamnolipid by these strains was determined by a spectrophotometric method in nutrient broth medium (NB). From the 18 strains screened, two Pseudomonas strains (Pseudomonas luteola B17 and Pseudomonas putida B12) which had produced the highest percentage yield of rhamnolipid were examined for rhamnolipid production at different incubation times (24, 48 and 72 hr) and different sugar beet molasses concentrations [1-5 % w/v concentration (1-5 g molasses/100 ml water)]. The rhamnolipid production increased with the increase in the concentration of molasses and maximum production occurred when 5 % (w/v) of molasses were used. At the same time, maximum rhamnolipid production occurred after 72 hr of incubation. When the amount of rhamnolipid produced at different incubation times (24, 48 and 72 hr) and with different concentrations of molasses [1-5 % w/v concentration (1-5 g molasses/100 ml water)] by Pseudomonas spp.; was compared, no significant difference in amount of production was seen. These studies show that the waste product from sugar industry may be suggested for important biotechnological processes such as rhamnolipid production.*

Key words: *Pseudomonas spp., Rhamnolipid, Sugar beet molasses*
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Introduction

Surfactants and emulsifiers are widely used in the petroleum, pharmaceutical, cosmetic and food industries. Most of these compounds are chemically synthesized and it is only in the past few decades that surface-active molecules of biological origin have been described. At present biosurfactants are unable to compete with the chemical surfactants due to their high production costs. As biosurfactants are readily biodegradable and can be produced from renewable and cheaper substrates, they might be able to replace their chemically synthesized counter parts (Patel and Desai, 1997). Biosurfactants are extracellular macromolecules produced by bacteria, yeast and fungi and in particular by natural and recombinant bacteria when grown on different carbon sources. (Raza *et al.*, 2005). Several carbon sources such as ethanol, glucose, vegetable oil, and hydrocarbon have been used to produce biosurfactant. Specifically *Pseudomonas* species is well known for its ability to produce rhamnolipid biosurfactants with potential surface active properties when grown on different carbon substrates (Tahzibi *et al*., 2004) and rhamnolipid biosurfactants produced by these species have great potential for industrial application and bioremediation (Rashedi *et al*., 2006). There are two structure of rhamnolipid which are L-Rhamnosyl-Lrhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate and L- rhamnosyl - β -hydr oxydeca noyl- β – hydroxydecanoate (Rashedi *et al.*, 2005). The rhamnolipids were produced when hydrocarbons, glycerol, glucose, or peptone was the substrate. Best production was obtained with hydrocarbons or glycerol (Rashedi *et al.*, 2006). On the other hand *Pseudomonas* spp. can use the various renewal resources, especially agroindustrial wastes, as the potential carbon sources. This leads to the greater possibility for economical productions and reduced pollution caused by those wastes (Maneerat, 2005). One of the important points in the biotechnologically process is obtained maximum metabolite production with a low cost substrate (Cazetta *et al.*, 2005). The rhamnolipids produced by *Pseudomonas* species from different carbon sources have been extensively studied. Molasses is used in some of these studies as carbon sources since it is cheap (Rashedi *et al.*, 2005; Maneerat, 2005; Rashedi *et al.*, 2006; Raza *et al.*, 2007). Molasses, a sweet, dark brown thick liquid that is produced in the process of beet, presents a high sucrose concentration, other important substances for the fermentation process and low cost (Cazetta *et al.*, 2005).

The present study was conducted with the following objectives: screening of eighteen strains of *Pseudomonas* spp. for biosurfactant production, selecting of two strains produced the highest percentage yield of rhamnolipid from the 18 strains screened and examining for rhamnolipid production at different incubation times and different molasses concentrations.

Materials and Methods

Bacterial strains: *Pseudomonas* spp. were obtained from the culture collection of the Biotechnology Laboratory of Gazi University, Department of Biology Faculty of Arts and Science, in Turkey. Stock culture was maintained on nutrient agar slants (Atlas and Parks, 1997) at 4°C and the bacterial strains were stored frozen at -20°C in 10% glycerol broth to supply a stable inoculum for this study and subcultured twice before use in the manipulations.

Table - 1: The production of rhamnolipid of the some *Pseudomonas* spp. in nutrient broth medium

Bacteria	Rhamnolipid production (g l^{-1})
P. aeruginosa B3	0.30 ± 0.0
P. aeruginosa B16	0.30± 0.0
P. aeruginosa B19	0.25 ± 0.0
P. aeruginosa B20	0.32 ± 0.0
P. fluorescens B4	0.24 ± 0.1
P. fluorescens B5	0.33 ± 0.0
P. fluorescens B6	0.20 ± 0.0
P. fluorescens B7	0.32 ± 0.0
P. stutzeri B8	0.30 ± 0.0
P. stutzeri B9	0.30 ± 0.1
P. stutzeri B10	0.24 ± 0.0
P. stutzeri B11	0.22 ± 0.2
P. cepacia B13	0.20 ± 0.0
P. cepacia B14	0.31 ± 0.0
P. putida B12	0.36 ± 0.0
P. putida B15	0.21 ± 0.3
P. putida B18	0.20 ± 0.0
P. luteola B17	0.38 ± 0.0

Values are the means ±SD of triplicate experiments

Medium: All the *Pseudomonas* spp. were cultivated firstly in nutrient broth (NB) (Atlas and Parks, 1997). The cultures were incubated in this media at 37°C for 72 hr. Cells grown in nutrient broth (NB) were initially used for rhamnolipid production as the inoculum (1%, v/v). From the 18 strains cultivated, two *Pseudomonas* strains (*Pseudomonas luteola* B17 and *Pseudomonas putida* B12) were selected which had produced the highest percentage yield of rhamnolipid in NB medium. Rhamnolipid production by these cultures was carried out 250-ml Erlenmeyer flasks containing 50 ml of the molasses, followed by incubation on a rotary shaker (130 rpm) at 37±2°C. Molasses was diluted with distilled water to required concentrations, pH adjusted to 7.0 and then sterilized in an autoclave (Joshi *et al*., 2008).

Rhamnolipid isolation and purification: The pH of the culture supernatant fluid (3 ml) obtained after removal of the cells by centrifugation at 6,000 rpm for 10 min was adjusted to 2.0 and allowed to stand overnight at +4°C, followed by extraction with a mixture of $CHCl_3$ and CH_3OH (2:1 v/v). The solvent was evaporated and residue dissolved in 0.1 mol l^{-1} $NaHCO_3$ (3 ml) (Patel and Desai, 1997).

Determination of rhamnolipid concentration: Rhamnolipid concentration was determined according to Dubois *et al.* (1956) by the colorimetric phenolsulphuric acid method at 480 nm by the spectrophotometer Hitachi UV-VIS.

Statistical analysis: Results are average of at least three independent trials. Statistical significance was assessed by ANOVA followed by Tukey HSD software package (SPSS version 11.0). The level of significance was defined at $p<0.05$.

Results and Discussion

Biosurfactants can be produced with high yield by some microrganisms, especially *Pseudomonas* spp. (Maneerat, 2005). These strains produce rhamnolipid biosurfactants during growth on molasses as the primary carbon sources (Sudhakar Babu *et al*., 1996). Sudhakar Babu *et al.* (1996) have reported rhamnolipid production by *P. aeruginosa* from industrial wastes (distillery and whey wastes) as substrates. The *Pseudomonas* spp. used in this study, produced rhamnolipid biosurfactants when grown with molasses as the carbon and energy source. Few reports have been published on the use of waste as substrates for rhamnolipid production. From distillery waste and whey milk, a production of 1.85 gl^{-1} and 1.78 gl^{-1}, respectively, was reported by Maneerat (2005). Patel and Desai, (1997) produced 0.24 gl^{-1} from molasses. In this study we investigated firstly the production of rhamnolipid biosurfactant of eighteen strains of *Pseudomonas* spp. in NB. Table 1 shows the production of rhamnolipid in these strains. While the rhamnolipid productivity percentage in *P. luteola* B17 and *P. putida* B12 were the highest (0.4 gl^{-1}), the lowest rhamnolipid productivity was found in *P. stutzeri* B10 (0.1 gl^{-1}) (Table 1).

From the 18 strains screened, two *Pseudomonas* strains (*P. luteola* B17 and *P. putida* B12) which had produced the highest percentage yield of rhamnolipid were examined for rhamnolipid production at different incubation times (24, 48, 72 hr) and different sugar beet molasses concentrations [1-5 % w/v concentration (1-5 g molasses/100 ml water)] (Table 2). There was not any difference in the production of rhamnolipid biosurfactant over 5 % molasses concentrations and incubation beyond 72 hr, for this reason these conditions were selected as upper concentration.

In our study, when compared to control, the rhamnolipid production increased with the increase in concentration of molasses and maximum production obtained when 5 % (v/v) of molasses was used. Maximum rhamnolipid production occurred after 72 hr of incubation. However, according to incubation times (24, 48 and 72 hr) with molasses concentrations [1-5 % w/v concentration (1-5 g molasses/100 ml water)], rhamnolipid produced of *P. luteola* B17 and *P. putida* B12 strains, any significant difference in amount of production was not observed. The statistical results indicated that rhamnolipid biosurfactant increased at all concentrations [1-5 % w/v concentration (1-5 g molasses/100 ml water)] at three time interval (24, 48, 72 hr) in a concentration-dependent manner, when compared with the control ($p<0.05$). As a result of this study 24 hr incubation period may be enough for production of rhamnolipid biosurfactant at different molasses concentrations. Therefore, *Pseudomonas* spp. showed the ability to utilize molasses for growth and rhamnolipid production and did not require the presence of additional nutrients.

Patel and Desai, (1997) used the molasses and cornsteep liquor as the primary carbon and nitrogen source to produce rhamnolipid biosurfactant from *P. aeruginosa* GS3. The biosurfactant production reached the maximum when 7 % (v/v) of molasses and 0.5 (v/v) of cornsteep liquor were used. Maximal surfactant production occurred after 96 hr of incubation, when cells reached

Table - 2: The production of rhamnolipid of the *P. luteola* B17 and *P. putida* B12 strains in different incubation times and in different molasses concentrations

Strains	Molasses	Rhamnolipid production gl^{-1}		
		24 hr	48 hr	72 hr
P. luteola B17	Control (NB)	0.17±0.05	0.23±0.00	0.38±0.00
	1%	0.40±0.01	0.43±0.05	0.45±0.05
	2%	0.44±0.02	0.45±0.00	0.47±0.00
	3%	0.46±0.05	0.46±0.00	0.48±0.00
	4%	0.49±0.00	0.51±0.01	0.51±0.01
	5%	0.51±0.03	0.53±0.02	0.53±0.02*
P. putida B12	Control (NB)	0.18±0.02	0.24±0.05	0.36±0.00
	1%	0.44±0.00	0.46±0.00	0.47±0.00
	2%	0.45±0.00	0.48±0.05	0.48±0.05
	3%	0.46±0.00	0.49±0.00	0.49±0.00
	4%	0.47±0.01	0.49±0.01	0.51±0.01
	5%	0.50±0.03	0.51±0.04	0.52±0.04*

Values are the means ±SD of triplicate experiments, * : The highest production, NB : Nutrient broth medium

the stationary phase of growth. Rashedi *et al.* (2006) studied biosurfactant production using medium A, with varying concentrations of molasses being used as the sole source of carbon. The biosurfactant production increased with the increase in the concentration of molasses and maximum production occurred when 7% (v/v) of molasses was used. Both two authors reported that increasing the concentration of molasses did not affect surfactant production significantly. Our studies showed which is in agreement with both studies reported in the literature.

Based on the experimental results it can be concluded that *Pseudomonas* spp. can utilize molasses as carbon sources for rhamnolipid production. Therefore, it is feasible to use cheaper substrates for rhamnolipid production.

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