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Full Length Research Paper

Determination of antimicrobial activity and production of some metabolites by *Pseudomonas aeruginosa* B1 and B2 in sugar beet molasses

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In the present study, exopolysaccharide (EPS), pyocyanin, rhamnolipid productions, total cell proteins and antimicrobial activities were investigated in two strains of *Pseudomonas aeruginosa* (B1 and B2). Exopolysaccharide, pyocyanin and rhamnolipid production of strains were determined in nutrient broth medium (NB) as control and different sugar beet molasses concentrations (1-5 % w/v) at different periods (24, 48 and 72 h) by a spectrophotometric method. The exopolysaccharide, pyocyanin and rhamnolipid productions of both strains increased after incubation when 5% (w/v) of molasses were used. Therefore the results disclose the correlations between the increasing molasses concentrations and EPS, pyocyanin and rhamnolipid productions of *P. aeruginosa* B1 and B2 strains (*P*≤0.01). *Bacillus subtilis* ATCC 6633 was more sensitive than *Escherichia coli* 11230 to the inhibitory effect of *P. aeruginosa* B1 and B2. Moreover, these strains exhibited the highest antimicrobial activity against both test bacteria in sugar beet molasses (5% w/v) at 72 h. Significant increases in total cell protein and antimicrobial activity of *P. aeruginosa* B1 and B2 were observed with increasing the molasses concentrations (*P*≤0.01). The results also indicated that strains having high total cell protein were higher antimicrobial activity, EPS, pyocyanin and rhamnolipid productions.

Key words: *Pseudomonas aeruginosa*, exopolysaccharide, pyocyanin, rhamnolipid, antimicrobial activity, sugar beet molasses.

INTRODUCTION

Pseudomonas aeruginosa is gram-negative bacterium found in almost every ecological niche, including soil, water and plants (Deziel et al., 2001). It is frequently isolated from contaminated sites and is capable of producing metabolites (i.e., alginate, rhamnolipid, pyocyanin) that enhance its competitiveness and survival. For example, the water-soluble secondary metabolite pyocyanin (1-hydroxy-5-methylphenazine) has demonstrated antimicrobial activity against a variety of microorganisms (Norman et al., 2004). The antimicrobial action of pyocyanin is bactericidal in nature (Baron and Rowe, 1981). This pigment has been used diagnostically to describe both the physiology and the pathogenicity of this bacterium. The synthesis of pyocyanin is affected by carbon and nitrogen sources in growth media (Cox, 1986). Pyocyanin production may give *P. aeruginosa* a selective advantage in certain growth situations (Baron and Rowe, 1981). However, exopolysaccharides are essential to the biological success of most bacteria living within the biofilm in the varied natural environments in which they are observed since they can concentrate nutrients from water flow and protect the bacteria from antibacterial agents and from predators. Exopolysaccharides-producing microorganisms are emerging as a new and industrial important source of polymeric materials. Several bacterial strains produce EPS that can be referred to as capsular polysaccharide, when attached to the cell wall as a capsule, or as slime EPS or free EPS when produced as loose unattached

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material. EPSs are of great ecological importance with respect to bacterial growth and survival strategies in different environments (Fusconi and Godinho, 2002). A wide range of bacteria, from clinical and environmental habitats, is known to produce complex and diverse exopolysaccharides (EPSs), occurring as capsular polysaccharides intimately associated with the cell surface or as slime polysaccharides, loosely associated with the cell. These are distinguished by the degree of cell association following centrifugation (Royan et al., 1999). Also, biosurfactants are extracellular macromolecules produced by bacteria, yeast and fungi and in particular by natural and recombinant bacteria when grown on different carbon sources. The microbial surfactants have gained attention in recent years due to their commercial importance, diverse desirable characteristics such as biodegradability, selectively effectiveness, low toxicity, ecological acceptability and their ability to be produced from cheaper substrates (Raza et al., 2005). Among the heterogeneous group of biosurfactants, are the rhamnose-containing glycolipids produced by *Pseudomonas* spp. Rhamnolipid have been known as biosurfactant which is produced by *P. aeruginosa* in fermentation process. Several carbon sources such as ethanol, glucose, vegetable oil, and hydrocarbon have been used to produce rhamnolipid. Microbial surfactants are generally less toxic and more biodegradable than synthetic surfactants. Rhamnolipid biosurfactants specifically produced by *P. aeruginosa* have great potential for industrial application and bioremediation (Rashedi et al., 2006).

The various advantages of producing EPS, pyocyanin and rhamnolipid from Pseudomonads include independence from weather conditions, easy and fast growth, colors of different shades, competitiveness and survival, function in evasion of host defense mechanisms, bacterial adhesion and resistance to antibacterial agents and growth on cheap substances (Norman et al., 2004; Joshi et al., 2003; Cerantola et al., 2000). EPS, pyocyanin and rhamnolipid-producing *Pseudomonas* spp. are finding vast potential applications in environmental protection, petroleum, food, mining, agriculture, pharmaceutical, textile, leather and other industries (Royan et al., 1999; Raza et al., 2005).

Pseudomonas spp. can use the various renewal resources, especially agro-industrial 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 that it has obtained maximum metabolite production with a low cost substrate (Rashedi et al., 2006). Molasses is used in the studies as carbon sources since it is cheap (Maneerat, 2005; Wu et al., 2001; Page, 1989; Page, 1992a,b; Beaulieu et al., 1995). 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 aim purpose of this study is to determine of antimicrobial activities, total cell proteins, exopolysaccharide, pyocyanin and rhamnolipid productions in different sugar beet molasses concentrations of *P. aeruginosa* B1 and B2 strains.

MATERIALS AND METHODS

Bacterial strains, media and growth conditions

P. aeruginosa B1 and B2 were obtained from the culture collection of the Biotechnology Laboratory of Gazi University, Department of Biology Faculty of Arts and Science, in Turkey. The bacterial strains were stored frozen at -80ºC in 10% glycerol broth to supply a stable inoculum for this study and subcultured twice before use in the manipulations.

Exopolysaccharide, pyocyanin and rhamnolipid productions of *P. aeruginos*a B1 and B2 were carried out 250-ml Erlenmeyer flasks containing 100 ml of the molasses and nutrient broth medium (NB) (Atlas and Parks, 1997) (as control) followed by incubation on a rotary shaker (130 rpm) at 37±2˚C, after active cultures inoculated as 1% (v/v).

Bacterial growth was measured in terms of whole cell protein, for which cells were harvested by centrifuging (5.000 *g*, 10 min) and the supernatant protein concentrations were determined according to Bradford (Amresco, E535-KIT, Protein Assay Bradford Method) (Bradford, 1976).

Isolation and quantification of EPS

The strains were cultivated in molasses (concentration 1-5 g molasses/100 ml water). The production of EPS by these strains was investigated in different concentrations of beet molasses at different periods (24, 48 and 72 h). After inoculation, broth cultures were incubated at 37ºC for 24 h. The cultures were boiled at 100ºC for 10 min. After cooling, they were treated with 17% (v/v) of 85% trichloracetic acid solution (Merck) and centrifuged (Frengova et al., 2000). Removal of cells and protein was done by centrifugation. EPS was precipitated with ethanol. It was recovered by centrifugation at 4ºC at 14.000 rpm for 20 min. Total EPS (expressed as mg/L) was estimated in each sample by phenol-sulphuric method (Dubois et al., 1956) using glucose as standard (Torino et al., 2001).

Determination of rhamnolipid

Bacterial cells were removed from rhamnolipid containing medium by centrifugation at 6.000 rpm for 10 min. This crude extract was dried with the aid of a rotary evaporator under vacuum (Yılmaz and Sıdal, 2005).

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).

Pyocyanin purification

Pyocyanin was extracted from the broth culture of *P. aeruginosa* B1 and B2 as previously described (Essar, 1990). The bacteria were removed by centrifugation after incubation. The culture supernatant was mixed with chloroform to remove most nonpyocyanin pigments. The blue pigments in chloroform were extracted by 10 mM HCl followed by neutral water. The pyocyanin was partitioned to the HCl aqueous phase, which was taken for quantification at OD_{520} (Dubois et al., 1956).

Inhibitory effect by the agar-well diffusion method

B. subtilis ATCC 6633, *E. coli* ATCC 11230, *Staphylococcus aureus* ATCC 25923, *Candida albicans* ATCC 10239 strains were used in this study. These strains were cultivated in nutrient broth (NB) medium and *C. albicans* was cultivated in YEPD broth medium (Atlas and Parks, 1997). The inhibitory effect of *P. aeruginosa* B1 and B2 strains were determined with agar diffusion method (Torino et al., 2001). Petri dishes with 20 ml of Nutrient (NA) agar and YEPD agar (Oxoid) were prepared. *B. subtilis*, *E. coli*, *S. aureus*, *C. albicans* cultures was added to culture medium, and 100 µl of these cultures was inoculated in each Petri dishes. Once solidified, the dishes were stored for 2 h at 4°C. Four wells (≈ 6 mm) were made and the wells were filled with 100 µl culture filtrate. The inoculated plates were incubated for 24 h at 37°C. The diameter of the inhibition zone was measured with calipers (Attaie et al., 1987; Okereke and Montville, 1991).

Statistical analysis

Statistical analysis was performed by SPSS (Version, 11.0). Person's correlation was used for determining any significant difference between whole cell protein of the strains and EPS, pyocyanin and rhamnolipid production amount of the strains. Also, Person's correlation was used for determining any significant difference between pyocyanin and rhamnolipid production of the strains and antimicrobial activity of the strains. Significance was determined at α = 0.01 level. If significant differences were indicated among treatment means ($P \leq 0.01$), means were differentiated using the least square mean test at $\alpha \leq 0.01$.

All analyses were run in triplicate for each of replications. Results are reported as mean ± standard deviation (S.D.).

RESULTS

In the present study, *P. aeruginosa* B1 and B2 were allowed to grow for different times (24, 48 and 72 h) in nutrient broth (NB) medium as control and during these periods, exopolysaccharides (EPSs), pyocyanin and rhamnolipid biosurfactant productions of strains were determined. Also EPS, pyocyanin and rhamnolipid productions were studied, with varying molasses concentrations (1-5%) being used as the sole source of carbon. These studies showed that the amounts of EPS, pyocyanin and rham-nolipid produced by two strains in NB media (control) were lower than the amount of EPS, pyocyanin and rhamnolipid productions in molasses concentrations of 1, 2, 3, 4 and 5% (w/v). While the EPS, pyocyanin and rhamnolipid productions in 5% molasses by *P. aeruginosa* B1 after 72 h were the highest (0.74±0.4 g/L, 17.1±0.0 µg/mL, 0.55±0.04 g/L, respectively), the lowest EPS, pyocyanin and rhamnolipid productions were found in 1% molasses concentration in *P. aeruginosa* B1 (0.16±0.0 g/L, 0.6±0.2µg/mL, 10.6±0.2 µg/mL, 0.47±0.01 g/L, respectively) after 24 h. In both strains, increasing the molasses concentration led to an increase in the EPS, pyocyanin and rhamnolipid productions. EPS, pyocyanin and rhamnolipid productions by *P. aeruginosa* B2 in 5% molasses after 72 h were the highest obtained (0.70±0.0 g/L, 5.8±0.0 µg/mL, 0.58±0.03 g/L, respectively). As shown in Table 1, the amounts of EPS, pyocyanin and rhamnolipid produced by *P. aeruginosa* B2 in 1% molasses (0.24±0.04 g/L, 0.3±0.3 µg/mL, 0.52±0.04 g/L, respectively) were lower than the amount of EPS, pyocyanin and rhamnolipid productions in molasses concentrations of 3, 4 and 5% after 24 h. No conclusive differences for both strains were determined at 5 and 6% molasses concentrations at 72 and 96 h. The results indicated that increasing molasses concentrations have a significant effect on growth and EPS, pyocyanin and rhamnolipid production ($P \leq 0.01$). The highest metabolite productions of strains occurred after 72 h of incubation, when 5 % (w/v) of molasses were used (Table 1). However, the biomass increased with the increase in the concentration of molasses, as evident from the whole cell protein (Table 1). At the same time, our study showed that molasses is more effective in the production of EPS and pyocyanin than rhamnolipids. Further increasing the concentration of molasses did not affect rhamnolipid biosurfactant production significantly. There is a positive correlation between EPS, pyociyanin and rhamnolipid productions quantity of the strains and whole cell protein and their correlation is significant at the 0.01 level.

On the other hand, in this study, *P. aeruginosa* B1 and B2 strains were assayed for ability to produce inhibitory substances against the growth of *B. subtilis*, *E. coli*, *S. aureus* and *C. albicans*. The results concerning the determination of the antimicrobial effect by the agar diffusion method are presented in Table 2. In this method, *P. aeruginosa* B1 and B2 inhibited the growth of *B. subtilis and E. coli.* However, *P. aeruginosa* B1 and B2 strains did not inhibit the growth of *S. aureus* and *C. albicans.* Antimicrobial activity reached the maximum when 5% molasses were used and occurred after 72 h of incubation. The highest antimicrobial activity of *P. aeruginosa* B1 and B2 strains was 25.6 - 26.4 mm against *B. subtilis*, whereas the lowest antimicrobial activity were 6.0 - 4.2 mm against *E. coli,* respectively. According to these results, the antimicrobial activity increased with the increase in the concentration of molasses and incubation period. These results indicated that strains having pyocyanin and rhamnolipid productions have higher antimicrobial activity. Therefore, increasing molasses concentrations stimulated antimicrobial activity of *Pseudomonas* spp. positively. Positive linear relationships existed between antimicrobial activitiy against *E. coli* and *B. Subtilis* and rhamnolipid productions of the strains. Their correlation was found significantly ($P \leq 0.01$).

DISCUSSION

Molasses contains 20% water, 8% inorganic matters, 72% sugar and non-sugar organic substances such as organic acids, lipids and inorganic salts, invert sugar and macromolecules of high weight (starch, cellulose, hemicelluloses, lignin, pectin, tannin) (Toğrul and Arslan, 2004). Cheap substrates, in addition to improved fermentation strategies, low-cost media and easier downstream

Table 1. The productions of EPS, pyocyanin and rhamnolipid of the *P. aeruginosa* B1 and B2 strains in different molasses concentrations and at different incubation times.

MOLASSES		Whole cell protein (mg/ml)			EPS (g/L)			Pyocyanin (µg/ml)			Rhamnolipid (g/l)		
		24 h	48 h	72 h	24 h	48 h	72h	24 h	48 h	72 h	24 h	48 h	72 h
P. aeruginosa B1	Control (NB)	0.005±0.01	0.01±0.01	0.01±0.01	0.03±0.01	0.05±0.1	0.08±1.0	4.9±0.0	6.1±0.0	6.6±0.2	0.39±0.05	0.43±0.02	0.44±0.02
	1%	0.006±0.01	0.01±0.01	0.01±0.01	0.16±0.01	0.19±0.2	0.24±0.5	0.6±0.2	2.5±0.0	3.2±0.1	0.47±0.01	0.48±0.0	0.49±0.01
	2%	0.01±0.01	0.01±0.01	0.02±0.01	0.52±0.00	0.52±0.0	0.56±0.3	1.1±0.0	7.9±0.1	9.8±0.1	0.49±0.02	0.50±0.02	0.51±0.01
	3%	0.01±0.01	0.02±0.01	0.03±0.01	0.60±0.03	0.61±0.5	0.69±0.0	1.5±0.3	8.9±0.0	9.9±0.2	0.52±0.02	0.54±0.04	0.54±0.01
	4%	0.01±0.01	0.02±0.01	0.03±0.01	0.73±0.02	0.74±0.1	0.74±0.0	1.8±0.4	15.1±0.2	15.4±0.0	0.53±0.0	0.54±0.04	0.55±0.03
	5%	0.02±0.01	0.03±0.01	0.04±0.01	0.74±0.00	0.74±0.0	0.74±0.4	5.5±0.0	16.6±0.0	17.1±0.0	0.54±0.03	0.55±0.05	0.55±0.04
P. aeruginosa B2	Control (NB)	0.008±0.01	0.01±0.01	0.01±0.01	0.06±0.02	0.08±0.4	0.09±0.2	0.3±0.1	0.4±0.1	0.5±0.0	0.48±0.02	0.51±0.01	0.52±0.02
	1%	0.01±0.01	0.01±0.01	0.01±0.01	0.24±0.04	0.240.2	0.31±0.5	0.3±0.3	1.7±0.2	2.0±0.3	0.52±0.04	0.53±0.03	0.53±0.02
	2%	0.01±0.01	0.01±0.01	0.02±0.01	0.52±0.02	0.52±0.3	0.53±0.0	0.8±0.2	1.9±0.2	2.1±0.2	0.53±0.02	0.54±0.01	0.54±0.01
	3%	0.01±0.01	0.02±0.01	0.03±0.01	0.56±0.02	0.60±0.1	0.60±0.0	1.1±0.0	3.2±0.1	3.4±0.4	0.55±0.01	0.55±0.01	0.55±0.01
	4%	0.01±0.01	0.02±0.01	0.03±0.01	0.65±0.00	0.69±0.1	0.69±0.5	1.4±0.5	5.5±0.0	5.6±0.2	0.56±0.01	0.56±0.0	0.56±0.04
	5%	0.01±0.01	0.02±0.01	0.04±0.01	0.67±0.03	0.70±0.0	0.70±0.0	1.6±0.1	5.6±0.0	5.8±0.0	0.56±0.03	0.57±0.0	0.58±0.03

Values are the means ±SD of triplicate measurements.

processes are required and can be obtained from agricultural wastes like whey or molasses, which are often used for fermentations (Omar et al., 2001). Molasses is effective in growth medium since it includes vitamins and minerals and has a significant growth stimulatory effect (Page, 1989).

Some specific metabolite production by *Pseudomonas* spp. has been found to be affected by carbon sources in medium (Rashedi et al., 2006; Osman et al., 1986). *Pseudomonas* BOP 100 has the capabilities for production of rhamnolipid and phenazine when grown on wastes as sole carbon source (Osman et al., 1996). The *P. aeruginosa* strains used in this study, produced some metabolites (EPS, pyocyanin and rhamnolipids) when grown with molasses as the carbon source.

Patel and Desai (1997) used the molasses and corn steep liquor as the primary carbon and nitrogen source to produce rhamnolipid biosurfactant from *P. aeruginosa* GS3. The biosurfactant production reached maximum when 7% (v/v) of molasses and 0.5 (v/v) of cornsteep liquor were used. Maximal surfactant production occurred after 96 h of incubation, when cells reached the stationary phase of growth (Rashedi et al., 2006). Biosurfactant production was studied using medium A, with varying molasses concentrations 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% molasses was used. Both authors reported that increasing the concentration of molasses did not affect surfactant production significantly. However, the biomass increased with the increase in the concentration of molasses, as evident from the whole cell protein. In the present study we used the molasses as the primary carbon source. Our studies is in agreement with both studies reported in the literature.

Several authors reported that pyocyanin and rhamnolipid substances had antimicrobial effect against some bacteria (Norman et al., 2004; Omar et al., 2001; Asthana et al., 1997). In this report, the antimicrobial activity of *P. aeruginosa* strains against pathogen bacteria increased with the increase in the concentration of molasses and incubation period (Table 1). *B. subtilis* ATCC 6633 was more sensitive than *E. coli* 11230 to the inhibitory effect of *P. aeruginosa* B1 and B2. Yılmaz and Sıdal (2005) reported that the antimicrobial activity against Gram positive bacteria was more effective than against Gram negative bacteria. In the present study, relation between antimicrobial activity and pyocyanin, rhamnolipid production was determined. This may be explained by the fact that the Gram negative bacteria have membranes that are hardly permeable to hydrophobic and amphipatic molecules. It is well-known that, the Gram-positive and Gram-negative bacteria have different chemical structure of the cell wall.

Table 2. Antimicrobial activity (inhibition zone diameter, mm) of *P. aeruginosa* B1 and *P. aeruginosa* B2 strains against *E. coli* ATCC 11230 and *B. subtilis* ATCC 6633.

Strain	Molasses	*E. coli* ATCC 11230*			*B. subtilis* ATCC 6633*		
		24 h	48 h	72 h	24 h	48 h	72 h
P. aeruginosa B1	CONTROL (NB)	NI	NI	NI	15.8±0.0	18.4±0.0	19.2±0.0
	1%	NI	NI	NI	17.8±0.0	19.0±0.0	21.2±0.0
	2%	6.0±0.0	7.0±0.0	11.8±0.0	19.4±0.0	19.6±0.0	22.0±0.0
	3%	6.0±0.0	8.2±0.0	13.0±0.0	22.8±0.0	23.0±0.0	23.2±0.0
	4%	8.0±0.0	12.2±0.0	13.6±0.0	22.0±0.1	22.8±0.0	23.4±0.0
	5%	10±0.0	12.8±0.0	13.8±0.0	25.0±0.0	25.2±0.0	25.6±0.0
P. aeruginosa B2	CONTROL (NB)	NI	NI	NI	16.0±0.0	16.0±0.0	18.0±0.0
	1%	4.2±0.0	5.0±0.0	6.0±0.0	16.0±0.0	17.0±0.0	18.4±0.0
	2%	6.8±0.0	6.8±0.0	7.0±0.0	19.6±0.0	19.8±0.0	20.0±0.0
	3%	7.8±0.0	10.4±0.0	10.4±0.0	21.0±0.0	22.0±0.0	23.2±0.0
	4%	9.4±0.0	11.4±0.0	11.4±0.0	22.0±0.0	22.6±0.0	23.8±0.0
	5%	11.4±0.0	11.4±0.0	13.2±0.0	24.6±0.0	24.8±0.0	26.4±0.0

NI: No inhibition.
*Values are the means ± standard deviations of triplicate measurements.

Conclusion

Molasses can be used as substrate for production of specific metabolites and general bacterial growth. Based on the experimental results it can be concluded that *P. aeruginosa* B1 and B2 can utilize molasses and produce effective EPS, pyocyanin and rhamnolipid. Therefore, it is feasible to use relatively inexpensive and renewable sources for industrial production.

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