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Enhanced Production of Biosurfactants by Marine *Pseudomonas aeruginosa*

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BIOSURFACTANTS are valuable microbial amphiphilic molecules with effective surface-active and biological properties applicable to several industries and processes. Microbes synthesize them, especially during growth on water-immiscible substrates, providing an alternative to chemically prepared conventional surfactants. The objective of this research was to evaluate factors affecting surfactant production from *Pseudomonas aeruginosa* which was dependent on some factors including carbon source, medium composition, pH, temperature and duration of incubation. Use of glucose as a carbon source enhanced the production more than the other carbon sources evaluated. Plackett-Burman statistical design was used to optimize culture conditions and evaluate the most significant variables affecting surfactant production. $(NH_4)_2SO_4$(5g/l) and Na_2HPO_4 (2g/l) were the most significant variables affecting biosurfactant production (emulsification activity) which was increased up to 1.2 fold. The optimum medium composition for biosurfactant production is as follows (g/l) : $(NH_4)_2SO_4$, 5; KH_2PO_4 , 5; $MgSO_4.7H_2O$, 1.5 ; glucose, 25; Na_2HPO_4 , 2; Na Cl, 2 and yeast extract , 0.2 ٠The optimum temperature and pH were 35°C and 6, respectively. Two days of incubation gave the highest production and increased biosurfactant yields up to 1.3 fold. Biosurfactant crude extract showed remarkable stability over a wide range in pH (6-10) and temperature (50-90ºC).

Keywords : Biosurfactant, *Pseudomonas aeruginosa*, Carbon sources, Plackett, Burman design.

Research on biosurfactant production has expanded in recent years due to its potential use in several different areas, including food industry, agriculture, pharmaceutics, the oil industry, petro-chemistry and paper industry (Soumen *et al*., 2006). Microbial-derived surfactants are amphipatic molecules produced by a wide variety of bacteria, yeasts and filamentous fungi (Krespky *et al.*, 2007). Due to increasing environmental concerns biological surfactants have been considered as alternative to chemical manufactured compounds. Some of the advantages of

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biosurfactants over those produced synthetically include lower toxicity, biodegradability, selectivity, higher specific activity at extreme temperatures, pH and salinity, the possibility of their production through fermentation, their potential applications in environmental protection and management, crude oil recovery, as antimicrobial agents in health care and food processing industries (Banat *et al*., 2000). Moreover, they can be modified by biotransformation to generate new products for specific requirements (Deleu & Paquot, 2004). Microbial surfactants show promising applications in bioremediation and waste treatment for removal of hazardous materials (Mulligan, 2005).

The genus *Pseudomonas* is capable of using a wide range of carbon substrates, such as glycerol, mannitol, fructose, glucose and vegetable oils, to produce rhamnolipid-type biosurfactants (Parthasarathi & Sivakumaar, 2009). The properties showed by rhamnolipids depend on their homologue composition and distribution, which are determined by the bacterial strain, culture conditions and medium composition (Guerra-Santos *et al*., 1984). The aim of this work was to: (i) Optimize the fermentation factors that enhance biosurfactant productivity from marine *P. aeruginosa*, and (ii) Study the stability of the produced biosurfactant.

Materials and Methods

Organisms , maintenance and biosurfactant production medium

The examined strains (*Bacillus subtilis, Vibrio fluvialis, Pseudomonas aeruginosa, Streptococcus faecalis and Streptoverticillium morookaense*) were isolated and identified in previous studies by Abou-elela *et al.* (2005, 2009a,b and El-Sersy *et al.* (2006). The cultures were maintained on nutrient agar medium. The biosurfactant production medium consisted of (g/l): $(NH_4)_2SO_4$, 7 ; Na_2HPO_4, 3.8 ; KH_2PO_4 ,3.5; $MgSO_4.7H_2O$, 0.7; NaCl , 5; yeast extract, 0.5 with 2% glucose (Carrillo *et al*., 1996). All media preparations were dissolved in 50% distilled water: 50% sea water.

Biosurfactant activity assays

All measurements were made in triplicate on cell-free broth obtained by centrifuging the cultures at 10,000 *g* for 25 min (Bodour & Maier, 1998).

Biodegradation of crude oil

Colonies of each strain were inoculated into 100 ml of Marine Broth 2216 (Difco, USA) containing 2 drops of weathered crude oil (Dutta & Harayama, 2000) and incubated with continuous shaking (200 rpm) for 24-48 hr at 28ºC . Those strains possessing biosurfactant producing activity, as evidenced by emulsification of weathered crude oil, scored as positive.

Haemolytic activity

The strains were screened on blood agar plates, which contained 5% (v/v) human blood, incubated at 28ºC 24 hr. Haemolytic activity was detected as a defined clear zone around the colony (Carrillo *et al*., 1996).

Drop collapsing test

2 µl of mineral oil was added to each well of a 96-well microtiter plate lid (Nunc, Roskilde, Denmark). The lid was equilibrated for 1 hr at room temperature, and then 5 µl of the cultural supernatant was added to the oil surface. The shape of the drop on the oil surface was inspected after 1 min exposure. Biosurfactant-producing cultures giving flat drops were scored as positive and those cultures with rounded drops were scored as negative, which is indicative of a lack of biosurfactant production (Youssef *et al*., 2004).

Oil displacement test

15 µl of weathered crude oil were placed on the surface of distilled water (40µl) in a petri dish (150 mm diameter) to which 10 µl of the culture supernatant was gently placed on the center of the oil film. The diameter and area of the clear halo seen under day light were measured and calculated after 30 sec as described by Morikawa *et al.* (1993).

Surface tension measurement

The surface tension measurement (s) of the cell free supernatant was determined by surface tensiometer (TD 1 Lauda tensiometer, Germany).

Emulsification measurement

The ability of the biosurfactant to emulsify liquid hydrocarbons as different substrates, including hexadecane, kerosene, hexane and parafin oil, was measured according to the method of Cooper & Goldenberg (1987) with a slight modification (Abouseoud *et al*., 2008). To 4 ml of culture supernatant, 4 ml of hexadecane was added and vortexed at high speed for 2 min. The mixture was allowed to stand for 10 min prior to measurement. The emulsification activity was defined as the height of the emulsion layer divided by the total supernatant height and was expressed as a percentage.

Biosurfactant production improvement

Effect of different carbon sources

P. aeruginosa was grown on the medium previously described for biosurfactant production except that different carbon sources were substituted for glucose. Each carbon source, fructose, rhamnose, mannitol, arabinose, galactose and molases, was added at a 2% concentration and prepared as follows: each carbon source (2 g) was dissolved in 50 ml water (A) and the nutrient salts were dissolved in 50 ml water (B) ; after sterilization , A was added to B. Fermentations were carried out in 500 ml Erlenmeyer flasks which were incubated at 30°C with an agitation speed of 200 rpm (Carrillo *et al*., 1996).

Optimization of growth medium

Plackett Burman experimental design : The Plackett- Burman design (Plackett & Burman, 1946 and Yu *et al*., 1997) was applied to identify the relative importance of various fermentation factors involved in the production of surfactant by *P. aeruginosa*. For each variable high (+) and low (-) levels were tested. The

examined variables in this experiment and their levels are shown in Table 4. Eight different trials were performed in duplicate. Rows in Table 5 represent the different trials (Row no. 9 represents the basal control). The main effect of each variable was determined with the following equation: Exi = (Mi+ – Mi-) / N, where Exi is the variable main effect, and Mi+, Mi- are the emulsification activity (%) and biomass yields in the trials, where the independent variable was present in high and low concentrations, respectively, and N is the number of trials divided by 2. Statistical *t-values* for equal unpaired samples were calculated using Microsoft Excel to determine the variable significance.

Verification experiment : A verification experiment was carried out in duplicate to compare the predicted optimum levels of the independent variables to the basal condition setting and to the averages of emulsification activity and biomass yield.

Optimization of fermentation conditions

The affect of temperature on biosurfactant production was determined over the range of 20 to 50°C. The effect of pH was determined by adjusting the medium pH from 5 to 9 with sterilized 1 M NaOH or 1 M HCl at room temperature (Subasioglu & Cansunar, 2008). The effect of duration of incubation period from 24 to 72 hr was also investigated (Dehghan-Noudeh *et al.,* 2009).

Biosurfactant stability

Effect of pH and temperature on biosurfactant stability

The effect of pH on surface tension and emulsifying activity was carried out by adjusting the supernatant pH to values ranging from 4 to 12 obtained by addition of HCl or NaOH at room temperature (Zhang & Miller, 1992). The heat stability of the culture supernatants was studied by incubating the supernatants at different temperatures (30-100ºC). The emulsifying activity and surface tension were determined after 24 hr.

Biomass yield

The culture broth was centrifuged at 10,000 *g* for 25 min and the cell pellets were washed twice with phosphate buffer (0.1 M, pH 7.0). The cell mass was dried at 80°C to a constant weight (Bidlan *et al*., 2007).

Results

Screening of biosurfactant producing marine bacteria

All strains showed haemolytic activity and exhibited a response to the oil displacement test (Table 1) and all, except *Streptoverticillium morookaens*e, lowered the surface tension. *B. subtilis* , *P. aeruginosa* and *S. faecalis* recorded positive results toward the drop collapse test. *P. aeruginosa* and *Streptoverticillium morookaens*e emulsified weathered crude oil within 24 hr incubation while the other strains emulsified weathered crude oil within 48 hr. *P.aeruginosa* was chosen for further study.

TABLE 1. Characteristics of biosurfactant producing strains.

Strain	Haemolysis	Oil displacement	Drop collapse	Surface tension	Emulsification of crude oil (24 hr)
Bacillus subtilis	+	+	+	+	-
Vibrio fluvialis	+	+	-	+	-
Pseudomonas aeruginosa	+	+	+	+	+
Streptococcus faecalis	+	+	+	+	-
Streptoverticillium morookaense	+	+	-	-	+

(-) means negative response to test .
(+) means positive response to test.

Emusification effect of the biosurfactant produced by P.aeruginosa to different substrates

These hydrocarbons examined served as substrates for the biosurfactant produced and were emulsified (Table 2). Kerosene and paraffin oil were the lowest emulsified substrates (59 and 40%, respectively) whereas hexadecane and hexane were the substrates most readily emulsified (68 and 66%).

TABLE 2. Emulsification activity (%) of biosurfactant for different substrates.

Emulsified substrate	Emulsification activity (%)
Hexadecane	68
Hexane	66
Kerosene	59
Paraffin oil	40

Biosurfactant production improvement

Effect of the different carbon sources on biosurfactant production

Though *P.aeruginosa* was able to grow on all of the carbon sources tested, the emulsification activity and surface tension values using hexadecane as the emulsified substrate varied (Table 3). *P. aeruginosa* showed the lowest biosurfactant production using fructose as a sole carbon source where the emulsification activity was 55.2 %. Biosurfactant production using rhamnose and arabinose as substrates gave higher values (65.2%), while using of glucose as a sole carbon source achieved the highest emulsification activity (68%). All the produced biosurfactants lowered the surface tension. The surface tension was lowered to (38.5 mN/m) using glucose as the sole carbon source.

TABLE 3. Emulsification activity (%) and surface tension of *P. aeruginosa* growth on different carbon sources after 48 hr incubation.

Carbon sources	Emulsification activity (%)	Surface tension (mN/m)
Glucose	68.0	38.5
Fructose	55.2	46.0
Rhammnose	65.2	40.2
Arabinose	65.2	41.5
Galactose	59.4	40.7
Mannitol	59.5	47.4
Molases	64.0	42

Optimization of growth medium

Plackett-Burman design : This statistical test was applied to evaluate the significance of the nine different fermentation conditions (Table 4). All experiments were performed in duplicate and the mean emulsification activity (%) and biomass yield are presented as the response (Table 5). The main effect of each variable on surfactant production (emulsification activity and biomass yield) as well as *t-values* were estimated for each independent variable (Table 6) and are graphically presented in Fig.1 a, b. The results indicated that high levels of KH_2PO4, $MgSO_4.7H_2O$ and glucose in the growth medium would enhance emulsification activity positively as would the presence of $(NH_4)_2\ SO_4$, Na_2HPO_4 , NaCl and yeast extract at their lowest levels. On the contrary, the presence of high levels of $(NH4)_2SO_4$, Na_2HPO_4, NaCl and yeast extract in the growth medium affects biomass yield positively and the presence of KH_2PO_4, $MgSO_4.7H_2O$ and glucose at their lowest levels also would result in high biomass yield.

Verification experiment

In order to evaluate the accuracy of the applied Plackett-Burman statistical design, an experiment was applied to compare predicted optimum levels of independent variables and the basal condition settings. It was found that the production of surfactant (expressed as emulsification activity %) increased to (80%) with a 1.2 fold increase when compared to its production under the basal conditions (68%). On the basis of the calculated *t-test* (Table 6), $(NH_4)_2SO_4$ and Na_2HPO_4 were the most significant variables affecting surfactant production. Their interaction (Fig. 2) showed an increase of surfactant production with the decrease of their concentrations, and indicated an indirect relationship between these two factors for high yields of surfactant.

TABLE 4. Factors examined in the Plackett-Burman design as independent variables affecting emulsification activity and biomass yield.

Variable (g/l)	Symbol	Level		
		-1	**0**	**+1**
$(NH_4)_2SO_4$	NH	5	7	10
Ka_2HPO_4	Na_2	2	3.8	5
KH_2PO_4	KH	1.5	3.5	5
$MgSO_4.7\ H_2O$	Mg	0.3	0.7	1.5
NaCl	Na	2	5	7
Glucose	G	15	20	25
Yeast extract	YE	0.2	0.5	1

TABLE 5. Results of the Plackett-Burman experimental design for 7 factors.

Trials	Factors							Emulsification activity (%)	Biomass yield (g/l)
	NH	**Na_2**	**KH**	**Mg**	**Na**	**G**	**YE**		
1	-1	-1	-1	1	1	1	-1	79.3	2.1
2	1	-1	-1	-1	-1	1	1	72.5	2.5
3	-1	1	-1	-1	1	-1	1	68.5	3.1
4	1	1	-1	1	-1	-1	-1	68.5	3.3
5	-1	-1	1	1	-1	-1	1	78.3	2.4
6	1	-1	1	-1	1	-1	-1	75.4	2.84
7	-1	1	1	-1	-1	1	-1	75.5	2.67
8	1	1	1	1	1	1	1	66.5	3.0
9	0	0	0	0	0	0	0	68	2.9

See Table 1 for explanation of factor symbols.
Results were obtained after 48 hr incubation.

TABLE 6. Statistical analysis of the Placket t- Burman design.

Variable	Emulsification activity (%)		Biomass yield (g/l)	
	Main effect	***t*-value[1]**	**Main effect**	***t*-value**
$(NH_4)_2SO_4$	**-4.65**	**-1.47**	**0.3**	1.26919
Ka_2HPO_4	**-6.65**	**-2.68**	**0.56**	2.767749
KH_2PO_4	1.7	0.47	-0.02	-0.07406
$MgSO_4.7\ H_2O$	0.2	-0.05	-0.08	-0.25639
NaCl	-1.25	-0.34	0.06	0.140063
Glucose	0.75	0.20	**-0.3**	**-1.26919**
Yeas textract	-3.2	-0.93	0.03	0.074064

t-value significant at the 1% level = 3.70
t-value significant at the 5% level = 2.446
t-value significant at the 10% level = 1.94
t-value significant at the 20% level =1.372
Standard *t*-values are obtained from Statistical Methods by Snedecor & Cochran (1989) .

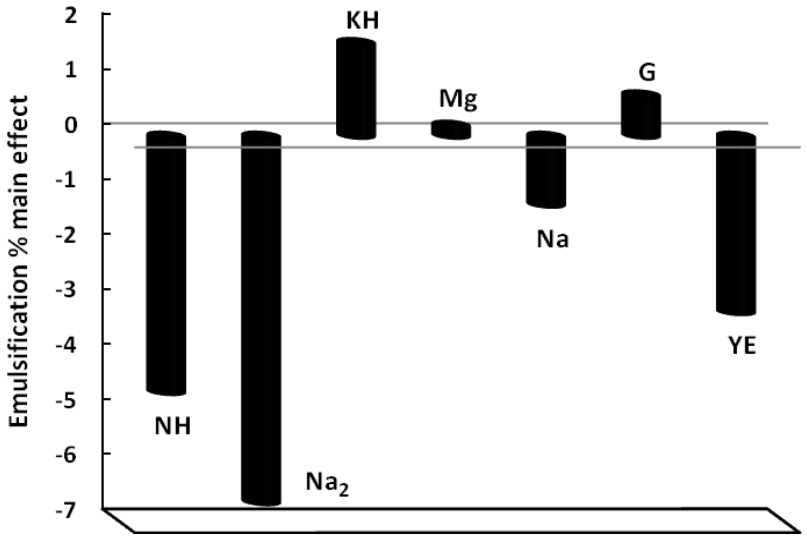

Fig. 1a. Elucidation of fermentation conditions affecting emulsification activity (%) by *P.aeruginosa.*

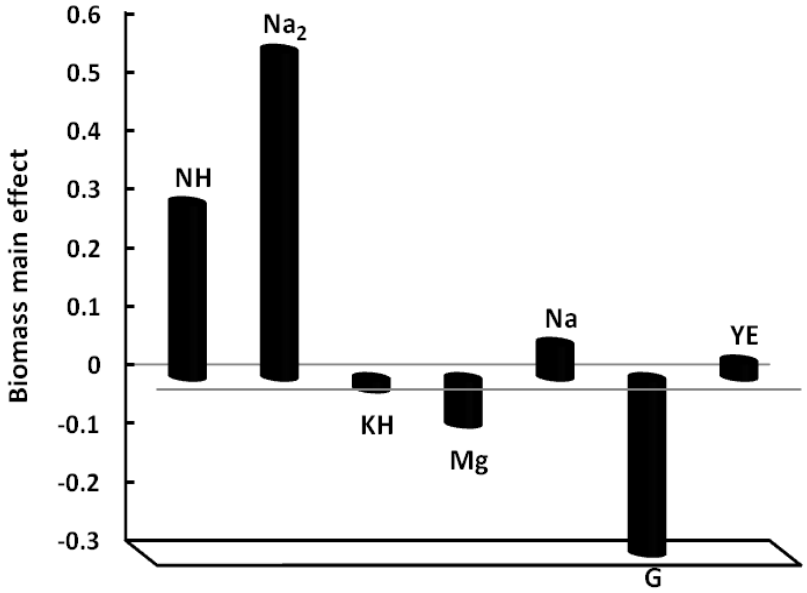

Fig.1b. Elucidation of fermentation conditions affecting biomass yield (g/l) by *P.aeruginosa.*

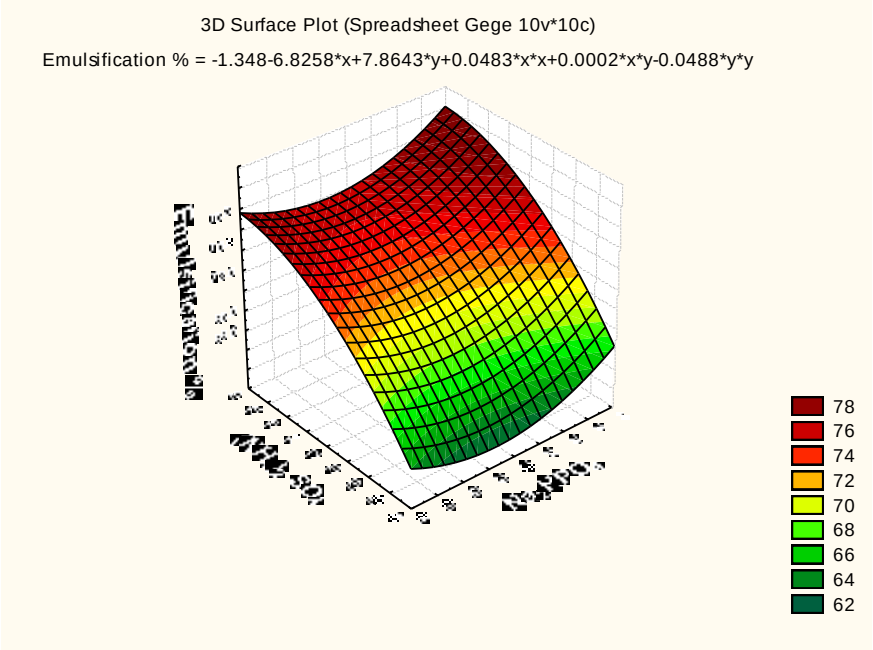

Fig. 2. Interaction between $(NH_4)_2SO_4$ and Na_2HPO_4 concentrations on emulsification activity (%).

Effect of fermentation conditions on biosurfactant production

Maximum emulsification activity and biomass yield was at 35°C for *P. aeruginosa* (Fig.3 a, b, c) and higher and lower values were not suitable for this strain. One of the parameters investigated in this study was the effect of pH on emulsification activity and biomass yield. Emulsification activity was most suitable at pH 6 and lower or higher pH values decreased surfactant production. *P. aeruginosa* was allowed to grow for different incubation periods (24, 48 and 72 hr) in the modified medium at 35°C and pH 6. The highest emulsification activity (86%) achieved after 48 hr.

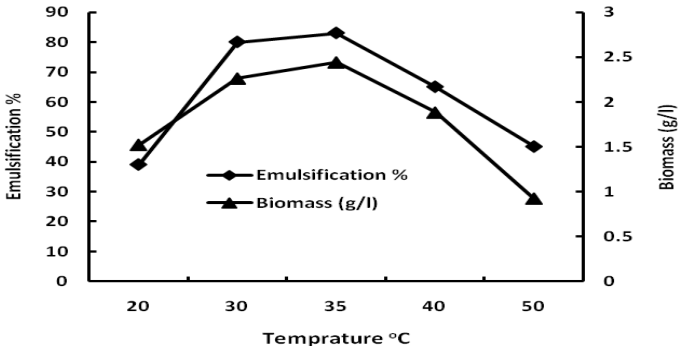

Fig. 3a. Effect of incubation temperature on emulsification activity(%) and biomass yield(g/l).

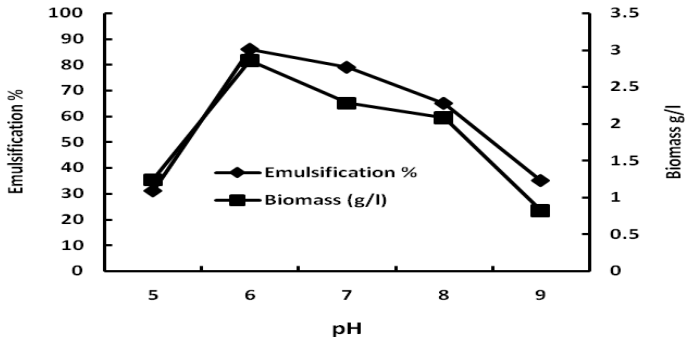

Fig.3b. Effect of pH on emulsification activity(%) and biomass yield(g/l).

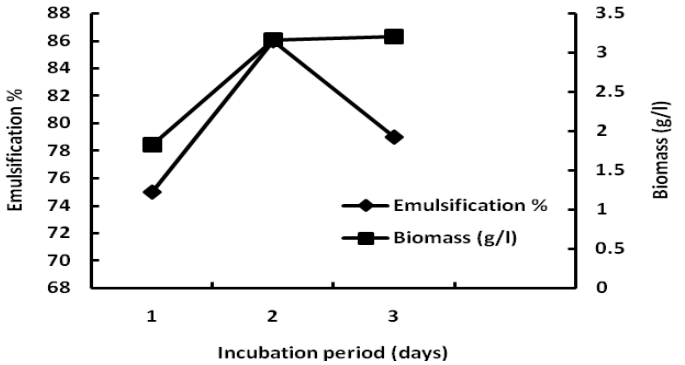

Fig. 3c. Effect of length of incubation periods on emulsification activity(%) and biomass yield (g/l).

Effect of pH and temperature on biosurfactant stability

Maximum emulsification activity and lowest surface tension were obtained at pH 6 (Fig.4a). The biosurfactant was stable at pH ranging from 6 to10 but at higher pHs, the emulsification activity decreased sharply and surface tension increased. At 40 ºC the maximum emulsification activity and the lowest surface tension were detected (Fig.4b). Even at higher temperatures (50-90 ºC), remarkable stability in the surface tension and emulsification activity was noticed whereas at lower temperatures the quality of the surfactant was negatively affected.

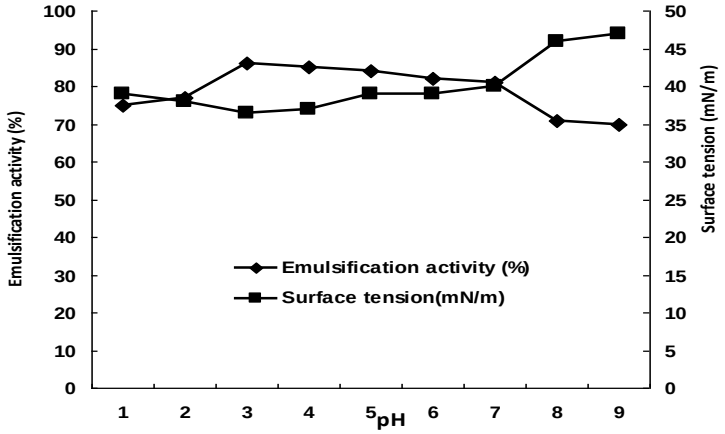

Fig.4a. Effect of pH on surface tension and emulsification activity.

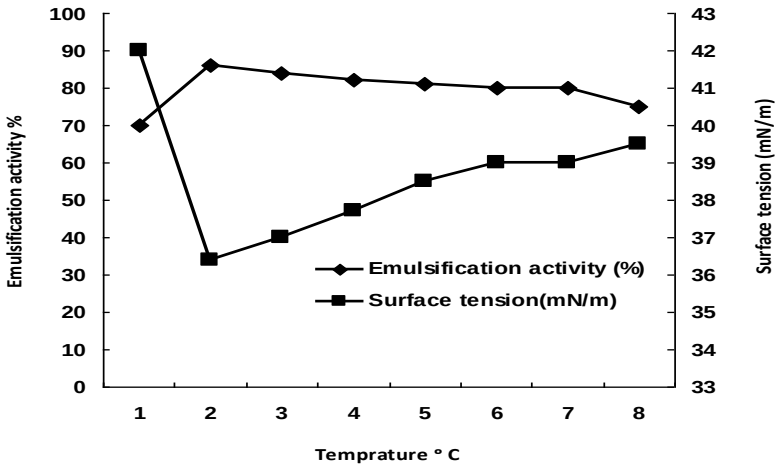

Fig.4b. Effect of temperature on surface tension and emulsification activity.

Discussion

The type of biosurfactant is generally governed by the type of microorganism (Desai & Banat, 1997) and in this study *P. aeruginosa* was found to be the best candidate for biosurfactant production. dos Santos *et al*. (2010) reported that *Pseudomonas* spp. strains were able to produce biosurfactants efficiently using renewable carbon resources and could grow and emulsify crude oil after 24 hr. A cell-free culture of *P.aeruginosa* grown in liquid medium showed the fastest time to collapse on the oil surface and also reduced the surface tension. In addition, it displayed the highest haemolytic action on blood agar. Mulligan *et al*. (1984) concluded that biosurfactant caused ß-haemolysis due to red blood cell lysis. However, Youssef *et al*. (2004) has reported that blood agar may give a large number of false results. This was due to other microbial products such as virulence factors which could also lyse blood cells or because of insufficient amount of biosurfactant produced, which poorly diffused into the agar and failed to lyse the blood cells. Furthermore, reduction of surface tension was indicative of biosurfactant production by the microbes (Dehghan-Noudeh *et al*., 2003 and Batista *et al*., 2006). Bodour & Maier (2002) reported that rhamnolipids secreted by *P. fluorescens* HW-6 reduced surface tension of water from 72 mN m^{-1} to nearly 32 mN m^{-1}, in agreement with this study.

Mata *et al.* (2006) have reported the emulsifying properties of polysaccharides produced by halophilic bacteria including *P.aeruginosa*. Recently, Pacwa-Płociniczak *et al*. (2011) and Techaoei *et al.* (2011) studied the production of biosurfactants by *P.aeruginosa* under standardized conditions for maximum biosurfactant production. Syldatrk & Wagner (1987) demonstrated that different carbon sources in the medium affected the composition of the biosurfactants produced. Guerra-Santos *et al.* (1984) reported that microorganisms can carry out biosurfactant production when grown either on insoluble substrates (such as hydrocarbons, oils and waxes) or on soluble ones (carbohydrates). For industrial processes, media that use carbohydrates as carbon sources are preferred. Hence, the isolation of microbial strains capable of biosurfactant production using soluble substrates is of interest. *P.aeruginosa* was able to utilize glucose, fructose, arabinose, galactose , mannitol and molases but the highest emulsification activity and the lowest surface tension were achieved using glucose as a carbon source. Rodrigues *et al*. (2006a) reported that, *L.lactis* 53 and *S.thermophilus* had a good performance for glucose or lactose to biosurfactant fermentation using the more costly MRS and M17 broths, which contain yeast extract and peptone.

Statistical experimental designs are powerful tools for identifying the key factors from a multivariable system, and minimizing the error in determining the effect of parameters (El-Sersy, 2007 and Abou-elela *et al*., 2009C). According to the results obtained, the optimum medium composition for surfactant production by *P.aeruginosa* is as follows (g/l) : $(NH_4)_2SO_4$, 5; KH_2PO_4 , 5; $MgSO_4.7H_2O$, 1.5 ; glucose, 25; Na_2HPO_4 , 2; NaCl , 2 and yeast extract , 0.2 . On the contrary, the optimum medium composition for biomass production is as follows (g/l) :

$(NH_4)_2SO_4$, 10; KH_2PO_4 , 1.5; $MgSO_4.7H_2O$, 0.3 ; glucose, 15; Na_2HPO_4 , 5; NaCl , 7 and yeast extract , 1 . Rodrigues *et al.* (2006b) optimized the medium components by response surface optimization for the production of biosurfactants by probiotic bacteria and concluded that it was possible to determine optimal operating conditions to obtain a higher cellular growth, thus a higher biosurfactant production yield. Maneerat & Phetrong (2007) reported that the production of biosurfactant was growth-associated as indicated by the growth and biosurfactant production kinetics. Bidlan *et al.* (2007) reported that the optimum conditions for biomass production did not result in highest biosurfactant production and these results are in accordance with the results of this study. It is clear that the conditions for both parameters were independent of each other. Low levels of $(NH_4)_2SO_4$ and Na_2HPO_4 enhanced high emulsification activity, while high levels of the same factors scaled up biomass productivity.

Subaşıoğlu & Cansunar (2008) observed maximum rhamnolipid production and cell growth at 34.5^oC .There was a sharp decrease above 36^oC and *P.aeruginosa* showed similar behaviour, the highest emulsification activity and biomass productivity were detected at 35 ^oC and pH 6. These results also agreed with Subaşıoğlu & Cansunar (2008) who studied pH dependent rhamnolipid production by *P. aeruginosa* and concluded that values between 6.5 and 7.0 were the most suitable points for maximum rhamnolipid production. Lower or higher pH caused a rapid decrease. Temperature and pH are the factors that effect rhamnolipid production through their effects on cellular activity. Sepahy *et al.* (2005) reported that BiBiB5 and Ilam E3P4 strains grown at a pH between 6.5 and 8.5 showed little difference in growth and biosurfactant production, but both were markedly lower at a medium pH of 4.5.

Incubation for 48 hr gave the highest emulsification activity and biomass, while after 72 hr both the values decreased because of the decrease in pH, probably caused by the production of secondary acid metabolites such as uronic acid (Healy *et al*., 1996). Dehghan-Noudeh *et al.* (2009) reported that, *P. aeruginosa* PTCC 1074 is a better biosurfactant producer and maximum biosurfactant production was achieved after 48 hr of incubation. The biosurfactants formed by *P.aeruginosa* retained surface active properties after exposure to high temperature (90 ^0C) and pH (6-10), which is agreement with dos Santos *et al.* (2010). It can be concluded that marine *P.aeruginosa* is a potent candidate in the bioremediation process of crude oil contaminated sea water.

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زيادة أنتاج المشتتات العضوية بواسطة ***Pseudomonas aeruginosa*** البحرى

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تكمن أهمية هذ البحث فى تقييم العوامل المؤثرة على أنتاج المشتتات العضوية بواسطة *Pseudomonas aeruginosa* و التى تمثلت فى المصدر الكربونى و تركيب الوسط الغذائى و الأس الأيدروجينى و درجة الحرارة و فترة التحضين و كذلك الوصول لأعلى أنتاجية من هذا المشتت العضوى ودراسة بعض خصائصه٠

تعتبر المشتتات العضوية جزيئات محبة للماء حيث تتميز بالأسطح النشطة ذات الصفات البيولوجية الخاصة والتى لها تطبيقاتها فى العديد من الصناعات و يمكن الأ ستفادة منها فى التخلص من البقع الزينية فى البحار والخلجان٠ تستطيع الميكروبات تخليقها أثناء نموها على بعض الأوساط الغذائية وتستخدم هذه المشتات العضوية كبديل للمركبات الكيميائية المعتادة٠

تم عمل مسح لستة أنواع من البكتيريا *Bacillus subtilis, Vibrio fluvialis, Pseudomonas aeruginosa, Streptococcus faecalis and Streptoverticillium morookaense* لأختيار أفضلهم لأنتاج المشتتات العضوية واتضح أن أفضلهم هو *Pseudomonas aeruginosa* ٠

أثبتت النتائج أن أستخدام الجلوكوز يزيد الأنتاج أكثر من مصادر الكربون الأخرى ، كما تم أستحدام النظام الأحصائى Blacket-Burman لتحقيق ظروف الأنتاج المثلى و تقييم أهم العوامل التى تؤثر على أنتاج المشتت العضوى٠وأثبتت الدراسة أن أهم العوامل المؤثرة فى زيادة الأنتاج هى تركيزات كلا من : $(NH_4)_2SO_4$ و Na_2HPO_4 و التى أدت تركيزاتها الأقل لزيادة قدرها ١,٢ مرة٠ونستخلص أن التركيب المثالى للوسط الغذائى المعدل هو (جم /لتر) : $(NH_4)_2SO_4$, 5; KH_2PO_4 , 5; $MgSO_4.7H_2O$, 1.5 ; glucose, 25; Na_2HPO_4 , 2; NaCl , 2 and yeast extract , 0.2 . وذلك عند التحضين فى درجة حرارة ٣٥ مئوية وأس هيدروجينى ٦ قى حين أن أستخدام التركيزات الأقل من نفس المركبين Na_2HPO_4 و $(NH_4)_2SO_4$ أدى إلى زيادة كتلة الكائن وكان تركيب الوسط الغذائى المقترح هو: $(NH_4)_2SO_4$, 10; KH_2PO_4 , 1.5; $MgSO_4.7H_2O$, 0.3 ; glucose, 15; Na_2HPO_4 , 5; NaCl , 7 and yeast extract , 1.

أن فترة التحضين لمدة يومين أعطت أعلى نتيجة لأنتاج المشتت العضوى و التى زادت حتى ١,٣ مرة٠كما وجد أن المستخلص الخام من المشتت العضوى المنتج له ثبات عند أس هيدروجينى يتراوح من ٦-١٠ ودرجة حرارة تتراوح من ٥٠-٩٠ درجة مئوية٠