Int. J. Environ. Sci. Tech., 3 (3): 297-303, 2006
ISSN: 1735-1472

Production of rhamnolipids by *Pseudomonas aeruginosa* growing on carbon sources

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Received 16 October 2005; revised 7 January 2006; accepted 15 March 2006; available online 25 June 2006

ABSTRACT: Arhamnolipid producing bacterium, *Pseudomonas aeruginosa* was previously isolated from Iranian oil over years. Isolated strain was identified by morphological, biochemical, physiological and 16 sr RNA (1). Glycolipid production by isolated bacterium using sugar beet molasses as a carbon and energy source was investigated. Biosurfactant production was quantified by surface tension reduction, Critical Micelle Dilution (CMD), Emulsification Capacity (EC), and Thin Layer Chromatogeraphy. biosurfactants during growth on waste Dates as the primary carbon and nitrogen sources, respectively. After 48 h of growth the culture supernatant fluid had a rhamnose concentration of 0.18 g/L and surface tension was reduced to 20 mN/m (%).(reduced the interfacial tension against crude oil from 21 mN/m to 0,47 mN/m) Result from the study showed that the growth of the bacteria using molasses as carbon sources is growth-associated. The specific production rate of rhamnolipid with 2 %, 4 %, 6 %, 8 % and 10 % of molasses are 0.00065; 4.556; 8.94; 8.85; and 9.09. respectively The yield of rhamnolipid per biomass with 2%,4%,6%,8% and 10% molasses are 0.003;0.009;0.053;0.041 and 0.213 respectively. The production of rhamnolipid (0.0531 g rhamnolipid/g biomass) is higher compare to the culture grown in aerobic condition (0.04 g rhamnolipid/g biomass) .The rhamnolipids were able to form stable emulsions with n-alkanes, aromatics, crude oil and olive oil. These studies indicate that renewable, relatively inexpensive and easily available resources can be used for important biotechnological processes.

Key words: *Pseudomonas aeruginosa, biosurfactant, waste dates, sugar beet molasses*

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 readily biodegradable and can be produced from renewable and cheaper substrates, they might be able to replace their chemically synthesized counter parts. (Arino, 1996). Among the heterogeneous group of biosurfactants, the rhamnose-containing glycolipids produced by *Pseudomonas*. Rhamnolipid has been known as biosurfactant which is produced by *Pseudomonas aeruginosa* in fermentation process. Several carbon sources such as ethanol, glucose, vegetable oil and hydrocarbon have been used to produce rhamnolipid. In this study, we are trying to use molasses which is a waste product from sugar industry as carbon source to produce rhamnolipid. The bacteria which was previously isolated from Iranian oil over years. Microbial surfactants are generally less toxic and more biodegradable than synthetic surfactants (Desai and Patel, 1994 and Guerra-Santos *et al*., 1984). Rhamnolipid biosurfactants specifically produced by *Pseudomonas aeruginosa* have great potential for industrial application (Reiling *et al*., 1986) and bioremediation (Banat, 1995). Four rhamnolipid analogs, R1-R4, have been isolated (Syldatk *et al*., 1985a). Rhamnolipid production has been examined with some carbon sources as *n*paraffin, *n*-tetradecane or glucose (Syldatk *et al*., 1985b and Robert *et al*., 1989) and plant oil was also good carbon, source. Waste Dates is an attractive raw carbon source for microbial production of a variety of biochemicals (Juzlová *et al*., 1994). *Pseudomonas* BOP 100 has the capabilities for production of rhamnolipid and phenazine when grown on Waste Dates as sole carbon source (Osman *et al*., 1996). In this paper, we describe the production of rhamnolipid

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by fed-batch culture using Waste Dates as sole carbon. Almost all surfactant currently in use are chemically derived from petroleum. however interest in microbial surfactant has been steadily increasing in recent years due to their diversity ,environmentally friendly characteristics, the possibility of their production through fermentation and their potential application in such areas as the environmental protection, surplus crude oil recovery, health care and the food processing industries. Therefore, it is necessary to know more about the producing microorganism's physiology and the process engineering to develop the technology for these production of molecules, the use of cheap substrates being of utmost importance. Interest in microbial surfactants has increased considerably in recent years, especially due to their potential application in enhanced oil recovery. The production of surface-active compounds by microorganisms is well established and has been a matter of discussion at different international meetings. Their potential for enhanced oil recovery is based on their application as agents for rock wetting, micellar flooding, emulsification, deemulsification, and viscosity reduction of heavy crude oils. The specific production rate and yield of rhamnolipid are explained as following:
Growth associated specific production rate:

$$Qp = Y_{P/X} \bullet \mu \quad (1)$$

$$Y_{P/X} = \frac{P2-P1}{X2-X1} \quad (2)$$

Mixed-growth associated specific production rate

$$Q_p = Y_{P/x} \bullet \mu + \beta \quad (3)$$

Qp = specific production rate of rhamnolipid (g/L.h)
Yp/x = yield of rhamnolipid
μ= specific growth rate (1/h)
P= rhamnolipid concentration (g/L)
X= biomass concentration (g/L)
â =production rate during stationary phase (g/L.h)

MATERIALS AND METHODS

Identification of the microorganism

The microorganism was isolated from oil wells in the southern of Iran. The method of serial dilutions of the sample and plate count in selective medium Cetrimide agar was used for isolation purposes. The plates were incubated at 30 ºC for 48 hr. The strain was activated in a triptic soyer agar medium (TSA), cultivated at 300 ºC for 48 h and transferred to a 250 mL flask, containing 50 mL of TSA. The flask was incubated at 300 ºC and 250 rpm during 20 hr. (shaker, Gallenkamp, England) Cells were havested by centrifugation at 6000 rpm during 20 minutes. (centrifuge, Shimadzu,Japan,). The centrifuged microbial mass was suspended in a culture medium (medium salt production - MSP) with the following composition (g/L): $(NH4)2SO_4$, 1.0; $KH2PO_4$, 3.0; $MgSO_4.7\ H_20$, 0.2. The pH was adjusted to 7.0 with a solution of KOH (1N) plus 1% v/v of glycerol P.A. (Merck) in order to obtain the initial inoculum concentration of 0.005, 0.075 and 0.1 g/L, in accordance with a calibration curve of dry weight versus absorbance. The production of rhamnolipids was studied during a seven days incubation period in flasks under agitation with the initial seeding material standardized in a culture medium, as mentioned previously, maintained at a temperature of 30 ºC and stirred in a rotary shaker at 120 rpm. The carbon sources used were n-hexadecane paraffin oil collected at flowing wells in the Khark island of Iran, consisting of 32 % saturated hydrocarbons, 23 % aromatics, 36 % of resins and 9.1% asphaltenes), glycerol (PA-Merck, Darmstadt) and molasses from pak company. In addition to the carbon sources studied, the C/N ratio varied with the following concentrations of glycerol: 0.5, 1, 2, 3, 4, 5 and 6% v/v, corresponding to C/N ratios of 20, 40, 60, 80, 100 table and 120. For evaluation of the most appropriate nitrogen sources for the production of biosurfactants, $NaNO_3$, $(NH_4)_2SO_4$ and CH_4N_2O were employed at the following concentrations: 1.45, 1.0, and 0.51 g/L and glycerol 3% v/v. rhamnolipid assay: 1 mL of culture broth was extracted with 1 mL of chloroform/methanol (1:1, v/v) mixture and the organic phase was analyzed by TLC on silica gel 60 F254 plate using a solvent of chloroform/methanol/water (65:25:4). Rhamnolipid spots were detected by orcinol reagent and quantified by the density (area) at 550 nm in the reflection mode with a TLC scanner. Glucose was used as a standard instead of rhamnolipid as described previously (Singer and Finnerty, 1990). In this assay, 1 mg rhamnolipid corresponded to 0.17 mg glucose. HPLC was carried out using a Shodex GS-310 (7.6 mm 250 mm) gel filtration column. Mobile phase used was 30 v/v% (v/v) methanol, run at 0.3 mL/min and monitored at 203 nm. Five mL of suitably diluted samples were injected. Retention time of rhamnolipid R1 and R2 was 15.1 min and 15.8 min, respectively. Waste Dates consumption during fermentation was assayed

according to the method as described previously (Nakata *et al*., 1995).

Biomass Concentration

Bacterial growth was monitored by measurement of absorbance at a wave length of 610 nm. Samples of 50 mL were removed from the flasks at regular intervals and centrifuged at 6000 rpm for 15 min. The centrifuged cells were suspended in 5 mL of distilled water and the biomass, expressed in dry weight (g/L), was obtained from a calibration curve.

Quantification of Rhamnose and Glycerol: The quantification of rhamnolipids expressed in rhamnose (g/L) was measured in the cell free culture medium, using the phenol sulfuric acid method. Glycerol was assessed by the enzymatic colorimetric method for triglyceride content evaluation.

Determination of the Critical Micelle Concentration (CMC): The surface tension of the biosurfactant was measured by the ring method using a CSC-Dunouy tensiometer (cole parmer instrument Co., Bunker, IL, U.S.A) at room temperature. The concenteration at which micelles began to from was represented as the CMC. At the CMC, sudden changes in surface tension, electrical conductivity and detergency were observed. The CMC was determined by plotting the surface tension as a function of the biosurfactant concentration and surface tension at this point was designated as CMC.

RESULTS

Biosurfactant production was studied using medium A, with % molasses (v/v) 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 were used (Figs. 1 and 2).

Table 1: Relation between molasses concenteration and specific production rate

Molasses	Qp (g/L.h)
2%	0.00065
4%	4.555674
6%	8.941563
8%	8.850229
10%	9.092182

Table 2: Effect of carbon source on rhamnolipid production

Carbon source (g/L)	Cell dry mass (g/L)	Rhamnolipid (R1) (g/cell dry mass)	Rhamnolipid (R2) (g/cell dry mass)
ethanol	0.9	2.2	1.8
glucose	7.1	0.030	0.010
sucrose	0.4	0	0
maltose	0.4	0	0
glycerol	0.4	0.8	0.8
dates	5.2	0.4	0.5

Culture condition: 30 g/L carbon source, 5 g/L yeast extract, 5 g/L Dates, 1g/L K_2HPO_4, 0.5 g/L Mg SO_4, $7H_2O$, pH 7 cultivative at 28 °C

Table 3: Effect of nitrogen source on rhamnolipid production

Nitrogen source(g/L)	Cell dry mass(g/L)	Rhamnolipid(R1) (g/cell dry massg)	Rhamnolipid (R2) (g/cell dry massg)
Dates	2.5	0.4	0.2
Yeast extract	1.5	0.7	0.5
(NH4)2So4	0	0	0
NH4NO3	0	0	0
Malt extract	0	0	0

Culture condition: 3 v/v % ethanol, 5 g/L nitrogen source, 1 g/L K_2HPO_4, 0.5 g/L Mg SO_4, $7H_2O$, pH 6.8 cultivative at 28° C for 7 days.

Table 4: Combination effect of yeast extract and other nitrogen source on rhamnolipid production

		Rhamnolipid								
		Dates			CSL			Poly pepton		
		2 g/L	5 g/L	10 g/L	2 g/L	5 g/L	10 g/L	2 g/L	5 g/L	10 g/L
Yeast extract	2 g/L	2.7	2.8	1.1	1.8	2.3	1	0	0	0
	5 g/L	3	3.7	2.5	2.8	0.2	0.003	0	0.5	0.7
	10 g/L	1.5	1.8	1.9	0.2	0	0.04	0	0	0

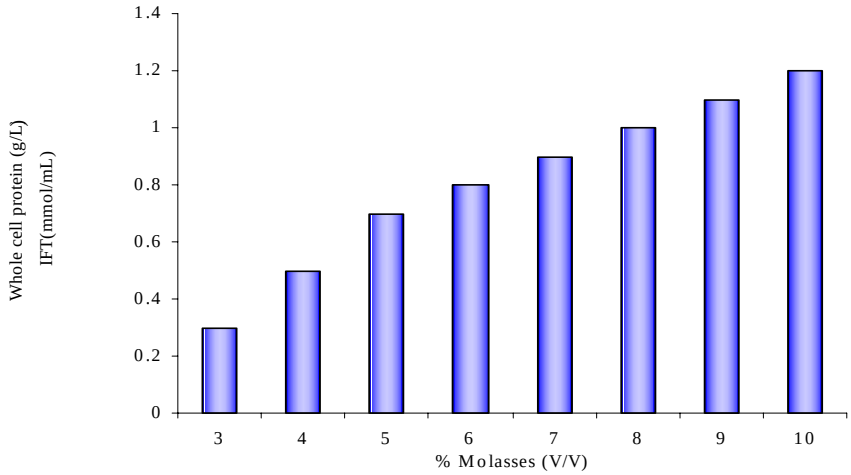

Fig. 1: Effect of concenteration of molasses on surfactant production were IFT between medium and crude oil was 21 mmol/lm

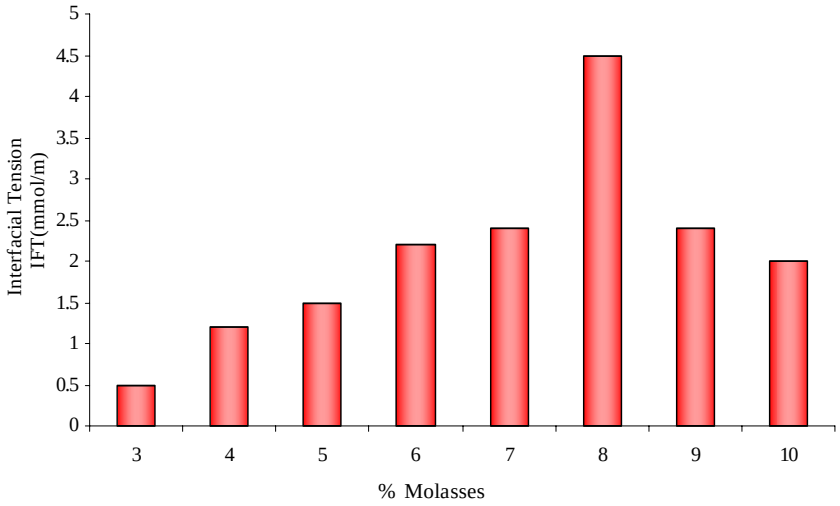

Fig. 2: Effect of concenteration of molasses on surfactant production were estimatated after 96 h of incubation IFT between medium and crude oil was 21 mmol /lm

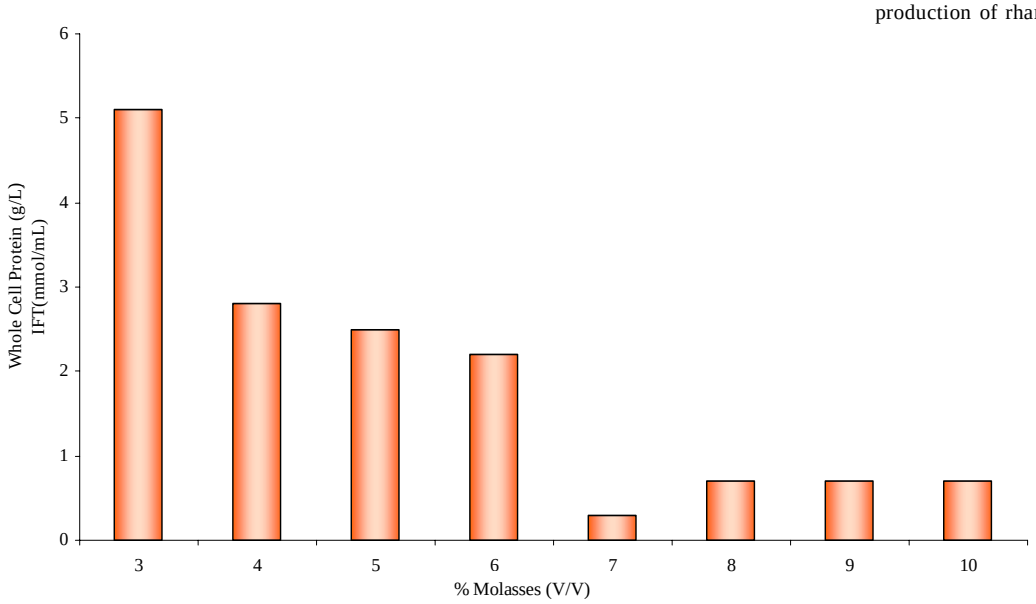

Fig. 3: Effect of concenteration of molasses on surfactant production were estimatated after 96 h of incubation IFT between medium and crude oil was 21 mmol/lm

Further increase in 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. (Fig.3). The increase in subtrate concentration (S) will result in the increase of specific growth rate (μ). This result can be seen in Fig. 2. Table 1 shows that the specific production rate of rhamnolipid (Qp) also related to the increase of subtrate concentration (S).

Effect of carbon and nitrogen source on rhamnolipid production

The experiments were conducted in the fermentation medium containing 3 v/v % waste dates and 5 g/L nitrogen source (Table 2). The effect of nitrogen source in production of rhamnolipid are shown (Table 3). Since yeast extract enhanced rhamnolipid production during early cultivation by 4 days , the effect of yeast extract plus other nitrogen source were examined (Table 4). The combination effect was the highest when soy flour was used as a pair of nitrogen sources. The optimal rhamnolipid production, 3.7 g/L, was obtained when yeast extract and soy flour were added 5 g/L each in 3% (v/v) Waste Dates medium.

Effect of waste dates feeding on rhamnolipid production

More than 3% (v/v) Waste Dates was toxic for growth of this strain. Therefore, we examined the effect of intermittent feeding of Waste Dates on rhamnolipid production using a fermentation medium containing 5 g/L each of yeast extract and soy flour (Fig. 1). Growth and rhamnolipid increased gradually in parallel for 7 day cultivation. Waste Dates was completely consumed at 7 days. Thirty two gram rhamnolipid/L was finally obtained from a total of 55.3 g Waste Dates/L. Substrate conversion rate was 58%.

Isolation and identification of rhamnolipids

The culture broth (450 mL) was centrifuged after adjusted to pH 3.0. The supernatant was applied to aDEAE-Toyopearl 650M column (2.5 48.5 cm) equilibrated with 0.5 M Tris-HCl buffer (pH 9.0). After washed with the buffer, the column was eluted with a linear gradient of 0 – 0.4 M NaCl in 0.5 M Tris-HCl buffer (pH 9.0). A flow rate was 2.3 mL/min and 7 mL each was fractionated, followed by rhamnolipid detection. The active fractions contained rhamnolipid R1 and R2 were pooled and extracted with equal volume of chloroform/ methanol (1:1, v/v) and the organic layers were evaporated to dryness. The recoveries of R1 and R2 from this chromatography were 91.5% and 89.8%, respectively. These compounds thus obtained were identified as rhamnolipid R1 and R2, by 1H-NMR and FAB mass analyses.

DISCUSSION AND CONCLUSION

Rhamnolipid production by *Pseudomonas aeruginosa* has been found to be affected by nutrient sources,

especially carbon sources in medium. Water soluble carbon sources such as glucose could be used for rhamnolipids production (Syldatk *et al*., 1985a), but water insoluble materials like alkane and oil are superior to them. It has been thus thought that the microorganism produces rhamnolipids to emulsify these water insoluble carbon sources for well assimilation. Nitrogen limitation caused an overproduction of rhamnolipids (Syldatk *et al*., 1985b) and the importance of C/N ratio in medium on rhamnolipid production (Guerra-Santos *et al*., 1984). The production of rhamnolipids was studied during a seven day fermentation period in flasks under agitation with the initial seeding material standardized in a culture medium, as mentioned previously, maintained at a temperature of 30 ºC and stirred in a rotary shaker at 120 rpm. The carbon sources used were N-hexadecane (Merck, Darmstadt), paraffinic oil collected at flowing, glycerol (PA - Merck, Darmstadt). In addition to the carbon sources studied, the C/N ratio varied with the following concentrations of glycerol: 0.5, 1, 2, 3, 4, 5 and 6% v/v, corresponding to C/N ratios of 20, 40, 60, 80, 100 and 120. For evaluation of the most appropriate nitrogen sources for the production of biosurfactants, $NaNO_3$, $(NH_4)_2SO_4$ and CH_4N_2O. According to their data, the optimum C/N ratio was 18 and rhamnolipids were no longer produced at the C/N ratio less than 11.Our results with rhamnolipid production by *Pseudomonas aeruginosa* HR revealed that Waste Dates gave the highest productivity (4 g/cell dry mass g) among several carbon sources tested (Table 1). Considering necessity of separation process of rhamnolipid from oil on recovery, it is clear that Waste Dates is superior to rape seed oil for rhamnolipid production. We achieved marked improvement in the yield by intermittent feeding of Waste Dates. Among some intermittently fed experiments tested, the result shown in Fig. 1 was best for rhamnolipid production. In our results, optimal nitrogen sources were a combination of 5 g/L yeast extract (Table 3). The C/N ratio was less than 3 in an non-feeding culture, but more than 50 in the feeding culture. This may be one of reasons why the feeding culture produces a considerable amount of rhamnolipids.

REFERENCES

Andre C. , Espuny, M. J., Robert, M. , Mercade, M. E., Manrresa, A., Guinea, J. and Leeuwenhoek A.V., (1998). Physicochemical characterization and antimicrobial properties of rhamnolipids produced by Pseudomonas aeruginosa 47T2 NCBIM 40044, J. Microbiol., in Press; **81** (3), 316-322.

Arino S. Marchai, R. and Van Decasteel, J. P., (1996). Identification and Production of a Rhamnolipidic Biosurfactant by a Pseudomonas Species , Appl Microbiol. Biotechnol; 45; 162-168.

Artiola, J. F., Ochoa-Loza, F. J. and Maier, R. M., (2001). Stability Constants for the Complexation of Various Metals with a Rhamnolipid Biosurfactant., J. Environ. Qual; **30** (2), 479-85.

Babu, P. S., Vaidya, A. N., Bai, A. S., kapur, R., Juwarkar, A. and Khanna, P., (1996). Kinetics of Biosurfactant, Production by Pseudomonas aeruginosa Strain BS2 from Industrial Wastes, Biotech. Letters; **18** (3), 263-268.

Bai, G., Mark, L., Brusseau, R. and Miller, M., (1998). Influence of Rhamnolipid Biosurfactants in: Biotechnology; 421-457.

Beeba, J. L. and Umbreit, W. W., (1971). Microbial production of biosurfactants and their importance, J. Bacteriol. **108,** 612.

Burger, M. M., Glaser, L., Burton, R. M., (1998). Biol. Chem.; **238**, 2595-2602.

Banat I. M., (1994) Bioresource Technol. 51, 1-12. Desai A. J. and Patel R. M. J. Sci. Ind. Res., **53**, 619-629.

Guerra-Santos, L., Käppeli, O. and Fiechter, A. (1984). Appl.Environ. Microbiol. **48**, 301-305.

Juzlová, P., Martínková, L., Lozinski, J. and Machek, F., (1994). Enzyme Microb. Technol; **16**, 996-1001.

Nakata, K., Hasegawa, J. and Okamura, K. (1995). Biosci., BiotechBiochem., **59**, 986-989.

Osman, M., Ishigami, Y., Someya, J. and Jensen, H. B., (1996). Oil Chem. So; **73**, 851-856.

Reiling, H. E., Thanei-wyss, U., Guerra-Santos, L. H., Hirt, R., Käppeli O. and Fiechter, A. (1986). Appl. Environ. Microbiol., **51**, 985-989.

Robert, M., Mercade, M. E., Bosch, M. P., Parra, J. L., Espuny M. J., Manresa, M. A. and Guinea, J. (1989). Biotech. Lett., **11**, 871-874.

Singer, M. E. and Finnerty, (1990). W. R. Can. J. Microbiol., **36**, 746-750.

Syldatk, C., Lang, S. and Wagner, F. Z., (1985a). Naturforsch., **40**, 51-60.

Syldatk, C., Lang, S., Matulovic, U. and Wagner, F. Z., (1985b). Naturforsch. 40c, 61-67.

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This article should be referenced as follows:
*Rashedi, H., Mazaheri Assadi, M., Jamshidi, E., and Bonakdarpour, B., (2006). Production of rhamnolipids by Pseudomonas aeruginosa growing on carbon sources. Int. J. Environ. Sci. Tech., **3** (3), 297-303.*