ScienSage

Journal of Advanced Scientific Research

Available online through http://www.sciensage.info/jasr

ISSN
0976-9595
Research Article

Optimization of Culture Conditions for Biosurfactant Production from *Pseudomonas aeruginosa* OCD_1

Sonali Sahoo, Sriparna Datta*, Dipa Biswas

Department of Chemical Technology, University of Calcutta, Kolkata, India

**Corresponding Author: sriparna_d@yahoo.com*

ABSTRACT

The air isolate *Pseudomonas aeruginosa* OCD_1 was studied for its maximal biosurfactant production in liquid Bushnell-Haas media with n-octadecane as the substrate. Several culture conditions were investigated to maximize biosurfactant production from the strain OCD_1. The optimum culture conditions were found to be 1% v/v inoculum, 2% v/v n-octadecane, 30°C temperature, 125 r.p.m. and pH 6.0 with extra 30% w/v $CaCl_2.2H_2O$ in growth medium. The strain *Pseudomonas aeruginosa* OCD_1 produced 0.98 mg/mL rhamnolipid in the culture broth at the stationary growth phase. Again, the biosurfactant production was enhanced by the addition of $ZnSO_4$ followed by $MnSO_4$ in the culture media at the optimized conditions.

Keywords: Biosurfactant, Optimization, Pseudomonas aeruginosa, Rhamnolipid

1. INTRODUCTION

Microbiologically-derived surfactants or biosurfactants are heterogeneous group of surface active molecules produced by a wide variety of bacteria, yeast and filamentous fungi, which either adhere to cell surface or are excreted extracellularly in the growth medium. Having both hydrophobic and hydrophilic moieties, biosurfactants are able to reduce surface tension and interfacial tension between two fluids at the surface and interface respectively. These are also able to form microemulsion where hydrocarbons can solubilize in water or where water can solubilize in hydrocarbon [1]. Microbial surfactants are complex molecules, comprising a wide variety of chemical structures, such as glycolipids, lipopeptides, fatty acids, polysaccharides-protein complexes, peptides, phospholipids and neutral lipids [2]. Biosurfactants have many industrial applications in different areas like oil industry, food industry, pharmaceutical sector etc. About a 30% increase in total oil recovery from underground sandstone by using trehalolipids from *Nocardia rhodochrous* has been documented [3]. Rhamnolipid from *P. aeruginosa* was found to remove a large quantity of oil from contaminated Alaskan gravel in the Exxon Valdez oil spill [4]. Biosurfactants are also useful in bioremediation of sites contaminated with heavy metals such as uranium, cadmium, lead etc [5]. In the food processing industries, improvement in dough stability, texture, volume and conservation of bakery products is obtained by addition of rhamnolipid [6]. Biosurfactants are also very attractive in the health care and cosmetic industries [7]. Some antimicrobial action against bacteria, fungi, algae, and viruses are observed by several biosurfactants. The lipopeptide iturin from *B. subtilis* showed potent antifungal activity [8].

Almost all surfactants being currently produced are derived from petroleum source. However, these synthetic surfactants are usually toxic and hardly degraded by microorganisms. These are potential source of pollution and damage to the environment. Therefore, in the recent years, much interest and attention have been directed towards biosurfactants over chemically synthesized surfactants due to their ecological acceptance, owing to their low toxicity and biodegradable nature [9]. Other advantages of biosurfactant are ease of synthesis, specific action, and effectiveness at extreme conditions viz., temperature, pH, salinity [10]. Even though interest in biosurfactants is increasing, these compounds do not compete economically with synthetic surfactants due to the higher production cost of biosurfactants. To reduce the production cost, different routes could be investigated with respect to the increase of yield and product accumulation, the development of economical engineering processes and the use of cost-free or cost-credit feed stock for growth of microorganism and biosurfactant production [11]. The optimization of culture conditions is one of the routes that could be investigated for maximum production of biosurfactant.

In this study, we have tried to optimize culture conditions for maximum production of rhamnolipid from *Pseudomonas aeruginosa* OCD_1, isolated from air.

2. MATERIALS AND METHODS

2.1 Organism, Media, Chemicals and Solvents

The organism *Pseudomonas aeruginosa* OCD_1 was isolated from air in this laboratory by the present author [12]. Bushnell-Haas (BH) and Nutrient agar media of Hi Media Laboratories Pvt. Ltd., Mumbai, India, were used for isolation, cultivation and maintenance of culture. n-Octadecane was procured from Lancaster. Other chemicals and solvents were of AR grade and purchased from local suppliers.

2.2 Surface Tension & Emulsification Index measurement

Surface tension of cell free and oil free culture broth was measured at 30°C by the application of du Nouy ring tensiometer locally made, graduated to 0.1 mN/m [13]. Culture broth was centrifuged at 10,000 rpm, (Remi C 30) and then separated from the

oil phase in a separating funnel. Emulsification activity was measured using the method described by Cooper and Goldenberg [14], where 2 ml of diesel was added to the same amount of cell free supernatant and vortexed at high speed for 2 min. The emulsion stability was determined after 24 h and the E_{24} index was calculated as the percentage of height of emulsified layer (mm) divided by total height of the liquid column (mm).

2.3 Study of optimization of growth conditions

The process parameters were optimized using liquid BH media in a series of experiments to obtain higher productivity of the biosurfactant. The observed variables are inoculum volume, hydrocarbon (C-source) percentage, temperature, incubation time, culture state and pH. The ranges of various parameters studied are presented in Table -1. Optical density (O.D.), surface tension, and emulsification index were measured with variation of the parameter under study, keeping other parameters constant at a time.

Table 1: Ranges of culture parameters for biosurfactant production by P. aeruginosa OCD_1

Factors	Ranges
Inoculum (vol %)	0.2, 0.5, 1.0, 3.0, 5.0, 10.0
Hydrocarbon (vol %)	0.25, 0.5, 1.0, 2.0, 4.0, 10.0
Temperature (°C)	25, 30, 35, 40
Culture state, r.p.m	0 (Static), 50, 100, 125, 150
pH	4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8

2.4 Study of supplementation and limitation of medium component in the growth media

Supplementation or limitation of medium component concentration in the growth media (BH broth) may increase the yield of biosurfactant. Here biosurfactant production was studied with the addition of 30%, 50% and 70% (w/v) of each medium component in the optimized growth media; limitation by N-source (NH_4NO_3) in the growth media was also studied.

2.5 Study of kinetics of biosurfactant production

The kinetic studies of biosurfactant production were carried out at optimized culture conditions with inoculum of fresh overnight culture and 5-days-old culture.

In this experiment, variations in biomass, surface tension, critical micelle dilution (CMD^{-1}, CMD^{-2}), and emulsification index were recorded at different time intervals over a period of 10 days. Biomass was expressed as optical density (O.D.) of culture broth measured by a colorimeter (CL 157, ELICO) at 600 nm. CMD^{-1} and CMD^{-2} are the surface tension of cell free culture broth diluted 10-times and 100-times respectively with distilled water.

2.6 Detection and quantification of biosurfactant

The biosurfactant produced from *Pseudomonas aeruginosa* OCD_1 was detected as rhamnolipid, a type of glycolipid, reported in our previous paper [12]. The quantification of rhamnolipid was done by orcinol assay [15]. This test was used for direct assessment of the amount of glycolipid in the culture broth. 0.5 ml of the culture supernatant was extracted twice with 1 ml mixture of chloroform and methanol (2:1 v/v). The extracted organic layer was evaporated to dryness and 0.5 ml of H_2O was added. To 100 µl of each sample with suitable dilution 900 µl of solution containing 0.19% orcinol (in 53% H_2SO_4) was added. After heating for 30 min at 80°C the samples were cooled to room temperature and the $OD_{422.5}$ was measured. The rhamnolipid concentration was calculated from a standard curve prepared with L-rhamnose monohydrate and expressed as rhamnose monohydrate equivalents (RME) (mg ml^{-1}).

2.7 Study of effect of multivalent cations on growth media

Sometimes it was observed that, limitation or supplementation of multivalent cation concentration in the growth media increases the biosurfactant production. Guerra-Santos et al [16] demonstrated that limiting the concentrations of salt of magnesium, calcium, potassium and trace elements resulted in a better yield of rhamnolipid in *P. aeruginosa* DSM 2659. The yield of biosurfactant production by *B. subtilis* MTCC 2423 was increased by adding the metal cations altogether than the individual addition of cations [17]. In our study, chloride and sulphate salts of some multivalent cations viz. $Al_2(SO_4)_3$, $MnSO_4$, $ZnSO_4$, $SnCl_2$ etc., were supplemented (0.03 gm/L) in the optimized culture media to observe the biosurfactant production efficiency. The biosurfactant production was denoted in terms of surface tension, CMD^{-1} and emulsification index.

3. RESULTS AND DISCUSSION

3.1 Optimization of culture parameters for biosurfactant production

The cell growth and accumulation of metabolic products were strongly influenced by medium composition and other growth factors viz., temperature, pH, culture state etc. Thus optimization process can give high yield of metabolites. The biosurfactant production was measured by surface tension reduction and emulsification index test. The results of optimization process are given in figures below.

Figure 1 indicates that production of biosurfactant from *P. aeruginosa* OCD_1 was affected by change in inoculum volume. Maximum reduction of surface tension and highest emulsification index were obtained with 1 vol% inoculum. Any change to both lower or higher inoculum volume gives poor result.

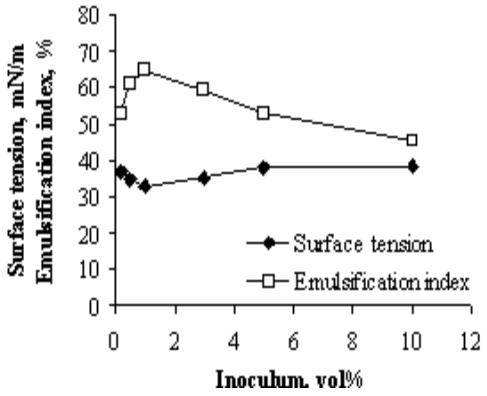


Figure 1: Optimization of inoculum vol. n-Octadecane = 2 vol%, Temp. = 35°C, Incubation time = 4days, shaking speed = 125 r.p.m, pH = 7.00

The biosurfactant production was affected by different concentration of n-octadecane(n-C_{18}) i.e. C-source (Fig 2). With increasing hydrocarbon from 0.25 to 10.0 vol%, initially surface tension decreased and emulsification index increased upto 2vol% n-octadecane, after that surface tension and emulsification index did not change significantly. Therefore, 2 vol% n-C_{18} was taken as optimum C-source.

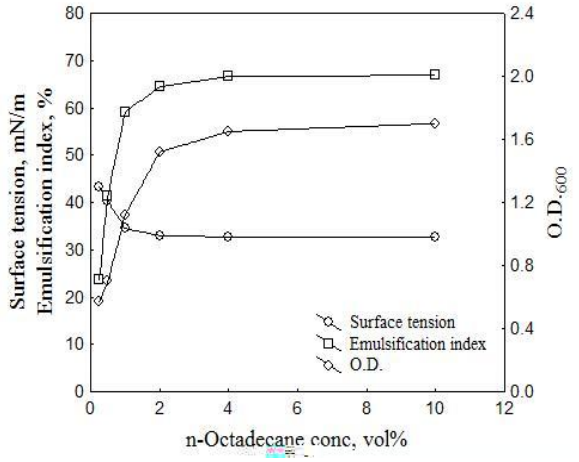


Figure 2: Optimization of hydrocarbon vol. Inoculum = 1 vol%, Temp. = 35°C, Incubation time= 4days, shaking speed = 125 r.p.m, pH = 7.00

Temperature is one of the critical parameter that greatly affected the culture growth and the biosurfactant production. Figure 3 indicates that optimum temperature for biosurfactant production was found to be between 30°C and 35°C. A decrease or increase in the incubation temperature leads to lower growth of organism and biosurfactant production.

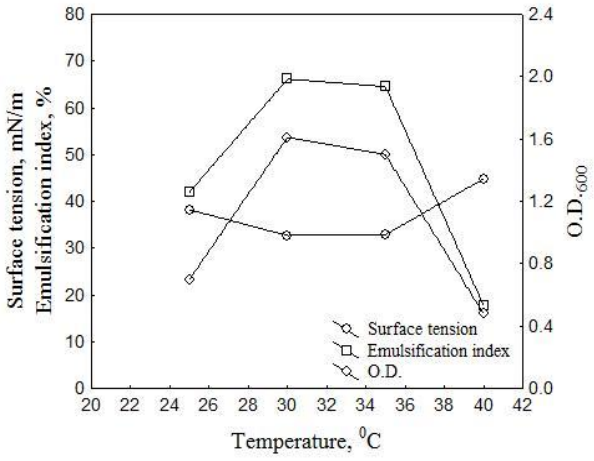


Figure 3: Optimization of temperature. Inoculum = 1 vol%, n-octadecane = 2 vol%, Incubation time = 4days, shaking speed = 125 r.p.m, pH = 7.00

Figure 4 shows that variation of biosurfactant production with culture state. The more biosurfactant was produced in shaking conditions than static. By increasing shaking speed upto 125 r.p.m, surface tension decreased and then again increased with increasing shaker r.p.m. At 125 r.p.m highest culture growth and emulsification index were obtained.

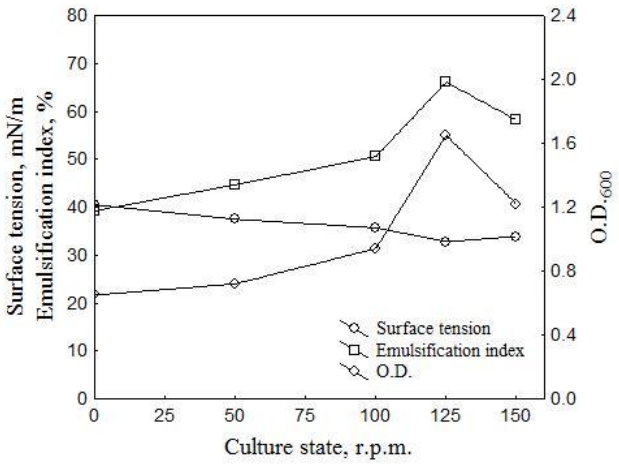


Figure 4: Optimization of shaker speed. Inoculum = 1 vol%, n-octadecane = 2 vol%, Temp. = 30°C, Incubation time= 4days, pH = 7.00

Another important characteristic of most organisms is their strong dependence on the pH for growth of organism and production of secondary metabolites. Figure 5 shows that highest biosurfactant production by *P. aeruginosa* OCD_1 was obtained at pH 6.00. Any change to both lower or higher pH values caused an appreciable drop in biosurfactant production indicated by surface tension reduction and emulsification index values.

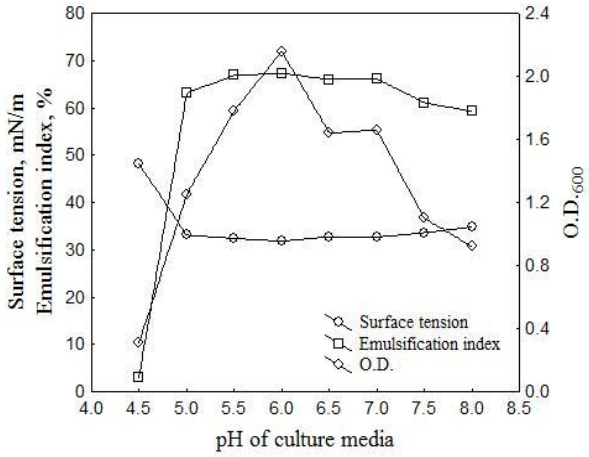


Figure 5: Optimization of pH of culture broth. Inoculum = 1 vol%, n-octadecane = 2 vol%, Temp. = 30°C, Incubation time= 4days, shaking speed = 125 r.p.m.

3.2 Supplementation and limitation of medium component in the growth medium

The study of supplementation and limitation of media component (BH broth) in the growth media at optimized conditions was presented in Table-2. From the result it was observed that, extra addition of $CaCl_2$ and KH_2PO_4 in the BH media give quite better result than BH media only at optimized conditions with respect to surface tension and emulsification index values. When NH_4NO_3 was added in the growth media the organism showed very low growth. Therefore, limitation of NH_4NO_3 was studied. But it didn't give significant result. Again, media selection for biosurfactant production was done from the result of Table-3. BH media at optimized

conditions with extra 30% (w/v) $CaCl_2$. $2H_2O$ was selected for production of biosurfactant.

3.3 Biosurfactant production kinetics

Kinetics of biosurfactant production was observed when the strain *P. aeruginosa* OCD_1 was cultivated in BH media at optimized conditions. Figure 6A shows the production of biomass and biosurfactant at different time intervals when fresh overnight culture was used as inoculum. The surface tension (ST), critical micelle dilution (CMD^{-1}, CMD^{-2}) and E_{24} values were recorded during growth of organism. Maximum biosurfactant production (expressed as rhamnose monohydrate equivalent, RME) of 0.98 mg mL^{-1} was observed in the stationary growth phase.

Table 2: Effect of supplementation and limitation of medium component in the growth medium

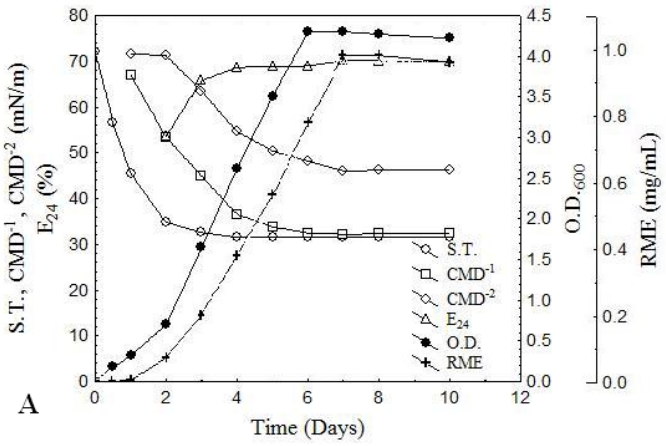
BH Media Component	30 % (w/v)		50 % (w/v)		70 % (w/v)	
	ST	E_{24}	ST	E_{24}	ST	E_{24}
Supplementation						
$MgSO_4$	32.2	66.92	33.0	63.16	35.8	48.89
$CaCl_2$. $2H_2O$	31.6	68.70	31.7	68.52	31.6	68.74
KH_2PO_4	31.9	68.22	31.7	68.45	31.8	68.40
K_2HPO_4	33.0	63.08	34.5	60.34	35.6	52.62
NH_4NO_3	48.50	7.35	52.8	-	55.6	-
$FeCl_3$	32.3	66.74	32.0	67.00	32.2	66.89
Limitation						
NH_4NO_3	46.5	15.74	55.6	-	58.2	-

Table 3 Media selection for higher biosurfactant production

Variables	$O.D._{600}$	ST	CMD^{-1}	E_{24}
BH media	2.16	31.8	38.8	67.32
BH media + 30% (w/v) $CaCl_2$. $2H_2O$	2.60	31.5	36.0	69.00
BH media + 50% (w/v) KH_2PO_4	2.55	31.7	36.6	68.45
BH media + 30% (w/v) $CaCl_2$. $2H_2O$ + 50% (w/v) KH_2PO_4	2.62	31.7	36.5	68.66

This observation suggests that the produced biosurfactant in the culture broth is a secondary metabolite. A similar type of observation was observed earlier by the other authors during the growth of *Pseudomonas putida* 21BN on n-hexadecane [18]. In the case when the culture was inoculated with an overnight culture an adaptation was required before reaching the stage of maximum biosurfactant production. This delay in the lag phase was expected since a number of different biochemical reactions are involved in alkane utilization including their terminal hydroxylation and β-oxidation [19]. However, enough rhamnolipid were produced to cause a drop in the ST of culture broth from 72 to 37 mNm^{-1} even after 36 hr of incubation. The surface tension reached a minimum of 31.5 in the stationary growth phase and did not decline further on. The CMD plot (a measure of biosurfactant concentration) showed that insufficient surfactant was initially present to form micelles. The minimum values of CMD^{-1} and CMD^{-2} were observed as 32.2 and 46.0 respectively after 7 days of incubation, i.e., in the stationary growth phase. E_{24} values increased with increasing cell growth, reaching the optimum of 70.10 at stationary growth phase and remaining constant until the end of incubation.

The time of lag phase was reduced when a 5 days-old culture was used as inoculum (Fig 6B). Biosurfactant production started more rapidly with the addition of old inoculum, and simultaneous reduction of surface tension and CMD values were observed. The surface tension decreased to 31.5 mNm^{-1} and emulsification index increased to 69.96 coincidently with the transition to the stationary growth phase i.e., 4 days of incubation. The shorter lag phase may be due to the enhancement of n-octadecane availability for the cells by the concomitant addition of biosurfactant with the inoculum. Moreover, the inoculum culture fluid may have contained diffusible autoinducers which regulate rhamnolipid synthesis in *Pseudomonas aeruginosa* [20].



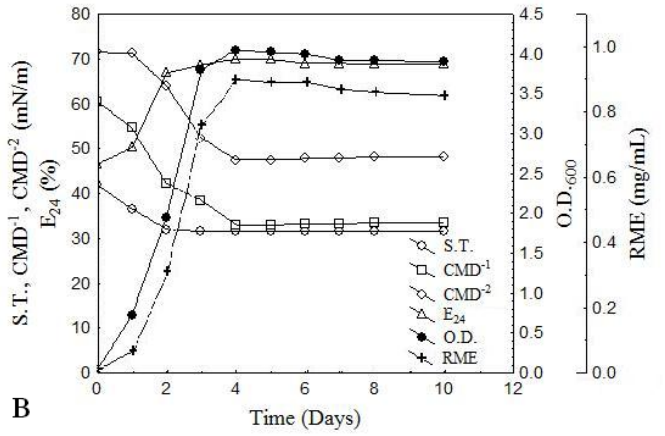


Figure 6: *Biosurfactant production kinetics at optimized culture conditions. (A) inoculation with overnight culture (B) inoculation with 5-days-old culture (on BH with 2 vol% n-octadecane). All values are averages from three experiments.*

3.4 Effect of multivalent cations on growth media

The effect of supplementation of multivalent cations in the culture broth is shown in Figure 7. In this study, biosurfactant production was enhanced by the addition of $ZnSO_4$ followed by $MnSO_4$ when observed at 3 days of incubation. This type of result was observed by Kiran et. al. [21] for biosurfactant production by *Aspergillus ustus* MSF3. Figure 7 also indicates that, the other salts $Al_2(SO_4)_3$ and $BaSO_4$ did not show better result than control flask. In contrast, $CuSO_4$, $SnCl_2$ and $CdCl_2$ addition showed lower growth of organism in the culture media.

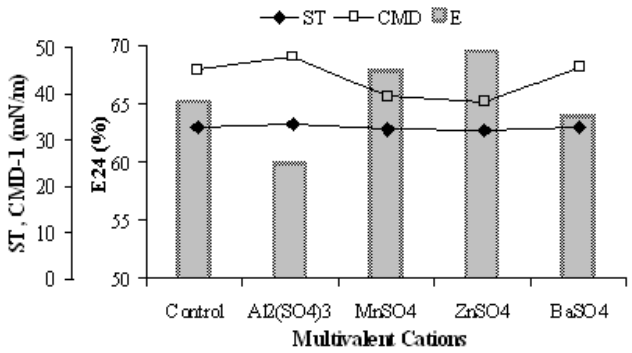


Figure 7 Effect of multivalent cations on biosurfactant production; ST, surface tension; CMD-1, critical micelle dilution (10-times diluted culture broth); E24, emulsification index; Incubation period: 3days at optimized conditions

4. CONCLUSION

The optimum culture condition for maximum biosurfactant production by *Pseudomonas aeruginosa* OCD_1 was obtained as 1% v/v inoculum, 2% v/v n-octadecane, 30°C temperature, 125 r.p.m. and pH 6.0 with extra 30% w/v $CaCl_2$. $2H_2O$. The strain is a potential source of rhamnolipid. The kinetics study revealed that maximum rhamnolipid production was obtained at stationary growth phase after 7 days of incubation with inoculum of overnight culture. But the delay of lag phase may be reduced with the inoculum of 5-days-old culture and the maximum rhamnolipid production was achieved within 4 days of stationary growth phase. Again, it was observed that, multivalent cations Zn and Mn have positive impact on biosurfactant production. In literature few workers reported that limiting the concentrations of salt of magnesium, calcium, potassium and trace elements resulted over production of rhamnolipid by *P. aeruginosa* DSM 2659 [16]. But, in our study supplementation of metal ions increased the biosurfactant production by *P. aeruginosa* OCD_1. There was no report in literature on over production of biosurfactant by *P. aeruginosa* OCD_1 with supplementation of metal ions. Therefore, our study gives new information in this respect. The rhamnolipid obtained from *Pseudomonas aeruginosa* OCD_1 may have various applications in different areas like bioremediation of polluted environment, pharmaceutical sector, food industry, cosmetics industry etc.

5. ACKNOWLEDGEMENT

Financial support of University Grants Commission (Govt. of India) is gratefully acknowledged.

6. REFERENCES

1. Desai JD, Banat IM. *Microbiol Mol Biol Rev,* 1997; **61**: 47 – 64.
2. Banat IM, Makkar RS, Cameotra SS. *Potential Appl Microbiol Biotechnol,* 2000; **53**: 495-508.
3. Rapp P, Bock H, Urban E, Wagner F et al. *DESCHEMA Monogr Biotechnol,* 1977; **81**: 177-185.
4. Harvey S, Elashvili I, Valdes J, Kamely D, Chakrabarty AM. *Bio Technology,* 1990; **8**: 228-230.
5. Miller RM. *Environ Health Perspect*, 1995; **103**: 59-62.
6. Van Haesendonck IPH, Vanzeveren ECA, Rhamnolipids in bakery products. *International application patent(PCT)* W. O. 2004/040984, 2004.
7. Klekner V, Kosaric N. Biosurfactants for cosmetics. In: Kosaric N editor. *Biosurfactants: production, properties, applications*. New York: Marcel Dekker Inc; 1993, p. 329-372.
8. Besson F, Peypoux F, Michel G, Delcambe L. *J Antibiot,* 1976; **29**: 1043-1049.
9. Karanth NGK, Deo PG, Veenanadig NK. *Curr Sci*, 1999; **77**: 116-126.
10. Tabatabaee A, Mazaheri Assadi M, Noohi AA, Sajadian VA. *Iranian J Env Health Sci Eng,* 2005; **2**: 6-12.
11. Mercade ME, Manresa MA. *JAOCS,* 1994; **71**: 61-64.
12. Sahoo S, Datta S, Biswas D, Banik Choudhury R. *J Oleo Sci,* 2010; **59**: 601-605.
13. Adria A, Bodour K, Drees P, Raina MM. *Appl Environ Microbiol,* 2003; **69**: 3280-3287.
14. Cooper DG, Goldenberg BG. *Appl Environ Microbiol*, 1987; **53**: 224-229.
15. Candrasekaran EV, Bemiller JN. Constituent analysis of glycosaminoglycans. In: Whistler RL, Wolfrom ML editors. *Methods in carbohydrate chemistry*. New York: Academic Press Inc; 1980, p. 89 – 96.
16. Guerra-Santos LH, Kappeli O, Fiechter A. *Appl Microbiol Biotechnol,* 1986; **24**: 443-448.
17. Makkar RS, Cameotra SS. *Appl Microbiol Biotechnol,* 2002; **58**: 428-434.
18. Tuelva BK, Ivanov GR, Christova NE. *Z Naturforsch*, 2002; **57**: 356-360.
19. Witholt B, de Smet MJ, Kingma J, van Beilen JB et al. *Trends Biotechnol,* 1990; **8**: 46-52.
20. Ochsner AR, Reiser J. *Proc Natl Acad Sci USA,* 1995; **92**: 6424 -6428.
21. Seghal Kiran G, Hema TA, Gandhimathi R, Selvin J et al. *Colloids and Surfaces B: Biointerfaces*, 2009; **73**: 250-256.