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Screening of Biosurfactants from Hydrocarbon Degrading Bacteria

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Abstract

Biosurfactants compounds are produced by microorganisms. These isolates reduced surface tension both in aqueous solutions and hydrocarbon mixtures. The objective of this study was to isolate and identify the biosurfactant producing bacteria from petrochemical soil wastes. Among the 16 isolates, two strains (BPB7: *Pseudomonas* sp and BPB13: *Serratia* sp) were shown high salt tolerance and successful production of biosurfactant in a range of pH 6.5 to 7.5 and at room temperature. To confirm the ability of isolates in biosurfactant production, different screening methods including hemolysis, emulsification, penetration, oil spreading test BATH assay and salt aggregation assay were assessed. This study was suggested that these two bacteria having potential for microbial enhanced oil recovery and also possessing Anti microbial and Anti adhesive property. The rich amount of biosurfactant compound like, glycolipid was found in these two strains. (BPB7 and BPB13).

1. Introduction

Biosurfactants are a leading group of valuable microbial natural products with unique biochemical properties. From a biotechnology prespective, the production of biosurfactants is important owing to their vast applications in food, cosmetics, pharmaceuticals, agricultural and the petrochemical industries [1,2]. Microbial surfactants, which are secreted by different groups of bacteria, composed of lipid, phospholipids, polysaccharide, protein and other biological macromolecules and contain various functional groups including carboxyl, amino and phosphate groups [3,4]. Practically all the usable surfactants are chemically synthesized at present. Surfactants have been used industrially as adhesives, deemulsifiers, flocculating, wetting and forming agents, lubricants and penetrants [5]. Because of their amphiphilic nature, surfactants tend to accumulate at interfaces (air-water and oil-water) and surfaces.

The total surfactant production has exceeded 2.5 million tonnes in 2002 for many purposes such as polymers, lubricants and solvents. The growth rate is related to the world demand in detergents since this sector uses over 50% of surfactant production [6]. The interest in biosurfactant has been steadily increasing in recent years due to the possibility of their production through fermentation and their potential applications in such areas as the environmental protection. The uniqueness with unusual structural diversity, the possibility of cost-effective ex-situ production and their biodegradability are some of the properties that make biosurfactant a promising choice for use in environmental application [7]. *Pseudomonas* species form the second largest group of bacteria producing biosurfactants. Many strains of *Pseudomonas* have been reported to produce glycolipids, especially rhamnolipids. Besides rhamnolipids *Pseudomonas* strain such as *Pseudomonas* sp. MIS38 have also been reported to produce arthrofactin, a lipopeptide type of biosurfactant. Other biosurfactants produced by *Pseudomonas* include viscosin produced by *Pseudomonas fluorescens*, putisolvin produced by *Pseudomonas putida* and amphisin produced by *Pseudomonas* sp. DSS73. Rhamnolipid is a glycolipid biosurfactant produced by many strains of *Pseudomonas* [8].

Serratia sp is a bacteria found in water, soil, plant and animals. It was once considered much a relatively harmless organism that pigment strains were used as a tracer of an aerosol in field experiments. Aim of this work is to isolate bacterial strains from petrochemical waste soil and screen for biosurfactant production using different screening methods. The potential for the degradation of diesel by these strains were studied and efforts to partially purify the biosurfactants were also carried out.

2. Materials and Methods

Bushnell Hass Mineral Salt Medium (BHMS) agar was used as an isolation medium for microorganisms obtained from various hydrocarbon contaminated sites. The plates were

incubated at 37°C for 24 h. After overnight incubation, the culture plates having colonies of several different microorganism species. Isolation of single colony was done by transferring a loop of individual colony that shared same color and morphological characteristic to BHMS agar plate. All isolates were sub-cultured three times before conducting experiments for screening, in order to obtain pure single isolate for each colony. Enrichment and isolation of oil degrading bacterial cultures were done using mineral salts medium with crude oil as substrate and a serial dilution-agar plating technique on nutrient agar medium (Hi-Media, Mumbai, India) respectively. The isolated bacterial cultures were characterized by their morphologically and biochemically.

The bacterial cultures (12 h old) were inoculated in Bushnell Hash Mineral Salt Medium with 1% of diesel as carbon source. They were kept in a shaker at 200 rpm at 30°C for a period of 7 days. The growth was monitored through culture densities, measuring absorption spectrophotometrically at 620 nm. The optimum pH, different carbon sources for the production of biosurfactant from *Pseudomonas sp and Serratia sp* was determined, so that the same conditions can be maintained for bulk production. Standardization of the conditions will enhance the growth of organism and biosurfactant production [9]. Preculture of bacteria strains were prepared in NB in OD_{600}=1.1ml of inocula were added to 100 ml MSS and 1% filtered oil as hydrocarbon source. The mixtures with control samples were incubated at 30°C on shaker at 150 rpm for 3days.

The identification of Biosurfactant by Hemolysis method was based on the fact that surfactants interact strongly with cellular membranes and proteins [10]. Exotoxins called haemolysins cause lysis of the red blood cells. Screening of biosurfactant producers via this method was previously outlined that only those isolates which showed haemolysis were considered to be the potential biosurfactant producing microbes [11].

The CTAB agar plate method is a semi quantitative assay for the detection of extra cellular glycolipids or other anionic surfactants. The microbes of interest are cultivated on a light blue mineral salts agar plate containing the cationic surfactant cetyltrimethyl ammonium bromide and the basic dye methylene blue. If anionic surfactants are secreted by the microbes growing on the plate, they form a dark blue insoluble pair with cetyltrimethyl ammonium bromide and methylene blue. Productive colonies are surrounded by dark blue halos. [12].

The degradation and consumption of hydrocarbons can be visualized by the colorimetric method developed by Hanson [13]. By adding a colored redox indicator, 2, 6 dichlorophenol indophenol to liquid cultures growing on hydrocarbons. The DCPIP is incorporated by bacteria that can degrade the hydrocarbons. It acts as electron acceptor and changes from blue (oxidized) to colorless (reduced).

The secondary screening of biosurfactant was done by Thin layer Chromatography method. Biosurfactant produced were characterized by thin layer chromatography using silica-gel (20cm x 20cm, Merck). The development of solvent systems used was differed based on the components tested. The TLC plates were spotted with biosurfactant extracts and developed with the following: solvent 1, petroleum ether - diethyl ether-acetic acid (80:20:1) for neutral lipids, solvent 2, chloroform-methanol-water (65:25:4) for polar lipids, solvent 3, *n*-buthanol-acetic acid-water (4:1:1) for amino acids and solvent 4, ethyl acetate-acetic acid-methanol-water (12:3:3:2) for carbohydrate compounds. After developing, the spots were visualized with standard reagents. The lipid components were detected as yellow spots after placing the plates in a closed jar saturated with iodine vapours. Using the ninhydrin solution followed by heating at 90ºC for 5 minutes, generated a red or purple color when the compound had an amine function. Carbohydrate components were detected as red spots on the plates after spraying with an alpha- naphthol solution followed by concentrated sulphuric acid.

The emulsifying capacity can be evaluated by measuring the emulsifying index following the method of Cooper and Goldenberg, 1987 [14].

The penetration assay is a simple, qualitative technique for screening large amounts of potential isolates [15]. The phenomenon by which silica gel is entering the hydrophilic phase from the hydrophobic phase is much faster if biosurfactants are present. This assay relies on the contacting of two insoluble phases which leads to a colour change.

The drop collapse assay relies on the destabilization of liquid droplets by surfactants. The stability of drops is dependent on surfactant concentration and correlates with surface and interfacial tension. This assay is rapid and easy to carry out by Bodour and Millel [16].

The diameter of the clearing zone on the oil surface correlates to surfactant activity, also called oil displacement activity. Pure biosurfactant has a linear correlation between quantity of surfactant and clearing zone diameter. It is rapid and easy to carry out by Morikawa [17].

The bacterial adhesion to hydrocarbon (BATH) assay was used to determine changes in cell surface hydrophobicity during growth on minimal salt medium with diesel as carbon source [18].

A salt aggregation assay is similar to the salting out of proteins. The cells are precipitated by increase sat concentration. The more hydrophobic the surface of the cell, the lower the salt concentration required to aggregate the cells. So the most hydrophobic cells precipitate first at low salt concentration [19].

The rate of degradation of diesel was analyzed by gas chromatography. The organism selected were sub cultured in BHMS medium and incubated in shaker for 14 days. After incubation the residual hydrocarbon was extracted using Dichloromethane and Hexane (1:1) ratio. The residue was analyzed by Gas Chromatography and pure diesel served as control.

The hydrocarbon degraders normally produce surfactants adhere effective contact with hydrophobic substrate many of the surfactants are reported to be basically lipids. Hence lipid content were analyzed which indicated the potential degradation of diesel [20].

For antimicrobial test, the concentrated culture supernatant of the strains BPB7 and BPB 13 were spotted on filter discs on top of an agar plate with freshly grown Gram negative (*Bacillus* sp) and Gram Positive (*Salmonella* sp) Bacteria [21]. The preconditioning of stainless steel surfaces with a biosurfactant obtained from the isolates BPB 7 and BPB 13 reduces the adhesion of the microbes on the surface. The bio-conditioning of surfaces through the use of microbial surfactants has been suggested as a new strategy to reduce Adhesion [22].

3. Result and discussion

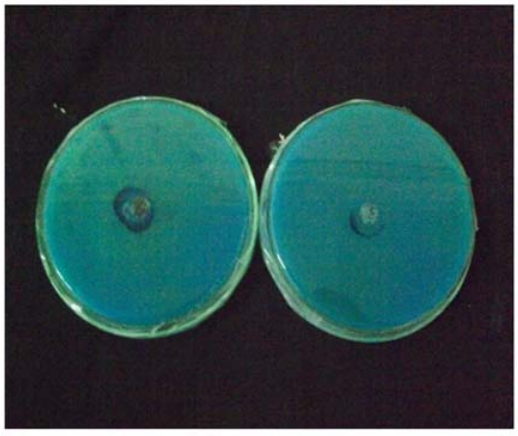
Bacterial strains identification and standardization

Sixteen morphologically distinct colonies were isolated, among these two strains (BPB7 and BPB13) were selected as the suitable biosurfactant producer. Morphological and microscopic observations revealed that the colonies were green, rod shaped and slick. Biochemical and physiological experiments on BPB7 and BPB13 suggested that there was Gram-negative, motile and non spore forming colonies. Based on these results, these strains were preliminarily identified as *Pseudomonas* sp and *Serratia* sp. Standardization of the conditions will enhance the growth of organisms and biosurfactant production. The two potential isolates BPB7 and BPB13 shown maximum growth at pH of 6.5 and 7.5 respectively. And there was also grown on different carbon source like diesel, kerosene, hexane, benezene and toluene. The strain BPB7 shown high growth rate on diesel and hexane carbon source compare to other sources. The isolate BPB13 shown the maximum growth on diesel, toluene, hexane, with benzene and kerosene.

The culture supernatants of BPB7 and BPB13 having higher rate of hemolysis activity of the diameter 10 and 11mm respectively. The same result was observed in the standard test of *Bacillus subtilis* inhibition test.

The anionic surfactant was determined on CTAB medium in which colonies were surrounded by the blue dark color halos (Fig.1)

Fig.1. CTAB agar test of BPB7 and BPB13



The DCPIP assay is incorporated by bacteria that can degrade the hydrocarbons. It acts as electron acceptor and changes from blue (oxidized) to colorless (reduced). The test isolates BPB7 and BPB 13 showed a colour change from blue to colorless.

TLC analysis showed that there was no red spot on silica gel plates when using ninhydrin as color developing reagent, suggesting that no lipopeptide was found in this biosurfactant. Meanwhile, the appearance of yellow spot proved the presence of glycolipid. Accordingly, the

biosurfactant excreted by strains BPB 7 and BPB 13 were glycolipids in nature.

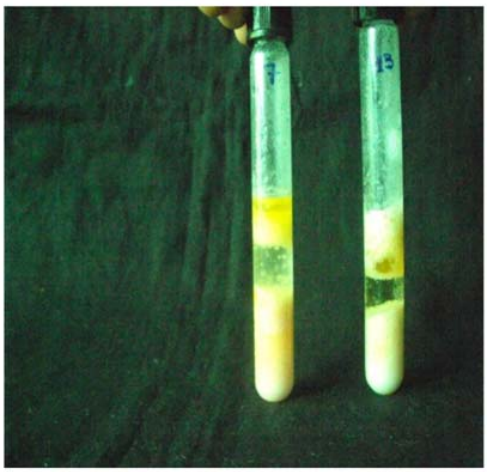
The emulsification index of the biosurfactants in diesel was determined and data are represented in Table1. The emulsification index of the crude biosurfactant from BPB7 and BPB13, were found to be 57 and 36%, respectively. The biosurfactant from BPB7 was the most stable with the decay constant of -0.0452 followed by BPB13 with the value of -0.0153. The emulsification index was calculated for both BPB 7 and BPB 13 isolates which shows 57% and 30% of emulsification respectively. The value above 30% was taken as positive result. (Table 1)

Table I. Emulsification index and emulsion stability of biosurfactants and chemical surfactant SDS (mean±SD)

Source of crude biosurfactant/chemical surfactant	Diesel	
	Decay constant (*Kd*)	Emulsification index (%)
SDS	−0.0788	45 ± 1.3
BPB 7	-0.0452	57±1.4
BPB 13	-0.0153	36±1.3

The penetration ability of the isolates BPB7 and BPB13 between two different phases were tested which resulted in mixing of the two distinct phases within 15 minutes. Both strains BPB 7 and BPB13 showed immediate spreading when a drop of isolate is placed on the surface of the oil. Figure 2 Shows the mixing of two different phases and penetration of silica gel from one phase to another phase which leads to the colour change.

Fig.2. Penetration assay of BPB7 and BPB13



Salt aggregation assay the stains BPB7 shows precipitation on adding 1M ammonium sulphate and BPB13 shows precipitation on adding 4 M ammonium sulphate.

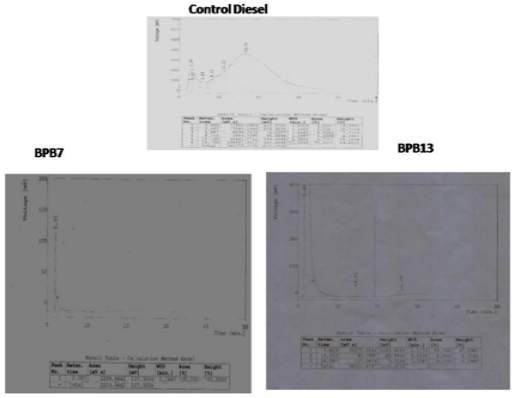
Additional experiments were done to evaluate influence of carbon source used in culture medium on cell surface hydrophobicity. The results obtained from BATH test showed that the cell surface hydrophobicity is changed in respect to the carbon source used in culture medium. The highest cell hydrophobicity was obtained when BHMS medium was supplied with diesel. The isolates were BPB7 shows 3.8% hydrophobicity and BPB13 shows 1.8% hydrophobicity. (Table 2).

Table 2. BATH test of BPB7 and BPB13

Strains	Initial absorbance(A)	After adding the hydrophobic layer (A0)	% of cell adhere to hydrophobic phase
BPB 7	1.187	1.014	1.8
BPB 13	0.996	1.223	3.8

Gas Chromatography analysis shows the complete degradation of the diesel in the BPB7 and BPB13 culture media after 14 days. Significant decrease of many carbon peaks when compared with the control. Pure diesel served as control. (Fig.3)

Fig. 3. Degradation of diesel in BPB7 and BPB13



Initially the lipid estimated in isolates BPB 7 is 30 mg/L and BPB 13 is 22 mg/L and the presence of lipid after 10 days was estimated to be 240mg/l in BPB7 and 160 mg/L in BPB13 culture.

All these results confirmed the production of biosurfactant. Further, the presence of hydrophobicity of the two isolates BPB 7 and BPB 13 were detected by using BATH and Salt aggregation assay. Where BPB 7 shows 3.8% hydrophobicity and BPB 13 shows 1.8% by which was calculated by bacterial adhesion to hydrocarbon. The more hydrophobic cell surface, the lower salt concentration was required to precipitate the cells where the BPB7 shows precipitation by adding 1M ammonium sulphate and BPB13 shows precipitation by adding 4M ammonium sulphate. As a matter of fact, the increased production of biosurfactant by *Pseudomonas sp* BPB7 and *Serratia sp* BPB13 may be occurred as a result of direct intake of amino acid as precursors for the surfactant biosynthesis, thus improving the biosurfactant yield. In a previous report, [23] showed that the some of the amino acids are excellent substrate as nitrogen sources for the production of surfactant from *Bacillus subtilis*. The two isolates BPB7 and BPB13 shows maxium growth at pH 6.5 and 7.5 it is also grown on different carbon source like Diesel, Kerosene, Hexane, Benezene and Toulene. This study reveals that the utilization of different PAH as carbon source. Utilization of different hydrocarbon substrates by *Pseudomonas* sp BPB7 and *Serratia* sp BPB13 is shown by increase in the number of cells. Gas chromatographic analysis revealed that 95% utilization of diesel substrate by the organisms in 48 h (Fig.3). The growth of bacteria on hydrocarbons is usually accompanied by the production of surfactants that helps in the adherence of the cells to oil droplets [24].

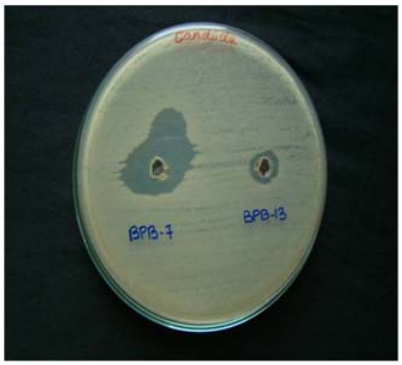
The hydrocarbon degraders normally produce surfactants which adhere effectively to hydrophobic substrate. Many of the surfactants are reported to be basically lipids. Hence lipid content were analyzed which indicated the potential degradation of diesel. Initially the lipid content was both in isolates BPB7 and BPB13 is 30 mg/l and 22 mg/l. The presence of lipid after 10 days was estimated to be 240 mg/l in BPB 7 and 160 mg/l in BPB 13 culture (Table 3). The optimum pH for growth and biosurfactant production was determined to be 8. Much work has been done on the role of biosurfactants as effective factors for emulsifying of hydrocarbons[25].

Table 3. Lipid content of BPB7 and BPB13

Stains	Initial concentration	Concentration after 10 days incubation
BPB 7	30mg/l	240mg/l
BPB 13	22mg/l	160mg/l

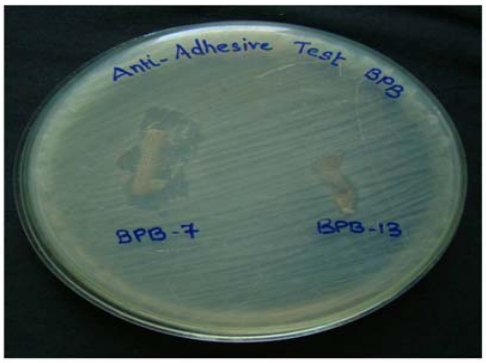
The biosurfactants extracted from the two test isolates BPB7 and BPB13 also found to have Antimicrobial and Antiadhesive activity. The zone of inhibition was found when inoculated against *Candida* sp*, Salmonella* sp *and Bacillus* sp the potent human pathogens (Fig.4).

Fig.4. Antimicrobial activity of BPB7 and BPB13



The biosurfactant coated steel was placed on bacteria inoculated plate which shows no growth on the surface of steel. Thus this property of antiadhesiveness can be used in surgical equipments for sterilization (Fig.5).

Fig.5. Anti adhesive activity of BPB7 and BPB13



The potential applications of biosurfactant in a variety of industries, the biotechnological production of this valuable biomolecule has been considered by investigators in recent years. The study also revealed that the biosurfactant from the organism emulsified different hydrocarbon substrates and aromatics. The interest in biosurfactant has been steadily increasing in recent years due to the possibility of their production through fermentation and their potential applications in such areas as the environmental protection. The uniqueness with unusual structural diversity, the possibility of cost –effective ex- situ production and their biodegrability are some of the properties that make biosurfactant a promising choice for use in environmental application. The ability of *Pseudomonas* sp BPB7 and *Serratia* sp BPB13 strains to utilize diesel as carbon source is the significant advantage as it provides an alternative for use of glucose, provided that its biosurfactant production can be enhanced economically to viable values. *Pseudomonas* sp BPB7 and *Serratia* sp BPB13 utilized hydrocarbons through emulsification of the substrate by producing glycolipid Biosurfactant. The study also revealed that the biosurfactant from the organism emulsified different hydrocarbon substrates and aromatics.

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References

1. Nguyen. T.T, Youssef. N.H, McInerney. M.J, Sabatinic, (2008). Rhamnolipid biosurfactant mixtures for environmental remediation, *Water Res*. 42:1735–1743.
2. Abouseoud. M, Maachi. R, Amrane. A, Boudergua. S, Nabi. A, (2008). Evaluation of different carbon and nitrogen sources in production of biosurfactant by *Pseudomonas fluorescens*, *Desalinatio.,* 223:143–151.
3. Desai, J.D. and Banat, I.M. (1997). Microbial production of Surfactant and Their

Commercial Potential. *Microbiology and Molecular Biology Review.,* 61: 47-64.
4. Christofi. N, Ivshina. I.B, (2002). Microbial surfactants and their use in field studies of soil remediation, *J. Appl. Microbiol.,* 93: 915–929.
5. Mulligan, C.N. and Gibbs, B.F. (1993). Factors Influencing the Economics of Biosurfactants". In: Kosaric, N. (ed.). "Biosurfactants Production, Properties and Applications". New York: Marcel Dekker, Inc. 329-371.
6. Deleu, M. and Paquot, M. (2004). From Renewable Vegetables Resources to Microorganisms: New Trends in Surfactants. *C.R. Chimie.*, **7**: 641-646.
7. Hua, Z., Chen, J., Lun, S. and Wang, X. (2003). Influence of Biosurfactants Produced by *Candida antarctica* on surface properties of microorganism and Biodegradation of *n*-alkanes. *Water Research*., 37: 4143-4150.
8. Lang, S. and Wagner, F. (1987). Structures and Properties of Biosurfactants". In: Kosaric, N., Cairns, W.L. and Gray, N.C.C. (eds) "Biosurfactants and Biotechnology". Surfactant Science Series. 25: 21-47.
9. Rahman,K.S.M.,Rahman,T.J.,Banat,I.M.,Lord, R.andStreet,G.,(2007).In Environmental Bioremediation Technologies (eds Singh,N.S and Tripathi.D.R). *Spinger Berlin* 391-398.
10. Pape, W. and Hoppe, U. (1988). "Evaluation of Acute Irritation Potentials of Tensides Using the In Vitro Alternative Red Blood Cell Test System". Second World Surfactants Congress, Paris. Proceedings, IV. 414-428.
11. Carrillo, P.G., Mardaraz, C., Pitta-Alvarez, S.J. and Giulietti, A.M. (1996). Isolation and Selection of Biosurfactant-producing Bacteria. *World J. Microbiol. Biotechnol.* 12: 82-84.
12. Siegmund I, Wagner F. (1991). New method for detecting rhamnolipids excreted by *Pseudomonas* species during growth on mineral agar. *Biotechnol Techniques.,* 5(4): 265–268
13. Hanson K, Desai J, Desai A. (1993). A rapid and simple screening technique for potential crude-oil degrading microorganisms. *Biotechnol Techniques.* 7(10): 745–748.
14. Cooper, D.G. and Goldenberg, B.G. (1987). Surface Active Agents from Two *Bacillus* Species. *American Society for Microbiology*. 224-227.
15. Maczek J, Junne S, Götz P. (2007). Examining biosurfactant producing bacteria—an example for an automated search for natural compounds. Application Note CyBio AG.
16. Bodour. A.A. and Miller-Maier, R.M. (1998). Application of a Modified dropcollapsing Technique for Surfactant Quantitation and Screening of Biosurfactant-producing Microorganisms., *Journal of Microbiological Methods.,* 32: 273-280.
17. Bodour, A.A. and Miller-Maier, R.M. (2002). Biosurfactants: Types, Screening Methods and Applications. *Encyclopedia of Environmental Microbiology*. Wiley, NY. 750-770.
18. Rosenberg M, Gutnick D, Rosenberg E. (1980). Adherence of bacteria to hydrocarbons a simple method for measuring cell-surface hydrophobicity. *FEMS Microbiol Lett.* 9(1): 29–33.
19. Lindahl M, Faris A, Wadstrom T. (1981). A new test based on salting out to measure relative surface hydrophobicity of bacterial cells. *Biochim Biophys Acta.,* 677(3-4): 471–476.
20. Zoellner, N and Kirsch, K, Z. (1962). fur die Gesampte., *Exp Med.,* 135:545.
21. Kitamoto, D., Yanagishita, H., Shinbo, T., Nakane, T., Kamisawa, C. and akahara, T., 1993. Surface active properties and antimicrobial activities of annosylerythritol lipids as biosurfactants produced by *Candida antarctic*a. *J. Biotechno*l., 29: 91–96.
22. Hood, S. K. and Zottola, E. A., (1995). Biofilms in food processing. *Food Control.,* 6: 9–18.
23. Sandrin. C, Peypoux. F, Michel. G, (1990). Coproduction of surfactin and iturin A, lipopeptides with surfactant and antifungal properties by *Bacillus subtilis*, Biotecnol., *Appl. Biochem.* 12. 370–375.
24. Rosenberg M, Rosenberg E. (1998). Role of adherence in growth of *Acinetobacter calcoaceticus* RAG-1 on hexadecane. *J Bacteriol.,* 148:51- 7.
25. Thavasi. R, Jayalakshmi. S, Balasubramanian. T, Banat. I.M, (2008). Production and characterization of a glycolipid biosurfactant from *Bacillus megaterium* using economically cheaper sources., *World J. Microbiol. Biotechnol.* 24:917–925.