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"Optimization of Process Conditions for Pesticide Specific Bioemulsifier Production in a Laboratory Fermentor"

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Abstract: Bioemulsifiers are valuable microbial amphiphilic molecules with effective surface-active and biological properties. Owing their structural diversity (i.e., glycolipids, lipopeptides, fatty acids etc), low toxicity and biodegradability these molecules are widely used in cosmetic, pharmaceuticals and food processing industry. A number of factors affect the production of these bioemulsifiers. Indeed the type, quality and quantity of bioemulsifier production are influenced by the producer microorganism and the nature of the carbon. Large-scale production of these molecules are hardly been realized because of low yields in production processes added to these the high recovery and purification costs. The scale-up of bioemulsifiers for industrial production is still challenging. In this study we attempt to optimize the fermentation conditions for a 1L lab fermentor using Burk's mineral media. We have deployed *Bacillus subtilis* FE-2 strain an Organophosphorus pesticide (Fenthion) degrading microorganism for the production of bioemulsifier specific to disperse the pesticide. The study emphasizes on the determination of optimum fermentation (1 L fermentor) condition for the production of pesticide specific bioemulsifiers which has a tremendous potential in agriculture sector. The ideal range of fermentation parameters viz., temperature, agitation and inoculum concentration was found to be 30°C, 250 rpm and 5% respectively.

Key words: Fenthion · Emulsifier Activity · Biosurfactant · Fermentation Condition

INTRODUCTION

Surfactants have long been recognized as the most versatile of process chemicals. Their market is extremely competitive and manufactures are busy in expanding their trades to develop products for this decade and beyond. In this regard, biosurfactants / bioemuslifiers are promising in addition to chemical surfactants. A large variety of microorganisms are known as biosurfactant / bioemulsifier producer [1]. The biosurfactant represent a diverse variety of chemical and molecular properties including the emulsifying properties. Biosurfactant exhibit high specificity and thus suit to varied applications [2] which include emulsification process too. Their yield greatly depends on nutritional conditions [3]. Several earlier studies [4] also affirm the fact that environmental factors and growth conditions such as pH, temperature, agitation and oxygen availability also affect bioemulsifier production. In view of widespread applications of bioemulsifier and biosurfactant, process development for large-scale production is an obvious necessity. Few scale up studies for the productions of biosurfactants and bioemuslifiers are reported. Margaritis *et al*. [5] worked on the production of biosurfactant in an air-lift Fermentor. Cooper and Paddock [6] reported production of sophorolipids in Fermentor. Guerra-Santos *et al*. [7] worked on the continuous production of biosurfactant in a compact loop reactor. The production of biosurfactant AP-6 by *P. fluorescence* 378 in a microcomputer controlled multicast fermentation system has been reported and this enabled simultaneous running of fermentor, Thereby greatly reducing the time and the equipment costs, compared to traditional laboratory Fermentor [8]. Zhou and Kosaric [9] studied the

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production of a biosurfactant, sophorolipids and scaled up to 1 L working volume bioreactor. Earlier Veenanadig *et al*. [10] had demonstrated the production of biosurfactant in SSF.

Nevertheless, biosurfactant are still unable to compete with chemically synthesized surfactants in the market. One striking reason for their inability to compete is their inefficient bioprocessing technology. Thus, it is very important to optimize the processing conditions and scale up the results of shake flask experiments to Fermentor and later to industrial scale. In this study we try to optimize the fermentation parameters such as temperature, agitation and percent of inoculums volume in lab fermentor.

MATERIALS AND METHODS

Organism and Inoculum Preparation: Bacillus subtilis FE-2 strain was deployed in the study. *Bacillus subtilis* obtained from Indian Institute of Sciences, Bangalore, India, was routinely maintained on mineral medium supplemented with Fenthion and subcultured every month. Five ml of the organism from the stock culture was transferred into the 100 ml Luria broth (10 g tryptone, 5 g yeast extract, 5 g NaCl in 1 l of distilled water). A teflon coated bar magnet sterilized with 70% ethyl alcohol was placed inside the inoculated culture flask. The culture was incubated at 30°C on a magnetic stirrer for 48 hrs (final OD of 2.8) and used as inoculum whenever mentioned.

Luria Broth (LB): The medium consisted of tryptone 10 g, yeast extract 5 g and NaCl 5 g in 1 L of distilled water. The pH was adjusted to 7.2 using 1N NaOH, Luria broth (100 ml) was taken into 250 ml Erlenmeyer flasks and sterilized by autoclaving at 121°C for 20 minutes. The seed culture (5%) was transferred into LB, grown for 24 hrs and then used as inoculum.

Burk's Mineral Media (BMM): Bacillus subtilis FE-2 strain was grown in modified Burk mineral medium (BMM) [11], containing (in grams per liter) K_2HPO_4, 0.2; KH_2PO_4, 0.8; $MgSO_4$ $7H_20$, 0.2; $CaCl_2$ $2H_20$, 0.1; $(NH_4)_2SO_4$, 1.0; yeast extract, 0.2; $Na_2MoO_4.2H_20$, 0.0033; $FeSO_4$ $7H_20$, 0.005. The media was autoclaved at 121°C and 101.3 kPa for 15 min. To the 100 ml of sterile media in 250 ml Erylen Meyer flask, 5 ml of inoculum was transferred in aseptic conditions and fortified with 0.5% Fenthion (200ul of 250mg/ml as acetone).

Fermentor Vessel: Bacillus subtilis Fe-*2* strain was grown in a 1.25 L "BioFloIIC" Fermentor (New Brunswick Scientific Co., Inc, New Jersey, USA) of 1 L working volume. The vessel parts consisted of a stainless steel head plate, a flanged glass tube vessel body which was detachable from the bottom – dished head. The dished head was jacked for circulation, of temperature controlled water. Ports were provided in the head plate for inoculation, base and acid addition. Thermo well was provided with a resistance temperature detector along with a temperature sensor a sparger, a harvest port, a sample port, an exhaust condenser, dissolved oxygen and pH electrodes, The drive bearing housing was also located in the head plate. A removable agitation servo motor located on top of h bearing housing was connected to the agitation shaft with a multi-jaw coupling, which could be easily disconnected, while autoclaving the vessel and was replaced after sterilization. The motor could provide an agitation speed range of 25-1000 (+/- 1 RPM). The culture temperature could be selected in the range from 20°C to 60°C and was controlled by a microprocessor based PI controller. The media temperature was sensed by an RTD (resistance temperature detector submerged in the thermo well. Sterile air was introduced into the medium through the ring sparger and was controlled by the needle valve of the flow meter. It was able to provide sterile air through 0.2 uM replacement cartridge filter. These filters were sterilizable with the vessel. The hooded sampler was attached to a sampling tube that extended to the bottom of the representative samples without contamination. A 25 ml screw-cap container served as a reservoir.

Fermentation Conditions: (until otherwise mentioned)

Fermentor (working volume: 1 L., aeration 1v/v/v., medium – Burk's mineral medium + Fenthion 50 m/ L + glucose 1g /L, pH of the medium 7, temperature was maintained at 28°C, agitation at 250 rpm, inoculum volume 50 ml along with 50 mg of Fenthion fortified.

Emulsifier Activity: The ability of an emulsifier to aid the formation of an emulsion is related to its ability to absorb and to stabilize the pesticide-water interface. The emulsifier activity was assayed by the standard method [10] i.e. measuring the turbidity due to the formation of pesticide emulsion by the bioemulsifier. To 5 ml of the culture supernatant taken in a test tube (25 ml capacity), 20 mg of Fenthion in acetone (0.2 ml) was added; the solution was then vortexed for 60 s and left undisturbed at room temperature for 24 hrs. The optical density (O.D) of the emulsion was read in a Shimadzu

UV-160A recording spectrophotometer at 660 nm. Similarly, a blank was prepared without Fenthion. The difference in absorbency between the sample and blank was taken as the Fenthion-specific bioemulsifier activity.

Growth Estimation: The culture media was harvested at specific time intervals. The OD of the sample at 600 nm was measured and recorded as growth and Biomass.

Effect of Agitation: The Fermentor was set to different agitation rates viz., 150, 250 and 500 rpm in order to study the effect of agitation on the production of bioemulsifier. The time course study for the production of bioemulsifier was carried out individually for each agitation rate.

Effect of Inoculum Volume: To one liter of medium, different volumes viz., 10, 30 and 50 ml was used as inoculum. Individually for each inoculum volume the time course study was carried out to optimize the inoculum volume for the maximum production of bioemulsifier.

Effect of Temperature on the Fermentation System: Like all chemical reactions, microbial growth is affected by temperature. The performance of the Fermentor was tested at three different temperatures viz., 23°C, 30°C and 35°C. IN separate experiments the time course was carried out and samples were collected and analyzed for bioemulsifier production.

RESULTS

Fermentor experiments were conducted using a BMM which had yielded the highest amount of the bioemulsifier in terms of activity 1.2 in the shake flask to optimize the conditions. The results obtained with Fermentor used in the present study were better than those with shake flask experiments in respect of both duration and bioemulsifier production. However, in the Fermentor, considerable quantity of foam was observed during the fermentation due to vigorous mixing of the medium which did not affect the process. Fig: 1 shows the pattern of emulsifier produced in optimized medium in the Fermentor and flask. It can be seen that the Fermentor system attained a peak emulsifier activity of 1.32 by 17.5 hours, where in the control (flask), a peak emulsifier activity of 1.2 was attained at 27.5 hours, which meant that almost 50% of the time was saved. In flask, OD was 50% when it was peak in Fermentor. A tendency to diauxy in flask was not observed in Fermentor as expected. Peak activity extended from 18 to 21 hours and this was not helpful.

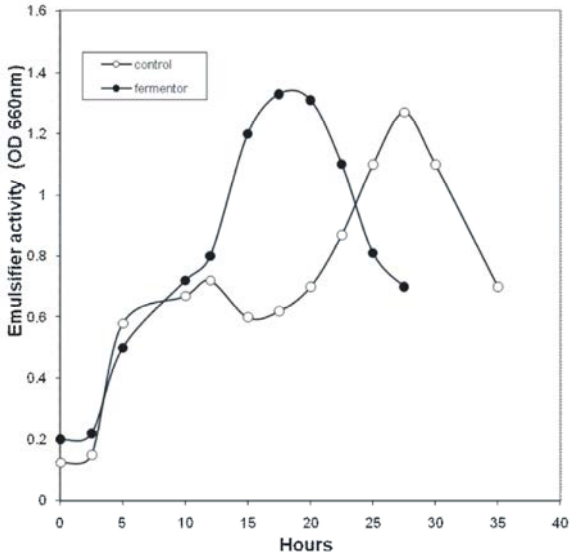

Fig. 1: Comparision of surfactant production in fermentor and flask

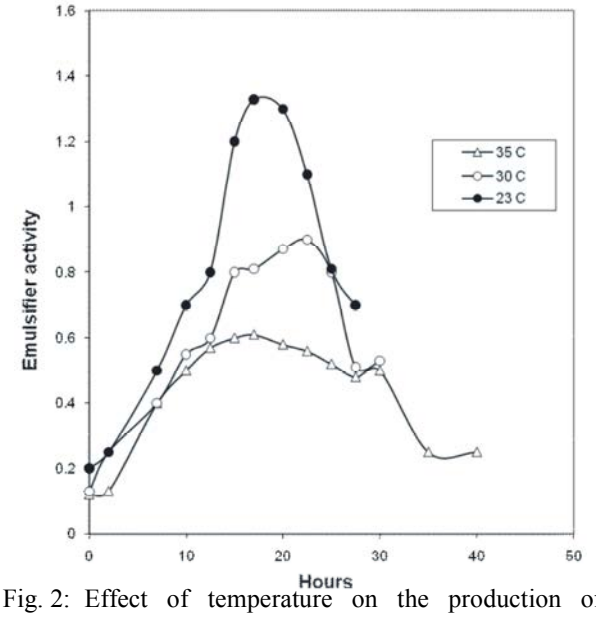

Fig. 2: Effect of temperature on the production of surfactan

Effect of Temperature on the Product Formation: Temperature is one of the critical parameter that greatly affected the biosurfactant production. The Fermentor was run at three different temperature viz., 25, 30 and 35°C as mentioned in methods and materials. The time course studies revealed typical results showing that the bioemulsifier production is temperature regulated (Fig: 2). The lowest temperature under the study i.e., 25° C hardly encouraged any bioemulsifier production. Throughout the time course study a maximum activity of 0.6 was observed at around 20 hours. On the contrary a maximum activity of

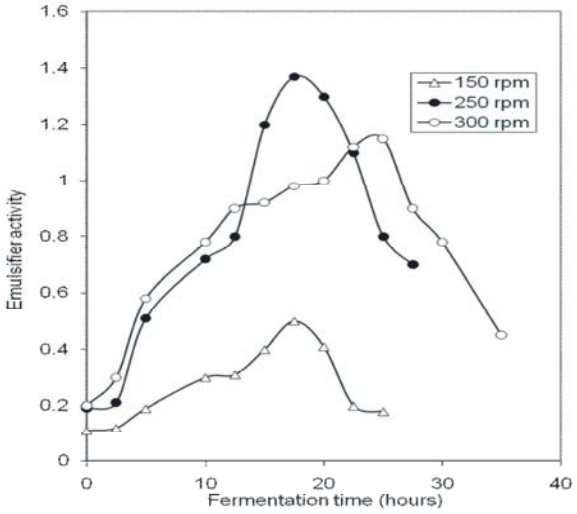

Fig. 3: Effect of agitation on the production of surfactant

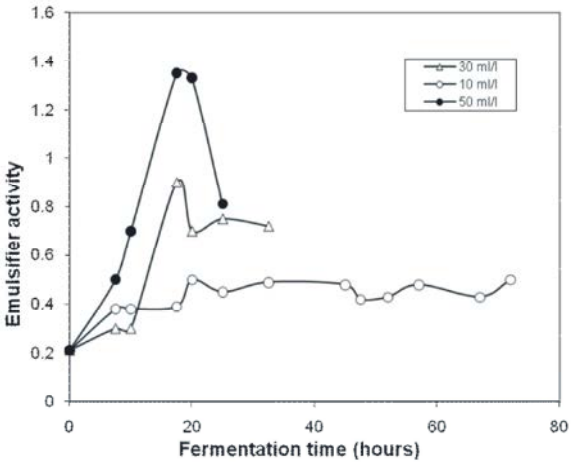

Fig. 4: Effect of inoculum on the production of biosurfactant

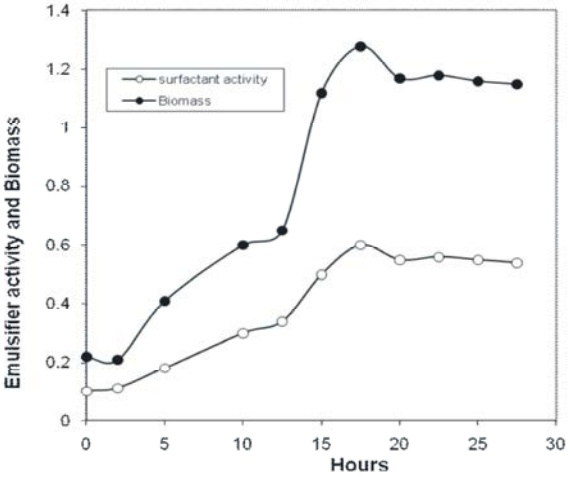

Fig. 5: Relation between surfactant production and Biomass

1.3 in about 18 hours was obtained when the temperature of the system was set at 30°C. When the temperature was increased to 35°C, the surfactant production not only decreased to 0.9 (about 40%) but the production peak also shifted from 18 to 24 hours. Based on the above observation, it was clear that the fermentation system was sensitive and the optimum temperature for the maximum emulsifier was 30°C.

Effect of Agitation on the Emulsifier Formation: Agitation is one of the important parameters controlling the product formation in aerobic fermentation systems. Since *Bacillus subtilis* used in the study is an aerobic strain, it was crucial to determine the agitation rate in promoting emulsifier production. Emulsifier production is an aerobic process as shown in Fig: 3. Agitation at 150 rpm, yielded very little emulsifier (0.5) in about 17 hours. When the agitation was increased to 250 rpm, the emulsifier production increased concomitantly to an activity of 1.3 in about 18 hours. When the system was agitated at still higher rate i.e., at around 500 rpm, the emulsifier activity was around 1.1 but the peak activity shifted to 25 hours. When the agitation conditions were at 300 and 150 rpm, a sharp peak was noticed. This was a limiting factor for downstream processing. However, when the agitation was at 250 rpm, an activity of more than 0.8 was obtained between 13-26 hours and this was very advantageous during the downstream processing. Thus, an agitation speed of 250 rpm was found o be ideal for emulsifier production.

Effect of Inoculum Concentration on the Product Formation: In the scale up studies of any bioreactor system, inoculum volume is one of the deciding factors. It is evident from Fig: 4, that 50 ml/l of inoculum produced the maximum emulsifier with a peak activity of 1.3. In the system, where inoculum volume was 10ml/l, there was a least emulsifier production. Moreover, when the system was supplied with 30ml/l inoculum, the peak activity never rose above 0.9. Therefore, based on the above observation, 50 ml/l inoculum volume was considered to be optimum.

Effect of Biomass on the Production of Bioemulsifier: The bioemulsifier production was coupled to the biomass build up. From the Fig: 5, it is clear that there was a direct co-relation between the biomass and yield. The highest emulsifier was produced at 17 hours, the highest biomass being 3g/l in terms of dry biomass.

DISCUSSION

The bioreactor is an important component and plays a key role in any biochemical process in which microbial, mammalian or plant cell systems are employed for the manufacture of a wide range of useful biological products. The main function of a properly designed bioreactor is to provide a controlled environment in order to achieve the optimal growth and / or product formation in the particular cell system employed. In industries, smaller bioreactors are used to gather data for optimal production conditions. Scale-up is then necessary to define appropriate working conditions in the larger vessels. Bioreactors are used in the studies of physiology and biochemistry. It is, therefore, an invaluable research tool to study different parameters. It is also easy to study the influence of one parameter at a time (either medium constituent or environmental condition). The laboratory bioreactor has the capacity for working at conditions that cannot be achieved on a larger scale. The development of a process should be initiated on this scale to determine the conditions for optimization and has to be evaluated for the working conditions available on the laboratory scale through experiments on a pilot plan scale.

In unicellular organisms such as bacteria, yeast or mould, oxygen for carrying out any oxidative reaction within the cell is generally incorporated through the intermediate state of the dissolved oxygen molecule. The importance of aeration and agitation in aerobic fermentation processes is well recognized by laboratory and industrial fermentation technologists. In aerobic processes, the main problem is to dissolve enough oxygen. Some fermentation is sensitive to the oxygen concentration. The primary objective is to supply the required oxygen to the microorganisms in order to achieve the proper metabolic activities. A secondary function of serration and agitation is to keep the microorganisms in suspension. Rates of oxygenation and agitation affect the biosurfactant production [12]. The bacterium *Nocardium erythropolis* has been shown to be sensitive to shear force and the agitation rate apparently affects the kinetics of the emulsifier production. Zhou and Kosaric [9] reported that the optimum agitation as 400 rpm and air flow of 13.5 /min. Guerr-santos *et al*. [13] showed that the yield of rhamnolipids was several folds higher, when a continuous process was used as compared to the batch culture. *B. licheniformis JF-2* under both aerobic and anaerobic conditions produced an anionic biosurfactant. Ramanna and Karanth [14] in their studies on surfactant production observed that the results obtained with shake flask experiments were consistent with those of the Fermentor run with respect to both biomass formation and biosurfactant production.

Macdonald *et al*. [15] studied the effect of agitation on biomass and biosurfactant production in *Nocardia erythropolis*. The agitation rate which was proportional to the shear rate at the tip of the impeller, affected the kinetics of growth and the final biomass concentration in the stationary phase. The lowest agitation rate of 250 rpm gave the best conditions for optimum biomass and biosurfactant production. In view of this, the system used in the present experiment should require 250 rpm for the maximum emulsifier production.

Temperature control is necessary. However, to carry out the fermentation processes at an optimum temperature for maximum bioemulsifier production of different microorganisms varies depending on certain process conditions. Continuous culture studies were performed in the temperature range of 28-39°C using *P. aeruginosa* [16]. The temperature optimum for bioemulsifier production was found to be between 31°C and 34°C. A decrease or increase in the incubation temperature led to changes in the microbial metabolism as evidence by lower bioemulsifier production. Pruthi and Camotra [17] isolated bioemulsifier from *A. peotophormiae*, under psychrophilic conditions (10°C). In the system adapted in the present study, the organism *Bacillus subtilis* appeared to be a mesophilic, since the optimum temperature for the maximum production of emulsifier was found to be 30°C.

In conclusion, production of bioemulsifier specific to Fenthion pesticide was faster and better in Fermentor compared to the production in flask. The fermentation parameters like temperature, agitation rate and inoculum volume played a crucial role in regulating the bioemulsifier production.

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REFERENCES

1. Priya, T. and G. Usharani, 2009. Comparative Study for Biosurfactant Production by Using *Bacillus subtilis* and *Pseudomonas aeruginosa* Botany Research International, 2(4): 284-287.
2. Deepika Lakshmipathy, T., A.S. Arun Prasad and Krishnan Kannabiran, 2010. Production of Biosurfactant and Heavy Metal Resistance Activity of Streptomyces Sp.VITDDK3-a Novel Halo Tolerant Actinomycetes Isolated from Saltpan Soil. Advances in Biological Research, 4(2): 108-115.
3. Parthasarathi, R and P.K. Sivakumaar, 2009. Effect of Different Carbon Sources on the Production of Biosurfactant by *Pseudomonas fluorescens* Isolated from Mangrove Forests (Pichavaram), Tamil Nadu, India. Global Journal of Environmental Research, 3(2): 99-101.
4. Roberta, B. Lovaglio, Siddhartha, S.G.V.A.O. Costa, Lireny A.G. Gonçalves and Jonas Contiero, 2010. Effect of C/N Ratio and Physicochemical Conditions on the Production of Rhamnolipids by *Pseudomonas aeruginosa* LBI. Libyan Agriculture Research Center Journal International, 1(2): 85-92.
5. Margaritis, A., K. Kennedy and J.E. Zajic, 1980. Application of an air-lift fermentor in the production of biosurfactants. Dev. Ind. Microbiol., 21: 285-294.
6. Cooper, D.G. and D.A. Paddock, 1984. Production of a biosurfactant from *Torulopsis bombicola*. Applied and Environmental Microbiology, 47: 173-176.
7. Guerra-Santos, L., O. Kappeli and A. Fiechter, 1984. *Pseudomonas aeruginosa* biosurfactant production in continuous culture with glucose as carbon source. Applied and Environmental Microbiology, 48: 301-305.
8. Persson, A., G. Molin, N. Andersson and J. Sjöholm, 1990. Biosurfactant yields and nutrient consumption of Pseudomonas fluorescens 378 studied in a microcomputer controlled multifermentation system. Biotechnol. Bioeng., 36: 252-255.
9. Zhou, Q.H., Vaclav Klekner and Nalma Kosaric, 1992. Production of sophorose lipids by *Torulopsis bombicola* from safflower oil and glucose Journal of the American Oil Chemists Society, 69(1): 89-91.
10. Veenanadig, N.K., M.K. Gowthaman and N.G.K. Karanth, 2000. Scale up studies for the production of biosurfactant in packed column bioreactor. Bioprocess Engineering, 22(2): 95-99.
11. Munnecke, D.M. and D.P.H. Hsieh, 1974. Microbial decontamination of parathion and p-nitrophenol in aqueous media. Appl. Microbiol., 28: 212-217.
12. Gerson DF and JE. Zajic, 1978. Surfactant production from hydrocarbons by *Corynebacterium lepus*, sp. nov. and *Pseudomonas asphaltenicus*, sp. Nov Dev. Ind. Microbiol., 19: 557-599.
13. Guerra-santos, L., O. Kappeli and A. Fiechter, 1984. *Pseudomonas aeruginosa* biosurfactant production in continuous culture with glucose as carbon source. Appl. Environ. Microbiol., 48: 301-305.
14. Ramanna, K.V. and N.G. Karanth, 1989. Factors affecting biosurfactant production using *Pseudomonas aeruginosa* CFTR-6 under submerged conditions Biotechnology Letters, 11: 437-442.
15. Macdonald, C.R., D.G. Cooper and J.E. Zajic, 1981. Surface-active lipids from *Nocardia erythropolis* grown on hydrocarbons. Applied and Environmental Microbiology, 41(1): 117-123.
16. Guerra-Santos, Luis H., Othmar Käppeli and Armin Fiechter, 1986. Dependence of *Pseudomonas aeruginosa* continuous culture biosurfactant production on nutritional and environmental factors. Applied Microbiology and Biotechnology 24(6): 443-448.
17. Pruthi, V. and Cameotra, Swaranjit Singh, 1997 Production of a biosurfactant exhibiting excellent emulsification and surface active properties by *Serratia marcescens*. World Journal of Microbiology & Biotechnology, 13(1): 137-139.