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Research paper

Biosurfactant Production by A Bacterial Strain Pseudomonas Aeruginosa MTCC 16036

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Abstract

Microbial compounds which has the potential to exhibit emulsification and surface activities are classified as termed as biosurfactants. Compared to that of a chemically synthesized surfactant, these biosurfactants are considered to be environmental friendly, exhibits lower toxicity and active even at extreme temperatures, pH and saline conditions. The current research is focused on biosurfactant production using *Pseudomonas aeruginosa* MTCC 16036 further it is evaluated for oil recovery from trapped petroleum reservoir. Studies on selection of best carbon and nitrogen sources were tested for enhanced biosurfactant production using nutrient medium. Among the tested sources, glucose and urea were found to be the best carbon and nitrogen source. The effect of initial pH was studied by varying pH from 5.0 – 8.0 and maximum biosurfactant production 2.8 g/L was achieved at pH of 8.0. Produced biosurfactant reduced interfacial tension (8mN/m) which is the key parameter for recovering residual oil. Further, the stability of produced biosurfactant wasinvestigated by varying pH (1.0 – 12.0) and temperature (40 – 120 °C).

Keywords: *Pseudomonas aeruginosa, interfacial tension, residual oil, biosurfactant.*

1. Introduction

The term biosurfactant is an extracellular compound produced by bacteria, fungi and yeasts which are surface active compounds[18]. The foremost advantages of biosurfactants in comparison to chemical surfactants is better biodegradability, eco-friendly, good foaming activity, zero toxicity at intense temperatures, pH and salinity [12]. Biosurfactants can be divided into two groups: low-molecular-mass, lower surface and interfacial tension; and high molecular-mass polymers, mainly used as emulsion stabilizing agents. Classification of surfactants areglycolipids, phospholipids and lipopeptides. Biological surfactantscan be either anionic or nonionicin nature, where the hydrophobic moiety attracted to long chain fatty acids[6].Certaintypes of bacteria produce low molecular weight molecules that efficiently reduce surface and interfacial tension such as glycolipids and lipopeptides [14]. The negative effects of the synthetic biosurfactant can be overcome by the microbial biosurfactants. Mostly all the microbes are capable of producing surfactants among which yeast are readily grown and are easy to cultivate in large scale level [10].

Surfactin and rhamnolipid are the most effective biosurfactants which are capable of reducing interfacial tension between water/oil [17]. Well known biosurfactants are synthesized by microbes grown on water immiscible hydrocarbons but few biosurfactants are produced on water-soluble substrates namely sucrose, glucose and ethanol [7]. Eventually, high product titerswith vegetable oil as sole carbon source in combination with Pseudomonas strains [9]. Crude oil behaves sluggish during measurement of IFT (interfacial tension) against aqueous phases as though they were a homogeneous hydro- carbon with a particular ACN (alkaline carbon number). This carbon number is referred to as the EACN (equivalent alkane carbon no.) of the crude oil[11].

Currently, research on biosurfactants has increased globally to enhance the present production rate of microbial surfactants. Potential usage of biosurfactant in oil industries includes cleaning oil sludge, mobilizing heavy crude oil and managing oil spillage. In addition to this, biosurfactants are being used in food industries as additives and emulsifiers which are applied in agriculture and cosmetics [8]. Biosurfactant has the potential to retrieve unrecoverable oil from the trapped zone which are held by high capillary pressure. This is achieved by reducing the interfacial tension between oil and water and improving the recovery of oil [5]. Application of biosurfactants in microbial enhanced oil recovery depends on their stability at higher temperature and pH conditions [2]. One of the main problem faced by oil industries is to recover oil economically. *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Bacillus cereus*, *Bacillus licheniformis*, *Bacillus thuringiensis* and *Staphylococcus aureus* are generally used for production of biosurfactants. Among all *Bacillus subtilis* and *Pseudomonas aeruginosa* are well known bacteria for producing biosurfactant named surfactin and rhamnolipid which are applied in microbial enhanced oil recovery process [1]. Due to the spontaneous increasing demand for petroleum over recent years, application of biosurfactant in oil recovery plays a key role.

The major problem facing by oil industries is to recover oil to the maximum possible extent using economical methods. In this regard, microbial enhanced oil recovery with the aid of biosurfactants is promising. Hence, extensive identification and characterization of new suitable strain for biosurfactant production and degradation during oil spills is necessary. In our study, biosurfactant is produced by *Pseudomonas putida* MTCC 2467 and potentially applied to reduce the surface and interfacial tension which influence the enhancement of oil. The strain produce biosurfactant can be suitably used in oil fields, biomedical and environmental applications. This is the first report describing biosurfactant production using strain *Pseudomonas putida* MTCC 2467. In-spite of several

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advantages, MEOR at relatively low level because of the following factors: (a) understanding the mechanism on in-situ geo-environmental aspects of bacteria (b) stability of key parameters such as pH and water saturation on fundamental processes of MEOR process [16]. *Pseudomonas aeruginosa* (ATCC 9027) has the potential of producing biosurfactant which in turn helped in reduction of interfacial tension from 73 mN/m to 33 mN/m at 30° C and pH 8.0 [3]. *Pseudomonas putida* MTCC 2467 produced biosurfactant (2.7 g/L) when glucose was used as carbon source (2% w/v). Further, the stability of the biosurfactant was unaffected at high temperature and pH conditions [5]. Recent report on biosurfactant was production using *Candida lipolytica* UCP 0988 with cost effective medium formulation along with 2% of waste frying oil, 2% corn steep liquor and 5% molasses at 120 h, 30° C maintained at 180 rpm. Surface activities (ST and IFT) was reduced upto24 mN/m and 11 mN/m respectively [4].

2. Materials and Methods

2.1. Microbe and maintenance conditions

Pseudomonas aeruginosa MTCC 16036 was procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, India for the present investigations. The procured culture was maintained in nutrient agar plates with the following composition (g/L): beef extract, 1.0; peptone, 5.0; yeast extract, 2.0; NaCl, 5.0; agar, 15.0; pH 6.0 ± 0.2, storage temperature −2° C to −9° C.

2.2. Conditions for media cultivation

Nutrient broth was used for media cultivating with the following composition (g/L) was used for preparation of inoculum. Beef extract, 1.0; yeast extract, 2.0; peptone, 5.0; NaCl, 5.0. *Pseudomonas aeruginosa*(MTCC 16063) was grown in Nutrient broth for 8 – 12 h at 30 °C (A_{600nm}0.7) and 2% (v/v) inoculum was used forbiosurfactant production using mineral salt medium with the following composition (g/L) KNO_3, 0.3; Na_2HPO_4, 0.2; KH_2PO_4, 0.013; NaCl, 0.001; $MgSO_4$, 0.05; $CaCl_2$, 0.003; $FeSO_4$, 0.001.
To above mentioned mineral salt media, 0.1 ml of trace elements are added with following composition (g/L) $MnSO_4.4H_2O$, 1.78; $Na_2MoO_4.2H_2O$, 0.39; $CoCl_2.6H_2O$, 0.42; EDTA, 0.5; $NiCl_2.6H_2O$, 0.004; KI, 0.66; $ZnSO_4.7H_2O$, 2.32; H_3BO_3, 0.56; $CuSO_4.5H_2O$, 1.0;
Carbon and nitrogen sources with following composition were added (g/L) glucose, 20; urea, 3.0.

2.3. Effect of different fermentation parameters on biosurfactant production

In order to determine the best carbon sources glucose, sucrose and starch were tested to determine best carbon source for biosurfactant production using the strain *Pseudomonas aeruginosa*. 100 mL of production medium with 2% (w/v) as mentioned above. Carbon sources were grown separately at 30 °C and 180 rpm for 5 days. At a regular interval of 12 h samples were collected and analyzed for production of biosurfactant, growth and few other key parameters. Further to identify the best nitrogen source three different compounds such as urea, ammonium nitrate and ammonium sulphate with 0.3 % (w/v) were added to the medium containing 2% (w/v) sucrose as carbon source. With the above composition, fermentation reaction was carried out for 5 days at 30 °C and 180 rpm for 5 days. Every 12 h samples were collected and analyzed for biosurfactant production.Identifying the effect of initial pH on biosurfactant production to the production medium was adjusted to 5.0, 6.0, 7.0 and 8.0 using 3 M HCl and 3M NaOH.

2.4. Analytical methods

2.4.1 biomass analysis

In order to determine the biomass concentration, 10 ml of sample was subjected to centrifugation for 20 min at 10000 rpm and decant the supernatant. A precipitate is obtained which was further washed twice with NaCl (0.8%) and pelletedcum dried at 50° C overnight. The product was cooled in desiccator and dried weight of pellet was determined.

2.4.2. Biosurfactant analysis

The culture was further centrifuged at 10000 rpm to remove bacterial cells present in it. Supernatant was subjected to acid precipitation test by adding 6 N HCl at 4 °C and pH 2.0. Precipitate formed was further pelletedto centrifugation at 10000 rpm for 20 min followed by re-suspending in double distilled water and pH was adjusted to 7.0, which is again freeze dried and weighed. Dichloromethane was used to isolate the biosurfactant using rotary evaporator under vacuum. This concentrated liquid obtained was considered to be pure form of biosurfactant.

2.4.3. Interfacial tension measurement

Interfacial tension (IFT) measurement of the cell free broth along with 10 ml crude oil was mixed and these mixtureswere subjected to IFT analysis digital tensiometer K6 (Kruss GmbH, Germany), by plate method. Sample (10 ml broth + 10 crude oil) placed into the container provided. Measurements were doneusing automatic controller that pull the plate in downward and contacted sample liquid placed in the glass. The force acting on the rectangular plate with known length were measured and converted into surface tension digitally.

2.4.4. Biosurfactant stability analysis

Identifying the effect on pH and stability of biosurfactant, pH of the biosurfactant solution was adjusted to various pH (1.0 - 12.0) by adding 3 N NaOHalso 3 N HCl. Surface tension had been determined to check the stability of biosurfactant. In the similar manner the effect of temperature stability on biosurfactant, the samples were heated at different temperature conditions ranging from 40, 50, 60, 70, 80, 90,100, 110 and 120 °C for 2 hours and analyzed for surface tension measurements subjected before and after the heat treatment.

2.5. Statistical analysis

All experiments were performed three times and reported values are mean of three individual experiments with $p<0.005$.

3. Results and Discussion

3.1. Effect of carbon sources on biosurfactant production

For the production of biosurfactant, effect of different carbon sources such as glucose, sucrose and starch on biosurfactant production by *Pseudomonas aeruginosa*MTCC 16036had beencarried out. With all the tested carbon sources, glucosehad been identified to produce maximum of 2.5g/L biomass and 3.1 g/Lbiosurfactant (Fig. 1A & 1B). Further in order to confirm that biosurfactant was produced during fermentation, interfacial tension of medium was measured at regular intervals of time. Interfacial tension analysis showed that it got decreased with time and it is due to increased biosurfactant concentration only (Fig. 1C). It is attributed that decrease in interfacial tension and its was maximum when *P. aeruginosa* was grown in medium containing glucose as the carbon source (Fig. 1C). This is further confirmed that maximum biosur-

factant production can be obtained using glucose as the best carbon source. Hence, glucose of 2 % (w/v) was used as carbon source for further studies.

3.2. Effect of nitrogen sources on biosurfactant production

The effect of different nitrogen sources such as urea, ammonium sulphate and ammonium nitrate towards the production of biosurfactant by *P. aeruginosa*MTCC 16036 had been carried out. Production medium was supplemented with glucose as source of carbohydrates and the concentration of nitrogen source was 0.3% (w/v). Urea, the nitrogen source gave 2.5 and 2.8 g/L of biomass and biosurfactant respectively (Fig. 2A & 2B) which was found to be the best among all the tested nitrogen source. Ensuring the production of biosurfactant, the fermented samples that was collected had been subjected to IFT analysis. Results show increased concentration of biosurfactant reduced the surface and interfacial tension of medium (Fig. 2C). Surface and interfacial tension reduction was maximum when *P. aeruginosa* was cultivated in the presence of urea, ammonium sulphate and ammonium nitrate as three different nitrogen sources. Result obtained showed that the maximum biosurfactant production had been achieved using urea as nitrogen source. All further experiments were carried out by using urea 0.3% (w/v) as nitrogen source.

3.3. Effect of initial ph on biosurfactant production

To study the effect of initial pH on biosurfactant production by *P. aeruginosa* MTCC 16036, the initial pH of the medium was adjusted to 5.0, 6.0, 7.0 and 8.0. The medium was supplemented with glucose and urea as sole carbon and nitrogen sources. A maximum of 2.6 g/L of biomass and 2.7 g/L of biosurfactant was produced at pH 7.0. (Fig. 3A & 3B), was found to be the best among all other pH ranges. To reconfirm the biosurfactant production, fermented samples at were subjected to interfacial tension analysis. The results showed that decrease in interfacial tension was maximum when *P. aeruginosa*was grown in medium with pH 8.0 (Fig 3C). This confirmed that maximum biosurfactant production can be obtained at pH 8.0 and therefore pH 8.0 was used for further studies.

3.4. Stability of biosurfactant

Evaluating the potential of biosurfactant in oil recovery is further confirmed by determining the stability of the surfactant at different pH and temperature was studied. The effect of temperature stability was studied by incubating the biosurfactant at various temperatures between 40 to 120ºC for 2 h and measured for surface tension. It has been found that the surface tension of the biosurfactant remained constant between 40 – 120ºC suggesting that biosurfactant produced by *P. putida* was highly thermostable (Fig. 4A). Similarly, the pH of the purified surfactant solution was subjected to various ranging of pH from 1.0 to 12.0, incubated for 1 h and the surface tension was analyzed. The surface tension decreased up to pH 6.0 suggesting that the biosurfactant was not stable below pH 6.0 (acidic conditions) and then the surface tension remained unchanged till pH 12.0 (Fig. 4B) which shows clearly that stability of biosurfactant was stable between pH 7.0 to 12.0.

*Bacillus, Pseudomonas and Acinetobacter*genus are some of the promising bacterial genus capable of producing biosurfactant.These bacteria have the tendency to grow on hydrocarbons that are immiscible with water and other sources where, salt media which are enriched with carbohydrates.Studies on the effect of carbon sources such as glucose, sucrose, starch and other few hydrocarbons (heptadecane, dodecane and hexadecane) are routinely used for biosurfactant production[7]. Current research work is mainly focused production of biosurfactant by selecting the best carbon source(glucose, starch and sucrose) and nitrogen source (urea, ammonium sulphate and ammonium nitrate)using mineral salt medium, and also to check so that the produced biosurfactant is stable at temperature, pH and saline conditions. Result obtained from performed experiments shows that increase in cell biomass was relatively good for the tested carbon sources. It is inferred that the presence of 2% glucose when used as carbon source gave highest biomass (2.4 g/L) as well as biosurfactant (2.6 g/L).Report shows that by using *Bacillus subtilis* MTCC1427 in the presence of 2% sucrose, 3.3 g/L biomass and 1.1 g/L biosurfactant was produced [8]. The incubation temperature at 30 °C, production of biosurfactant was 1.9 g/L yield was achieved when glucose was used as sole carbon source.

Interfacial tension is the critical and crucial parameter in oil recovery techniques. This can only be achieved on production of biosurfactant. Extracted biosurfactant using *Pseudomonas aeruginosa*was able to reduce IFT at a highest value (10 mN/m). Reports have shown that the effect of nitrogen source on biosurfactant productionusing strains such as *Arthrobactersp* are preferred when urea and salts with ammonium ammoniumused as nitrogen source [13, 7]. Merely, in this study we used organic (urea) and inorganic nitrogen sources (ammonium sulphate and ammonium nitrate)for screening the best source of nitrogen. Our experimental results with urea was most potent among other two sources (ammonium nitrate and ammonium sulphate) and attributed as the best nitrogen source since maximum biomass (2.4 g/L) and biosurfactant (2.7 g/L) were obtained.In addition,the interfacial tension (44.3 to 14.2mN/m) got reduced with ureabeing used as a nitrogen source.This result obtained was in par with earlier report where bacteria *Bacillus subtilis* MTCC1427 where ammonium sulphate was used to produce biosurfactant [8].

Metabolic reaction occurs during the fermentation process which were inoculated with specific microbes is that are susceptible to pH value changes. Our results show that maximum biosurfactant (2.57g/L) was obtained at pH 8.0 at 120 h of fermentation.Biosurfactant production got reduced at acidic pH, the possible reason for this may beformation of precipitate. Stability studies on biosurfactant produced was analyzed to ensure that biosurfactant is stable over a broad range of temperature and pH conditions which will be actually prevailing in typical petroleum reservoir.Biosurfactant produced was subjected to different temperature ranges and had no significant effect on reduction of interfacial tension at tested temperature conditions (40 - 120° C) that better than earlier reports where strains showed stability from 40 – 100 °C [10]. The activity of biosurfactant reduced till pH 5.0 and then stabilized till pH 10.0. The results obtained were found to be similar with previous reports in which biosurfactant produced by Biosurfactant produced using *B. subtilis, P. aeroginosa, B. Cereus and R. erythropolis*was able to reduce the surface tension up to pH 6 and beyond this pH it got stabilized [2, 18]. Hence,*Pseudomonas aeruginosa*exhibits temperature as well as pH conditions.

4. Conclusions

Present study has proved that the strain *Pseudomonas aeruginosa*MTCC 16037is capable of producing biosurfactant. Further it optimized bysubjecting to different temperature and pH conditions. *P. aeruginosa*has the capability toproduce optimum amount of biosurfactant when grown in mineral salt medium using glucose and urea ascarbohydrate and nitrogen sources. Also, produced biosurfactant can reduce the interfacial tension from 52 mN/m to 8mN/m as at pH 8.0 which is the key parameter for oil recovery analysis. Further, stability studiesreveal that the produced biosurfactant is thermostable at higher temperature ranges.

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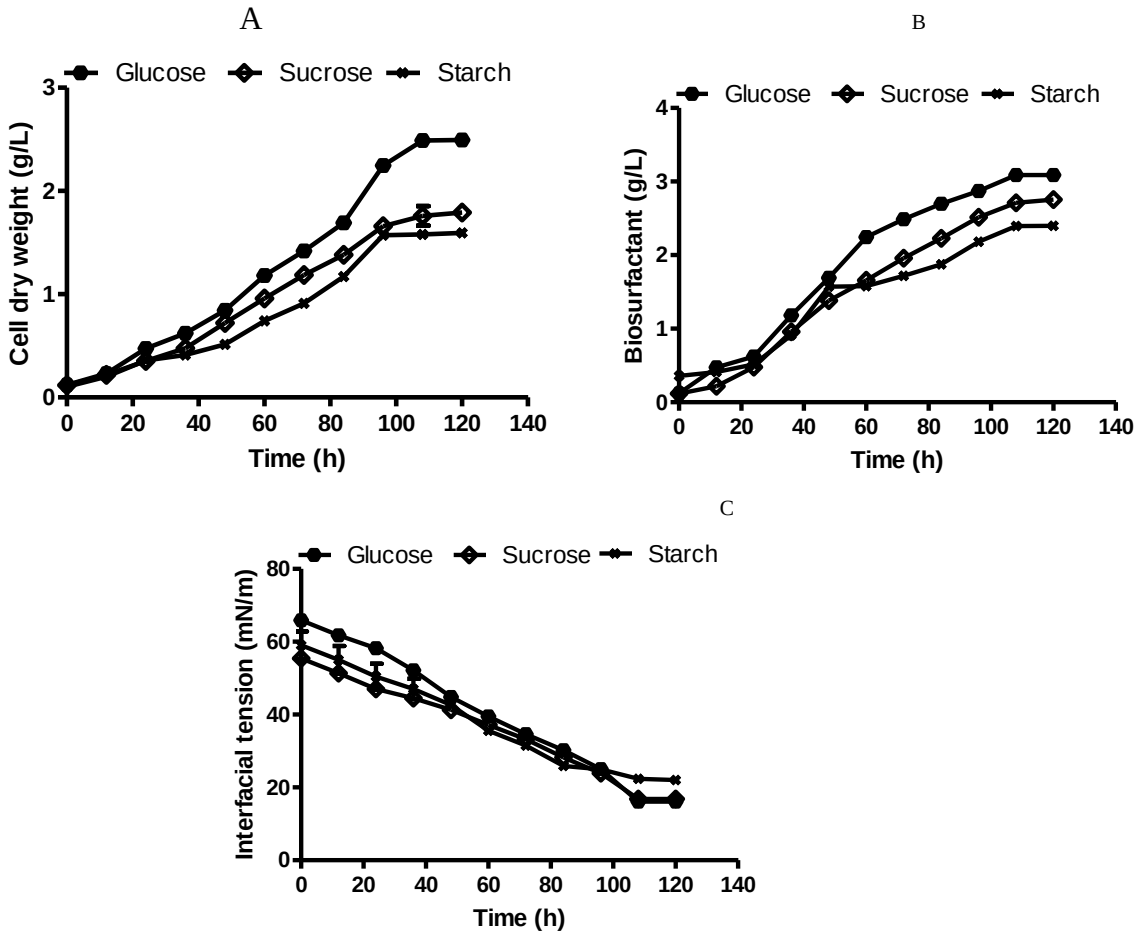

Figure 1: Effect of different carbon sources on biosurfactant production by *Pseudomonas aeruginosa* MTCC 16036 a) Cell dry weight b) Biosurfactant produced c) Interfacial tension. Each experiment was performed 3 independent times and error bars represent ± SE ($p<0.005$)

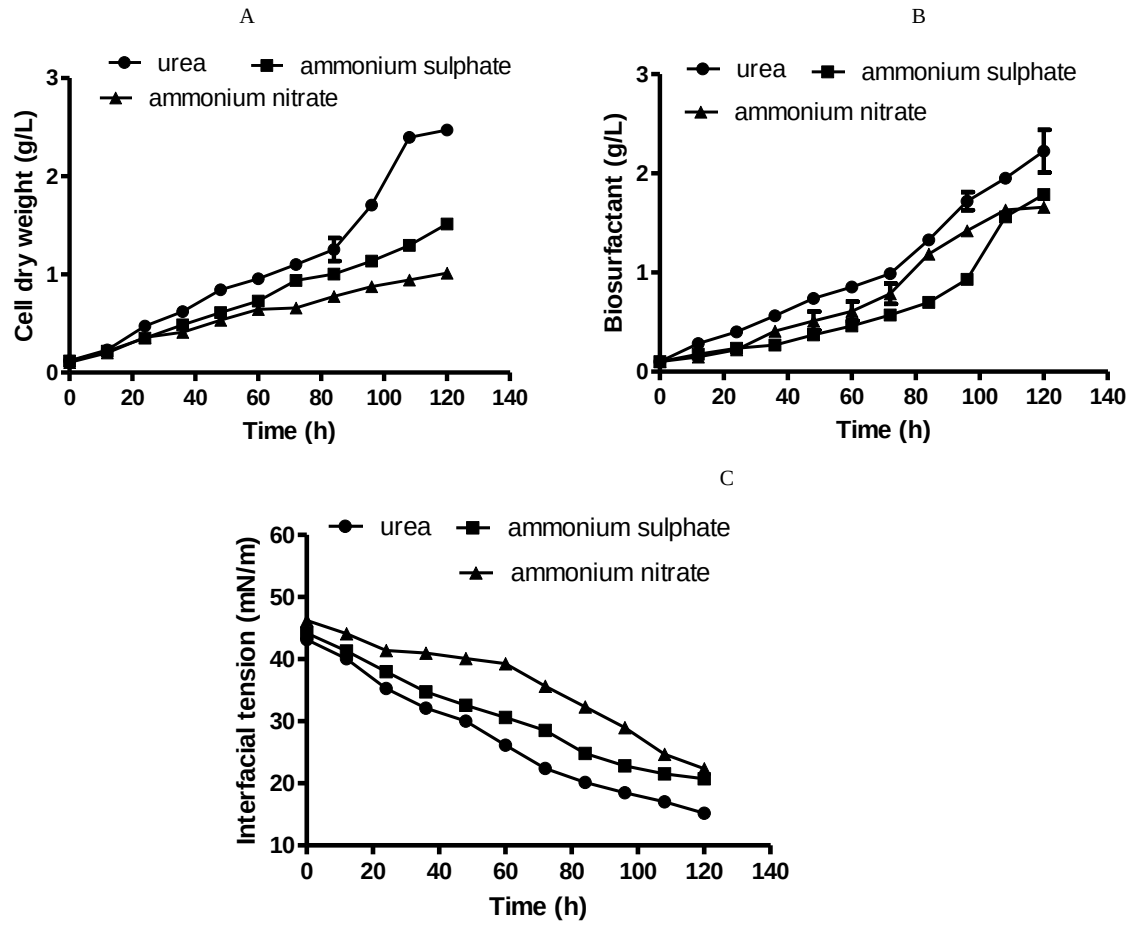

Figure 2: Effect of different nitrogen source on biosurfactant production by *Pseudomonas aeruginosa* MTCC 16036 a) Cell dry weight b) Biosurfactant produced c) Interfacial tension. Each experiment was performed 3 independent times and error bars represent ± SE ($p<0.005$)

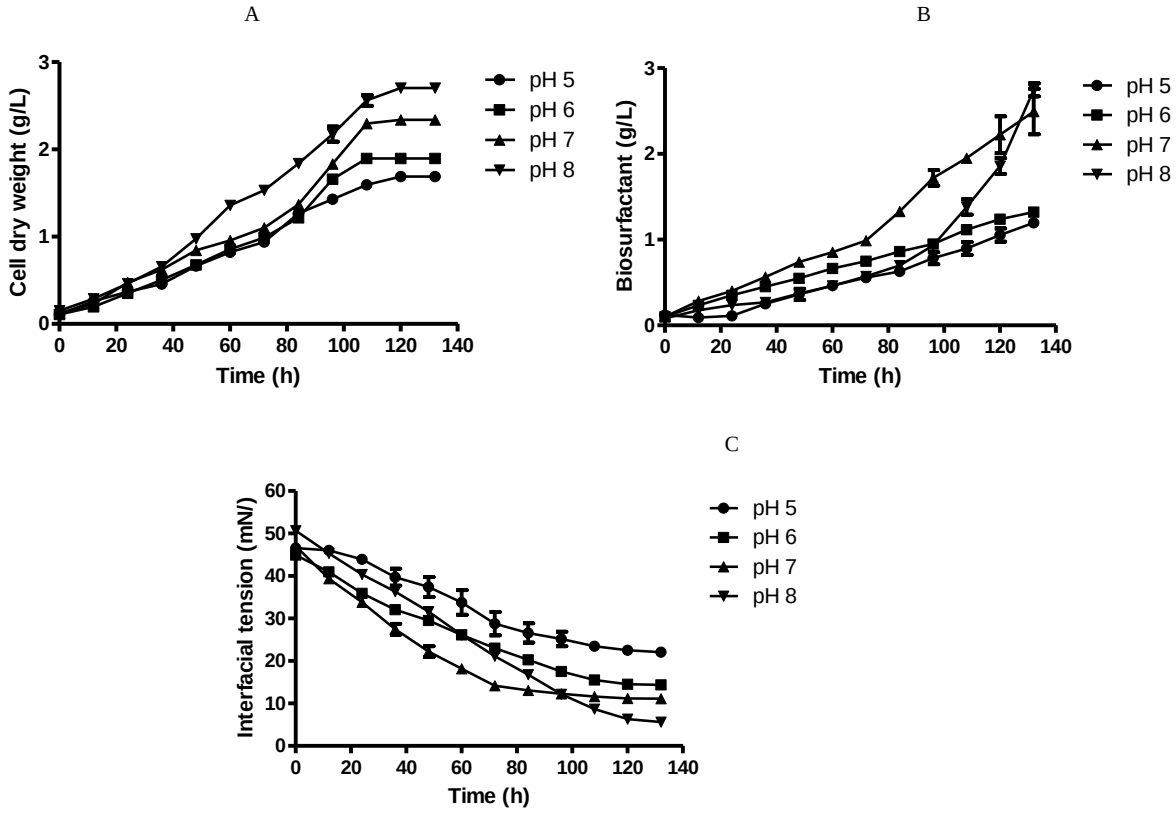

Figure 3: Effect of initial pH on biosurfactant production by *Pseudomonas aeruginosa* MTCC 16036 a) Cell dry weight b) Biosurfactant produced c) Interfacial tension profiles. Each experiment was performed 3 independent times and error bars represent ± SE ($p<0.005$)

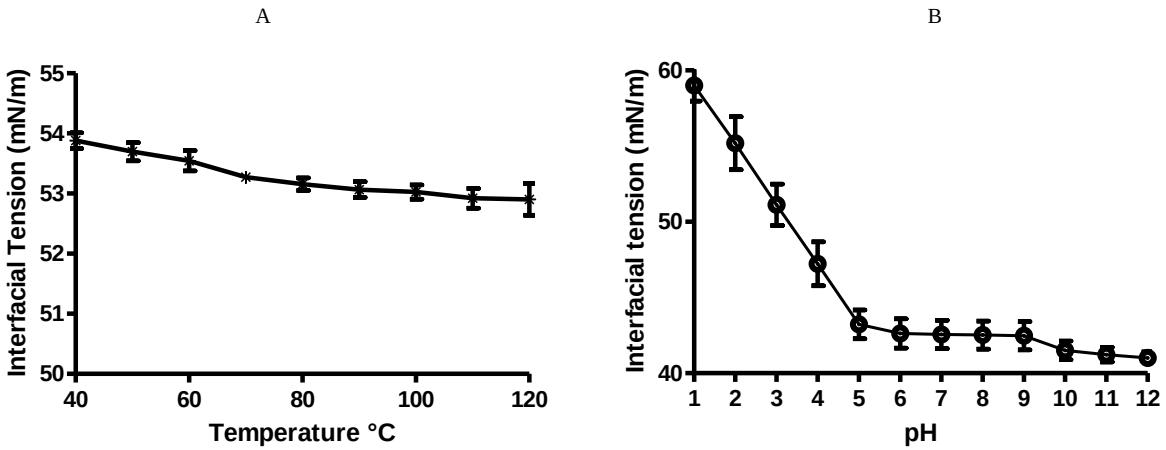

Figure 4: Stability of biosurfactant at a) temperature: produced biosurfactant sample was incubated for 1 h at various temperatures between 40 – 120 °C and analyzed for surface tension. b) pH: samples of produced biosurfactant was adjusted to various pHs ranging from 1.0 to 12.0 and incubated for 1 h and analyzed for surface tension. Each experiment was performed 3 independent times and error bars represent ± SE ($p<0.005$)