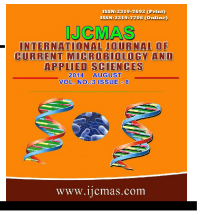
International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 9 (2014) pp. 140-151**
http://www.ijcmas.com



Original Research Article

Optimization of biosurfactant production from *Pseudomonas aeruginosa* PBSC1

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A B S T R A C T

In this present study, biosurfactant producing microorganism *Pseudomonas aeruginosa* PBSC1, was isolated from mangrove ecosystem in Pichavaram (Boat house), Tamil Nadu, India. The biosurfactant production was studied using a Minimal Salt Medium (MSM) with crude oil (1 %) as the hydrocarbon. The optimization study was carried out using various carbon, nitrogen sources, pH, temperature, hydrocarbons, and trace elements. The effect of dry cell biomass, biosurfactant production, emulsification activity and surface tension were studied. The glycerol and sodium nitrate was the best carbon and nitrogen source studied with the isolate. The environmental factors such as pH 7 and temperature 30^{o}C were found to be optimum for the biosurfactant production. Among the hydrocarbon tested the crude oil and n-hexadecane showed statistically on par results. The biosurfactant yield was higher in the presence of all the trace elements. In future study the optimization using Response Surface Methodology (RSM) will be helpful in studying the interaction between the variables.

Keywords

Pseudomonas aeruginosa, Biosurfactant, Hydrocarbons Emulsification activity, Surface Tension

Introduction

Biosurfactants are amphiphilic molecules mainly produced by microorganisms as a secondary metabolite. They possess both hydrophilic and hydrophobic moieties and are able to display a variety of surface activities and help to solubilize hydrophobic substrates (Mnif et al., 2013). The biosurfactants are used in cosmetic, pharmaceutical, chemical, food, agriculture, cleansers, enhanced oil recovery industries, and in bioremediation of oil contaminated sites, considering the advantages and characteristics as thermostability, tolerance to ionic strength, biodegradability and low toxicity (Jain et al., 2013a andb)

Biosurfactants exhibit such important advantages but they have not been yet employed extensively in industry because of relatively high production cost. One possible strategy for reducing cost is the utilization of

alternative substrates such as agro industrial wastes (Maria et al., 2014).

Cell growth and the accumulation of metabolic products are strongly influenced by medium compositions such as carbon sources, nitrogen sources, growth factors, and inorganic salts. Thus, it is difficult to search for the major factors and to optimize them for biotechnological processes as several parameters are involved. Environmental factors and growth conditions such as pH, temperature, agitation, and oxygen availability also affect biosurfactant production through their effects on cellular growth or activity. The classical method of medium optimization involves changing one variable at a time, keeping the others at fixed levels (Rodrigues et al., 2006). The present study involves the optimization of various parameters and its effect on growth and biosurfactant yield was determined.

Materials and Methods

The selected bacterial strain *P. aeruginosa* PBSC1 was studied for the biosurfactant production under the influence of certain physical and chemical factors. All the experiments were carried with five replicates. Biosurfactant production was determined by estimating the surface tension (ST) reduction and the emulsification assay (E24 %), dry cell biomass (DCBM).

Effect of carbon sources on the production and activity of biosurfactant

One hundred ml of MSM broth (pH 7.0) with six different carbon sources *viz.*, glucose, glycerol, fructose, sodium citrate, mannitol and starch were prepared in a different 250 ml Erlenmeyer flask and sterilized in an autoclave. It was added with1.0 ml of crude oil. To that 5.0 ml of inoculum of different bacterial isolates were added and incubated at 28 ± 2°Cfor 5 days over a shaker set at120 strokes min^{-1}. Extraction was done following the procedures of Cameotra (1995) and emulsification assay with crude oil was performed following the procedures of Banat (1995).

Effect of nitrogen sources on the production and activity of biosurfactant

One hundred ml of MSM broth (pH 7.0) with five different nitrogen sources like yeast extract, peptone, ammonium chloride, ammonium nitrate and sodium nitrate were dispensed in five 250 ml Erlenmeyer flasks respectively and sterilized in an autoclave. A quantity of 1.0 ml of crude oil and 5.0 ml of culture inoculum were added. The flasks were incubated at 28 ± 2°C for 5 days over a shaker set at 120 strokes min^{-1}. Extraction was done and emulsification assay was determined.

Effect of pH on the production and activity of the biosurfactant

One hundred ml of MSM broth was prepared of varying pH 5.0, 5.5, 6.0, 6.5, 7.0 7.5 and 8.0 and sterilized. It was added with one ml of crude oil. To that 5.0 ml of bacterial inoculation of different isolates were added and incubated at 28 ± 2°C for 5 days over a shaker set at 120 strokes min^{-1}. Extraction was done and emulsification assay was determined as detailed earlier.

Effect of temperature on the production and activity of biosurfactant

One hundred ml of MSM broth (pH 7.0) was prepared and sterilized. It was added with 1.0 ml of crude oil and different bacterial cultures were inoculated. The flasks were incubated at varying temperatures of 25, 30,

35, 40, 45 and 50°C in an incubator over shaker. Extraction was done and emulsification assay was determined as detailed earlier.

Effect of hydrocarbons on the production and activity of the biosurfactant

One hundred ml of MSM broth (pH 7.0) was taken in a clean 250 ml Erlenmeyer flask and sterilized. One ml of different heavy hydrocarbons *viz.*, n-hexadecane, heptane, xylene, kerosene, petrol, diesel and crude motor oil were added in different flasks. To that 5.0 ml of inoculum of different bacterial isolates were added and incubated at 28 ± 2°Cfor 5 days over a shaker set at 120 strokes min^{-1}. Extraction was done and emulsification assay was determined as detailed earlier.

Effect of trace elements

One hundred ml of MSM broth with 2 % glucose for *B. cereus*, glycerol for *P. aeruginosa* and pH 7.0 was prepared as follows; (a) without $MgSO_4$ (b) without $MnSO_4$, (c) without $FeSO_4$, (d) without $FeSO_4$ and $MnSO_4$, (e) without $MgSO_4$ and $FeSO_4$ (f) without $MgSO_4$, $MnSO_4$ and $FeSO_4$. One ml crude oil was added aseptically and to that 5 ml of inoculums from selected bacterial isolates was added and kept at room temperature 28 ± 2°C for 5 days over a shaker set at 120 strokes min^{-1}. Extraction was done and emulsification assay was determined as detailed earlier.

Statistical Analysis of Results

All the results related to determination of emulsification activity, biosurfactants quantity and CFU counts were the average of three replicates of two separate experiments for each cultural condition. They were statistically analyzed by SPSS software (version 100) using the Duncan test performed after analysis of variance (ANOVA).

Results and Discussion

Effect of carbon sources on the production and activity of biosurfactant

The isolate *P. aeruginosa* PBSC1 was able to utilize glycerol as a sole carbon source and produced higher amount of biosurfactant 5.14 g/l followed by 3.93 g/l by glucose (Table 1 and Fig. 1) The mannitol also utilized by the isolate and produced statistically on par results with the glucose (3.27 g/l). The lowest production was observed with the sodium citrate (0.48 g/l). The highest dry cell biomass produced was 4.38 g/lin case of glycerol followed by glucose with 3.92 g/l. The highest surface tension reduction ability and emulsification activity observed was 30.25 mN/m and 79.65 per cent respectively by the isolate. An abundant formation of foam was observed in the culture medium containing glycerol. Glycerol is a simple fatty acid precursor with high solubility in medium, so it is easily utilized by bacteria for their carbon and energy source.

Effect of nitrogen sources on the production and activity of biosurfactant

Sodium nitrate was the best sources of nitrogen for growth and biosurfactant synthesis of isolate *P. aeruginosa* PBSC1 (Table 2 and Fig. 2). The biomass formed was higher with a 4.34 g/l at 144 h of growth. The bosurfactant was produced higher with the value of 4.96 g/l and emulsification activity was recorded as 78.52 per cent. The lowest surface tension with higher production was measured as 30.28 mN/m. Nitrogen limitation has been reported to increase the rhamnolipid production. The rhamnolipid production of

the *P. aeruginosa* PBSC1 is dependent not only on carbon source but also on limiting portion of nitrogen sources. Although some of the researchers have studied nutrient effects on rhamnolipid production, microorganism strains, culture conditions and media are different in our study. Therefore, the production results are varied and higher. All experiments were repeated 3 times. In all instances, similar trends were observed. Because of their interesting properties, biosurfactants, especially rhamnolipids seem to have potential applications in future.

Effect of different pH

In case of *P. aeruginosa* PBSC1 the highest surface tension reduction was observed as 29.19 mN/m for pH 6.5 and emulsification activity was 75.12 per cent for pH 7 (Table 3 and Fig. 3). pH level 6.5 and 7 produced statistically on par results for the surface tension reduction and emulsification activity. The highest biosurfactant production observed by the isolate *P. aeruginosa* PBSC1 was 5.13 g/l at pH 7. 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.

Effect of different temperature

The optimum condition of temperature observed was 30 °C and followed by 25 °C. The highest biosurfactant production was recorded as 5.12 g/l for the *P. aeruginosa* PBSC1 at the temperature of 30°C (Table 4 and Fig. 4). The temperature 25°C and 35°C produced statistically on par results for the biosurfactant production, dry cell biomass weight, surface tension reduction and emulsification activity. The highest emulsification activity was measured as 78.45 per cent for the isolate *P. aeruginosa* PBSC1. Temperature is one of the critical parameter that greatly affected the culture growth and the biosurfactant production. When the incubation temperature increased to 40°C, bacterial growth and biosurfactant production were totally inhibited, indicating that the biosurfactant produced by *P. aeruginosa* PBSC1was temperature-dependent. Different strains of *P. aeruginosa* PBSC1 have different optimum pH and *P. aeruginosa* was shown to be a mesophilic bacterium that cannot survive at temperature more than 40°C.A decrease or increase in the incubation temperature leads to lower growth of organism and biosurfactant production.

Effect of hydrocarbons

In case of *P. aeruginosa* PBSC1 the crude motor oil enhanced the biosurfactant production (4.99 g/l) but produce on par results statistically with the n-hexadecane (4.76 g/l) (Table 5 and Fig. 5).The lowest surface tension was recorded as 30.98 mN/m for the crude oil and followed by n-hexadecane with 31.65 mN/m for the isolate *P. aeruginosa* PBSC1 and produce statistically on par results with the crude oil. To improve biosurfactants production yield, different oils and hydrocarbons were added and these have a significant role in the production of biosurfactant and increase the yield parameters. The highest emulsification activity was found to be 75.86 per cent followed by 72.42 per cent for the crude oil and n-hexadecane respectively. In case of isolate *P. aeruginosa* PBSC1, almost for all the hydrocarbons the emulsification percentage was above 60 except for the Xylene.

Effect of trace elements

The trace elements in the medium also

influence the biosurfactant production level. The maximum biosurfactant production was observed in the control that means in the presence of all three trace elements (Table 6 and Fig. 6). The medium without each trace elements produce on par results statistically. In case of isolate *P. aeruginosa* PBSC1 the highest biosurfactant recorded was 5.14 g/l. Due to the ability of biosurfactants to degrade aromatic compounds, addition of hydrocarbons into the culture medium-enhanced biosurfactants production for both isolates.

The carbon source was found to affect the cell mass to a great extent. As the biosurfactant is cell-wall associated, high cell density is desirable (Bicca et al., 1999). The carbon source, particularly the carbohydrate, has a major effect on the type of glycolipids formed. Glucose, fructose and sucrose lipids are formed by *Arthrobacter paraffineus* and several species of *Corynebacterium*, *Nocardia* and *Brevibacterium* during growth on the corresponding sugar (Suzuki et al., 1974).
NH_4NO_3 in presence of yeast extract was the best nitrogen source and the concentration 0.46 g/l NH4NO3 and 0.2 g/l yeast extract were the best concentration for biosurfactant production. The type of nitrogen present (Whether NH4 +, NO3-, urea or amino acid) influences the biosurfactant produced (Robert et al., 1989; Duvnjak et al., 1983; Haba et al., 2000). Interesting observations relate to the effect of nitrogen limitation that appears to stimulate biosurfactant production and overproduction by some microorganisms (Suzuki et al., 1974). The nitrogen source in the medium influences the production of biosurfactant (Desai et al., 1994). *Arthrobacter paraffineus* showed a preference of ammonium salts and urea as the nitrogen source (Duvnjak et al., 1983). Robert et al. (1989) while investigating rhamnolipid production by *Pseudomonas* 44Ti on olive oil reported that sodium nitrate was the best nitrogen source. Similar results have been noted for *Pseudomonas aeruginosa* (Ramana et al., 1989)

Hydrocarbons added to the fermentation medium are known to induce the production of biosurfactant (Bento and Gaylarde, 1996). Biosurfactant production has been demonstrated in the presence of water-soluble substrates, hydrocarbons and oils. The type of surfactant formed when growing on these carbon sources can be influenced (Makkar and Cameotra, 1999; Duvnjak, and Kosaric, 1985; Robert et al., 1989).

Table.1 Effect of different carbon * sources on the isolated organism's biomass, surface tension and emulsification activity

Carbon sources	***Pseudomonas aeruginosa* PBSC1**	
	DCBM (g/l)	**Emulsification Index (E_{24}) (%)**
Glucose	3.92 ± 0.18^{b}	75.17 ± 0.10^{b}
Glycerol	4.38 ± 0.08^{a}	79.65 ± 0.12^{a}
Fructose	0.95 ± 0.16^{e}	56.17 ± 0.22^{e}
Sodium citrate	1.54 ± 0.12^{d}	62.67 ± 0.18^{d}
Mannitol	3.34 ± 0.04^{c}	69.34 ± 0.04^{c}
Starch	0.66 ± 0.12^{f}	49.18 ± 0.24^{f}

*- 2 per cent concentration

DCBM – Dry cell biomass; E_{24} – Emulsification index

Values are mean of five determinants ± SD, within column different letter after values indicate that there is a significant difference at a 'p' value of 0.05 as determined by DMRT

Table.2 Effect of different nitrogen sources on the isolated organism's biomass, surface tension and emulsification activity

Nitrogen source	*Pseudomonas aeruginosa* PBSC1	
	DCBM (gL^{-1})	Emulsification Index (E_{24}) (%)
Yeast extract	3.46 ± 0.06^{b}	71.23 ± 0.16^{c}
Peptone	2.81 ± 0.12^{c}	66.54 ± 0.15^{d}
Ammonium chloride	1.85 ± 0.24^{e}	54.37 ± 0.09^{e}
Ammonium nitrate	3.92 ± 0.16^{d}	73.47 ± 0.18^{b}
Sodium nitrate	4.34 ± 0.10^{a}	78.52 ± 0.08^{a}

DCBM – Dry cell biomass; E_{24} – Emulsification index

Values are mean of five determinants ± SD, within column different letter after values indicate that there is a significant difference at a 'p' value of 0.05 as determined by DMRT

Table.3 Effect of different pH on the isolated organism's biomass**,** surface tension and emulsification activity

pH	*Pseudomonas aeruginosa* PBSC1	
	DCBM (gL^{-1})	Emulsification Index (E_{24}) (%)
5.0	2.23 ± 0.1^{e}	68.32 ± 0.08^{c}
5.5	3.91 ± 0.2^{b}	70.56 ± 0.10^{b}
6.0	2.98 ± 0.5^{d}	68.45 ± 0.23^{c}
6.5	4.56 ± 0.5^{a}	74.92 ± 0.18^{a}
7.0	4.94 ± 0.3^{a}	75.12 ± 0.20^{a}
7.5	3.34 ± 0.2^{c}	64.43 ± 0.14^{d}
8	2.76 ± 0.5^{d}	57.35 ± 0.22^{e}
8.5	1.84 ± 0.5^{f}	51.12 ± 0.12^{f}

DCBM – Dry cell biomass; E_{24} – Emulsification index

Values are mean of five determinants ± SD, within column different letter after values indicate that there is a significant difference at a 'p' value of 0.05 as determined by DMRT

Table.4 Effect of different Temperature on the isolated organism's biomass, surface tension and emulsification activity

Temperature (°C)	*Pseudomonas aeruginosa* PBSC1	
	DCBM (gL^{-1})	Emulsification Index (E_{24}) (%)
25	3.92 ± 0.22^{b}	75.18 ± 0.33^{b}
30	4.26 ± 0.10^{a}	78.46 ± 0.12^{a}
35	3.64 ± 0.12^{b}	70.22 ± 0.18^{c}
40	2.32 ± 0.08^{c}	53.31 ± 0.08^{d}
45	1.68 ± 0.14^{d}	49.18 ± 0.16^{e}
50	0.96 ± 0.00^{e}	48.64 ± 0.22^{e}

DCBM – Dry cell biomass; E_{24} – Emulsification index
Values are mean of five determinants ± SD, within column different letter after values indicate that there is a significant difference at a 'p' value of 0.05 as determined by DMRT

Table.5 Effect of different hydrocarbons on the isolated organism's biomass, surface tension and emulsification activity

Hydrocarbons (1%)	*Pseudomonas aeruginosa* PBSC1	
	DCBM (gL^{-1})	Emulsification Index (E_{24}) (%)
n-hexadecane	4.23 ± 0.13^{b}	72.78 ± 0.32^{b}
Heptanes	3.86 ± 0.25^{c}	69.46 ± 0.21^{c}
Xylene	1.55 ± 0.22^{f}	58.61 ± 0.40^{f}
Kerosene	3.76 ± 0.10^{c}	69.14 ± 0.12^{c}
Petrol	2.18 ± 0.18^{e}	61.82 ± 0.22^{e}
Diesel	2.84 ± 0.21^{d}	67.44 ± 0.10^{d}
Crude motor oil	4.87 ± 0.08^{a}	74.32 ± 0.52^{a}

DCBM – Dry cell biomass; E_{24} – Emulsification index
Values are mean of five determinants ± SD, within column different letter after values indicate that there is a significant difference at a 'p' value of 0.05 as determined by DMRT

Table.6 Effect of different trace elements on the isolated organism's biomass, surface tension and emulsification activity

Trace elements	*Pseudomonas aeruginosa* PBSC1	
	DCBM (gL^{-1})	Emulsification Index (E_{24}) (%)
Control	4.76±0.56[a]	71.13±0.22[a]
Mg_2SO_4(0.5 g/l) free	4.57±0.12[b]	65.42±0.18[b]
$MnSO_4$(trace) free	4.16±0.48[c]	65.57±0.20[b]
FeSO4 (0.01g/l) free	4.08±0.15[c]	62.82±0.12[b]
Mg2SO4 + FeSO4 free	3.64±0.59[d]	59.65±0.10[c]
MnSO4 + FeSO4 free	2.59±0.56[e]	59.22±0.16[c]
Mg2SO4 + MnSO4 + FeSO4 free	1.61±0.38[f]	55.12±0.21[d]

DCBM – Dry cell biomass; E_{24} – Emulsification index
Values are mean of five determinants ± SD, within column different letter after values indicate that there is a significant difference at a 'p' value of 0.05 as determined by DMRT

Fig.1 Effect of different carbon sources on the production of biosurfactant (g/l) and surface tension of *Pseudomonas aeruginosa* PBSC1

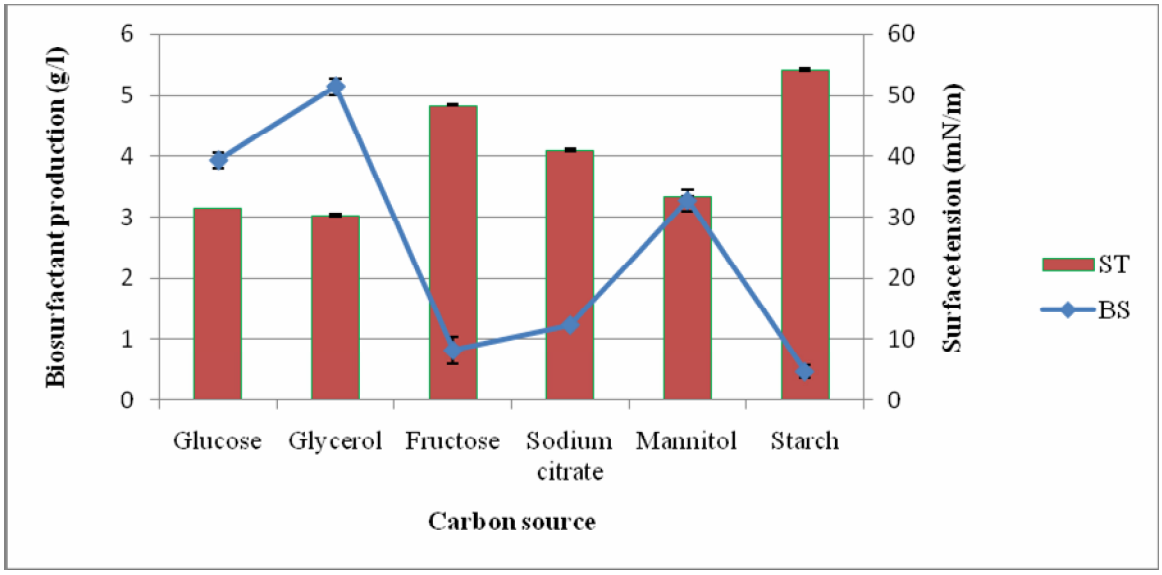


Fig.2 Effect of different nitrogen sources on the production of biosurfactant (g/l) and surface tension of *Pseudomonas aeruginosa* PBSC1

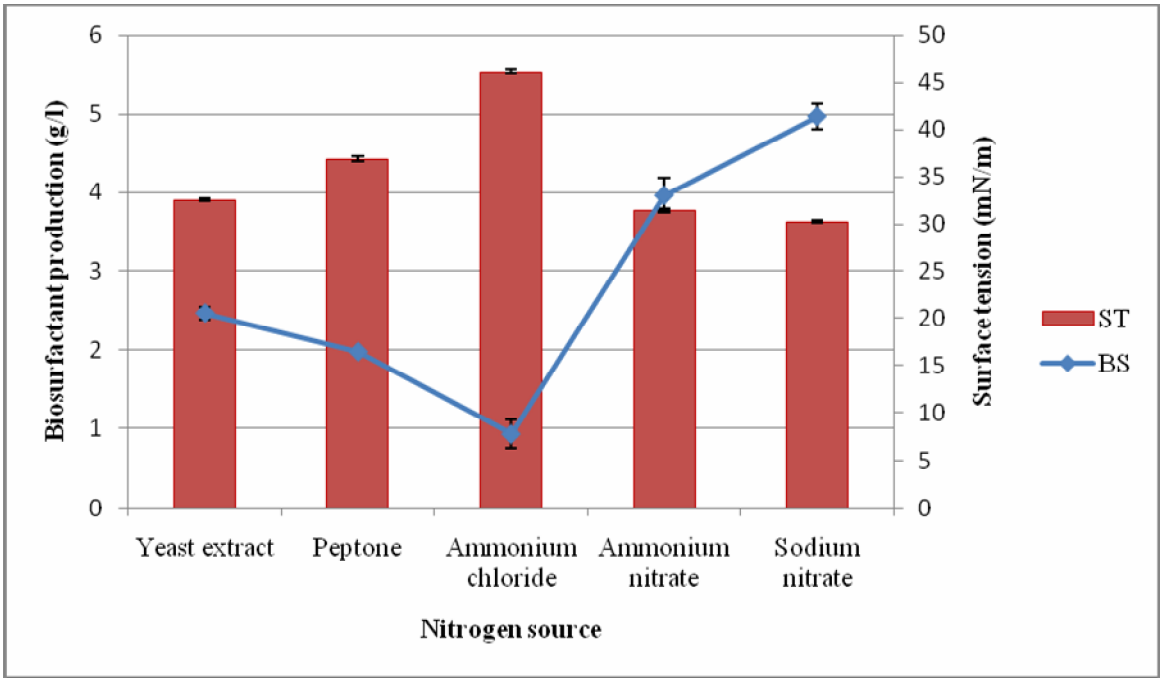


Fig.3 Effect of different pH on the production of biosurfactant (g/l) and surface tension of *Pseudomonas aeruginosa* PBSC1

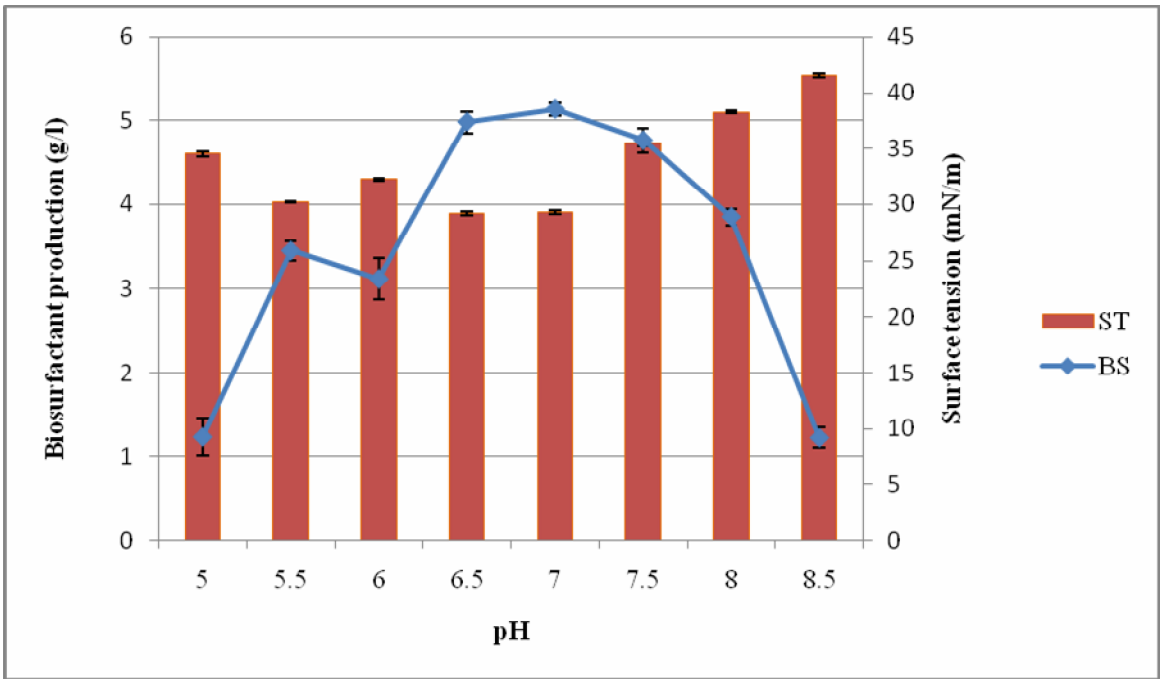


Fig.4 Effect of different temperature on the production of biosurfactant (g/l) and surface tension of *Pseudomonas aeruginosa* PBSC1

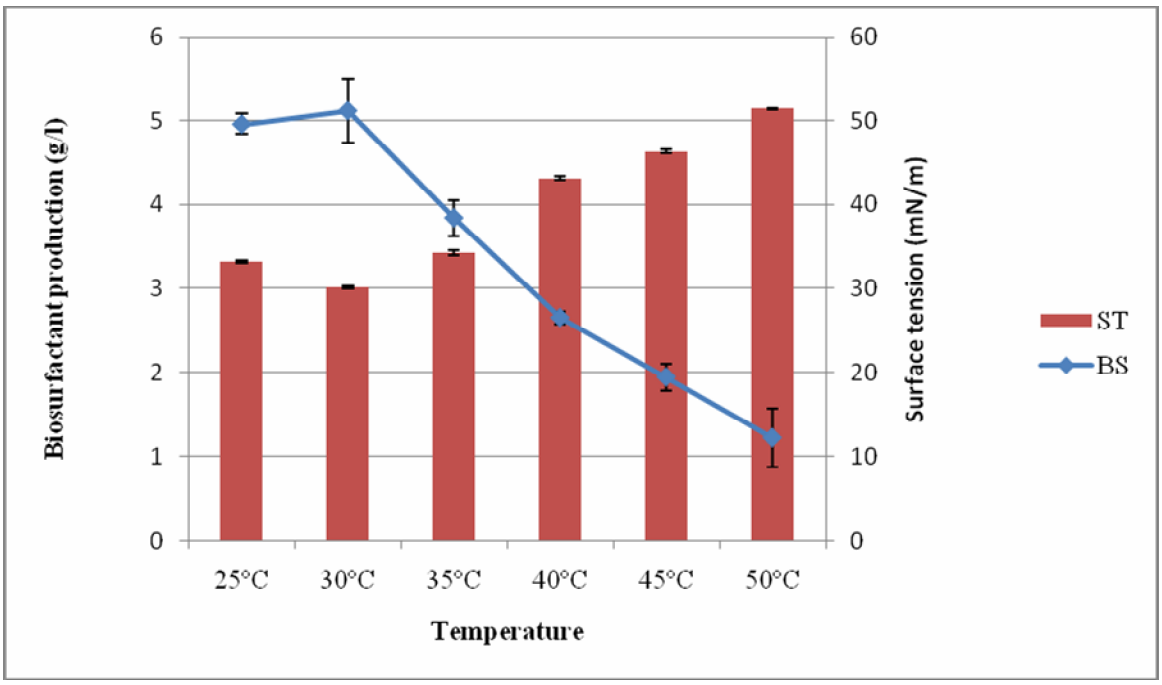


Fig.5 Effect of different hydrocarbons on the production of biosurfactant (g/l) and surface tension of *Pseudomonas aeruginosa* PBSC1

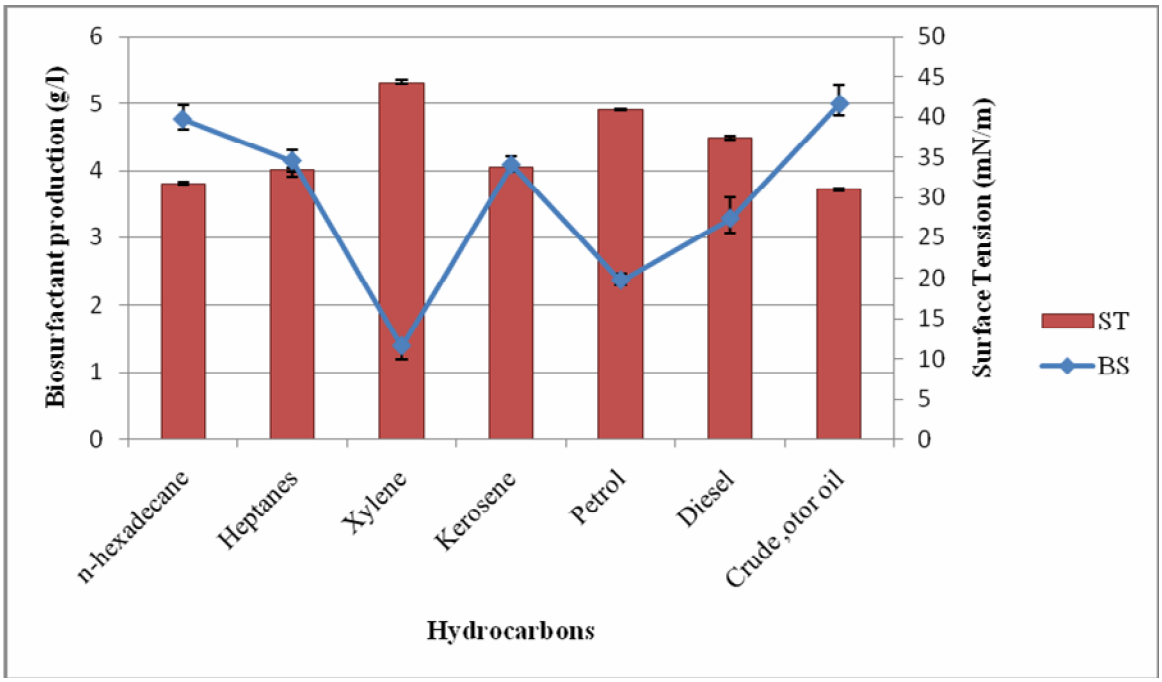
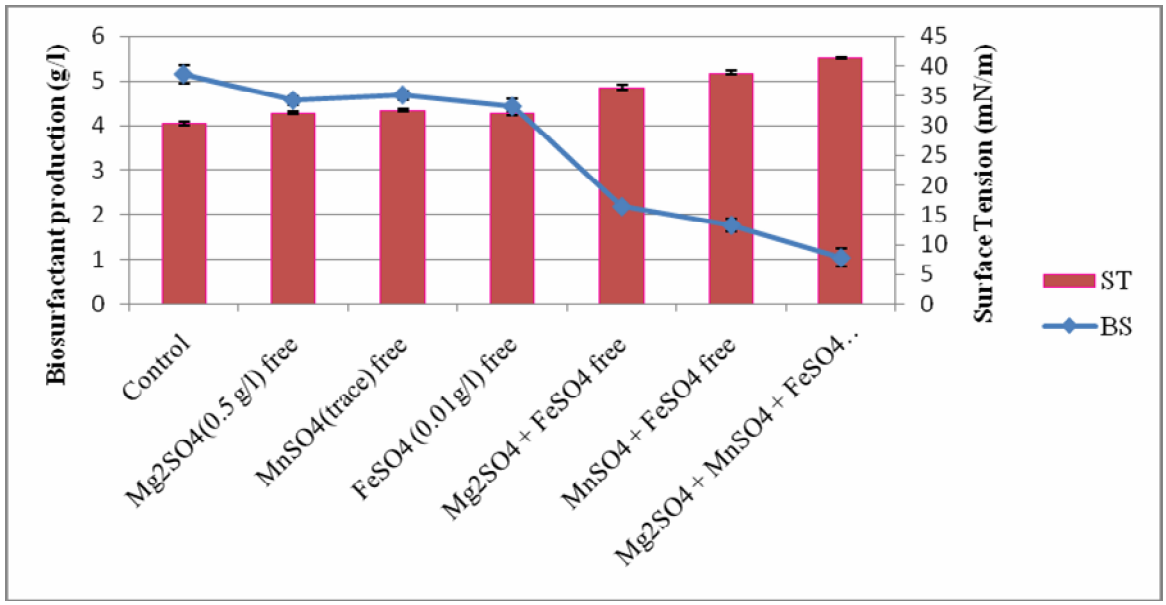


Fig.6 Effect of different trace elements on the production of biosurfactant (g/l) and surface tension of *Pseudomonas aeruginosa* PBSC1



Acknowledgment

The authors AJ and RP would like to acknowledge the University Grants Commision (UGC), Government of India, for the financial assistance and Annamalai University, Tamil Nadu, India for providing the necessary facilities to conduct the research experiment.

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