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Production and stability studies of biosurfactant produced by an isolated indigenous bacterial strain

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Abstract
For screening of potent biosurfactant producing bacterial strain, oil contaminated soil sample were collected from different locations. Blood hemolysis test of isolated bacterial strain confirmed its biosurfactant producing capacity. Culture conditions with varying carbon and nitrogen sources were studied at constant pH, temperature and revolution per minute, to obtain maximum biosurfactant production by the isolate. Surface tension measurement, bioemulsification assay and E24 activity of the broth was tasted to assure biosurfactant production. Crude coconut oil and phenyl alanine as carbon and nitrogen source respectively, C/N ratio 20, pH 7, temp. 35 ^{0}C and salt concentration of 2%. Showed enhanced biosurfactant production. Growth kinetics indicated that biosurfactant production was growth dependant showing reduced surface tension of the broth upto 38mN/m on 12th day of bacterial growth. Purified biosurfactant was found to stable at high temperature, at wide pH range and salt concentration, thus reflecting its applicability in bioremediation of oil spills, food, cosmetics and pharmaceutical industries. The biosurfactant characterization by TLC showed yellow spots on the plates indicating presence of glycolipid in biosurfactant. The biosurfactant producing indigenous isolates was identified as the *Pseudomonas* species by biochemical tests.

Keywords: Biosurfactant, blood haemolysis, emulsification index, surface tension

1. Introduction
Surfactants are amphipathic molecules which reduce surface tension, by acting as a bridge between the two materials meeting at the interface [1]. Biosurfactants are biological surface active, amphiphillic compounds produced, mostly by microbial cells on its surface. These are reported to be biodegradable non-toxic, and highly specific agents effective at extreme conditions of *p*H, temperature and salinity [2].
These molecules contain hydrophobic and hydrophilic moieties which reduces surface and interfacial tension between indivisual molecules [3]. It covers a wide range of chemical types including peptides fatty acids, phospholipids, glycolipids, antibiotics, lipopeptides etc [4]. In recent era research on biosurfactants production has expanded due to its potential use in different areas, such as food industry, agriculture, pharmaceuticals, oil industry, petrochemicals, paper and pulp industry. It also has also great importance for environmental protection through bioremediation and dispersion of oil spills.
Chemically synthesized surfactant have been used in the oil industry to clean up of oil spills as well as to enhance oil recovery from oil reservoir. These compounds are not biodegradable and can be toxic to environment [5, 6]. Biosurfactant have special advantage over their chemically synthesized surfactants because of their lower toxicity, biodegradable nature and effectiveness at extreme temperature and pH salinity and case of synthesis. Because of their broad range of potential industrial application, including emulsification, phase separation, wetting, foaming, emulsion stabilization and viscosity reduction of heavy crude oils, research in the area of biosurfactant synthesis is getting significant attention of researchers [7]. Biosurfactants are powerful natural emulsifier capable of reducing the surface tension of water. Most of the hydrocarbons are insoluble in water and their degradation using microorganisms has an important role in combating environmental pollution. One of the mechanisms used by these microorganisms for hydrocarbon degradation is through production of extracellular biosurfactant [8]. A variety of microorganism, including bacteria, fungi and yeasts has been reported to produce biosurfactant [9].
In the present study efforts were made to synthesize potent biosurfactant from indigenously isolated bacterial strain. Different culture conditions were optimized for enhanced biosurfactant production and the stability studies of purified biosurfactant were undertaken.

2. Materials and Methods

2.1 Collection of sediment sample

Total Five diesel contaminated soil sediment samples were collected from different locations, in Jalgaon city (MS, India). Collected soil sample was suspended in sterile water and mixed on rotatory incubator shaker (ORBITEK, Scigenics Biotech, India) at 150 rpm for 30 minutes.

2.2 Screening for biosurfactant producing bacterial strain

Various bacterial species from soil sediment samples were isolated and purified on nutrient agar medium. All the isolates obtained from above were subjected to biosurfactant production using following medium.

2.3 Inoculum preparation Media and culture conditions.

Isolation of biosurfactant producing bacterial strain was performed by liquid enrichment cultures in which diesel oil was taken as the sole carbon source [10]. 0.5 ml of 24 hour old inoculum of isolated bacterial strain was inoculated with 100 mL of the enrichment culture medium at 37 ^{0}C. The composition of the medium (g/L) used in this study was: $NaNO_3$ (7); K_2HPO_4 (1); KH_2PO_4 (0.5); KCl (0.1); $MgSO_4$. $7H_2O$ (0.5); $CaCl_2$ (0.01); $FeSO_4$ $7H_2O$ (0.01) and yeast extract, (0.1). The medium was supplemented with 0.05 mL of trace elements solution of the following composition (g L^{-1}): H_3BO_3 (0.25); $CuSO_4.5H_2O$ (0.5); $MnSO_4.H_2O$ (0.5); $MoNa_2O_4.H_2O$ (0.06) and $ZnSO_4.7H_2O$ (0.7) Diesel oil was sterilized thrice by filtering through 0.2 mm membrane filters (Vontron). Rest of the media were adjusted to pH 7.2 and autoclaved. After 42 hours all the bacterial culture obtained were tested for biosurfactant production. Maximum biosurfactant producing bacterial strain was maintained on glycerol nutrient broth medium. The experiment was conducted in triplicate. Biosurfactant production by isolates was confirmed by following three tests.

i. β -Haemolytic activity

An initial clue for the isolation of biosurfactant producing strain is its ability for haemolytic activity. For this Isolates were screened on blood agar medium (HIMEDIA) and incubated at 37 °C for 48 h. Hemolytic activity was detected as the presence of clear zone around the growth of colonies [11].

ii. Oil spreading technique

For Oil spreading activity, in a petri dish (15cm diameter) 30 ml of distilled water was added followed by the addition of 1 ml of oil and on its surface and 20 µl of supernatant from culture broth [3].

iii. Qualitative drop collapse tests performed on glass slide:

A thin coat of oil (ground nut) applied to slide. Then 3 of each supernatant was delivered in to centre of each glass slide. If the drop remained beaded after 2 minutes, the result was scored as negative. If the drop spread, the result was positive for the presence of biosurfactant [11].

2.4 Medium optimization

For medium optimization a series of set of experiments was conducted by changing one variable at one time and keeping other variables unchanged.

Bacterial cell growth as well as accumulation of biosurfactant as metabolic products were dependant on composition medium components such as carbon, nitrogen. Three factors were chosen for achieving higher production of the biosurfactant: optimization carbon source (C), nitrogen source (N) and C/N ratio. The carbon sources used were olive oil (2% w/v) (commercial type), coconut oil ((2% w/v) (commercial type), Starch (20 g/l), (Himedia, India) sucrose (20 g/l) (Himedia, India), glucose (20 g/l) (Himedia, India) and fructose (20 g/l) (Himedia, India) with ammonium chloride (NH4Cl) (Himedia, India) as nitrogen source.

The appropriate nitrogen sources for the production of biosurfactants was evaluated by taking, alanine, aspartic acid, phenyl alanine (Himedia, India), urea (Himedia, India), ammonium sulphate (Merck, India), NH_4Cl and sodium nitrate ($NaNO_3$)(Merck, India), were employed at a concentration of 1 g/l with the optimum carbon source. The C/N ratio (with optimized carbon and nitrogen sources) was varied from 10 to 40 by keeping a constant nitrogen source concentration 1g/l [7, 12].

2.5 Kinetic studies on biosurfactant production

For kinetics of biosurfactant production batch culture of 12 days was established at optimum conditions by measuring emulsification assay and surface tension and of supernatant samples obtained after cell separation [7].

2.6 Influence of pH, temperature, sodium chloride and aeration on biosurfactant production and activity

In order to study the effect of pH and temperature on the biosurfactant production, pH range was adjusted within 4 and 10 and the temperature was set at 10, 15, 25, 30, 35, 40, 45 and 60 °C. The pH of the medium was adjusted on a digital pH-meter (EUTECH, pH tutor, India). Effect of sodium chloride on biosurfactant production was tested in optimized medium; the sodium chloride was added in medium to achieve final concentrations of 1–10% (w/v). Effect of aeration on biosurfactant production of was evaluated by incubating the inoculated media at different aeration conditions such as 50, 75, 100, 125, 150, 175, 200, 225 and 300 rpm. Biosurfactant production was measured by emulsification assay and absorbance was measured at 400 nm [13].

2.7 Effects of oils, surfactants and hydrocarbon on biosurfactant production:

We tasted the effect of various crude oils, chemical surfactants and hydrocarbons on biosurfactant producing ability of isolate. For this emulcification activity index of the culture medium was measured by adding oils and surfactants separately. Different oils available in market like clove oil, eucalyptus oil, caster oil, coconut oil, sunflower oil, olive oil and ground nut oil were tasted. Impact of synthetic chemical surfactants was evaluated by taking 1% (V/V) Tween 20, tween 40, tween 60, tween 80, sodium dodicyl sulphate (SDS) (Thermo fisher scientific, USA), Cetyl trimethyl ammonium bromide (CTAB), (Himedia, India) and ethylene diamine tetra acetic acid (EDTA) (Himedia, India). Surface tension of the medium was measured as follows by adding each hydrocarbons in 1% (v/v) such as tolune, n hexane, disel, petrol and kerosene.

2.8 Quantitative measurement of surface tension

Quantitative measurement of surface tension was done by taking cell free supernatant (cell-free broth obtained by centrifuging the cultures at 10000 x *g* for 25 min) in a K6

tensiometer, using the du Nouy ring method [14]. The values reported were the mean of three measurements.

2.9 Quantitative measurement of bioemulsifier production

Production of bioemulsifier occurs by degradation of hydrocarbons. These are utilized as anutrient by bacteria in limitednutrient condition. (Kokre *et al*, 2007.). 3 ml of the supernatant was taken from shake flask culture and filter sterilized by using milipore 0.2µm membrane filter. 2 ml filtrate was taken in a screw caped test tube (15 x 120mm) and diluted with 0.1M sodium acetate buffer (pH 3.00). Then 1 ml of hexadecane wad added to it and test tube was capped. Mixture was shaken for 3 minutes at 25 ^{0}C with rate of 180-200 strokes per minute (stroke length 3cm). Resulting mixture was allowed to set for 30 mints at 25 ^{0}C. Aqueous phase was carefully removed and its absorbance was recorded at 540nm on ELICO SL 18 UV- visible spectrophotometer. An absorbance of 0.01 unit at 540 nm multiplied by dilution factor, if any was assumed as one unit emulsification activity per ml (EU/ml). Sterile fresh medium was used as the blank [15].

2.10 Quantitative measurement of Emulsification activity (E24) test

Oil emulsification activity (E24) of isolates was calculated with 2 ml of oil to the same amount of culture and mixing it in a vortex for a 2 min and leaving to stand for 24 hours. The E24 index is given as percentage of height of emulsified layer (cm) divided by total height of the liquid column (cm) [16].

$$E24 = \frac{\text{Height of emulsion formed}}{\text{Total height of the solution}} \times 100$$

2.11 Extraction of bio surfactant

To isolate the biosurfactant, the bacteria were removed by centrifugation and the remaining supernatant liquid was filtered through a 0.22 mm pore size filter (milipore). Biosurfactant was obtained by adjusting the supernatant PH 2.0 using 6N Hcl (Merk, India) and keeping it at 4 °C overnight. The precipitate thus obtained was pelleted by centrifugation for 20 min(10,000rpm,4 ^{0}C) Thus obtained crude surfactant fraction was further purified by dissolving in distilled water at PH 7.0 and dried at 60 °C. The dry product was extracted with Chloroform: Methanol (65:15), filtered and the solvent evaporated.

2.12 Stability studies

By considering the industrial importance of biosurfactant its stability was assessed. To determine its thermal stability cell free broth was maintained at a temperature range of 30-100 ^{0}C for 15 minutes and then it allowed to reach at room temperature. To determine pH stability cell free broth was adjusted to different pH using 1 N NaOH and 1 N HCl. Salt toloerence capacityof the biosurfactant was studied in different concentrations of Nacl. Then biosurfactant activity was measured in the form of emulsification index of the cell free broth.

2.13 Characterization of biosurfectants

Preliminary characterization of the biosurfactant was done by thin layer chromatography method. Silica gel plates were prepared by mixing the silica powder with their and poured on the glass plates. A spot of crude biosurfactant was placed on the silica plate. The Biosurfactant was separated on the plate using chloroform: methonal: water (10:0.5) Ninhydrin regent was sprayed to detect lipopeptide biosurfectants as red spots. Throne reagent was sprayed to detect glycolipid biosarfactant as yellow spot.

3. Results and discussions

Biologically synthesizes surfactants are having certain advantages over synthetic ones, which includes lesser toxicity, biodegradable nature, selectivity, specific activity at extreme temperatures, pH and salinity. In our laboratory, we have isolated three bacterial strain B1, B2, B3 from petrol bunk soil samples. Out of which one strain B1 showing positive blood haemolysis test (Fig. 1) was confirmed for biosurfactant production.

3.1 Growth characteristics B1 strain with biosurfactant production

Reducing surface tension of the sample was the criteria to access biosurfactant production which was dependant on cell growth [17]. Biosurfactant production initiated in early lag phase and continuously increased up to 10 days after inoculation. i e. up to decline phase of bacterial growth (Fig 2).

3.2 Optimization of cultivation medium

i. Effect of carbon source

Various carbon sources *viz.* olive oil (2% w/v) (commercial type), coconut oil ((2% w/v) (commercial type), Starch (20 g/l), (Himedia, India) sucrose (20 g/l) (Himedia, India), glucose (20 g/l) (Himedia, India) and fructose (20 g/l) (Himedia, India) were tasted to access its effect on qualitative biosurfactant production. For this emulsification activity (E24) of the broth samples with different carbon sources was calculated (Fig. 3). Use of vegetable oils like coconut oil and soya oils to produce biosurfactants seems to be suitable and cost effective alternative. Use of other sources like glucose, sucrose, starch might cause reduction in pH of the broth which seems to be inhibitory to biosurfactant production.

ii. Effect of nitrogen source

The appropriate nitrogen sources for the production of biosurfactants was evaluated by taking, alanine, aspartic acid, phenyl alanine (Himedia, India), urea (Himedia, India), ammonium sulphate (Merck, India) and sodium nitrate ($NaNO_3$)(Merck, India), were employed at a concentration of 1 g/l with the optimum carbon source. Isolate vacterial strain was able to use all nitrogen sources. However, Aspartate and phenyl alanine were found as good nitrogen source for biosurfactant synthesis by isolate (Fig. 4).

iii. Effect of C:N ratio

For the enhancement in biosurfactant synthesis ability by isolate, one necessary aspect was to optimise C/N ratio. It was done using soya oil and aspartic acid as carbon and nitrogen source respectively as both gave good result for biosurfactant production. C/N ratio 20 gave least value of surface tension (ST= 22) and maximum emulsification activity (EA=250U/ml). Other C/N ratios 35, 40, 50 does gave any significant difference in emulsification activity. These result showed the similarity with previous work by Khopade *et al*. [7].

3.3 Kinetics of biosurfactant production

Amount Biosurfactant synthesized and rate of decrease in surface tension of the medium were corelated with growth of

the cukture in the fermentation medium [18]. Along with increase growth of culture continuous dropping in the surface tension was observed and after 12 days lowest value of surface tension was 37 mN/m. (Fig. 5). Again it was noted that at about 4th day of growth, biosurfactant concentration started to increase reaching its maximum on 9th day. From 10th day continuous dropping in surface tension was not observed, which indicated that biosurfactant synthesis might stopped, probably due to release of secondary metabolites. These secondary metabolites produced in the medium might be interfering the biosurfactant synthesizing enzymes. These results also showed that biosurfactant production from soya oil was occurred mostly in exponential growth phase suggesting that biosurfactant was synthesized as primary metabolite along with increase cellular biomass. This property suggests us that biosurfactant could be successfully synthesized under chemostat condition or by immobilized cells.

3.4 Influence of pH, temperature, sodium chloride and aeration on biosurfactant production and activity

A slow increase in biosurfactant production by the isolate at pH 7 was observed, suggesting as optimum pH (fig. 6) Effect of NaCl concentration showed that maximum biosurfactant production the isolated bacterial was achieved with 2% NaCl although the bacterial strain retained its activity even in 10% w/v Nacl (fig 7). This result may indicate the halophytic behevior of the isolate. Optimum temperature noted for biosurfactant production was 35 ^{0}C, but the bacterial strain showed good growth even in up to 50 ^{0}C temperature range (Fig. 8). Thus the isolate was found as moderately thermostable. On rotatory incubator shaker rotation speed of 120-140 RPM was found suitable for maximum biosurfactant production.

3.5 Effects of oils, surfactants and hydrocarbon on biosurfactant production by the isolate

Further investigations on biosurfactant production by our indigenous isolate were carried out with addition of different concentrations of oils, surfactants, and hydrocarbons in the fermentation medium. Sunflower and ground nut oil, tween 80 and hexane showed maximum activity against all tasted oils, surfactants and hydrocarbons respectively. Ground nut oil and tween 80 showed emulsification activity as 185 EU/ml and 195 EU/ml respectively (Fig. 9, 10). Alkane hydrocarbon n hexane as the substrate showed maximum drop in surface tension of the medium (Fig. 11) suggesting maximum biosurfactant production in the medium.

3.6 Stability studies

i. Temperature stability

Role of biosurfactants in industrial fields for various applications is dependant on its stability at different physic-chemical conditions. Initially thermostability of the biosurfactant was tasted over a wide temperature range. As per our previous study our indigenous isolate was found as thermostable (Fig. 8). Heating of biosurfactant up to 1000C could not cause any signficant drop in immulcification activity ans altimately biosurfactant performance was also fairly stable (Fig. 12). This finding suggests the applicability of the produced biosurfactant in food, pharmaceutical as well as cosmetics indusries.

ii pH stability

Emulsification activity of our crude biosurfactant remained moderately stable over pH range of 7-12. (Fig. 13), showing stability in alkaline pH than acidic pH. At pH 12 the emulsification activity was 67% while at pH 6 it was 25%. Additionally bellow the pH 6 turbidity in the sample was noticed. It may be due to partial precipitation of biosurfactant [15]. Thus increasing pH gave positive impact on stability of biosurfactant. This may be due to better stability of fatty acid surfactant micelles in alkaline environment.

iii. Effect of salinity

Biosurfactant stability of in saline environment was studied by dissolving purified biosurfactant in distilled water containing specific concentration of NaCl. Here the biosurfactant showed stability over all used concentration of NaCl though, 3% NaCl concentration gave comparatively more stability by showing higher emulsification activity (Fig. 14). These findings recommends usefulness of such biosurfactant in alkaline environment. Illori *et al* has reported the stability of emulsion in the presence of salt is one of the property of biosurfactant [15].

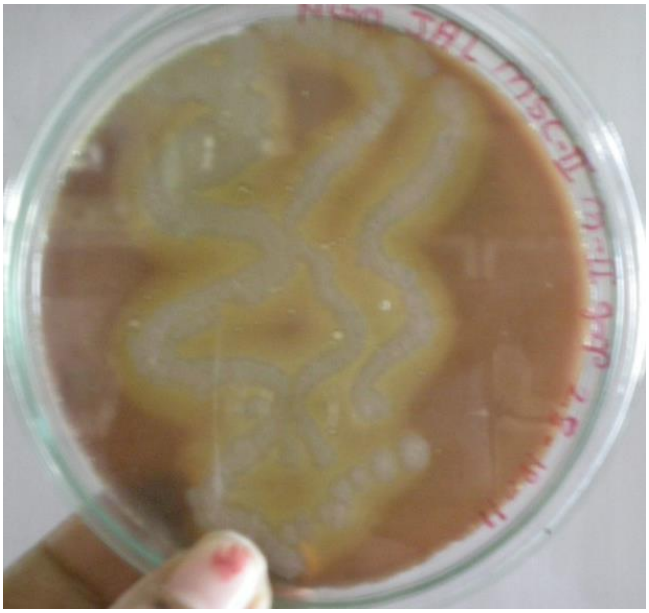

Fig 1: Blood hemolysis activity shown by isolate

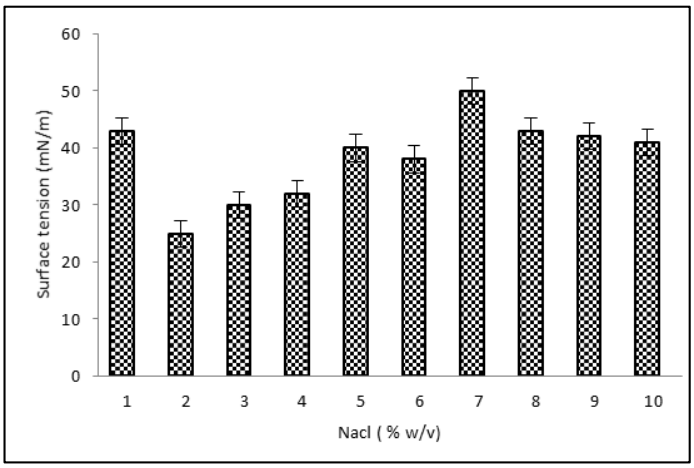

Fig 2: Effect of carbon source on biosurfactant

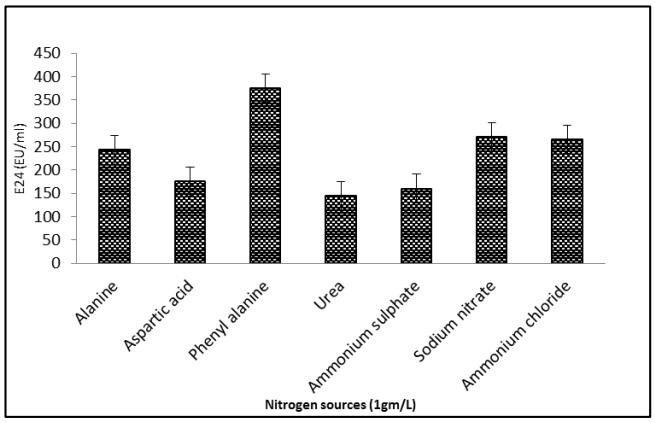

Fig 3: Effect of nitrogen source on biosurfactant production.

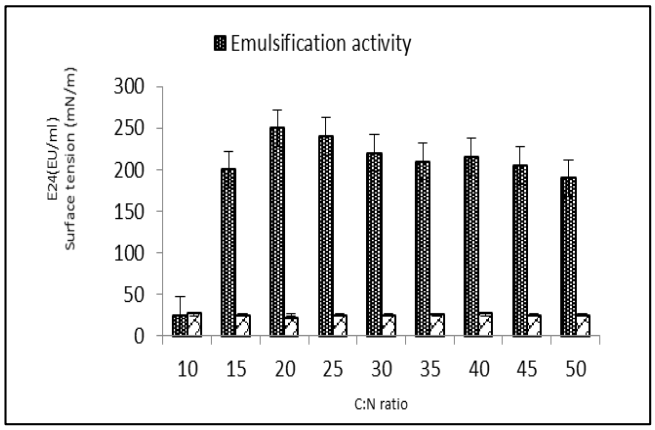

Fig 4: Effect of C:N ratio on biosurfactant production

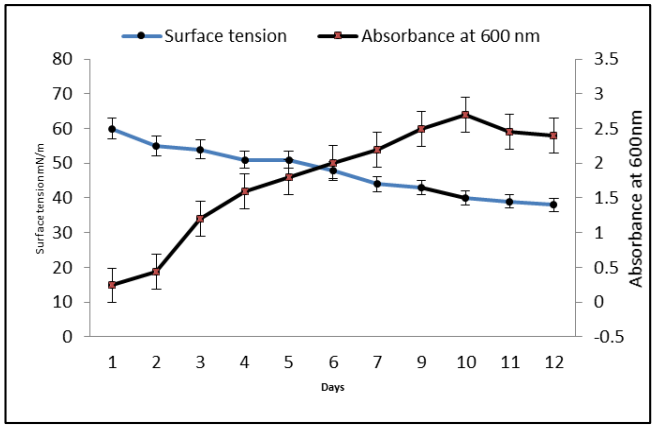

Fig 5: Growth kinetics of bacterial strain and biosurfactant production

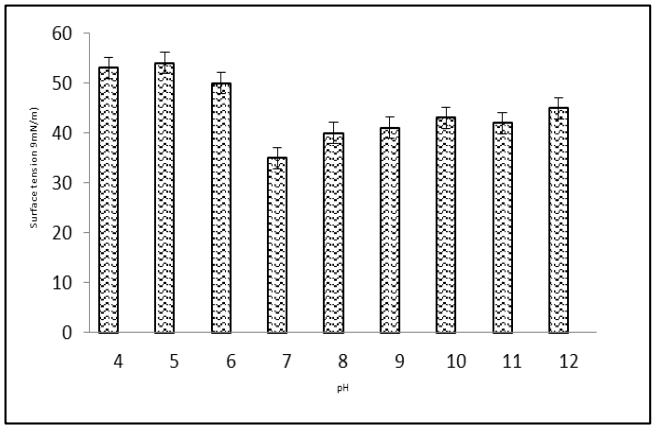

Fig 6: Effect of pH on biosurfactant production

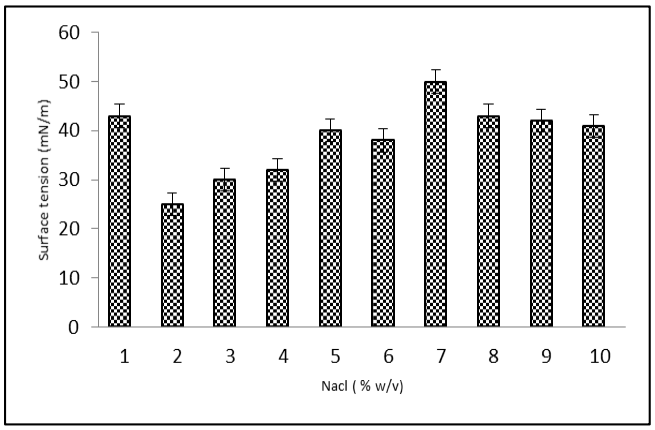

Fig 7: Effect of NaCl on biosurfactant production

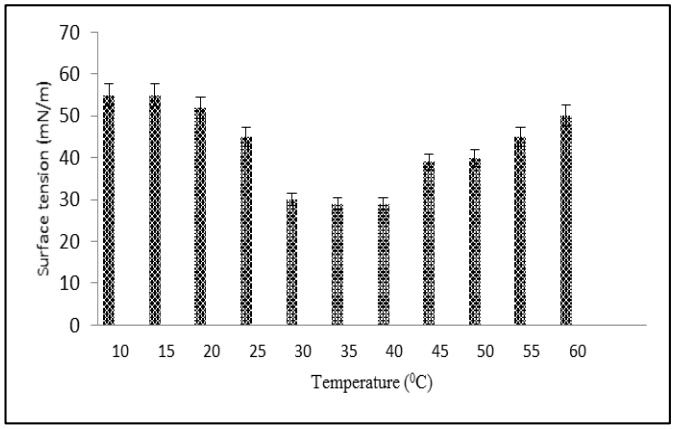

Fig 8: Effect of temperature on biosurfactant production

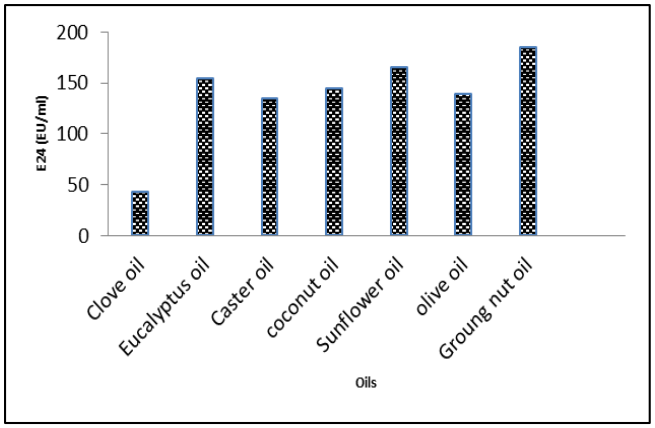

Fig 9: Effect of different oils on biosurfactant production

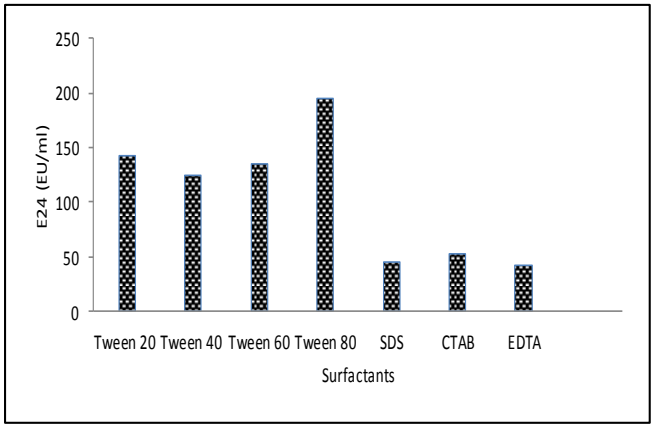

Fig 10: Effect of surfactants on biosurfactant production

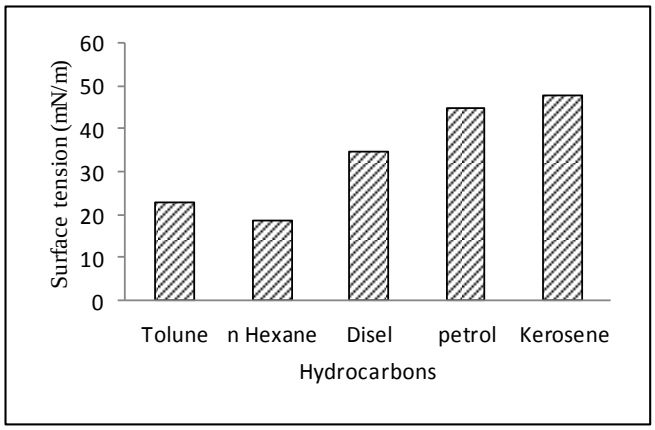

Fig 11: Effect of various hydrocarbons on biosurfactant production

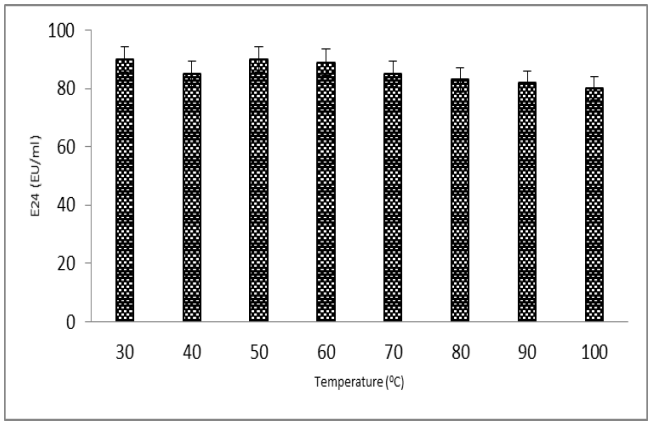

Fig 12: Effect of temperature on biosurfactant stability

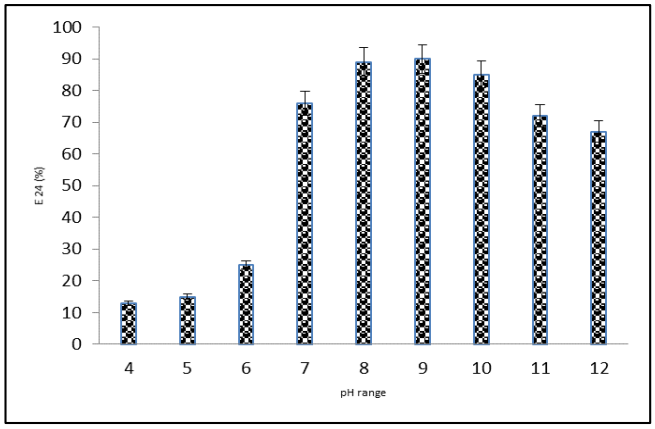

Fig 13: Effect of pH on biosurfactant stability

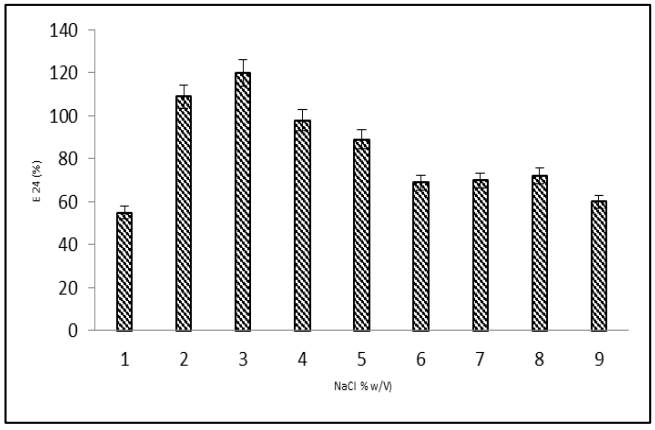

Fig 14: Effect of NaCl on biosurfactant stability.

4. Conclusion

In the present research studies biosurfactant isolated from our indigenous bacterial isolate showed good stability over a wide range of temperature, pH and salinity. While optimizing carbon and nitrogen source, crude coconut oil and phenyl alanine respectively gave maximum biosurfactant production. Thermo stability of our purified biosurfactant even at 100^0C indicated its industry oriented usefulness in detergent, pharmaceutical industry. Again for the bioremediation of oil contaminated water, there is great demand of such halotolerant strain for oil recovery

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