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Research Article

Optimization and characterization of biosufactant produced by *Pseudomonas aeruginosa* SCS2 isolated from oil contaminated soil samples

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Abstract

Biosurfactants are surface active compounds produced by microorganisms. These molecules reduce surface tension between aqueous solutions and hydrocarbon mixtures. The biosurfactant producing microorganisms isolated from different location of soil samples in Tamil Nadu, India. Seventy five strains were isolated from the oil contaminated soil samples. Among the total isolates only thirty strains was selected for the biosurfactant production based on the glass plate assay. The isolate SCS2 recorded maximum surface reduction ability (27.42 mN/m), followed by AWS4 (29.12 mN/m) and MSS4 (30.12 mN/m).The screening results reveals that the isolate *Pseudomonas aeruginosa* SCS2 was found to be efficient biosurfactant producer with higher activity, hence selected for the further experiments. Based on the biochemical and physiological test performed, the strain was identified as *Pseudomonas aeruginosa* SCS2. The bacterial isolates were grown in Mineral Salt Medium (MSM) with addition of 1% (V/V) oil as carbon source. The biosurfactants produced by the isolate was extracted using chloroform: methanol extraction method. The crude biosurfactant was further characterized by Thin layer chromatography and Fourier Transform Infrared Spectroscopy (FT-IR), NMR, Gas chromatogarphy mass spectrum (GC-MS), High pressure liquid chromatography (HPLC). The studies confirm the presence of rhamnose sugar moiety in the glycolipid chain. Glycolipid production by the isolated bacterium using different Carbon, Nitrogen, pH, Temperature, Trace element, hydrocarbon and Vegetable oil. The surface tension of the culture broth was determined initially and after 72 hrs. Dry cell biomass and the emulsification activity was determined after 72 hrs.

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Keywords: Biosurfactant, *Pseudomonas aeruginosa*, optimization, HPLC and GC-MS.

Introduction

Biosurfactants are a leading group of valuable microbial natural products with unique biochemical properties. From a biotechnology perspective, the production of biosurfactants is important owing to their vast applications in food, cosmetics, pharmaceuticals, agricultural and the petrochemical industries (Nguyen *et al*., 2008; Abouseoud *et al.*, 2008). Microbial surfactants, which are secreted by different groups of bacteria, composed of lipid, phospholipids, polysaccharide, protein and other biological macromolecules and contain various functional groups including carboxyl, amino and phosphate groups (Desai *et al*., 1997; Christofic *et al.*, 2002). Practically, all the usable surfactants are chemically synthesized at present. Surfactants have been used industrially as adhesives, deemulsifiers, flocculating, wetting and forming agents, lubricants and penetrants (Mulligan, *et al.*, 1993). Because of their amphiphilic nature, surfactants tend to accumulate at interfaces (air-water and oil-water) and surfaces (Nalini and Parthasarathi, 2013).

Microbiologically derived surfactants or biosurfactants are heterogeneous group of surface active molecules produced by a wide variety of bacteria, yeast and filamentous fungi, which either adhere to cell surface or are excreted extracellularly in the growth medium.

Having both hydrophobic and hydrophilic moieties, biosurfactants are able to reduce surface tension and interfacial tension between two fluids at the surface and interface respectively. These are also able to form microemulsion where hydrocarbons can solubilize in water or where water can solubilize in hydrocarbon (Desai *et al.,* 1997).

Microbial surfactants are complex molecules, comprising a wide variety of chemical structures, such as glycolipids, lipopeptides, fatty acids, polysaccharides - protein complexes, peptides, phospholipids and neutral lipids (Banat *et al*., 2000). Biosurfactants have many industrial applications in different areas like oil industry, food industry, pharmaceutical sector etc. About a 30% increase in total oil recovery from underground sandstone by using trehalolipids from *Nocardia rhodochrous* has been documented (Rapp *et al.,* 1977). Rhamnolipid from *Pseudomonas aeruginosa* was found to remove a large quantity of oil from contaminated Alaskan gravel in the Exxon Valdez oil spill (Harvey *et al.,* 1990).

Biosurfactants are also useful in bioremediation of sites contaminated with heavy metals such as uranium, cadmium, lead etc (Miller, 1995). In the food processing industries, improvement in dough stability, texture, volume and conservation of bakery products is obtained by addition of rhamnolipid (Van Haesendonck *et al.,* 2004). Biosurfactants are also very attractive in the health care and cosmetic industries Klekner *et al*., 1993). Some antimicrobial action against bacteria, fungi, algae, and viruses are observed by several biosurfactants.
Almost all surfactants being currently produced are derived from petroleum source. However, these synthetic surfactants are usually toxic and hardly degraded by microorganisms. These are potential source of pollution and damage to the environment. Therefore, in the recent years, much interest and attention have been directed towards biosurfactants over chemically synthesized surfactants due to their ecological acceptance, owing to their low toxicity and biodegradable nature (Karnath *et al.,* 1999). Other advantages of biosurfactant are ease of synthesis, specific action, and effectiveness at extreme conditions *viz.*, temperature, pH and salinity (Tabatabaee *et al.,* 2005).

Even though interest in biosurfactants is increasing, these compounds do not compete economically with synthetic surfactants due to the higher production cost of biosurfactants. To reduce the production cost, different routes could be investigated with respect to the increase of yield and product accumulation, the development of economical engineering processes and the use of cost - free or cost - credit feed stock for growth of microorganism and biosurfactant production (Mercade *et al.,* 1994). The optimization of culture conditions is one of the routes that could be investigated for maximum production of biosurfactant.

Various types of biosurfactants are synthesized by a number of microbes particularly during their growth on water-immiscible substrates. A majority of biosurfactants are produced by bacteria. Among the bacteria, the *Pseudomonas* species is well known for its capability to produce rhamnolipid biosurfactants with potential surface - active properties when grow on different carbon substrates. Rhamnolipid biosurfactants produced by *Pseudomonas aeruginosa*, in particular offer special advantages because of their potent emulsifying activity and low critical micelle concentration (Cooper *et al*., 1981).

Biosurfactant has been widely exploited in areas related to agriculture for enhancement of biodegradation of pollutants to improve the quality of agriculture soil, for indirect plant growth promotion as these biosurfactants have antimicrobial activity and to increase the plant microbe interaction beneficial for plants (Takenaka *et al.,* 2007; Lima *et al.,* 2011; Sachdev and Cameotra, 2013).

Agro-industrial wastes contain high levels of carbohydrate or lipids which enhance the growth of microorganisms and biosurfactant production (Nitschke *et al*., 2005). Thus, the use of such waste substrate for biosurfactant production is an ecologically acceptable option as it would reduce the waste treatment cost and add economic value to industrial residues (Oliveira *et al*., 2013).

One possible strategy for reducing cost is the utilization of alternative substrates such as agro industrial wastes (Mercade *et al*., 1994). The main problem related to use of alternative substrates as culture medium is to find a waste with the right balance of nutrients that permits cell growth and product accumulation (Makker *et al.,* 1999). Molasses (1997), peat hydrolysate (Sheppard *et al*., 1987) and potato process effluents (Fox *et al*., 2000) are examples of alternative substrates that have been suggested for biosurfactant production by *Pseudomonas aeruginosa.*

This particular bacteria (*Pseudomonas aeruginosa*) produces two types of glycolipids both containing

rhamnose as the carbohydrate moiety. These glycolipids are produced after attaining the stationary phase when the nitrogen is depleted in the medium (Cooper *et al.*, 1984). The genus *Pseudomonas* is capable of using different substrates, such as glycerol, mannitol, fructose, glucose, n-paraffins and vegetable oils, to produce rhamnolipidtype biosurfactants several studies have been carried out to define the best ratio between carbon, nitrogen, phosphorus and iron needed to obtain high production yields (Cooper *et al*.,1981; Finnerty *et al*.,1983).

Optimization of the carbon/nitrogen ratio in continuous cultures of *Pseudomonas aeruginosa* has been studied, indicating ratios between 15 and 23 as the optimum range for achieving high specific productivity of rhamnolipids, using glucose and vegetable oil as substrates, respectively. After nitrogen has been fully consumed, cell metabolism is directed to producing rhamnolipids, whose production increases after the exponential growth phase (Guerra-Santos *et al*., 1983; Maier *et al*., 2000).

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Sampling area and sampling

The soil samples were collected from various locations of Tamil Nadu, India. After collection, soil samples were air dried, sieved through 2 mm sieve and stored in airtight container for further use. Site description of the sample collected and the designated code was presented in Table - 1.

Production of culture medium

Mineral salts medium (MSM) used to enrich and isolate for biosurfactant producing microorganisms**.** The composition of the Mineral medium used was as follows (g L^{-1}): 4 g NH_4NO_3, 0.1g KCl, 5g KH_2PO_4, 1.0 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.01 g $CaCl_2$, and 0.01 g $FeSO_4 \cdot 7H_2O$ and supplemented with 1 ml trace element solution containing (L^{-1}): 0.75 g $ZnSO_4 \cdot 7H_2O$, 0.08 g $COCl_2 \cdot 6H_2O$, 0.075 g $CuSO_4 \cdot 5H_2O$, 0.5 g $MnSO_4 \cdot H_2O$, 0.15 g H_3BO_3, and 0.06 g $NaMoO_4 \cdot 2H_2O$. The pH of culture media was adjusted to 7.

Isolation and identification of biosurfactant producing microorganism

The soil samples were collected from different location of Tamil Nadu, India under aseptic condition by using sterile sampling bottle. Accordingly, a few grams of the soil sample were transferred to 100 ml of Mineral salt medium (MSM) in a 250 ml Erlenmeyer flask. The flasks were incubated at 30°C on a rotary shaker at 200 rpm for 7days. The isolates were screened for biosurfactant production. Control and replica plates were maintained. The biosurfactant producing isolates were determined by qualitative studies and identified by biochemical tests. All these results were compared with Bergey's Manual of Determinative Bacteriology to determine the genus (Holt *et al.,* 1994). The biosurfactant production of the isolates was evaluated by rapid tests *viz*., hemolytic activity (Carrillo *et al*., 1996), drop collapse test (Youssef *et al*.,2004) and oil displacement test were used for screening of biosurfactant producers. The surface tension was measured using Du-Nouy ring tensiometer (Krüss, GmbH, Hamburg, Germany). The distilled water and un-inoculated medium was used as negative control and Tween-20 was used as positive control. The measurements were repeatedly taken thrice and the average value was used to express the surface tension of the sample.

Biosurfactant production

Isolates were grown in 500 ml Erlenmeyer flasks containing 100 ml mineral salt medium adjusted to pH 7.0 was used as culture medium. The flasks were incubated at 37°C on a shaker incubator for 7 days. To extract the biosurfactant, the bacterial cells were removed by centrifugation and the remaining supernatant liquid was filtered through a 0.22 mm pore size filter (milipore).

The cell free supernatant was acidified by using 6N HCl and the pH was adjusted to 2 and keeping it at 4°C overnight. The precipitate thus obtained was pelleted by centrifugation for 20 min, dried and weighted.

For further purification the crude surfactant was dissolved 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

Biosurfactant characterization

Biochemical characterization

Determination of Protein

Protein estimation was done using crude surfactant by Lowry's method (Lowry *et al*., 1951). Protein presents in the sample form a blue coloured complex and protein as estimated with standard graph, using bovine serum albumin as standard solution.

Carbohydrate estimation

The presence of carbohydrates in the cell free extract was estimated by the method described by Chaplin and Kennedy (1994). To each hundred µl extract of *Pseudomonas aeruginosa* SCS2 were mixed with 1 ml of 5 per cent phenol and 2.5 ml of 96 per cent of concentrated H_2SO_4 and it was mixed well and incubated for 15 min. Then the absorbance was recorded at 490 nm. The distilled water with all reagents served as control. The experiment was performed in five replicates.

Estimation of free fatty acids

The free fatty acid content in the cell free extract of *Pseudomonas aeruginosa* SCS2 were estimated by titrating the extract against 0.1 N KOH using the indicator phenolphthalein until a pink colour was obtained. The free fatty acid was attained by using the formula (Sadasivam and Manickam, 1991).

$$\text{Acid value} = \frac{\text{Titre value} \times \text{Normality of KOH} \times 56.1}{\text{Weight of the sample}}$$

The sterile distilled water with all reagents served as blank. The experiment was performed in five replicates.

Rhamnolipid Estimation

The orcinol assay (Chandrsekaran and Bemiller, 1980) was used for the directly assessing the amount of glycolipids in the concentrated extract of *Pseudomonas aeruginosa* SCS2. Extracellular glycolipids concentration was evaluated in five replications. One hundred µl of each sample mixed with 900 µl of a solution containing 0.10 per cent orcinol (Prepared in 53 per cent H_2SO_4). After heating for 30 min at 80°C, the samples were cooled to room temperature and the optical density at 421 nm was measured. The sterile broth of respective culture medium without inoculum served as blank.

Activity characterization

Foaming properties of the biosurfactant from the selected isolate

The foam was produced by hand shaking a 5 g/L of crude biosurfactant solution from *Pseudomonas aeruginosa* SCS2 for several minutes. The stability of the foam was monitored by observing them for 2 hrs duration.

CMC value of the biosurfactant from the selected isolate

The CMC value was used to measure the surface tension dilutions of the cell free culture broth (Cooper *et al*., 1981; Zajic and Seffens, 1984). The CMC value was determined from the break point of the surface tension versus dilution times curve. The dilution reduces the biosurfactant levels below the CMC value, at a point in which the surface tension of the media increase suddenly. The selected isolate and the reference strain were grown in the MSM media containing 2 per cent glucose for 72 hrs in an orbital shaker at 120 rpm (M/s, Elico, India). Then, the surface tension of the media was determined earlier section after making dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}. The dilution at which the surface tension suddenly increases is the break point (CMC).

Stability characterization

To determine the thermal stability of the biosurfactant, cell free-broth of *Pseudomonas aeruginosa* SCS2 were maintained at a constant temperature range of 20 - 100°C for15 min, followed by cooling at room temperature (28 ± 2ºC).

To determine the effect of pH on biosurfactant activity, the pH of biosurfactant was adjusted (2.0 - 12) prior to filter sterilization (Whatman qualitative filter paper 42, 125 mm diameter).

The study the effect of addition of different concentration of NaCl on the activity of the biosurfactant, the biosurfactant was re-dissolved after purification with distilled water containing the specific concentration of sodium chloride (5 - 20 per cent, w/v). The surface tension and emulsification index (E_{24}%) values of each treatment were performed.

Comparison of the biosurfactant with synthetic surfactant

To determine whether the biosurfactant produced by the isolate *Pseudomonas aeruginosa* SCS2 was effective than the synthetic surfactant, the emulsification activity of the biosurfactant produced by reference strain and the isolate were compared with the synthetic surfactant standards such as SDS and Tween 80. The emulsification activity was performed.

Chemical characterization of the biosurfactant
Preparative column chromatography

For the purification of biosurfactant by column chromatography, a column of dimensions 30 x 4 cm (length x diameter) was prepared with 60 g of activated silica gel (200 - 245 mesh, 0.035 - 0.075 mm) and mixed in enough methanol. The slurry was stirred well to remove trapped air. Then, the slurry was poured into a column and packed tightly with a continuous flow of methanol. The column was allowed to stand for 24 hrs for settling. After that 1 gram of the crude biosurfactant was dissolved in 5 ml of methanol, and it was filtered with whatman filter paper (3 µm) and loaded on the silica gel column with the help of Pasteur pipette. The fraction was eluted with step wise elution using 65 - 100 per cent methanol at a flow rate of 0.5 ml/min, at room temperature (28 ± 2 ºC). The active fractions were collected and confirmed by the emulsification activity as per the procedure described in the section 3.6.8 and the purity was checked by thin layer chromatography (TLC). The purification of extract for *Pseudomonas aeruginosa* SCS2 was performed separately.

Thin layer chromatography

The preparative silica gel plates were prepared by adding 10 g of silica gel (200 -245 mesh) in enough distilled water and mixed well. The slurry was coated on a microscopic slide and allowed to dry. Ten µl column purified fractions of *Pseudomonas aeruginosa* SCS2 was separately applied at the point of origin near the bottom of the preparative silica gel plate. The silica gel plates containing column purified biosurfactant were eluted using a solution mix of chloroform-methanol-water (65:15:2, *v/v*/v), then visualized using a 50:1:0.05 (*v/v*/v) mixture of the solution glacial acetic acid-sulfuric acid-anisaldehyde at 90ºC for 30 min. The R_f value of each macromolecule was noted using the formula:

$$R_f = \frac{\text{Distance travelled by the substance}}{\text{Distance travelled by the solvent}}$$

The fraction was scrapped and eluted with chloroform: methanol (1:2, *v/v*) mixture. The solvent fraction was centrifuged for 10 min to remove the silica gel. The aliquots were micro-filtered and concentrated by air-drying. Emulsification activity was performed again for the confirmation of the biosurfactant activities.

Fourier Transform infrared (FT-IR) spectroscopy

The IR spectra of TLC-purified biosurfactant were recorded in a FT-IR spectrometer (Thermo Niocolet, AVATAR 330 FFT-IR system, Madison WI 53711 - 4495) in the 4000 - 400 cm^{-1} spectral region using potassium bromide (kBr) solid cells. The analysis was done in The Department of Chemistry, Annamalai University, India. The concentrated biosurfactant samples of *Pseudomonas aeruginosa* SCS2 were grounded with a purified potassium bromide salt (Sigma-Aldrich) to remove scattering effects from large crystals. This powdered mixture was then pressed in a mechanical press to form a translucent pellet through which the beam of the spectrometer can be passed. The spectra were recorded for the different pellets and analyzed using the standard methods described by the previous authors (Lang and Wagner, 1987; Jenny, 1988; Lin *et al.*, 1994; Yin *et al.*, 2008; Pornsunthorntawee , 2009).

HPLC analysis

The TLC fraction was further tested for their purity by an HPLC analysis. The gradient HPLC (SHIMADZU RF-10 AXL, Japan) containing C18 column (250 mm x 4.6 mm x 5 µm 1.d.), UV/visible detector set at 244 nm. One mg of extract of *Pseudomonas aeruginosa* SCS2 was dissolved in 1 ml distilled water and 1 ml acetonitrile containing 2- bromoaceto-phenone and trimethylamine in the molar ratio of 1:4:2 (extract:2-bromoacetophenone:Et3N) and heated for 1 h at 80°C. The mixture was filtered through 0.22 µm syringe filter to remove particulate materials. The mobile phase was prepared with solution A (CH_3CN) and solution B (3.3 mM H_3PO_4). The gradient condition set as follows; 50 per cent A and 50 per cent B for 3 min, then to 100 per cent mobile phase A over 19 min and held for 5 min, followed a change to 50 per cent B over 3 min and held for 10 min. The flow rate was set at 1.0 ml min^{-1} with an injection volume of 50 µl. Standard curves was drawn using biosurfactant obtained from Hi-media, India.

Gas Chromatography-Mass Spectrum (GC-MS)

The C18 column purified biosurfactant of the isolate *Pseudomonas aeruginosa* SCS2 was subjected to GC-MS. A GC-MS (SHIMADZU, model QP 5050 A) system equipped with a fused silica capillary tube was used to analyse the components in the sample. The column condition was programmed as column oven temperature 150°C (4 min) - 4 ºC/ min, temperature of inject port 250ºC and detector port 280ºC. The peaks of the gas chromatography were subjected to mass-spectral analysis. The spectra were analyzed by NIST MS search (version 2.0) (included with NIST'02 mass-spectral

library, Agilent p/n G1033A). The analysis was done at IIT, Chennai.

Nuclear Magnetic Resonance (NMR)

The C18 column purified biosurfactant of the isolate *Pseudomonas aeruginosa S*CS2 was subjected to NMR. Complete assignments of the glycolipid signals were carried out using ^{1}H and^{13}C NMR (coupled and decoupled) by ^{1}H, ^{1}H correlation spectroscopy (COSY),^{1}H,^{1}H total correlation spectroscopy (TOCSY), and ^{1}H, ^{13}C heteronuclear multiple quantum coherence (HMQC) programs in a Bruker Avance DRX 400 MHz spectrometer with a 5 mm inverse probe. The purified samples were deuterium-exchanged by repeated evaporation in chloroform – methanol – DMSO (1:1:1, *v/v*). The NMR spectra were determined in chloroform - deuterated methanol (MeOD) (2:1, *v/v*) at 30ºC, using tetramethylsilane (TMS) as the internal standard. The analysis was done in the Department of Chemistry, Annamalai University, India.

Kinetics of biosurfactant producing isolate

The kinetics studies were carried out in batch culture for biosurfactant production from a pure substrate by both the reference strain *Pseudomonas aeruginosa* SCS2. One hundred ml of MSM broth (pH 7.0) with 2 percent glucose was prepared in different 250 ml Erlenmeyer flasks and sterilized in an autoclave. After autoclaving, each flask was added with one ml of kerosene aseptically. Then 5.0 ml of inoculum of *Pseudomonas aeruginosa* SCS2 were separately added and incubated for 3 days at room temperature over an orbital rotary shaker set at 120 rpm min^{-1}. In these experiments, variations in biomass, substrate and product concentrations were recorded over a period of 20 hrs with an interval of every 2 hrs starting from the zero hour. Five batches were maintained in order to take samples at an appropriate hour and that does not affect the statistical and experimental significance. Kinetic study was performed according to the Monad model. The experiment was designed in such a way to calculate, specific growth rate (μ).

The specific growth rates (μ) of culture was then calculated respectively, based on the plot of In (cell dry weight at log phase) versus time (hour). Values are recorded as mean from five batches. Substrate consumption ($Y_{p/s}$), product formation related to dry cell biomass ($Y_{p/x}$), bacterial growth related to substrate consumption ($Y_{x/s}$), specific substrate utilization rate (q_s) and specific product yield (q_p). Biomass were measured by dry weight method as described in section 3.7 and residual sugar and consumed sugar in the fermentation broth was estimated by Dinitrosalicyclic acid method (Miller, 1972) and expressed in g/l. Crude biosurfactant was recovered from the cell free broth as described in section 3.10 and expressed as g/l. Simultaneously surface tension reduction of the medium was recorded.

Influence of different factors on the production and activity of biosurfactant

Cell growth and the accumulation of metabolic products are strongly influenced by medium composition such as carbon sources, nitrogen sources, salinity and other growth factors. The selected isolate *Pseudomonas aeruginosa* SCS2 were 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 (E_{24}%) Further, dry cell biomass (DCBM) was estimated according to the methods

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Effect of different carbon sources and nitrogen sources

Bacterial inoculums was inoculated in MSM at optimized (pH 7.0)to which was added (2 per cent) *viz*., glycerol, sucrose, maltose, glucose, manitol, fructose, starch, sodium acetate with l ml of kerosene and incubated at room temperature (28 ± 2° C) for 3 days at 120 rpm min^{-1} in an orbital rotary shaker.

Same cultural condition added different nitrogen sources peptone, sodium nitrate, ammonium chlorde, yeast extract, ammonium nitrate, beef extract, potassium nitrate, ammonium sulphate 1 ml of keosene were added and incubated at room temperature (28 ± 2°C) for 3 days at 120 rpm min^{-1} in an orbital rotary shaker. The surface tension of the culture broth was determined initially and after 72 h. Further, dry cell biomass and the emulsification activity were determined after 72 h.

Effect of pH and temperature

For determination of optimal pH, the standardised inoculums was inoculated in MSM medium on 2 per cent glucose were prepared with different pH range (4.0, 5.0, 6.0, 7.0, 7.5, 8.0 and 8.5), then incubated at37°C on an orbital rotary shaker at 150 rpm.

After optimal pH had been determined, the bacteria were grown in MSM at optimized pH and incubated at

different temperature (25, 30, 35, 40, 45, 50°C) on an orbital shaker at 150 rpm for 5 days. The surface tension of the culture broth was determined initially and after 72 hrs. Dry cell biomass and the emulsification activity was determined after 72 hrs.

Effect of trace elements and hydrocarbon

Two hundred and fifty ml of six different types of modified MSM broth with 2 percent glucose (pH 7.0) were prepared and sterilized as described below; i) without $MgCl_2$, ii) without $MnCl_2$, iii) without $FeSO_4$, iv) without $FeSO_4$ and $MnCl_2$, v) without $FeSO_4$ and $MgCl_2$, vi) without $MgCl_2$, $MnCl_2$ and $FeSO_4$. MSM broth was prepared (pH 7.0) with 2 percent glucose as control. All the seven broths including control were added with one ml of kerosene aseptically. To that, 5.0 ml of inoculum from selected isolate *Pseudomonas aeruginosa* SCS2 was added and incubated at room temperature (28 ± 2° C) for 3 days over an orbital shaker set at 120 rpm min^{-1}.

Same cultural condition Two ml of different heavy hydrocarbons (Cyclopendane, n-hexadecane, heptanes, octane, xylene, benzene and kerosene) (Sigma-aldrich, India) were filtered, sterilized through 0.4 µm size filter and added to the flask aseptically. To that, five ml inoculum of the selected isolate *Pseudomonas aeruginosa* SCS2 was added and incubated at room temperature (28 ± 2°C) for 3 days over an orbital rotary shaker set at 120 rpm min^{-1}. Surface tension reduction of the culture broth was determined initially and after 72 hrs. Further, cell free broth emulsification activity and dry cell biomass were estimated after 72 hrs.

Results

Isolation and screening of biosurfactant producing microorganisms

The soil samples screened for biosurfactant producers were collected from oil contaminated soils of different location in Tamil Nadu India. Totally thirty strains were isolated. Among them, *Pseudomonas aeruginosa* had highest biosurfactant production and surface activity was selected for further study (Fig - 1). The isolate showed β-hemolytic activity (Fig - 3), Oil displacement test (3.2 mm) and reduction in surface tension (27.42 mN/m) in Mineral salt medium. The isolate was examined based on colony morphological and biochemical characteristics presented in Table - 2. Morphological observation revealed that the colony was circular and convex with an entire margin. The isolate was characterized as Gram negative and rod shaped bacterium. The biochemical test was carried out according to Bergey's Manual (Holt *et al.*, 1994) clearly identified the strain to be *Pseudomonas aeruginosa* SCS2.

Biosurfactant production

Biosurfactant production of the isolate was preliminary screened by haemolytic activity, the drop collapse test, oil displacement test and measuring the surface tension of the solution. In drop collapse test a flat drop was observed and in oil displacement test, a clear diameter was 3.2.The surface tension of cell-free culture, decreased from 64.78 to 27.42 mN/m. Surface tension measurement would be the best method for quantifying the biosurfactant production. The surface tension remained invariable even after 72 hrs.

Extraction of biosurfactant

The partially purified product was considered as the crude biosurfactant. For further purification, the crude biosurfactant was dissolved in 0.05 M sodium bicarbonate. After filtration, the pH of this solution was adjusted to 2.0 mL using 6 M HCl and then the solution was kept at 4°C for 24 hours. The precipitate was finally collected by centrifugation at 12500 rpm for 15 min, freeze - dried, and stored in airtight container for further use.

Biosurfactant characterization

Estimation of macromolecules

Total protein was estimated at 75.29 µg/0.1 ml, carbohydrate as 256.37 µg/ 0.1 ml and lipid as 276.0 µg/0.1 ml. Thus the overall percentage of the macromolecules is as follows: 12.5 per cent protein, 31.3 per cent carbohydrate and 56.2 per cent lipid. *Pseudomonas aeruginosa* SCS2 showed total glycolipid concentration of 2046.4 µg/0.1 ml.

Biosurfactant activity characterization

The biosurfactant produced from the isolate *Pseudomonas aeruginosa* SCS2, showed good foaming stability, the disappearance of the foam was detected after 2 hrs. The isolate *Pseudomonas aeruginosa* SCS2 showed maximum emulsification activity (72.85 per cent), the CMC value was found as 72 mg/l (Table 3)

Table 1: Isolation of biosurfactant producing bacterial isolate from crude oil enriched soil samples

Soil sample	Heterotrophic bacterial population*	Bacterial population after crude oil addition*	Bacterial isolates producing biosurfactant*	Percentage of biosurfactant producing bacterial isolates
AWS	14.13 ± 0.1	10.19 ± 0.2	6.40 ± 0.2	60.74 ± 0.4
BMS	10.64 ± 0.2	10.10 ± 0.1	6.50 ± 0.1	47.32± 0.1
SCS	14.30 ± 0.1	11.20 ± 0.3	6.30 ± 0.2	64.35 ± 0.1
MSS	12.80 ± 0.1	6.10 ± 0.3	2.40 ± 0.1	38.40 ± 0.3
CAS	10.54 ± 0.2	10.02 ± 0.2	6.25 ± 0.1	62.34 ± 0.1
KKS	8.19 ± 0.2	9.40 ± 0.3	3.20 ± 0.3	40.70 ± 0.2
STS	9.20 ± 0.2	4.20 ± 0.2	1.10 ± 0.5	20.10 ± 0.1
MPS	8.20 ± 0.2	10.40 ±0.2	4.20 ± 0.3	42.67 ± 0.2

Values are a mean of five replicates ± SD
*Population expressed in CFU × 10^6 g^{-1} dry weight of soil within column different letter after values indicate that there is a significant difference at a P value of 0.05 as determined by DMRT.

Stability characterization

The stability of the biosurfactant was tested over a wide range of temperature, different concentration of sodium chloride and at different pH. The effect of pH on the biosurfactant properties revealed that an increase in pH showed a positive effect on the emulsification activity. When pH was lowered below 4.0 it had a negative effect on biosurfactant activity. However, an increase in pH up to 12.0 did not affect the emulsification activity of the biosurfactant produced by *Pseudomonas aeruginosa* SCS2 (Table - 4).

The effect of temperature on the biosurfactant revealed that, the heat treatment of biosurfactant obtained from *Pseudomonas aeruginosa* SCS2 upto 121ºC did not affect the emulsification activity. Thus, the biosurfactant produced by the *P. aeruginosa* SCS2 was found to be thermostable. Likewise, cold treatment also had a little effect on the biosurfactant property.

The effect of sodium chloride added to the biosurfactant solutions of the isolate *Pseudomonas aeruginosa* SCS2 revealed that the emulsification activity of was not affected upto 20 per cent of sodium chloride concentration. However, above 20 percent the sodium chloride concentration affected the biosurfactant property of this strain. The emulsification activity of the biosurfactant from *Pseudomonas aeruginosa* SCS2 reduced to below 30 per cent when treated with 25 per cent sodium chloride (Table 4).

Thin Layer Chromatography

After solvent extraction, crude biosurfactant were subjected to preparative column chromatography. The various percentage of solvent methanol (55 -100 per cent) were used for elution. The biosurfactant was eluted in 85 per cent methanol. The E_{24}% was found to be 73 per cent. The active fraction was subjected to TLC. Rhamnolipid consists of one or two units of rhamnose linked to one or two fatty acid chains with C8-C14 carbon atoms, which may or may not be saturated.

The product was separated on TLC plates, it showed two predominant Ocharacteristic spots, the lower spot consisted of di-rhamnolipids with R_f value of 0.45, while the higher spot consisted of mono-rhamnolipids with the R_f value of 0.64. The spots of the *Pseudomonas aeruginosa* SCS2 were compared with the results predicted by Gunther (2005). Based on the comparison of the spot and R_f value obtained revealed the compound as rhamnolipid (Fig.5).

FT-IR spectral analysis

Fourier Transform Infrared (FT-IR) spectral analysis of the biosurfactant produced by *Pseudomonas aeruginosa* (SCS2) surface active compounds and of the referent rhamnolipids (Fig.6). In the region 3000 - 2700 cm^{-1} several C-H stretching bands of CH_2 and CH_3 groups were also observed. The deformation vibration at 1234 cm^{-1} also confirmed the presence of alkyl groups. Carbonyl stretching band was found at 1654 cm^{-1} a2 and 1072 cm^{-1} which is the characteristic peak for ester

compound. The wave number 1072 cm^{-1} indicated the presence of C-O bonds. FT-IR results that, the peak observed at 1072.5, 1234.6, 1382.5, and 2853.35, 2922.23, 3207.8 indicated the presence of protein and lipid moieties (Fig.6).

High Performance Liquid Chromatography

The major spots on TLC plates of the isolate *Pseudomonas fluorescens* MFS scraped off and extracted with chloroform: methanol (1:2, v/v) mixture, were further analyzed by the HPLC method to check their purity and relative abundance. The representative chromatogram was shown in Fig. 11. The results showed that, the *tr* of 4.737 was observed for the rhamnolipid.

Gas Chromatography-Mass Spectrometry analysis

The fatty acid compositions of the purified fractions of *Pseudomonas aeruginosa* SCS2 was determined by GC-MS. The most abundant fatty acid (around 35 per cent) was 3-hydroxydecanoic acid (C_{10}). Other fatty acids were found in smaller amounts and identified as 3-hydroxydodecanoic ($C_{12:0}$), 3-hydroxydodecenoic ($C_{12:1}$) and 3-hydroxyoctanoic acids ($C_{8:0}$). The alditol acetate derivatives of the fractions showed rhamnitol, confirming the presence of rhamnose in the glycolipid. Methylation analysis of the fraction gave rise to the derivative 2,3,4-Me_3-Rha, as indicated by the primary (*m/z* 115, 135) and secondary fragments (*m/z* 155) which indicates the presence of monorhamnolipids in which the lipid moiety is attached to the anomeric carbon. The derivatives of 3,4- Me_2-Rha, (*m/z* 185) suggests a di-rhamnolipid, with one rhamnose being substituted at O-2 by a non-reducing end unit of rhamnose .

NMR analysis

The structure of the compound in the purified fraction of the isolate *Pseudomonas aeruginosa* SCS2 was confirmed by ^{1}H and ^{13}C NMR analysis, based on HMQC, COSY, TOCSY and ROESY sequence. The ^{13}C and ^{1}H shifts are shown in Figs. 13 and 14. In the ^{13}C NMR spectrum, the signal at δ 30.6 indicated the presence of CH_2 and CH_3 groups, and the signal at δ 206.7 indicates the presence of ester and carboxylic groups. HMQC analysis showed a single anomeric signal in the region signal at δ 4.594/79.17, suggesting a β-L-Rhap-hydroxyfatty acid linkage. The configuration of the Rhap was determined by Coupled ^{13}C NMR. The most important signals were obtained by ROESY analysis, space connection was obtained by a ROESY cross peak at δ 4.594/4.517, which confirms the β-L-Rhap hydroxyfatty acid linkage and the O-substituted of the β-L-Rhap unit was confirmed at (δ 79.17) respectively.

Kinetics of biosurfactant producing isolates

The specific growth rate of *Pseudomonas aeruginosa* SCS2 was 0.36 $\mu\ h^{-1}$. The bacterial growth related to substrate consumption ($Y_{x/s}$) was 0.802 at 2 hrs for *Pseudomonas aeruginosa* SCS2 (Tables 5). Product formation related to dry cell biomass ($Y_{p/x}$) was 2.010 for *Pseudomonas aeruginosa* SCS2 at 8 hrs. The cellular production increased after 4 hrs in the isolates.

The product formation related to substrate consumption ($Y_{p/s}$) of the *Pseudomonas aeruginosa* SCS2 was found to be 0.022 at 4 hrs. It has been evident from the present study that the production yield depends on the bacterial strain as well as of nature of the carbon substrate. The specific product yield (q_s) was 0.737 *Pseudomonas aeruginosa* SCS2. Specific substrate utilization rate (q_p) of 1.441 at 20 hrs was recorded by *Pseudomonas aeruginosa* SCS2.

Effect of various factors on the production and activity of biosurfactant

Influence of different carbon source

Among the carbon source tested, the isolate *Pseudomonas aeruginosa* SCS2 with glucose as carbon source recorded the maximum dry cell biomass (4.51 g/l) followed by glycerol which recorded 3.47 g/l by *Pseudomonas aeruginosa* SCS2 and mannitol showed 3.35 g/l for *Pseudomonas aeruginosa* SCS2.The maximum surface tension reduction was recorded by the isolate *Pseudomonas aeruginosa* SCS2 (25.70 mN/m) when grown with glucose as carbon source. The next best carbon source responsible for the maximum surface tension reduction was found to be glycerol followed by Mannitol (Table 6). The highest emulsification activity (E_{24}%) was recorded in glucose. The isolate *Pseudomonas aeruginosa* SCS2 recorded 75.81 per cent. Glycerol and mannitol also enhanced the emulsification activity of the isolates.

Influence of different nitrogen source

Literature revealed that the limitation of nitrogen source leads to increase the production of biosurfactant (Ramana and Karanth, 1989). Among the inorganic nitrogen sources, sodium nitrate recorded the maximum cell growth of the isolate *Pseudomonas aeruginosa*

SCS2 (3.80 g/l). The next best nitrogen source was found to be ammonium nitrate recorded 2.65 g/l by *Pseudomonas aeruginosa* SCS2. However, ammonium chloride did not support any growth (Table 14). Among the different organic nitrogen sources evaluated yeast extract recorded higher cell dry biomass of 2.22 g/l by *Pseudomonas aeruginosa* SCS2. The next best nitrogen was found to be glutamic acid recorded 2.13 g/l by *Pseudomonas aeruginosa* SCS2. The maximum surface tension reduction of 32.06 mN/m was recorded by *Pseudomonas aeruginosa* SCS2 when grown with sodium nitrate as a nitrogen source. The next maximum surface tension reduction was recorded by the isolate *Pseudomonas aeruginosa* SCS2 (34.18 mN/m) when grown in peptone supplemented medium (Table 7). The maximum emulsification activity (E_{24}%) of 79.39 per cent by the isolate *Pseudomonas aeruginosa* SCS2 when grown with sodium nitrate as nitrogen source.

Influence of pH

The effect of pH on the production and activity of biosurfactant of the selected isolate revealed that the pH of 7.0 has shown significant influence on cell biomass, production and activity of biosurfactant. The maximum cell dry biomass was achieved by *Pseudomonas aeruginosa* SCS2 (3.94 g/l). The high surface tension reduction was recorded at a pH of 7.0 for the isolate *Pseudomonas aeruginosa* SCS2 (28.11 mN/m). The reduction in surface tension was observed in the pH range of 5.5 to 8.0 by the cultures tested. The maximum emulsification activity (E_{24}%) of 76.75 per cent was recorded for the *Pseudomonas aeruginosa SCS2* at pH7.0. The tested culture recorded less than 60 per cent emulsification activity at pH 8.0 and above (Table 8).

Influence of temperature

The temperature above 45ºC did not support cell biomass growth and the production of biosurfactant for the isolate. The maximum cell dry biomass was achieved at a temperature of 30ºC by *Pseudomonas aeruginosa* SCS2 (3.78 g/l) respectively. Temperature range 25ºC and 35ºC have been found to be suitable for production of biosurfactant. The temperature level of 30ºC was found to favour the surface tension reduction of both the isolates as they recorded the maximum surface tension reduction of the media 26.11 mN/m by the isolate *Pseudomonas aeruginosa* SCS2. The maximum emulsification activity (E_{24}%) of the biosurfactant was recorded by the isolate 75.11 per cent by *Pseudomonas aeruginosa* SCS2 when grown at a temperature of 30ºC (Tables - 9).

Influence of trace element

The presence of all the three trace elements in the media composition was greatly influenced the production of biosurfactant of the isolate tested. However, it was found that the absence of ferrous sulphate did not affect the cell biomass growth and production of biosurfactant. Whereas, magnesium chloride free media drastically reduced the quantity of biosurfactant production at 72 hrs. A maximum biosurfactant production of *Pseudomonas aeruginosa* PA2G recorded 8.72 g/l of biosurfactant production and 4.13 g/l of cell dry biomass in the medium containing all the three trace elements. It may be concluded that the absence of all the three trace elements reduced the dry cell biomass and biosurfactant production in the cultures used in the study (Table -10).

Influence of hydrocarbons

Among the different hydrocarbons tested, n-hexadecane significantly increased the dry cell biomass of 2.64 g/l for *Pseudomonas aeruginosa* SCS2. The maximum surface tension reduction was influenced by the hydrocarbon n-hexadecane. This was evidenced in the reduction of surface tension of the media when grown *Pseudomonas aeruginosa* SCS2 (31.24 mN/m). The maximum emulsification activity (E_{24}%) was recorded in n-hexadecane 69.12 per cent by *Pseudomonas aeruginosa* SCS2. The next best hydrocarbon found to be octane which was followed by kerosene, cyclopentane and heptanes in the decreasing order of preference. However, the hydrocarbons benzene and xylene did not increased the dry cell biomass of the culture tested in the study (Table 11).

Influence of vegetable oils

The effect of different vegetable oils on the dry cell biomass, production and activity of biosurfactant was presented in Table 20. Among the vegetable oils tested, soybean oil recorded the maximum dry cell biomass of *Pseudomonas aeruginosa* PA2G. The maximum surface tension reduction was achieved in soybean oil by the isolate 29.13 mN/m by *Pseudomonas aeruginosa* PA2G.

Table 2: Characterization of bacterial isolates from different soil

Isolated soil sample collected area code	Gram's reaction	Shape of the cell	Spore formation	Mobility	Carbohydrate utilization													
					Fructose	Galactose	Glucose	Inositol	Lactose	Mannitol	Maltose	Mannose	Rhamnose	Sorbitol	Sucrose	Trehalose	Xylose	Starch
AWS-1	-	Rod	-	+	-	-	-	-	-	-	-	-	+	-	+	-	-	-
AWS-2	-	Rod	-	+	-	+	+	-	-	+	-	-	-	-	-	-	+	+
AWS-3	-	Rod	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AWS-4	-	Rod	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AWS-5	-	Rod	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BMS-1	-	Rod	-	+	-	+	-	-	-	-	-	-	-	+	+	+	+	+
BMS-2	+	Rod	+	+	-	-	-	-	-	+	-	-	-	-	-	-	-	+
BMS-3	+	Rod	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-
SCS-1	-	Rod	-	+	-	+	+	-	-	+	-	-	-	-	-	-	+	+
SCS-2	-	Rod	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SCS-3	+	Rod	+	+	-	-	-	-	-	-	-	-	-	+	+	-	-	-
SCS-4	-	Rod	-	+	-	-	-	-	-	-	-	-	+	-	+	-	-	-
MSS-1	+	Rod	+	+	-	-	-	-	-	+	-	-	-	-	-	-	-	+
MSS-2	-	Rod	-	-	-	-	+	-	-	+	-	-	-	+	+	-	-	-
MSS-3	+	Cocci	-	-	-	-	+	-	+	+	+	-	-	-	-	-	-	+
MSS-4	-	Rod	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CAS -1	-	Rod	-	+	-	-	+	-	-	+	-	-	-	-	-	-	-	-
CAS -2	-	Rod	-	+	-	+	+	-	-	+	-	-	-	-	-	-	+	+
CAS-3	-	Rod	-	+	-	-	+	-	+	+	-	-	-	-	-	-	-	-
KKS-1	-	Rod	-	+	-	-	-	-	-	-	-	-	+	-	+	-	-	-
KKS-2	+	Rod	+	+	-	-	-	-	-	+	-	-	-	-	-	-	-	+
KKS-3	+	Rod	+	-	-	-	+	-	+	-	-	-	-	+	-	-	-	-
STS-1	-	Rod	-	+	-	+	+	-	-	+	-	-	-	-	-	-	+	+
STS-2	-	Rod	-	-	-	-	+	-	+	+	-	-	-	-	-	-	-	-
STS-3	-	Rod	-	+	-	-	-	-	-	-	-	-	+	-	+	-	-	-
STS-4	-	Rod	-	+	-	+	-	-	-	-	-	-	-	+	+	+	+	+
MPS-1	+	Rod	+	-	-	-	+	-	+	-	-	-	-	+	-	-	-	-
MPS-2	-	Rod	-	+	-	-	-	-	-	-	-	-	+	-	+	-	-	-
MPS-3	-	Rod	-	-	-	-	+	-	-	+	-	-	-	+	+	-	-	-
MPS-4	-	Rod	-	-	-	-	+	-	-	+	-	-	-	+	+	-	-	-

Table (Contd.)2a: Characterization of bacterial isolates from different soil

Isolated soil sample collected area code	Gelatin	Catalase	Casein	Nitrate reductase	Indole production	Methyl red	Voges-prosteauer	Citrate utilization	H_2S production	Urease test	Litmus reaction	Pigments	Cyst formation	Capsular slime production	Fluorescens	Tentative characterisation
AWS-1	-	+	-	-	-	-	-	-	-	-	-	Green	-	-	+	*Psedomonas fluorescens*
AWS-2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*Pseudomonas sp.*
AWS-3	+	-	-	-	-	-	-	-	+	-	-	+	-	-	-	*Corynebacterium sp*
AWS-4	+	+	-	-	+	+	-	-	-	-	-	Red	-	-	-	*Pseudomonas aeruginosa*
AWS-5	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	*Bacillus subtilis*
BMS-1	+	+	+	-	+	+	-	+	-	-	-	-	-	-	-	*Bacillus circulans*
BMS-2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*Pseudomonas sp.*
BMS-3	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	*Bacillus cereus*
SCS-1	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	*Bacillus megaterium*
SCS-2	+	+	-	-	+	+	-	-	-	-	-	Red	-	-	-	*Pseudomonas aeruginosa*
SCS-3	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	*Bacillus subtilis*
SCS-4	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	*Citrobacter sp.*
MSS-1	+	-	+	+	-	-	-	+	-	-	-	-	-	-	-	*Staphyllococcus sp.*
MSS-2	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	*Escherchia coli*
MSS-3	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	*Acetobacter sp.*
MSS-4	+	+	-	-	+	+	-	-	-	-	-	Red	-	-	-	*Pseudomonas aeruginosa*
CAS -1	+	-	-	-	-	-	-	-	-	-	-	Yellow	-	-	-	*Xanthomonas sp.*
CAS -2	-	+	-	-	-	-	-	-	-	-	-	Green	-	-	+	*Psedomonas fluorescens*
CAS-3	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	*Bacillus subtilis*
KKS-1	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	*Bacillus sp.*
KKS-2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*Pseudomonas sp.*
KKS-3	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	*Escherchia coli*
STS-1	-	+	-	-	-	-	-	-	-	-	-	Green	-	-	+	*Pseudomonas fluorescens*
STS-2	+	+	-	-	+	+	-	-	-	-	-	Red	-	-	-	*Pseudomonas aeruginosa*
STS-3	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	*Bacillus sp.*
STS-4	-	+	-	-	-	-	-	-	-	-	-	Green	-	-	+	*Psedomonas fluorescens*
MPS-1	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	*Citrobacter sp.*
MPS-2	+	+	-	-	-	-	-	-	-	-	-	orange	-	-	-	*Serrtia sp.*
MPS-3	+	+	-	+	-	+	-	-	-	-	-	Yellow	-	-	-	*Micrococcus sp*
MPS-4	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	*Flavobacterium*

Table 3: Screening result of isolates for biosurfactant production

Isolated soil sample collected area	Blood agar lysis	Drop collapse test	Oil preading (cm0	Surface tension (mN/m)	Hydrophobicity Index(%)	CTAB agar plates	Lipase activity	Emulsification activity(E24%)
AWS-1	-	+	3.2	29.11	0.87	+	+	74.56
AWS-2	+	+	1.81	51.01	17.52	+	+	65.23
AWS-3		-	1.4	40.21	4.23	-	-	42.13
AWS-4	+	+	3.2	29.12	0.98	+	+	73.58
AWS-5	+	+	0.7	64.78	83.12	+	+	61.25
BMS-1	+	+	0.5	70.01	84.21	+	-	60.89
BMS-2	-	+	2.1	40.32	2.67	+	+	70.13
BMS-3	+	+	0.9	71.56	96.02	+	+	63.52
SCS-1	+	+	1.7	42.02	8.90	+	+	64.58
SCS-2	+	+	3.3	27.42	0.81	+	+	75.86
SCS-3	+	+	0.6	64.91	83.56	+	+	60.23
SCS-4	-	+	1.6	46.02	9.52	-	-	43.85
MSS-1	+	-	0.7	68.53	82.95	-	-	35.36
MSS-2	-	-	2.9	32.15	2.12	-	-	39.12
MSS-3	+	-	1.6	51.02	24.16	-	-	41.22
MSS-4	+	+	2.9	30.12	2.67	+	+	72.53
CAS -1	+	-	1.9	75.23	98.56	-	-	38.53
CAS -2	-	+	3.1	27.56	0.84	+	+	74.85
CAS-3	+	+	-	64.13	83.25	+	+	69.52
KKS-1	+	-	0.9	62.13	80.24	-	+	67.52
KKS-2	+	-	1.9	40.26	1.67	+	+	70.56
KKS-3	-	+	2.7	32.16	2.06	-	-	41.23
STS-1	+	-	1.2	75.54	98.51	-	-	74.54
STS-2	+	+	3.6	29.31	0.86	+	+	70.35
STS-3	+	+	0.6	63.81	81.23	-	+	68.28
STS-4	-	+	3.0	29.34	0.93	+	+	72.65
MPS-1	-	-	1.2	47.12	9.64	-	-	51.32
MPS-2	+	+	1.5	29.11	0.87	+	+	72.45
MPS-3	-	+	0.7	51.01	17.52	-	+	45.67
MPS-4	-	-	-	40.21	4.23	-	-	34.87

Table 4: Characterisation of biosurfactant from the selected isolate

Isolates	Emulsification activity (E_{24}%)	CMC value (mg/l)	Rhamnose test	Foam stability	Emulsification activity (E_{24}%)															
					pH						Temperature					Sodium chloride concentration				
					2	4	6	8	10	12	0°C	25°C	50°C	100°C	121°C	0%	5%	10%	15%	20%	25%
Pseudomonas aeruginosa SCS2	72.50 ± 0.2	74	+	Very good	31.50 ± 0.2	41.30 ± 0.2	72.23 ± 0.2	71.02 ± 0.2	70.56 ± 0.2	69.32 ± 0.3	69.56 ± 0.3	72.56 ± 0.2	73.76 ± 0.1	71.70 ± 0.1	71.23 ± 0.1	70.42 ± 0.1	72.13 ± 0.1	72.06 ± 0.1	71.15 ± 0.2	71.02 ± 0.2	26.08 ± 0.2

Values are a mean of five replicates ± SD

Table 5: Biosurfactant production kinetics of *Pseudomonas aeruginosa SCS2*

Time (h)	Dry cell biomass (g)	Sugar consumed (g)	Yx/s(gg^{-1})	BS production (g/l)	Yp/x (gg^{-1})	Yp/s (gg^{-1})	q_s (gg^{-1} h)	q_p (gg^{-1} h)	ST (mN/m)
0	0.002	0.0	0.0	0.002	0.0	0.0	0.0	0.0	65.32
2	0.93	0.90	0.802	0.010	0.012	0.012	0.005	0.441	60.12
4	1.40	1.80	0.775	0.040	0.020	0.022	0.008	0.476	55.13
6	3.85	11.64	0.320	5.37	1.285	0.456	0.512	1.124	50.15
8	4.10	13.89	0.279	7.32	2.010	0.558	0.737	1.337	30.52
10	4.56	16.94	0.264	8.70	1.955	0.514	0.723	1.405	30.32
12	4.73	18.76	0.258	8.75	1.810	0.466	0.670	1.437	30.31
14	4.85	19.08	0.254	8.80	1.812	0.461	0.671	1.456	30.31
16	4.87	19.26	0.252	8.82	1.811	0.457	0.670	1.463	30.31
18	4.89	19.53	0.250	8.85	1.809	0.453	0.669	1.478	30.10
20	4.90	19.68	0.248	8.86	1.808	0.450	0.668	1.441	29.30

BS- Biosurfactant; Yx/s – Bacterial growth related to substrate concentration; BS production Yp/x – product formation related to dry cell biomass; Yp/s – product formation related to substrate consumption; qs – specific substrate utilization; qp – specific product yield; ST –surface tension.

Table 6: Effect of carbon* source on cell biomass, biosurfactant production and activity

Carbon source (2%)	ST (mN/m) (Control)	*Pseudomonas aeruginosa* SCS2		
		DCBM (g/l)	ST (mN/m)	E_{24} (%)
Fructose	69.21 ± 0.2	2.42 ± 0.2[c]	32.33 ± 0.3[e]	53.19 ± 0.5[e]
Glucose	67.13 ± 0.2	4.51 ± 0.1[a]	25.70 ± 0.6[a]	75.81 ± 0.5[a]
Starch	67.12 ± 0.1	1.46 ± 0.4[d]	42.60 ± 0.2[f]	35.67 ± 0.4[f]
Mannitol	68.31 ±0.2	3.35 ± 0.3[b]	34.52 ± 0.4[b]	65.80± 0.2[c]
Glycerol	67.35 ± 0.3	3.47 ± 0.2[b]	32.11 ± 0.1[b]	66.43 ± 0.3[b]
Sucrose	69.12 ± 0.2	3.12 ± 0.1[b]	30.36 ± 0.3[c]	60.19 ± 0.2[d]
Maltose	69.81 ± 0.1	2.46 ± 0.1[c]	31.33 ± 0.3[d]	51.20 ± 0.2[d]
Sodium acetate	69.81 ± 0.1	3.56 ± 0.1[b]	31.23 ± 0.4[c]	65.18 ± 0.1[c]

*- 2 per cent concentration

DCBM – Dry cell biomass; ST – Surface tension; 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 7: Effect of nitrogen* source on cell biomass, biosurfactant production and activity

Nitrogen (1%)	ST (mN/m) (Control)	*Pseudomonas aeruginosa* SCS2		
		DCBM(g/l)	ST(mN/m)	E_{24}(%)
NH_4Cl	62.17 ± 0.7	1.61 ± 0.4[e]	50.32 ± 0.5[d]	41.23 ± 0.6[d]
$NaNO_3$	62.84 ± 0.6	3.80 ± 0.3[a]	32.06 ± 0.3[a]	79.39 ± 0.3[a]
NH_4NO_3	61.27 ± 0.2	2.65 ± 0.1[b]	52.17 ± 0.2[e]	31.26 ± 0.2[e]
Yeast extract	62.40 ± 0.3	2.22 ± 0.3[c]	36.31 ± 0.2[c]	49.12 ± 0.1[c]
Peptone	62.02 ± 0.1	1.96 ± 0.1[d]	34.18 ± 0.3[b]	56.12 ± 0.1[b]
Glutamic acid	62.06 ± 0.3	2.13 ± 0.2[c]	54.32 ± 0.2[f]	30.14 ± 0.1[f]
Beef extract	62.05 ± 0.1	1.56 ± 0.1[f]	55.18 ± 0.3[g]	29.12 ± 0.1[g]
Potassium nitrate	62.12 ± 0.3	2.10 ± 0.2[c]	52.12 ± 0.2[f]	30.12 ± 0.1[f]

*- one per cent concentration

DCBM – Dry cell biomass; ST – Surface tension; 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 8: Effect of pH on cell biomass, biosurfactant production and activity

pH	ST (mN/m) (Control)	*Pseudomonas aeruginosa* SCS2		
		DCBM(g/l)	ST(mN/m)	E_{24} (%)
5.0	61.70 ± 0.4	2.14 ± 0.1[d]	34.18 ± 0.1[d]	67.21 ± 0.2[c]
5.5	60.24 ± 0.2	3.20 ± 0.2[b]	30.10 ± 0.2[b]	70.13 ± 0.1[b]
6.0	61.19 ± 0.2	2.56 ± 0.5[c]	32.18 ±0.3[c]	68.15± 0.1[c]
6.5	61.03 ± 0.2	3.70 ± 0.5[a]	28.17 ±0.3[a]	75.10± 0.4[a]
7.0	61.24 ± 0.2	3.94 ± 0.3[a]	28.11± 0.3[a]	75.16 ±0.5[a]
7.5	61.55 ± 0.2	2.25 ± 0.2[d]	35.25 ± 0.3[d]	64.23 ± 0.3[d]
8.0	61.71 ± 0.5	1.83 ± 0.5[e]	38.17 ±0.3[e]	57.18 ± 0.2[e]
8.5	61.82 ± 0.5	1.80 ± 0.5[e]	41.13 ± 0.2[f]	51.11 ± 0.2[f]

DCBM – Dry cell biomass; ST – Surface tension; 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 9: Effect of temperature on cell biomass, biosurfactant production and activity

Temperature (°C)	ST (mN/m) (Control)	*Pseudomonas aeruginosa* SCS2		
		DCBM (g/l)	ST (mN/m)	E_{24} (%)
25	62.12 ± 0.2	3.56 ± 0.5[a]	30.13 ± 0.2[b]	70.16 ± 0.3[a]
30	61.30 ± 0.3	3.78 ± 0.5[a]	26.11 ± 0.4[a]	75.11 ± 0.2[a]
35	62.33 ± 0.2	2.60± 0.5[b]	31.12 ± 0.3[b]	67.14 ± 0.3[b]
40	62.40 ± 0.2	2.10 ± 0.1[c]	56.15 ± 0.5[d]	45.21 ± 0.2[c]
45	62.46 ±0.3	1.29 ± 0.4[d]	43.14 ± 0.2[c]	44.16 ± 0.1[c]
50	60.13 ± 0.2	0.51 ± 0.2[e]	42.16 ± 0.2[c]	42.21 ± 0.4[d]

DCBM – Dry cell biomass; ST – Surface tension; 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 10: Effect of trace elements on cell biomass, biosurfactant production and activity

Trace elements	*Pseudomonas aeruginosa* SCS2		
	DCBM (g/l)	E_{24} (%)	BS production (g/l)
Control	4.13 ± 0.1[a]	76.16 ± 0.3[a]	8.72 ± 0.5[a]
$MgCl_2$ (0.2 g/l) free	3.52 ± 0.1[b]	56.14 ± 0.2[c]	3.61 ±0.1[c]
$MnCl_2$ (0.75 g/l) free	3.24 ± 0.1[b]	69.23 ± 0.3[b]	6.20 ± 0.2[b]
$FeSO_4$ (0.001 g/l) free	3.47 ± 0.3[b]	70.32 ± 0.2[a]	7.38 ± 0.2[b]
Fe^{2+}/Mn^{2+} free	1.83 ± 0.3[d]	51.16 ± 0.1[c]	3.78 ± 0.1[d]
Fe^{2+}/ Mg^{2+} free	2.58 ± 0.4[c]	43.21 ± 0.4[d]	2.11 ± 0.1[e]
$Mg^{2+}/Fe^{2+}/ Mn^{2+}$ free	1.54 ± 0.3[d]	38.62 ± 0.3[e]	0.21 ± 0.1[f]

DCBM – Dry cell biomass; BS – Biosurfactant production; 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 11: Effect of hydrocarbons on cell biomass, biosurfactant production and activity

Hydrocarbon source	ST (mN/m) (Control)	*Pseudomonas aeruginosa* SCS2		
		DCBM (g/l)	ST(mN/m)	E_{24}(%)
n-hexadecane	60.12 ± 0.5	2.64 ± 0.5[a]	31.24 ± 0.4[a]	69.12 ± 0.3[a]
Octane	56.81 ± 0.5	2.46 ± 0.4[a]	32.16 ± 0.2[a]	67.23 ± 0.6[b]
Kerosene	57.11 ± 0.5	1.97± 0.2[b]	35.52 ± 0.3[c]	66.14 ± 0.1[c]
Cyclopentane	56.13 ± 0.1	1.86 ± 0.2[b]	34.16 ± 0.1[c]	63.14 ± 0.1[d]
Benzene	58.15 ±0.1	0.73 ± 0.2[c]	32.14 ± 0.1[b]	56.14 ± 0.1[e]
Xylene	51.64 ± 0.1	0.68 ± 0.3[c]	37.18 ± 0.1[d]	49.24 ± 0.1[f]
Heptane	61.23 ± 0.1	2.34 ± 0.1[a]	31.18 ± 0.1[a]	67.09 ± 0.2[b]

DCBM – Dry cell biomass; ST – Surface tension; 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 12: Effect of Vegetable oils on cell biomass, biosurfactant production and activity

Vegetable oils	ST (mN/m) (Control)	*Pseudomonas aeruginosa* SCS2		
		DCBM (g/l)	ST (mN/m)	E_{24} (%)
Soybean oil	51.45 ± 0.3	3.72 ±0.3[a]	29.13 ±0.1[a]	77.21 ± 0.1[a]
Olive oil	50.92 ± 0.1	2.26± 0.1[b]	30.16 ± 0.1[a]	70.41 ± 0.1[b]
Groundnut oil	46.25 ± 0.2	2.09 ± 0.1[b]	32.82 ± 0.1[b]	68.11 ± 0.1[c]
Sunflower oil	52.16 ± 0.1	2.24 ± 0.1[b]	31.07 ± 0.1[b]	68.22 ± 0.1[c]
Gingelly oil	51.83 ± 0.2	0.83 ± 0.2[d]	33.65 ± 0.2[c]	61.23 ± 0.2[d]
Palm oil	54.12 ± 0.1	1.55 ± 0.3[c]	33.72 ± 0.1[c]	61.24 ± 0.2[d]
Castor oil	50.16 ±0.1	0.69 ± 0.2[d]	33.21 ± 0.2[c]	61.23 ± 0.2[d]
Coconut oil	53.22 ± 0.2	1.10 ± 0.1[c]	44.22 ± 0.1[d]	30.32 ± 0.2[e]

DCBM – Dry cell biomass; ST – Surface tension; 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

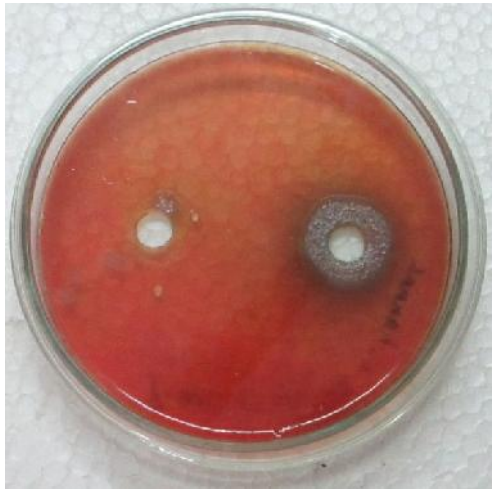

Fig: 3 Heamolysis in Blood Agar

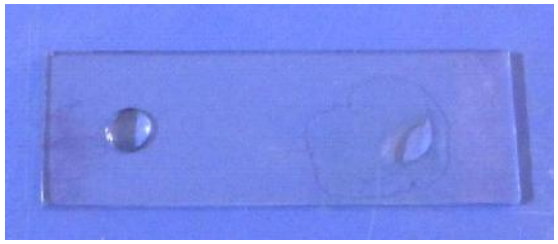

Fig .4 Drop collapse method

Fig : 5 Thin Layer Chromatography

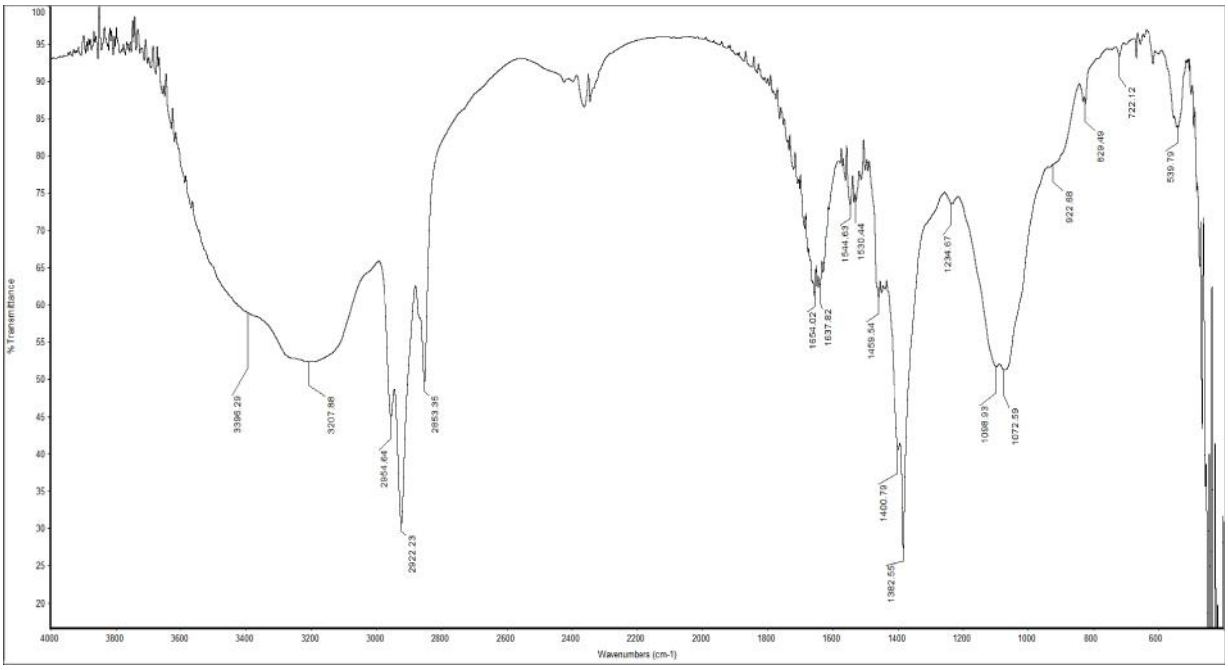

Fig: 6 FTIR spectra analysis of biosurfactant

The higher emulsification activity was recorded by the isolate *Pseudomonas aeruginosa* SCS2 (77.21 per cent) when grown in soybean oil. The second best vegetable oil influenced the growth and activity of both the cultured used in the study was olive oil, that was followed by sunflower oil and groundnut oil in the decreasing order of merit. Whereas, castor oil and palm oil did not influence the growth and activity of the culture studied (Table - 12).

Discussion

The single screening method is not suitable to identify all types of biosurfactants and recommended more than one screening methods should be included in the primary screening as to identify potential biosurfactant producers (Satpute *et al*., 2008). Therefore, in the present study the selected isolates were performed with different screening test to check the biosurfactant producing ability and to find the efficient biosurfactant producer by following the standard methods described by the earlier authors *viz.*, drop collapse test (Jain *et al.,* 1991), hemolytic activity (Yonebayashi *et al.,* 2000), CTAB plate assay (Siegmund and Wagner, 1991), oil spreading technique (Morikawa *et al.,* 2000), surface tension measurement (Haba *et al.,* 2000), glass-slide test (Persson and Molin, 1987), cell surface hydrophobicity (Pruthi and Cameotra, 1997), emulsification activity (Makkar and Cameotra, 1998) and lipase activity (Kiran *et al*., 2009).

The hemolytic activity was used as a primary method to screen the biosurfactant production (Carrillo *et al*., 1996). Blood agar lysis has been used to quantify biosurfactant (Moran *et al*., 2002) and rhamnolipids (Johnson and Boese - Marrazzo, 1980). Youseff *et al.* (2004) reported that some organisms excluded the haemolytic activity, so other screening methods are followed for the confirmation of biosurfactant production. In the present study also only seventeen isolates had shown positive for the hemolysis. Hence, it is clear from the present study that the organisms turned negative on blood agar lysis may be positive for surface tension reduction and emulsification activity

The oil spreading techniques measures the diameter of clear zones caused when a drop of a biosurfactant containing solution is placed on oil - water surface (Bodour and Miller-Maier, 1998; Morikawa *et al*., 2000). This method is easy and can be used to screen a large number of samples (Bodour *et al*., 2003). The reduction of surface tension in the liquid- liquid interface leads to complete spreading of liquid drop over the surface of oil (Yosuff *et al.,* 2004). So, the drop collapsing and oil displacement tests are the easiest and effective method to screen biosurfactant producers.

In the present study three isolates showed negative for oil spreading technique and eighteen isolates showed positive for drop collapse test

The measurement of surface tension has traditionally been used to detect biosurfactant production and most of the other methods measure the surface properties of biosurfactant. Hence, the surface tension reduction method had been used by various earlier researchers (Willumensen and Karlson, 1997; Makkar and Cameotra, 1998, 1999a). Regarding the surface tension reduction ability, in the present study, all the isolates lowered the surface tension of the medium when compared to the control and for the emulsification activity all the isolates showed positive results. The similar results of emulsification activity of crude oil by an alkaline - oxidizing *Rhodococcus* sp. reported was by Kuyukin (2001). Based on the screening results, the isolate *Pseudomonas aeruginosa* SCS2 was selected for the further experiment as they showed maximum biosurfactant producing ability, which is evident by the highest surface tension reduction measurements.

The TLC chromatogram of biosurfactant produced by the isolate *Pseudomonas aeruginosa* SCS2 revealed that, the lower spot consisted of di-rhamnolipids with R_f value of 0.48, while the higher spot consisted of mono-rhamnolipids with the R_f value of 0.65. This data fully comply with the previously reported results of TLC of rhamnolipids from different strains of *Pseudomonas aeruginosa* SCS2 (Silva *et al.,* 2010; Hoskova *et al.,* 2013).

The FT-IR spectrum produced by the isolate *P. aeruginosa* SCS2 suggested that, the functional group present were of glycolipid type. The spectra were recorded and analyzed using the standard methods described by the earlier authors (Lin *et al.,* 1994; Yin *et al*., 2008; Pornsunthorntawee *et al.,* 2009). The authors suggested characteristic peaks for rhamnolipids as the important adsorption bands to be located at 3468, 2922, 2853, 1743 and 1300-1100 cm^{-1} which will indicate chemical structures identical to those of rhamnolipids. The characteristic peak displayed at 1743 cm^{-1} should be related to the C=O stretching vibrations of the carbonyl groups while the C-O stretching bands at 1300 - 1000 cm^{-1} will confirm the presence of the bonds formed between carbon atoms and hydroxyl groups in the chemical structures of the rhamnose rings. The biosurfactant produced by the isolate strain *P. aeruginosa* SCS2 in the present study were quite similar to these descriptions. The results obtained in the present study on the biosurfactant production by the isolate were further corroborates with the findings of Rodrigues *et al.* (2006); Pornsunthorntawee *et al.* (2008) and Oliveria *et al.* (2009b).

In the HPLC analysis, the representative chromatogram from the isolate *Pseudomonas* aeruginosa SCS2 showed the peak with the retention time of 4.737 was observed for the rhamnolipid. Similar results were observed in some of the previous studies revealed that the active ingredient of biosurfactant produced by *P. aeruginosa* is a rhamnolipid (Mulligan, 2005; Wei *et al.,* 2005; Bodour *et al*., 2004).
In the present study, the fatty acid compositions of the purified fractions were determined by GC-MS. The most abundant fatty acid (around 35 per cent) was 3-hydroxydecanoic acid (C_{10}). Other fatty acids were found in smaller amounts and identified as 3-hydroxydodecanoic ($C_{12:0}$), 3-hydroxydodecenoic ($C_{12:1}$) and 3-hydroxyoctanoic acids ($C_{8:0}$). The alditol acetate derivatives of the fractions showed rhamnitol, confirming the presence of rhamnose in the glycolipid. Methylation analysis of the fraction at m/z 115,135 and 155 indicates the presence of monorhamnolipids and the observation at m/z 185 suggest the presence of di-rhamnolipids. Similar results, at least at a qualitative level, were reported by Monteiro *et al.* (2007). They found decanoic acid in an amount of 68 per cent total fatty acids. The present results also support the earlier reports of Kiran *et al*. (2010a) and Jain *et al*. (2012a).
The NMR analysis of the biosurfactant from the isolate *Pseudomonas aeruginosa* revealed that, the signal at δ 30.6 indicated the presence of CH_2 and CH_3 groups, and the signal at δ 206.7 indicates the presence of ester and carboxylic groups in ^{13}C NMR spectrum. HMQC analysis showed a single anomeric signal in the region signal at δ 4.594/79.17, suggesting a β-L-Rhap-hydroxyfatty acid linkage. ROESY cross peak at δ 4.594/4.517, which confirms the β-L-Rhap hydroxyfatty acid linkage and the O-substituted of the β-L-Rhap unit was confirmed at (δ 79.17), respectively. NMR characteristic spectral peaks of biosurfactant were in accordance with the previous studies of Lin *et al.* (1994), Mishra *et al*. (2011) and Jain *et al.* (2012b).

The carbon source plays an important role in biosurfactant production (Itoh and Suzuki, 1974). Slight differences in the maximum cell biomass and biosurfactant production could be observed as the initial glucose concentration increased above the optimum level (Guerra Santos *et al.,* 1986). The type of carbon substrate used for production has been reported to influence both the quality and quantity of biosurfactants (Panilaitis *et al.,* 2007; Abouseoud *et al*., 2008).

The choice of nitrogen source has been reported to affect the biosurfactant production (Abouseoud *et al.,* 2008). Reports have shown that rhamnolipid production is more efficient under nitrogen-limiting conditions (Benincasa *et al*., 2002; Kim *et al*., 2006). In the present study, the influence of inorganic and organic nitrogen sources plays an important role in the production of biosurfactant. Sodium nitrate with 1 per cent concentration recorded maximum dry cell biomass. Among the organic nitrogen sources, yeast extract recorded comparatively higher cell dry biomass by the isolates. Yeast extract was found to be required for glycolipid production by *Torulopsis bombicola*, but was very poor in *P. aeruginosa* (Syldatk *et al.,* 1985; Desai and Desai, 1993). Whereas in the present study, yeast extract recorded maximum dry cell biomass by both the isolate and the reference strain. This may be selective induction of biosurfactant from the use of yeast extract. This study was lending evidence for the yeast extract mediated production of biosurfactant from the much elaborate works of Mulligan (2005) and Desai and Banat (1997).

The temperature level of 30ºC was found to favour the surface tension reduction of both the isolate and the reference strain, as they recorded the maximum surface tension reduction of the media 26.11 mN/m *Pseudomonas aeruginosa* SCS2.The maximum emulsification activity (E_{24}%) of the biosurfactant was recorded by the isolate *Pseudomonas aeruginosa SCS2* of 76.02 per cent, when grown at a temperature of 30ºC. This study supports the previous work of Pornsunthorntawee *et al.* (2008). Likewise Similar work as the present was reported by Guerra-Santos *et al.* (1986). The maximum rhamnolipid production by *Pseudomonas aeruginosa* cultivated in manitol at 34.5ºC with a great reduction in temperatures above 36ºC.

In the present study, the effect of trace elements on the maximum biomass, higher surface tension reduction and the maximum emulsification activity was observed in the presence of all the three elements in the media composition. This result supports the report suggested by Wei *et al.,* (2007), that the trace elements Mg^{2+}, K^{+}, Mn^{2+}, and Fe^{2+} were found to be more significant factors affecting surfactin production by *B. subtilis*.

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