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ORIGINAL ARTICLE

Isolation of biosurfactant-producing *Pseudomonas aeruginosa* RS29 from oil-contaminated soil and evaluation of different nitrogen sources in biosurfactant production

Rashmi Rekha Saikia · Suresh Deka · Manab Deka · Ibrahim M. Banat

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Abstract An efficient biosurfactant-producing native *Pseudomonas aeruginosa* RS29 has been isolated from crude oil contaminated soil. Isolation was followed by optimization of different factors to achieve maximum production of biosurfactant in terms of surface tension reduction (STR) and emulsification index (E24). The isolated strain produced highest biosurfactant in the presence of glycerol after 48 h of incubation at 37.5°C, with pH range of 7–8 and at salinity <0.8% (w/v). The extent of STR and the E24 of medium with different nitrogen sources were investigated and found to be maximal for sodium nitrate (26.3 mN/m, E24=80%) and potassium nitrate (26.4 mN/m, E24=79%). The production of biomass by the designated strain was found to be maximal in ammonium-nitrate-containing medium as compared to the other nitrogen sources. A kinetic study revealed that biosurfactant production is positively correlated with growth of *P. aeruginosa*, and highest STR was achieved (27.0 mN/m) after 44 h of growth. The biosurfactant was produced as a primary metabolite and 6 g/L crude biosurfactant was extracted by chloroform:methanol (2:1). The critical micelle concentration of the biosurfactant was 90 mg/L. The absorption bands of the FTIR spectra confirmed the rhamnolipid nature of the biosurfactant. The biosurfactant was thermostable (up to 121°C for 15 min) and could withstand a wide range of pH (2–10) and NaCl concentration (2%–10% w/v). The extracted biosurfactant had good foaming and emulsifying activities and was of satisfactory quality in terms of stability (temperature, pH and salinity) and foaming activity.

Keywords Isolation · Biosurfactant · *Pseudomonas aeruginosa* RS29 · Rhamnolipid

R. R. Saikia (✉) · S. Deka
Life Sciences Division, Institute of Advanced Study in Science and Technology,
Paschim Boragaon,
Guwahati 781035 Assam, India
e-mail: rashmi.saikia@gmail.com

M. Deka
Department of Biotechnology, Gauhati University,
Guwahati 781014 Assam, India

I. M. Banat
Faculty of Life and Health Sciences, University of Ulster,
Coleraine BT52 1SA Northern Ireland, UK

Introduction

Biosurfactants are diverse groups of surface-active molecules/chemical compounds synthesized by microorganisms (Desai and Banat 1997). These are amphipathic molecules with both hydrophilic and hydrophobic domains. Biosurfactants reduce surface tension and critical micelle dilution (CMD) in both aqueous solution and hydrocarbon mixtures. These properties create micro-emulsions in which micelle formations occur in which hydrocarbons can solubilize in water or water in hydrocarbons (Banat 1995). Microorganisms have been reported to produce several classes of biosurfactants, such as glycolipids, lipopeptides, phospholipids, neutral lipids or fatty acids and polymeric biosurfactants (Cooper and Zajic 1980; Cooper 1986; Kosaric 1993). These compounds are metabolic products produced during the growth of microorganisms on water-soluble and water immiscible substrates (Sheppard and Mulligan 1987; Jenny et al. 1993; Ron and Rosenberg 2001).

The most commonly isolated and the best studied groups of biosurfactants are mainly glycolipids and phospholipids

in nature. Nevertheless, rhamnolipids are glycolipid compounds produced by *Pseudomonas* sp. that can reduce water surface tension and emulsify oil (Babu et al. 1996; Deziel et al. 1999; Lang and Wullbrandt 1999; Mata-Sandoval et al. 1999; Rahman et al. 2002; Perfumo et al. 2006). Biosurfactants are environmentally friendly and have potential industrial and environmental applications.

When compared to synthetic surfactants, biosurfactants have several advantages, including high biodegradability, low toxicity, low irritancy, and compatibility with human skin (Banat et al. 2000; Cameotra and Makkar 2004). Therefore they are superior to their synthetic counterparts.

Many studies have been conducted with microorganisms to ensure the production and activity of biosurfactants, emphasizing the importance of the need for these compounds. However, we have failed to identify any literature report dealing solely with native bacterial isolates. Exploring native strains for biosurfactant production could be of great importance since native strains can be assumed to perform better in their native environment than other exotic strains. Therefore, the present experiment was conducted to isolate potent biosurfactant-producing native bacteria and to identify the optimal environmental factors—temperature, pH, salinity, nitrogen source and carbon source—for maximum production of biosurfactant. Besides other objectives, the nature of the produced biosurfactant was also characterized, along with production kinetics and efficacy studies.

Materials and methods

Soil sample

Crude oil-contaminated soil samples were collected from the Lakowa oil fields of Assam, India. Soil samples were collected at a depth of 0–10 cm from the soil surface and stored at room temperature (25±2°C) for subsequent use.

Isolation and screening of biosurfactant-producing bacteria

Bacteria were isolated by adding 1 g collected soil sample to a flask containing nutrient broth and mineral salt solution at a 1:1 ratio and crude oil (2% w/v) was provided as a sole carbon source; the enriched flask was incubated at 35°C for 4 days in a rotary shaker Scigenics Biotech, ORBITEK LJEIL at 150 rpm. From this original flask, 5 mL cultures were subsequently used to inoculate a second flask containing fresh medium, and this flask was maintained under the same conditions. This process was repeated four times, and each time same amount of culture was withdrawn from the older flask and added to a new flask with fresh medium. Cultures from the last enriched flask were plated on nutrient agar using a serial dilution technique. Morphologically different individual bacterial colonies were isolated from the agar plates and streaked on nutrient agar to obtain pure cultures of the isolates. The isolates were grown in nutrient broth for 48 h at 35°C at 150 rpm and used as inoculum for further experiments.

Proper screening of the biosurfactant-producing bacterial isolates was carried out by adding 10 mL inoculum of each isolate and growing them in 500 mL flasks containing 100 mL sterilized mineral medium with glucose as the sole carbon source. Cultivations were performed in triplicate. The composition of the mineral medium used was as follows (g/L): NH_4NO_3 (4.0), KCl (0.1), KH_2PO_4 (0.5), K_2HPO_4 (1.0), $CaCl_2$ (0.01), $MgSO_4{\cdot}7H_2O$ (0.5), $FeSO_4{\cdot}7H_2O$ (0.01), Yeast extract (0.1) and 10 mL of trace element solution containing (g/L): 0.26 g H_3BO_3, 0.5 g $CuSO_4{\cdot}5H_2O$, 0.5 g $MnSO_4{\cdot}H_2O$, 0.06 g $(NH_4)6Mo_7O_{24}{\cdot}4H_2O$ and 0.7 g $ZnSO_4{\cdot}7H_2O$. The pH of the medium was adjusted to 7.0±0.2 and the flasks were kept in a shaking incubator at 35°C and 150 rpm for 7 days. Screening was based on surface tension reduction (STR); surface tension was measured every day up to the 7th day using a K11 tensiometer (Kruss, Hamburg, Germany) and the plate method. All chemicals were purchased from Merck (Mumbai, India).

Identification of efficient isolates

The most efficient bacterial strain was identified by (1) studying the morphological and physiological characteristics (Cappuccino and Sherman 1999), and (2) sequencing 16S rDNA and aligning the sequence in the NCBI GenBank and RDP database (performed by Bangalore GeNei, India). Following a standard protocol, genomic DNA was extracted from a pure culture, and a 16S rDNA fragment of ~1.5 kb was amplified using *Taq* DNA polymerase and consensus primers. The PCR product was bi-directionally sequenced using the forward, reverse and an internal primer. Sequence data was aligned and analyzed to find the closest homolog of the isolated bacterial strain.

Optimization of growth conditions

Different carbon sources were tested to select the one most suitable for maximum STR. The carbon sources used in the experiment were glucose, glycerol, mannitol, n-hexadecane and sludge. These were added to the mineral medium to 2%. The inoculated mineral medium was incubated in a rotary shaker at 35°C and 150 rpm for 4 days and the surface tension was recorded every day. To compare STR, flasks with the same carbon sources but no bacterial culture (control) were also studied.

A range of temperatures was investigated to determine the optimum temperature for maximum production of biosurfactant. Production of biosurfactant was measured in terms of STR and emulsification index (E24). Efficient strains growing in mineral medium with a suitable carbon source were incubated at the following temperatures: 25, 27.5, 30.0, 32.5, 35.0, 37.5, 40.0, 42.5 and 45.0°C at 150 rpm. The initial pH of the mineral medium with a suitable carbon source was investigated by adjusting the pH of the medium within the range of 2–9. The pH of the medium was adjusted using 1 M HCl and 1 M NaOH solutions. The cultures were kept in the shaking incubator at the optimum temperature at 150 rpm. Optimum salinity was determined by changing the concentration of NaCl of the mineral medium from 0.2% to 5% (w/v). The efficient strain was grown in medium with a suitable carbon source. The initial pH of the medium was adjusted and the culture was incubated in the rotary shaker at 150 rpm at the optimum temperature. Surface tension and E24 were measured in each flask. E24 was measured by mixing 3 mL whole bacterial culture with an equal volume of n-hexadecane and vortexing at high speed for 2 min. The sample was left undisturbed for 24 h. The E24 is expressed as the percentage of the height of the emulsified layer (mm) divided by the total height of the liquid column (mm) (Cooper and Goldenberg 1987; Abouseoud et al. 2008a).

Optimization of growth conditions was followed by a kinetic study of biosurfactant production as well as production of biomass. These parameters were investigated by growing the efficient strain in mineral medium with a suitable carbon source and maintaining all the optimum conditions. The kinetics of biosurfactant production were studied in batch cultures over a period of 72 h. Surface tension and optical density at 460 nm (OD_{460}) were measured at 4-h intervals. Surface tension was measured in tensiometer and OD_{460} was measured in a SHIMADZU UV-1800 UV-spectrophotometer. E24 was also recorded. Dry cell biomass was determined by centrifugation of an aliquot (100 mL) of 48-h-old bacterial culture at 8,000 rpm for 20 min. The cell pellet was washed with n-hexane to remove any slimy materials attached to the cell surface that might cause error in the assessment. The washed cells were resuspended in sterilized distilled water and centrifuged again. The pellet was oven-dried at 105°C for 4 h and weighed (Raza et al. 2007).

Evaluation of nitrogen sources

Different nitrogen sources, namely ammonium nitrate, ammonium chloride, ammonium sulfate, sodium nitrate and potassium nitrate, were investigated for maximum production of biosurfactant. For this experiment, the nitrogen salts were used at 4% (w/v) concentration. A control was also studied where no nitrogen salts were added to the mineral medium. Variation in pH of the culture medium, the E24, OD as well as the surface tension of the medium were measured.

Biosurfactant extraction and characterization

Crude biosurfactant was extracted from cell-free culture broth (CFCB) by chloroform:methanol (2:1) extraction three times. CFCB was obtained by centrifuging 48-h-old bacterial culture grown on glycerol at 8,000 rpm for 20 min at 4°C. The combined extracts were then transferred to a round-bottom flask connected to a rotary evaporator. The concentration process was continued at 40°C until a yellowish-brown-colored viscous and consistent extract was obtained. The crude biosurfactant was then dried and weighed.

The extracted biosurfactant was dissolved in distilled water at concentrations ranging from 1.0 to 200 mg/L for calculation of critical micelle concentration (CMC). This is a direct measurement of surfactant concentration corresponding to the concentration of an amphiphilic component at which the formation of micelles is initiated in the solution (Abouseoud et al. 2008a). The CMC of the produced biosurfactant was determined following standard methods (Kim et al. 1997; Bonilla et al. 2005). The critical micelle dilution (CMD) is defined as the solubility of a surfactant in an aqueous phase and is commonly used to measure the efficiency of a surfactant (Desai and Banat 1997). CMD^{-1} and CMD^{-2} were determined by measuring the surface tensions of cell free supernatant diluted 10-times and 100-times in distilled water (Kosaric 1993). Cell free supernatant was obtained by centrifuging a 48-h-old culture of the strain grown in mineral medium with glycerol at 8,000 rpm for 20 min at 4°C.

Carbohydrate moieties in the biosurfactant molecule were assayed using rhamnose (Dubois et al. 1956) and Molisch's test. The rhamnose test was performed by adding 0.5 mL cell supernatant to 0.5 mL 5% phenol solution and 2.5 mL sulfuric acid and incubating the sample for 15 min before measuring absorbance at 490 nm. Molisch's test was performed by adding 3 mL cell free supernatant to 1 mL 10% α-naphthol. This was followed by the addition of 1 mL concentrated sulfuric acid to the sample without disturbing it.

The crude biosurfactant extracted with chloroform: methanol was analyzed by thin layer chromatography (TLC). The TLC tank was filled with a solvent mixture of chloroform:methanol:acetic acid:water (25:15:4:2 v/v/v/v). The chromatogram was sprayed with α-naphthol and sulfuric acid. The crude biosurfactant was also analyzed

in a Bruker Vector 22 FTIR-Spectrometer. The spectral region used was 4,000–400 cm^{-1} at a resolution 4 cm^{-1} using a KBr plate of 0.26 mm thickness.

Activity characterization

Foam was produced by hand shaking a 2-day-old culture supernatant for a few minutes. The stability of the foam was monitored by observing it for 48 h. Thermal stability of the biosurfactant was determined by autoclaving cell-free culture supernatant at 121°C for 15 min. The effect of pH and salinity on stability of the biosurfactant was evaluated by altering the pH (2–10) and the concentration of NaCl (2%–10%) of the cell free culture supernatant and measuring the surface tension (Bordoloi and Konwar 2008).

Results and discussion

Isolation, screening and identification of biosurfactant-producing bacteria

Table 1 lists 29 morphologically different bacterial isolates that were screened out from the collected soil samples. Among these, isolate no. 29, which could reduce the maximum surface tension of the mineral medium from 71.1 to 31.4 mN/m, was selected as the most potent biosurfactant-producing strain. The morphological and physiological patterns of the strain showed a high similarity to *Pseudomonas aeruginosa* (99%). When the partial 16S rDNA gene sequence was aligned with the NCBI GenBank and RDP databases, ten of the top ten matches were to *Pseudomonas aeruginosa* strains. Since the sequence similarities to *Pseudomonas aeruginosa* were uniformly 100%, the unknown strain was confirmed as *Pseudomonas aeruginosa* RS29.

Growth conditions optimization

Glycerol was found to be the best carbon source on which to reduce maximum surface tension (62%). Glucose and mannitol could reduce the surface tension of mineral medium to 57% and 56%, respectively. Growth of the strain on hexadecane resulted in a 37% decrease in surface tension and a poor result was recorded for sludge (19%) (Table 2). These results indicated that the strain produces maximum biosurfactant on water-soluble substrates. Biosurfactant production reached its maximum at 37.5°C (Fig. 1), with an optimum initial pH range of 7.0–8.0 (Fig. 2). This is in agreement with a previous report that maximum biosurfactant production by *Pseudomonas aeruginosa* 181 was achieved after 120 h of incubation at pH 7.0 and 37°C (Al-Araji and Issa 2004). For *Bacillus subtilis*, the optimal production of biosurfactant was observed at pH 7 (Makkar and Cameotra 2002). Addition of NaCl to the mineral medium had no influence on the enhancement of biosurfactant production. On the contrary, NaCl supplementation lowered production at concentrations <0.8% (w/v) (Fig. 3), suggesting that either the strain cannot tolerate salinity or that it cannot produce biosurfactant at higher salinity. This is in agreement with a result reporting that limiting the concentrations of salts of magnesium, calcium, potassium, sodium and trace elements resulted in a better yield of rhamnolipid in *P. aeruginosa* DSM 2659 (Guerra-Santos et al. 1986).

The surface tension dropped rapidly at about 20 h of growth, reaching its lowest value (27.0 mN/m) during exponential phase after about 44 h of growth. At the stationary phase of growth, no further decrease in surface tension was recorded. Surface tension gradually increased at this phase of bacterial growth (Fig. 4). As seen in Fig. 4, with the growth of the bacteria, the E24 gradually increased with the decrease in surface tension. The E24 reached a maximum (76%) at 68–72 h of growth and then began to decrease with the increase in surface tension. The E24 plot, a measure of the biosurfactant concentration, also showed that the surfactant was not present initially in sufficient amount to form micelles (Abouseoud et al. 2008b), but with the growth of the bacterial culture, the concentration of surfactant in the medium increased, thus increasing the E24. These results indicated that biosurfactant production in glycerol occurred predominantly during the exponential growth phase, suggesting that the biosurfactant was produced as a primary metabolite accompanying cellular biomass formation (growth-associated kinetics). The dry cell biomass obtained was 3.7 g/L.

Evaluation of nitrogen sources

Maximum STR of the medium was achieved in 24 h of bacterial growth using sodium or potassium nitrate as nitrogen source (Table 3). With ammonium nitrate, maximum STR was obtained in 48 h; thereafter surface tension started increasing to a certain extent. The control with no nitrogen source showed a gradual decrease in surface tension. STR for ammonium chloride- or ammonium sulfate-containing media was less prominent compared with other nitrogen sources.

As seen in Table 3, the pattern of change in pH of the mineral medium was different for different nitrogen sources. With ammonium chloride and ammonium sulfate, pH of the medium decreased abruptly from 7.5 to approximately 5.0 within 24 h of bacterial growth. In the control, the initial pH of the medium was retained almost for the whole period of 96 h. With ammonium, sodium and potassium nitrate, pH of the medium increased by 24 h then

Table 1 Surface tension of 29 bacterial isolates on glucose-containing mineral medium over a period of 7 days. Results are presented as mean ± SD of three replicates

Isolate no.	Day							
	0	1	2	3	4	5	6	7
C[a]	71.1±0.30	71.0±0.24	71.0±0.40	69.9±0.30	69.9±0.23	70.0±0.20	69.8±0.32	69.8±0.04
1	65.0±0.25	60.0±0.20	55.8±0.50	46.3±0.40	45.0±0.43	45.0±0.33	48.9±0.30	50.0±0.30
2	65.5±0.50	56.3±0.09	56.0±0.20	53.9±0.24	53.0±0.32	53.2±0.22	48.3±0.20	40.8±0.30
3	67.9±0.40	69.4±0.44	66.5±0.43	65.0±0.31	65.0±0.33	63.7±0.26	62.2±0.40	60.0±0.21
4	68.8±0.16	64.0±0.23	61.8±0.43	61.0±0.33	62.2±0.43	63.0±0.27	60.7±0.44	69.5±0.20
5	71.2±0.19	71.0±0.30	70.4±0.41	70.1±0.50	69.0±0.50	69.0±0.30	69.8±0.32	57.2±0.20
6	71.3±0.25	71.0±0.51	54.1±0.40	53.4±0.30	52.4±0.30	55.5±0.15	57.0±0.20	44.5±0.22
7	60.3±0.16	45.9±0.21	47.0±0.20	46.0±0.30	45.0±0.20	43.2±0.30	41.4±0.24	56.4±0.32
8	69.8±0.12	68.5±0.17	60.1±0.24	60.0±0.43	62.7±0.41	59.8±0.16	57.7±0.25	69.5±0.40
9	69.3±0.50	69.5±0.46	70.6±0.34	70.6±0.40	70.0±0.30	69.7±0.40	69.5±0.30	54.0±0.30
10	68.3±0.25	52.2±0.30	53.7±0.40	51.8±0.30	45.0±0.16	48.3±0.30	53.5±0.40	62.2±0.31
11	69.9±0.51	56.6±0.26	60.2±0.40	60.0±0.30	60.0±0.37	61.7±0.40	62.0±0.21	61.8±0.40
12	60.8±0.15	57.7±0.21	60.4±0.16	57.8±0.23	57.0±0.16	59.5±0.24	61.0±0.40	57.1±0.22
13	70.9±0.50	70.2±0.47	61.2±0.50	61.0±0.35	55.5±0.25	55.0±0.34	57.1±0.23	44.7±0.40
14	69.4±0.16	59.2±0.24	58.6±0.22	57.1±0.35	56.2±0.40	55.0±0.08	54.7±0.12	57.4±0.05
15	65.9±0.06	63.8±0.17	61.3±0.07	55.3±0.11	55.0±0.22	45.0±0.17	46.3±0.41	54.5±0.15
16	71.2±0.40	70.5±0.30	66.5±0.31	66.2±0.23	65.0±0.33	60.9±0.06	57.0±0.32	57.0±0.17
17	69.4±0.21	66.0±0.16	62.3±0.45	59.0±0.09	59.3±0.24	59.3±0.32	59.0±0.05	51.6±0.32
18	68.8±0.06	58.9±0.17	55.5±0.30	53.9±0.20	50.4±0.31	50.0±0.40	49.2±0.09	67.6±0.21
19	69.9±0.05	68.0±0.21	67.0±0.31	67.1±0.22	68.2±0.09	68.3±0.26	68.9±0.17	54.4±0.33
20	68.0±0.15	60.9±0.18	60.0±0.09	59.4±0.12	56.6±0.47	56.0±0.06	55.7±0.27	47.0±0.20
21	69.4±0.50	55.9±0.45	46.7±0.30	45.0±0.30	44.0±0.24	44.2±0.22	45.9±0.40	57.1±0.14
22	69.3±0.30	65.0±0.08	62.9±0.30	58.1±0.50	57.4±0.06	57.0±0.31	59.3±0.16	66.8±0.31
23	67.8±0.31	64.6±0.22	64.0±0.06	56.6±0.31	55.2±0.21	60.8±0.22	63.5±0.40	56.8±0.20
24	69.0±0.16	65.4±0.07	59.8±0.21	52.3±0.30	50.1±0.20	50.0±0.21	55.5±0.30	57.3±0.20
25	68.9±0.22	66.6±0.18	60.7±0.16	54.9±0.20	53.0±0.20	53.2±0.31	51.2±0.40	52.0±0.21
26	68.2±0.40	54.7±0.09	48.3±0.23	47.2±0.30	45.0±0.21	52.2±0.30	51.4±0.30	57.7±0.40
27	70.2±0.21	64.0±0.34	60.6±0.32	58.2±0.16	58.6±0.21	58.0±0.26	70.2±0.19	44.0±0.24
28	66.7±0.04	47.0±0.30	50.2±0.30	49.0±0.31	48.2±0.30	44.2±0.20	43.3±0.50	35.6±0.16
29[b]	60.5±0.23	55.9±0.33	31.4±0.12	35.5±0.15	35.3±0.23	35.3±0.33	35.5±0.34	35.6±0.22

[a] Abiotic control

[b] Isolate reducing surface tension to 31.4 mN/m, the lowest value amongst all isolates

decreased at 48 h, with this change being more abrupt for ammonium nitrate than the other two N sources. This was again followed by an increase in pH of the medium in the subsequent hours for all three N sources.

Table 2 Surface tension reduction (STR) with different carbon sources. Surface tension values represented mean ± SD of three independent experiments

Carbon source	Surface tension of control (mN/m)	Surface tension of sample (mN/m)	Surface tension reduction (%)
Glucose	69.3±0.2	29.7±0.30	57
Glycerol	70.2±0.14	28.4±0.33	60
Mannitol	68.7±0.31	30.4±0.12	56
n-Hexadecane	55.0±0.22	37.0±0.22	33
Sludge	47.0±0.32	39.0±0.34	17

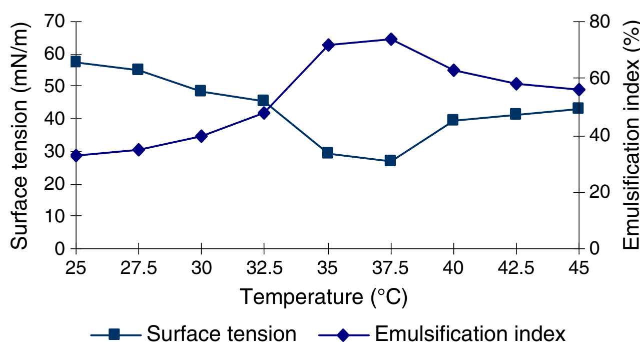


Fig. 1 Effect of temperature on biosurfactant production

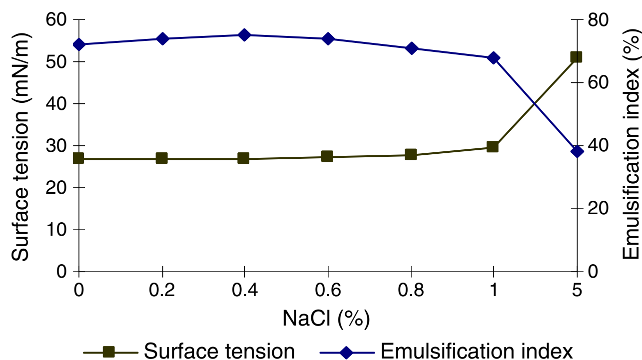


Fig. 3 Effect of NaCl concentration on biosurfactant production

Maximum bacterial growth was observed in ammonium nitrate-containing mineral medium, with almost equal growth being recorded for the rest of the four nitrogen sources. The lowest bacterial growth was observed in the control (Table 3).

E24 was maximal, and almost the same, for sodium nitrate (80%) and potassium nitrate (79%) followed by ammonium nitrate (72%). A minimum value was observed for ammonium chloride (36%) and ammonium sulfate (36%). The E24 for the control was 50% (Table 3).

In summary, *P. aeruginosa* RS29 could utilize all five tested nitrogen salts for growth. However, maximum growth (OD_{460} 4.0) was recorded in medium containing ammonium nitrate as the nitrogen source. Although growth of the strain was lower in sodium and potassium nitrate than ammonium nitrate, the former two were best for production of biosurfactant in terms of STR and E24 (26.3 mN/m and 26.4 mN/m, with E24 of 80 and 79%, respectively). This might be because nitrogen was less available from these two salts because nitrate first undergoes dissimilatory nitrate reduction to ammonium followed by assimilation by glutamine-glutamate metabolism. This means that the assimilation of nitrate as a nitrogen source is so slow that it would simulate conditions of limiting nitrogen (Hisatsuka et al. 1971; Itoh and Suzuki 1972; Guerra-Santos et al. 1983). It had been reported that nitrogen limitation enhances biosurfactant production (Suzuki et al. 1974; Ramana and Karanth 1989).

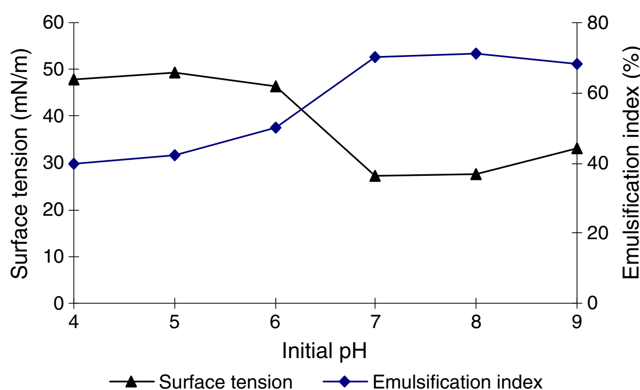


Fig. 2 Effect of initial pH on biosurfactant production

In the case of ammonium nitrate, biomass growth of *P. aeruginosa* RS29 was supported more than biosurfactant synthesis. After maximum STR of the medium, surface tension again started to increase with time, indicating that the concentration of biosurfactant decreased in the medium. The observed disappearance of biosurfactant might be related to the development of competence. There are three possible mechanisms responsible for the decline in the biosurfactant concentration in stationary phase: (1) the biosurfactant was degraded by the enzymes in the culture, (2) the biosurfactant might be adsorbed on the cell surface, or (3) the biosurfactant was reinternalized and processed intracellularly (Lin et al. 1993). Although ammonium sulfate and ammonium chloride supported bacterial growth, production of biosurfactant was very poor. It can be deduced that, at low pH of the culture medium, bacteria could not efficiently synthesize biosurfactant (Fig. 2). The increase in pH in the presence of ammonium-, sodium- and potassium-nitrate implied accumulation of compounds like siderophores in the culture medium (Varma and Chincholkar 2007). The color of the medium also changed to brown for these three salts. The change in color of the transparent mineral medium to brown color after 48 h of incubation was also an indicator of the accumulation of siderophores in the medium (Nair et al. 2007). The pH of the medium increased during the growth period, in accordance with the siderophore concentration, suggesting that alkalinity is important to avoid siderophore destruction (Díaz de Villegas et al. 2002). The control also exhibited a gradual decrease in surface tension. The observed bacterial growth and biosurfactant production in the control was because of bacterial utilization of yeast extract in the mineral medium as a nitrogen source.

Biosurfactant recovery and characterization

The yield of biosurfactant (6 g/L) from *P. aeruginosa* RS29 strain in the presence of glycerol as sole source of carbon

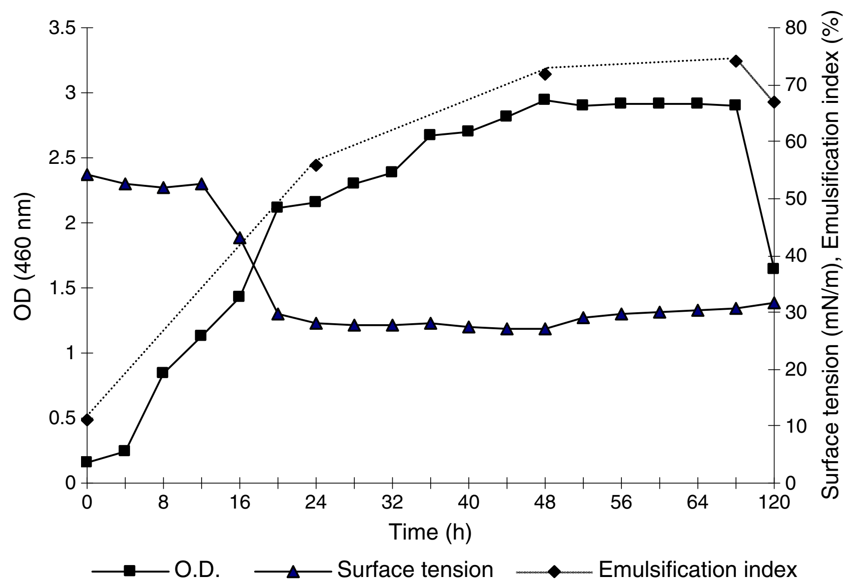


Fig. 4 Kinetics of biosurfactant production by *Pseudomonas aeruginosa* RS29 under optimized conditions

Table 3 Effect of nitrogen source on surface tension reduction (STR), pH, optical density (OD) and emulsification index (E24) of the mineral medium during biosurfactant production by *Pseudomonas aeruginosa* RS29. Results represent the mean ± SD of three independent experiments

Mineral medium with different nitrogen sources	0 h	24 h	48 h	72 h	96 h
Control					
Surface tension	62.0±0.12	51±0.15	36.1±0.62	32.2±0.3	28.9±0.8
pH	7.5±0.02	6.7±0.5	6.8±0.4	7.1±0.30	7.0±0.30
OD_{460}	0.138±0.15	1.18±0.03	1.39±0.02	1.47±0.02	1.5±0.03
E24 (%)			50±1.20		
Ammonium nitrate					
Surface tension	63.2±0.10	35.5±0.5	27.2±1.0	30.9±0.8	31.1±0.7
pH	7.5±0.03	8.2±0.12	5.54±0.20	8.3±0.40	8.3±0.45
OD_{460}	0.165±0.05	2.33±0.04	3.87±0.05	4.0±0.03	4.0±0.04
E24 (%)			72±1.0		
Ammonium chloride					
Surface tension	62.0±0.02	50.5±0.15	52.2±0.5	50.6±0.3	47.0±0.40
pH	7.5±0.02	4.8±0.20	4.6±0.45	5.3±0.4	4.8±0.23
OD_{460}	0.162±0.1	2.039±0.02	2.12±0.02	2.15±0.04	2.15±0.03
E24 (%)			36±0.6		
Ammonium sulfate					
Surface tension	62.3±0.05	49.3±0.34	51.5±0.4	52.6±0.42	51.1±0.50
pH	7.5±0.01	5.1±0.20	5.53±0.2	5.0±0.3	4.8±0.20
OD_{460}	0.165±0.22	1.96±0.02	2.06±0.01	2.12±0.02	2.11±0.02
E24 (%)			36±0.5		
Sodium nitrate					
Surface tension	62.2±0.01	26.3±0.1	26.3±0.12	26.5±0.1	27.0±0.20
pH	7.5±0.02	8.5±0.10	7.4±0.15	8.6±0.2	8.65±0.20
OD_{460}	0.160±0.07	2.18±0.01	2.36±0.02	2.36±0.01	2.32±0.01
E24 (%)			80±1.0		
Potassium nitrate					
Surface tension	62.1±0.03	26.4±0.2	26.4±0.4	26.5±0.4	26.8±0.30
pH	7.5±0.03	8.3±0.10	7.4±0.12	8.8±0.20	8.53±0.30
OD_{460}	0.163±0.2	2.08±0.01	2.28±0.02	2.31±0.01	2.25±0.01
E24 (%)			79±0.6		

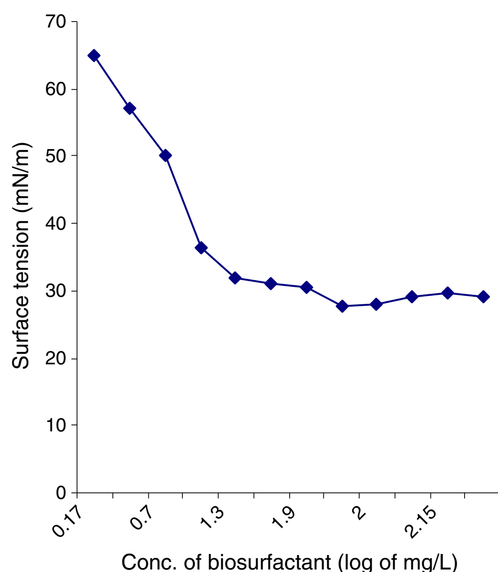


Fig. 5 Critical micelle concentration (CMC) of extracted biosurfactant produced by *Pseudomonas aeruginosa* RS29

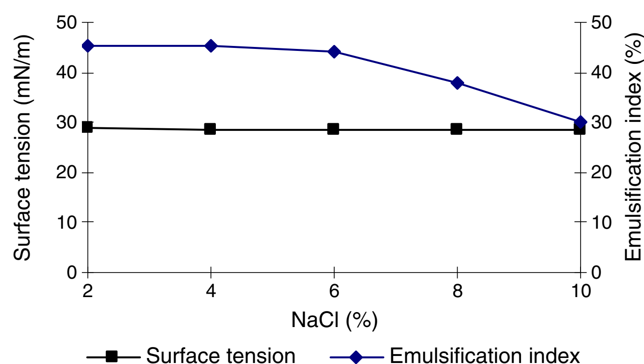
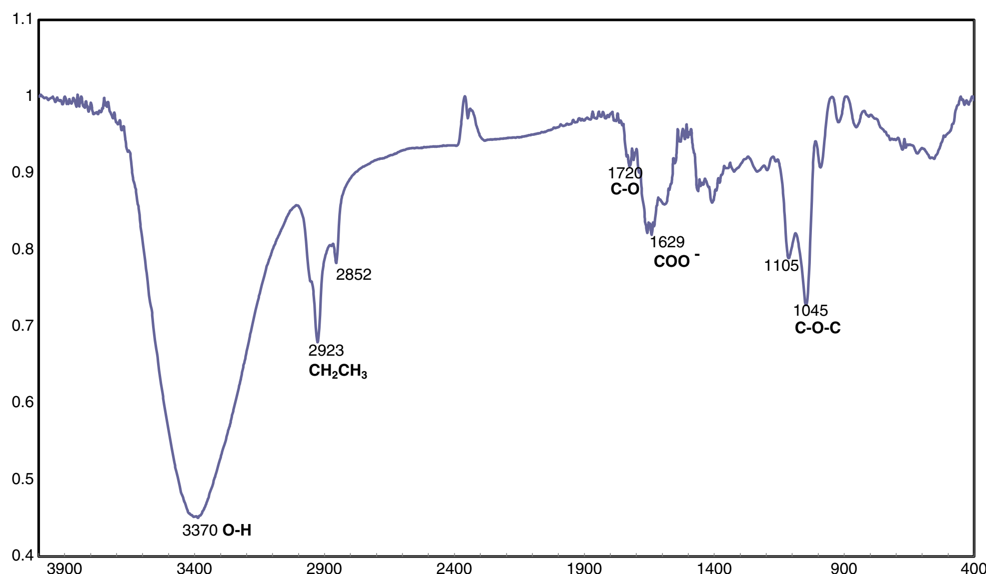


Fig. 7 Effect of salinity on biosurfactant activity

was higher than the reported biosurfactant production by *P. aeruginosa* NM strain (5 g/L) grown in glycerol (Das and Mukherjee 2005), and almost the same as with a previous report for *P. aeruginosa* M strain (Das and Mukherjee 2005) and other *Pseudomonas* sp. (Pruthi and Cameotra 1995).

As seen in Fig. 5, the surface tension is dependent on the concentration of crude biosurfactant, and the CMC corresponded to a sudden change in the surface tension (Abouseoud et al. 2008b). The CMC for the isolated biosurfactant calculated from the breakpoint of surface tension versus the log of its concentration curve was 90 mg/L, and the corresponding surface tension was 27.8 mN/m. Biosurfactant concentrations above the CMC could not decrease the surface tension further, indicating that biosurfactant molecules had begun to aggregate (Karsa et al. 1999; Meylheuc et al. 2001). The CMC obtained was in agreement with previous reports (Bordoloi and Konwar 2009). In the case of *P. aeruginosa* SP4, the excreted biosurfactant in the culture supernatant could decrease the surface tension of pure water from 72.0 to 28.3 mN/m, and the CMC was estimated to be 120 mg/L (Pornsunthorntawee et al. 2008). For *Pseudomonas fluorescens*, the CMC recorded for the isolated biosurfactant was 290 mg/L and the corresponding surface tension was 32 dynes/cm. The CMC value of the chemical surfactant sodium dodecyl sulphate (SDS) is 140 mg/L (Bordoloi and Konwar 2009). So, the biosurfactant produced by *P. aeruginosa* RS29 exhibits better properties in terms of higher STR and a lower CMC. The results of CMD^{-1} and CMD^{-2} of the biosurfactant containing cell-free medium were 28.3 mN/m and 40.0 mN/m, respectively, depicting little change in efficiency. The results suggest that a sufficient amount of biosurfactant was present in the culture medium, and thus its surface activity was retained even at such a high dilution. These values are appreciably better than the values reported for *P. aeruginosa* strains

Fig. 6 Fourier transform infrared (FTIR) spectra of the biosurfactant extracted from *Pseudomonas aeruginosa* RS29



(MTCC8165, MTCC7815, MTCC7812 and MTCC7814) (Bordoloi and Konwar 2008).

The rhamnose test was positive, indicating that the separated biosurfactant could be of the glycolipid type. Molisch's test showing a clear purple ring between the layers of solvent and the sample, indicating that the sample contained sugar moieties. Red spots appeared on the TLC plate after spraying with α-naphthol and sulfuric acid, indicating the presence of carbohydrates in the sample. The production of glycolipid-type biosurfactant was previously reported for *Pseudomonas* sp. (Persson et al. 1988; Wilson and Bradley 1996; Patel and Desai 1997).

Spectral analysis (Fig. 6) showed strong absorption bands at 3,370 cm^{-1}. This was observed due to stretching vibration of the –OH group. The absorption band observed at 2,923 cm^{-1} confirmed the presence of alkyl (CH_2 and CH_3) groups. A carbonyl stretching band characteristic of ester compounds was found at 1,720 cm^{-1}. The absorption at 1,629 cm^{-1} was because of stretching of the COO– group. The spectra also showed an absorption band at 1,045 cm^{-1} due to stretching vibration of –C–O–C. The pattern of absorption bands observed for this particular strain was reported previously for rhamnolipids (Bordoloi and Konwar 2009). These latter authors explained that strong absorption bands at 3,443 cm^{-1} occurred due to stretching vibration of the –O–H group and absorption at 1605–1625 cm^{-1} due to either stretching of –C=C or >C=O, i.e., stretching of the carboxylate anion. It was also reported that absorption at 1,625 cm^{-1} occurred due to presence of carboxylate anion, and absorption at 1,120 cm^{-1} due to stretching vibration of –C–O–C of the ether linkage of Rha-C8-C10 and Rha-C10-C8 molecules.

Activity characterization

Biosurfactant containing culture supernatant showed good foaming stability. The foam produced was stable for 48 h. Stable foam indicates that the produced biosurfactant can be used as a good foaming agent. Similar findings were reported for 48-h-old culture of *P. aeruginosa* PTCC 1561 grown in nutrient broth, which showed foam stability for 48 h (Noudeh et al. 2010). The biosurfactant produced by *P. aeruginosa* RS29 was shown to be thermostable. Autoclaving it at 121°C did not destroy its foaming properties. The surface tension measured before autoclaving the sample was 27.0 mN/m. The value was retained and recorded as 27.6 mN/m after autoclaving the sample. The E24 before autoclaving the sample was 72% and 70% after autoclaving. This indicated that STR and the E24 were stable up to quite a high temperature, in contrast to synthetic surfactants such as SDS, which exhibits a significant loss of emulsification activity above 70°C (Kim et al. 1997). Thus, our product is better than synthetic surfactants in terms of temperature stability. The effect of addition of NaCl on surface tension and E24 of biosurfactant is shown in Fig. 7. Little change was observed in either parameter with addition of up to 10% (w/v) NaCl. The surface tension of the biosurfactant was stable at different pH values ranging from 2 to 10, and pH increase had a positive effect on E24 (Fig. 8). This could be caused by the higher stability of fatty acids-surfactant micelles in presence of NaOH and the precipitation of secondary metabolites at higher pH values (Abouseoud et al. 2008a). Similar findings were reported for *P. aeruginosa* isolate Bs20, which exhibited excellent stability at high temperature (heating at 100°C for 1 h and autoclaving at 121°C for 10 min), salinities up to 6% NaCl, and pH values up to pH 13 (Abdel-Mawgoud et al. 2009). The properties of the biosurfactant produced by native *P. aeruginosa* RS29 are promising for its application in different industries.

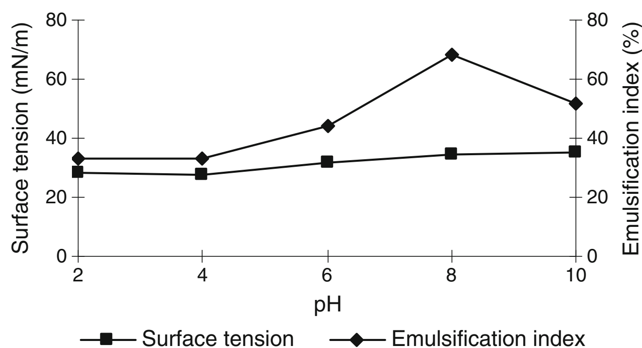


Fig. 8 Effect of pH on biosurfactant activity

Conclusion

The isolated *P. aeruginosa* RS29 is a very potent biosurfactant-producing native strain. Strain RS29 produces maximum biosurfactant in sodium- or potassium-nitrate-containing medium where surface tension was reduced from 70.0 to 26.3 mN/m and 26.4 mN/m, respectively. The amount of crude biosurfactant recovered (6 g/L) from the culture medium is very promising. The CMC value (90 mg/L) of the produced biosurfactant is superior to many other biosurfactants. The tensioactive properties and stability of the biosurfactant to high temperature, pH and salinity reveal good prospects for this product in industrial applications. The emulsifying and foaming activity of the biosurfactant indicate that it can be used as a good emulsion-forming and foaming agent in different industries.

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