Effect of Nutrients on Optimal Production of Biosurfactants by *Pseudomonas putida*—A Gujarat Oil Field Isolate

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ABSTRACT: Nutritional requirements for maximal production of biosurfactant by an oil field bacterium *Pseudomonas putida* were determined. The optimal concentrations of nitrogen, phosphate, sulfur, magnesium, iron, potassium, sodium, calcium, and trace elements for maximal production of biosurfactants were ascertained, and a new "Pruthi and Cameotra" salt medium was formulated. Data show that maximal biomass (2.4 g L^{-1}) and biosurfactant production (6.28 g L^{-1}) takes place after 72 h of growth on 2% hexadecane. The biosurfactant was produced optimally over pH and temperature ranges of 6.4–7.2 and 30–40°C, respectively. That the highest biosurfactant yield was obtained during late log phase of growth indicates that the biosurfactant is a secondary metabolite.

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KEY WORDS: Biosurfactant, maximum, nutrients, *Pseudomonas putida* (a Gujarat oil field isolate).

The term "biosurfactants" refers to a large group of structurally diverse molecules produced by microorganisms. The common feature of these molecules is that they possess surface-active properties. Recently, microbial surface-active agents have gained considerable importance in the fields of food, pharmaceuticals, cosmetics, and the petrochemical industries (1–5). These amphiphilic molecules, containing polar (hydrophilic) and nonpolar (hydrophobic) parts together with their specific chemical identity, impart particular properties to different surfactants that render them capable of reducing surface and interfacial tensions (6). Moreover they are nontoxic, biodegradable, and highly selective. They can be active at extremes of temperature, pH, and salinity and can be synthesized from renewable feedstocks besides, making an ecofriendly environment (7–10). Biosurfactant-producing microbes are distributed among a number of genera. The type, effectiveness, and efficiency of biosurfactants are influenced by the nature of the carbon sources and the concentrations of nitrogen, phosphorus, magnesium, iron, and sulfur ions in the medium. Biosurfactant activities are also influenced by changes in culture conditions, such as pH, temperature, agitation, and dilution rate in continuous culture. In this paper we report the role of nutrients in optimizing growth and biosurfactant production by a Gujarat oil field bacterial isolate *Pseudomonas putida*. On the basis of this study a new "Pruthi and Cameotra" salt medium has been developed.

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Abbreviations: CMD, critical micelle dilution; E_{24}, emulsification index (%); IFT, interfacial tension; ST, surface tension.

MATERIALS AND METHODS

Microorganism and medium. The microorganism used in this study was isolated earlier from Gujarat oil fields, India. Based on biochemical and morphological characteristics, the bacterium was identified as *P. putida* (11). The organism was grown in 50 mL of minimal medium supplemented with 2% hydrocarbon or 2% water-miscible substrate in a 500-mL Erlenmeyer flask (12). The initial pH of the medium was adjusted to 6.8. Incubation was carried out at 30°C on a rotary shaker at 200 rev min^{-1}. Hydrocarbons used in the study were obtained from Sigma Chemical Co. (St. Louis, MO). Chemicals for identification and optimization of the nutrients for the organism were purchased from Difco Laboratories (Detroit, MI) and Hi-Media (Mumbai, India).

Dry biomass estimation. Dry biomass of hydrocarbon-grown *P. putida* was determined by centrifugation of 50 mL of culture broth at 19,300 × *g* for 20 min. The sedimented cells were then extracted with a mixture of acetone/hexane (3:1) to remove the adhering hydrocarbon. This was followed by centrifugation with hexane (10 mL) and drying at 80°C overnight to obtain dry biomass. When the strain was grown on water-miscible substrate, dry biomass was estimated by centrifuging the culture broth at 16,300 × *g* for 20 min, drying overnight, and weighing (13).

Measurement of surface activities. Surface tension (ST), interfacial tension (IFT), and relative concentration of biosurfactants expressed in terms of critical micelle dilution (CMD) were measured by a Du Nouy Tensiometer (CSC No. 70535; CSC Corp., Albany, NY) at different times intervals. In the case of hydrocarbon-grown culture, ST of the cell-free culture broth were measured after removing the unemulsified hydrocarbons, as reported earlier (11). IFT of the cell-free broth were measured against *n*-hexane. Similarly for determining the CMD, the cell-free broth was diluted 10-fold (CMD^{-1}) and 100-fold (CMD^{-2}) with distilled water. The ST of these were then recorded, as described previously (13).

Measurement of emulsification activities. For measuring emulsification activity, 4 mL of culture broth diluted with 6 mL of kerosene oil was vortexed at high speed for 2 min. The emulsion stability and emulsification index, E_{24} (%), was recorded after 24 h, as reported previously (14).

Isolation of biosurfactants. The isolation scheme used for biosurfactant extraction, employing acetone precipitation from the culture broth of *P. putida* grown on hexadecane, is according to our previous protocol (11).

RESULTS AND DISCUSSION

During growth of the Gujarat oil field bacterial isolate *P. putida* on a water-immiscible substrate such as dodecane, hexadecane, or pristane, maximal reduction in ST values (32.2, 30.6, and 36.4 dyn cm^{-1}, respectively) were recorded after 72 h incubation. In comparison, the water-miscible substrates glucose, yeast extract, and peptone showed surface tension values of 54.6, 42.2, and 40.6 dyn cm^{-1}, respectively, after 24 h. These results are in accordance with earlier reports on the uptake of water-immiscible substrates, such as *n*-alkanes, and production of biosurfactants (15,16). Medium constituents other than carbon source also affect the production of biosurfactants. Among the inorganic salts tested, nitrate supported maximal reduction in surface tension (32.2 dyn cm^{-1}) in comparison to ammonium sulfate (36.4 dyn cm^{-1}) and ammonium hydroxide (42.8 dyn cm^{-1}). Emulsifying activities of 36, 28, and 10%, recorded for the culture grown on sodium nitrate, ammonium sulfate, and ammonium hydroxide, respectively, as nitrogen source, showed that sodium nitrate is the most suitable nitrogen source for this organism. Nitrate has also been rated as the best source for biosurfactant production by *Pseudomonas* strain 44T1 and *Rhodococcus* strain ST-5 grown on olive oil and paraffin, respectively (17,18).

The effect of different concentrations of nitrogen, phosphorus, sulfur, and magnesium on *P. putida* grown for 72 h on 2% hexadecane is depicted in Figure 1. The results show that optimal growth and biosurfactant activities (ST and E_{24}) were obtained at N, P, S, and Mg concentrations of 330, 800, 80, and 15 ppm, respectively. The lowest surface tension, 32 dyn cm^{-1}, was obtained using 330 ppm N as nitrate. With phosphate, maximal growth and emulsification activity (E_{24}) were obtained at 800 ppm P. An increase in the concentration of phosphorus in the growth medium was not associated with any remarkable change in growth, emulsification activity, and ST of the culture broth (Fig. 1). The results also indicated that the requirement for Mg^{2+} (15 ppm) is low in comparison with other biosurfactant-producing bacteria (19).

From Figure 2 one can see that 12 ppm Fe^{3+}, 1000 ppm Na^{+}, 15 ppm Ca^{2+}, and 1 ppm trace elements are optimal for biosurfactant production by *P. putida* on hexadecane. A tolerance to high concentrations of salts for production of biosurfactant is not surprising since organisms producing biosurfactant are capable of growth at high concentrations of NaCl (10%), as reported for psychrophilic *Arthrobacter protophormiae* (14). An increase in the total trace element

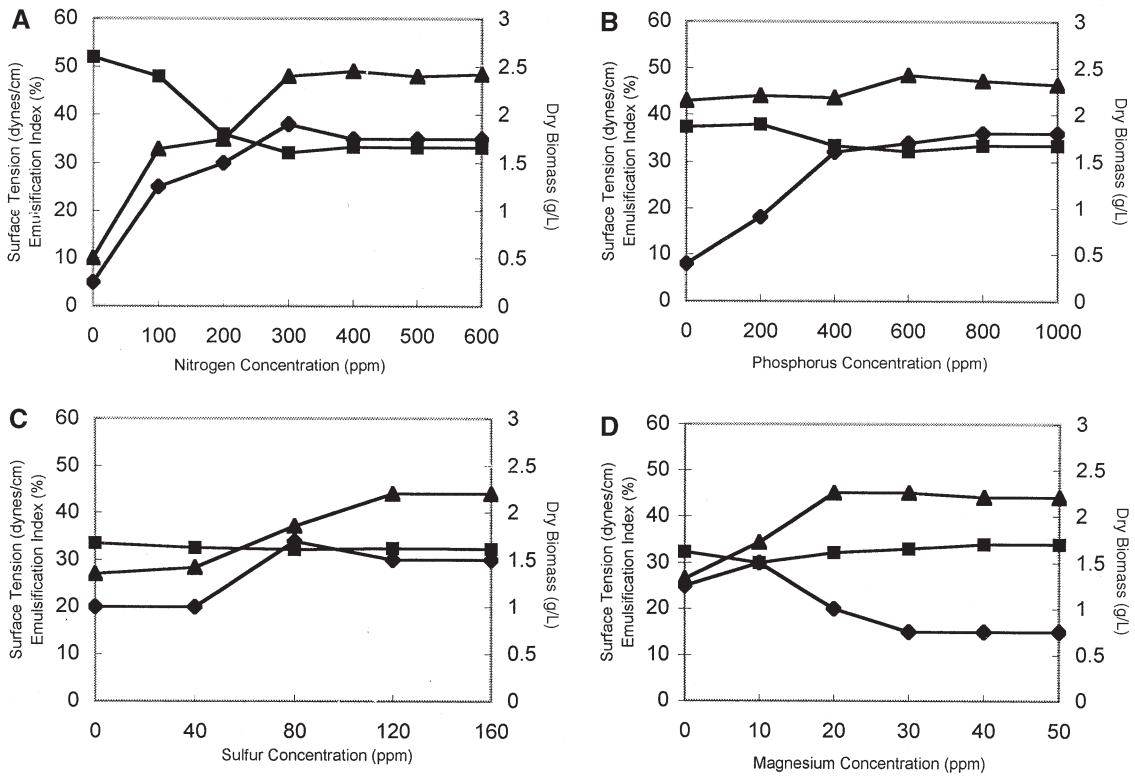

FIG. 1. Effect of different concentrations of (A) nitrogen, (B) phosphorus, (C) sulfur, and (D) magnesium on growth and biosurfactant activities of *Pseudomonas putida*. ■, Surface tension; ◆, emulsification index; ▲, dry biomass.

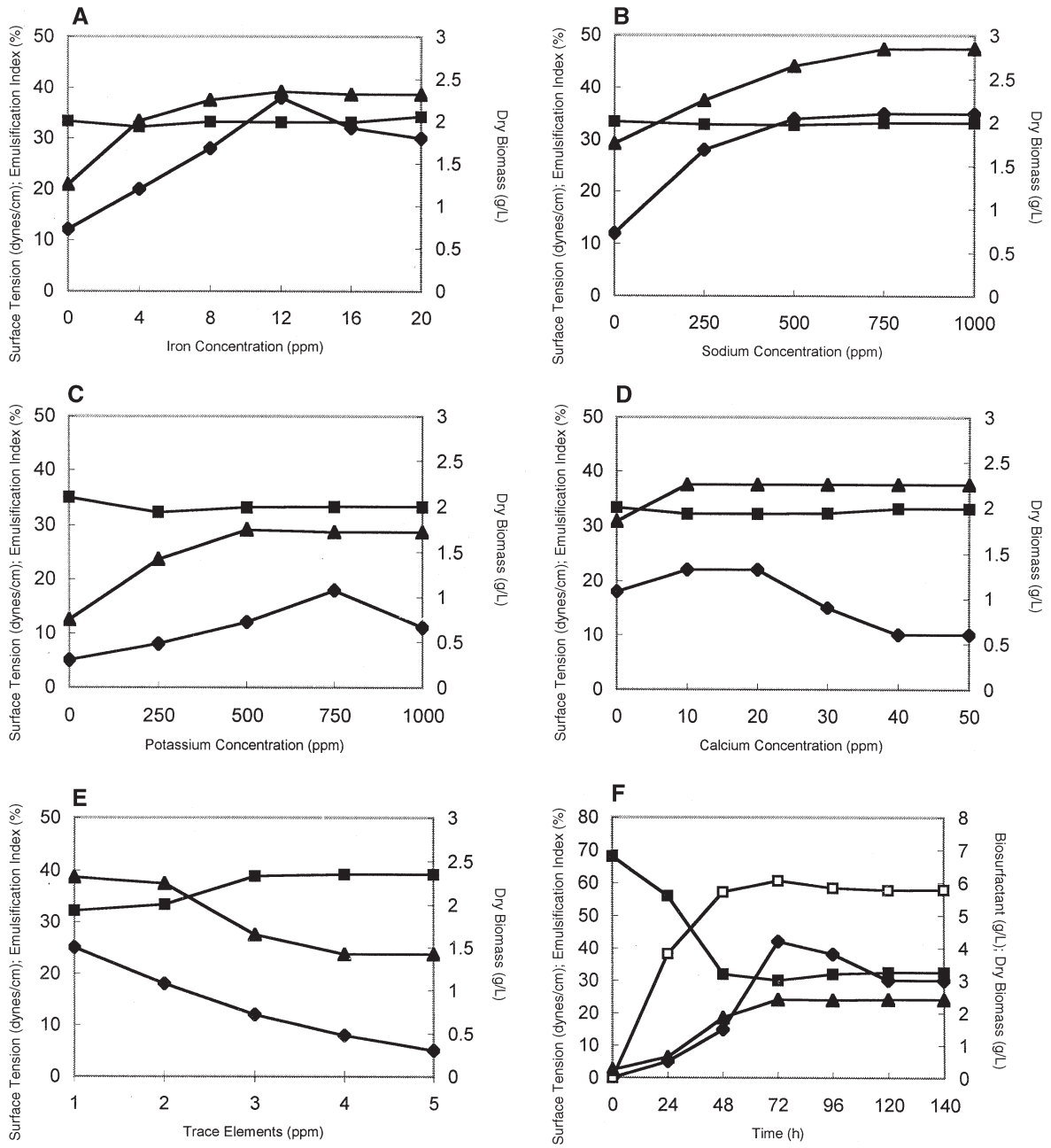

FIG. 2. Effect of different amounts of (A) iron, (B) sodium, (C) potassium, (D) calcium, and (E) trace elements on growth and biosurfactant activities of *Pseudomonas putida*. (F) Production of biosurfactants, dry biomass, and values of surface tension and emulsification index during growth of *P. putida* on "Pruthi and Cameotra" salt medium supplemented with 2% hexadecane. ■, Surface tension; ◆, emulsification index; ▲, dry biomass; □, biosurfactant.

concentration results in reduction in cell yield and emulsification activities but has no effect on ST values.

Based on these results, a new "Pruthi and Cameotra" salt medium was formulated. The medium contained the following nutrient amounts (in mg L^{-1}), which were found optimal in biosurfactant synthesis: KH_2PO_4 2000; $Na_2HPO_4{\cdot}2H_2O$ 2000; K_2SO_4 350; $NaNO_3$ 2000; $MgSO_4{\cdot}7H_2O$ 150; NaCl 100; $FeSO_4{\cdot}7H_20$ 50; $CaCl_2$ 50; and 1 mL of the trace elements containing (in mg L^{-1}): $ZnSO_4{\cdot}7H_2O$ 525; $MnSO_4{\cdot}4H_2O$ 200; $CuSO_4{\cdot}5H_2O$ 705; $Na_2MoO_4{\cdot}2H_2O$ 15; $CoCl_2{\cdot}6H_2O$ 200; H_3BO_3 15; $NiSO_4{\cdot}6H_2O$ 27.

By the use of this salt medium, maximal dry biomass (2.40 g L^{-1}) and emulsification activity (42%) were recorded after 72 h of cultivation (Fig. 2F). At this point maximal reduction in ST value (30.6 dyn cm^{-1}) and maximal production of biosurfactants (6.28 g L^{-1}) were observed (Fig. 2F). These data correspond to our previous report on lipopeptide biosurfactant production during the late log phase of growth by *P. putida* (11).

Table 1 shows the effect after 72 h of pH and temperature on biomass production and biosurfactant activities of the strain grown on 2% hexadecane. It is interesting to note that maximal production of biosurfactant, as determined by ST, CMD, IFT (against *n*-hexane), and emulsification

TABLE 1
Growth and Biosurfactant Activities of *Pseudomonas putida* Grown on 2% Hexadecane After 72 h at Different pH and Temperature Values[a]

	Dyn cm^{-1}					
	Surface tension[b]	CMD^{-1}	CMD^{-2}	IFT	E_{24} (%)	Dry biomass (g L^{-1})
pH						
6.0	38.5 (68)	56.8	59.4	5.5	12	0.81
6.4	32.0 (68)	34.2	46.3	5.2	40	1.93
6.8	30.6 (68)	34.2	46.3	4.6	42	2.31
7.2	32.0 (68)	36.6	51.8	4.1	42	2.32
7.6	34.0 (68)	39.6	54.2	4.4	38	2.28
Temperature (°C)						
25	38.6 (68)	56.2	60.1	5.6	28	1.86
30	31.2 (68)	34.4	46.6	4.3	40	2.32
35	33.8 (68)	35.9	47.2	4.7	38	2.02
40	35.8 (68)	38.3	48.6	5.2	35	2.12
45	44.2 (68)	62.6	63.4	10.8	18	1.06

[a]Abbreviations: CMD^{-1} and CMD^{-2}, critical micelle dilution of broth diluted 1:10 and 1:100 times, respectively; IFT, interfacial tension against *n*-hexane; E_{24}, emulsification index.
[b]Values in parentheses indicate surface tension of the cell-free broth at day 0.

index, was maintained over a pH range (6.4–7.2). These findings suggest that biosurfactants can be effectively used for large-scale production where unexpected changes in pH can occur. As listed in Table 1, the optimal growth and surface activities were obtained at 30°C. At this temperature, maximal biomass yields of 2.32 g L^{-1}, E_{24} of 40%, ST of 31.2 dyn cm^{-1}, and CMD of 34.40 dyn cm^{-1} were recorded. These data suggest that a change in temperature (lower and higher) essentially has a depressing effect on growth and biosurfactant production. The effect is more evident at 45°C, where the biomass production decreased to 1.06 g L^{-1}, which is less than half the biomass obtained at 30°C. However, the biosurfactant properties showed slight variation within a temperature range of 30 to 40°C.

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