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Optimization of biosurfactant production from Hydrocarbonoclastic Bacteria *Pseudomonas putida*

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Abstract

Biosurfactant are surface active agent that are produced extracellularly or as part of the cell membrane by bacteria, yeast and fungi. In the present study, hydrocarbonoclastic bacteria were isolated from oily contaminated sites of Ennore horbour. Screening of biosurfactant producing potential strain was done by haemolytic assay, drop collapse and oil spread assay and the potential strain was identified by biochemical tests as *Pseudomonas putida*. The ability of strain *Pseudomonas putida* to grow and produce biosurfactant on different carbon sources, temperatures and pH was studied. In this study, the biosurfactat productivity was 7% inoculum in cultures grown in a medium supplemented with corn oil, sodium nitrate adjusted to pH 7.0 and incubated at 35º. This approach may be useful during the initial isolation of biosurfactant producing bacteria to reduce the number of strains to be screened.

Keywords: *Pseudomonas putida*, Biosurfactant, Ennore, optimization.

Introduction

Biosurfactants are surface active compound that reduce the interfacial tension between two liquids, or that between a liquid and a solid. Their unique property like nontoxic, easily biodegradable, eco-friendly and high stability, and wide variety of industrial application makes them highly useful group of chemical compound. Biosurfactants produced by a variety of microorganisms mainly bacteria, fungi and yeasts are diverse in chemical composition and their nature and the amount depend on the type of microbes producing a particular biosurfactant (Kevin *et al*., 2008).

The vast majority of commercially employed surfactants are synthesized from petroleum derivatives. Biosurfactants are increasing interest for commercial use because of the continually growing spectrum of available substances. There are many advantages of biosurfactants compared to their chemically synthesized counterpart (Krishnaswamy *et al*., 2009). The enormous market demands for surfactants are currently met by numerous synthetic mainly petroleum-based, chemical surfactants. These compounds are usually toxic to the environment and non-degradable. Tightening environmental regulations and increasing awareness for the need to protect the ecosystem have effectively resulted in an increasing interest in biosurfactants as possible alternative to chemical surfactants (Banat *et al*., 2000). Furthermore, biosurfactants have the potential to be used as anti adhesive biological coatings for medical insertional materials,thus reducing hospital infections and use of synthetic drugs and chemicals. They may also be incorporated into probiotics preparations to combat urogenital tract infections and pulmonary immunotherapy (Batista *et al*., 2006).

The present study focuses on the screening, isolation and identification of the biosurfactant producing bacterium from soil obtained from Ennore horbour. We report optimization of media and growth conditions for biosurfactant production by the isolate *Pseudomonas putida.*

Materials and Methods

Collection of soil samples and enrichment of microorganisms:

Oil contaminated soil and water samples from Ennore horbour were collected and enriched by inoculating into sterile mineral salt medium (MSM), individually. one gram/ml of each soil and water sample were inoculated into 50 mL of minimal salt medium (Tahzibi *et al.*, 2004) containing (g/L); 15 g $NaNO_3$, 1.1 g KCl, 1.1 g NaCl, 0.00028 g $FeSO_4.7H_2O$, 3.4 g KH_2PO_4, 4.4 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$, 0.5 g yeast extract at 37°C in shaker incubator (100 rpm). After 48 h of incubation, the samples were serially diluted using sterile saline (0.85% NaCl) and different bacterial isolates were selected based on the colony morphology on nutrient agar. The selected isolates were screened for the production of biosurfactants using following screening methods.

Screening for biosurfactant production

Hemolytic activity

Pure culture of bacterial isolates were streaked on the freshly prepared blood agar and incubated at 37°C for 48-72 h. Results were recorded based on the type of clear zone observed i.e. α-hemolysis when the colony was surrounded by greenish zone, β-hemolysis when the colony was surrounded by a clear white zone and γ-hemolysis when there was no change in the medium surrounding the colony (Carrillo *et al.*, 1996).

Drop collapsing test

Screening of biosurfactant production was performed using the qualitative drop-collapse test described by Bodour and Maier (1998). Crude oil was used in this test. Two microlitres of oil was applied to the well regions delimited on the covers of 96-well micro plates and these were left to equilibrate for 24 h. Five micro liters of the 48 h culture, before and after centrifugation at 12,000 g for 5 min to remove cells, was transferred to the oil-coated well regions and drop size was observed after 1 min with the aid of a magnifying glass. The result was considered positive for biosurfactant production when the drop was flat and those cultures that gave rounded drops were scored as negative, indicative of the lack of biosurfactant production (Youssef *et al.*, 2004).

Oil spreading assay

Oil spreading experiment was performed using the method described by Morikawa et al., 2000. In brief, 20 ml of distilled water was added to a plastic Petri dish followed by addition of 20 µl of crude oil to the surface of the water. 10 µl of cell free culture broth was then added to the oil surface. If biosurfactant is present in the cell free culture broth, the oil will be displaced with an oil free clearing zone and diameter of this clearing zone indicates the surfactant activity, also called oil displacement activity. A negative control was maintained with distilled water (without surfactant), in which no oil displacement or clear zone was observed and Triton X-100 was used as the positive control.

Surface tension measurement

Surface tension measurement of cell-free culture broth from each strain was determined in a K6 tensiometer (Krüss GmbH, Hamburg, Germany), using the du Nouy ring method. Triton X-100 solution prepared at 1mg/ml concentration was used as a standard.

Emulsification assay

Cell free culture broth was used as the biosurfactant source to check the emulsification of crude oil. 1 ml of cell free culture broth was added to 5 ml of 50mM Tris buffer (pH 8.0) in a 30 ml screw-capped test tube. Five milligram of hydrocarbon was added to the above solution and vortex-shaken for 1 minute and the emulsion mixture was allowed to stand for 20 minutes. OD of the emulsion mixture was measured at 610 nm in a spectrophotometer and the results were expressed as D610 (Rosenberg *et al*., 1979). A negative control was maintained only with buffer solution and crude oil and Triton X-100 was used as the positive control.

Identification of bacteria

The most potential biosurfactant producing strain was identified following Bergey's manual of determinative bacteriology (Buchanan JB and Warwick, 1974). Morphologically distinct colonies were isolated and purified by replicating on the same solid medium to obtain pure cultures.

Optimization

For determination of optimal pH, the standardized inoculum was inoculated in MSM at different pH (5.0, 5.5, 6.0, 6.5, 7.0 and 7.5), then incubated at 37°C on an orbital shaker at 150 rpm. After optimal pH had been determined, the bacteria were grown in MSM at optimized pH and incubated at different temperatures (25, 30, 35, 40, 45 and 50°C) on an orbital shaker at 150 rpm for 5 days.

Bacterial inoculum was inoculated in MSM at optimized pH to which was added different carbon sources comprising Corn oil, Coconut oil, Sesame oil, Sunflower oil and Kerosene at 1% (v/v), then incubated on an orbital shaker at 150 rpm and at the predetermined optimized temperature for 5 days.

To determine the best nitrogen source for optimized production of biosurfactant, the bacterial inoculum was inoculated in MSM at optimized pH with added different nitrogen sources, namely, Peptone, Yeast extract, ammonium chloride (NH4Cl), Sodium nitrate and Potassium nitrate, (4 g/L) then incubated on an orbital shaker at 150 rpm and at the predetermined optimized temperature for 5 days.

Results and Discussion

A total of 169 bacterial strains were isolated from the sea water (74) and sediment sample (95). Among these 169 strains only 26 strains were selected based on the test like drop collapse, oil displacement capacity, emulsifying property and hemolytic capacity (table 1).

Table 1. Screening results of ten isolates for the production of biosurfactant.

Organism	Hemolytic	Drop collapse	Oil spread	Surface tension (m N m-1).	Emulsification index (% EI24)
ES4	β	++	+	55.9	36
ES7	β	++	+	53.2	29
ES8	α	+	+	61.9	35
ES9	α	+	+	43.2	27
ES15	α	++	+	59.6	42
ES24	α	+	++	45.3	38
ES27	α	+	++	48.2	41
ES38	β	+	+	54.5	20
ES39	α	++	++	38.5	72
ES41	β	+	+	54.1	34
ES44	α	++	+	52.3	38
ES50	α	+	+	53.9	40
ES63	β	+	++	64.7	51
ES81	β	++	++	55.1	26
EW15	α	+	+	51.3	46
EW20	α	+	+	39.8	35
EW23	α	++	+	50.5	61
EW25	β	+	++	42.1	46
EW31	β	++	+	49.3	32
EW37	α	+	+	41.7	30
EW56	α	++	+	54.5	57
EW61	β	++	++	47.2	49
EW68	β	++	+	52.1	28
EW70	α	+	+	40.8	23
EW71	α	++	++	59.6	35
EW74	β	++	++	67.8	49

α = Reduction of hemoglobin to met , hemoglobin
β = Lysis of RBC, medium around the colony becomes colourless;
Nil = Negative test; + = Positive test; ++ = Good activity;
Emulsification >30% is indicated in bold to denote high emulsification activity.

From the collected samples, the highest Emulsification activity E24 value was observed in ES39 strain for selected and based morphological and physiological characters it was identified as the name of the strain was designated as *Pseudomonas putida ES39* (Table 2). Dominant existence and biosurfactant producing Pseudomonas strains in hydrocarbon polluted environment was reported by many researchers (Yateem *et al*., 2002; Bodour *et al*., 2003; Das and Mukherjee, 2005) supporting the results obtained in this study.

Table 2: Identification of the ES39 bacterial culture isolates, morphological and biochemical identification

S.No	Test	ES39 results
1	Oxidase	+
2	Catalase	+
3	Carbohydrate	--
4	DNA	--
5	Gelatin	
6	Urea	+
7	Citrate	+
8	MrVp	+/--
9	Nitrate	--
10	Starch	--
11	Indole	--
12	Grams stain	--
13	Shape	Rod
14	Size	short
15	Color	cream
16	Margin	undulate
17	Elevation	Concave
18	Surface	Irregular
19	Opacity	Opaque
20	Strains identified	*Pseudomonas putida*

Optimization studies for biomass production were carried out and different pH (5-7.5), temperature (25-50° C), different carbon and different nitrogen sources were tested. Growth in different pH values showed maximum at pH 7 and maximum biosurfactant concentrations achieved in 35° C (Table 3). Different species of *Pseudomonas* have been found to produce biosurfactant at different pH. For example, *P. aeruginosa* S6 isolated from sludge containing oil produced biosurfactant when grown in MSM with added 5.0 g/L of glucose at pH9.0 reducing surface tension to 33.9 dynes/cm (Yin et al. 2009). Meanwhile, *Pseudomonas* sp. isolated from oil-contaminated soil produced maximum biosurfactant at pH 7.0 when grown in medium with 3% (v/v) when added (Praveesh *et al*., 2011). Praveesh *et al*., (2011) showed *P. aeruginosa* sp. produced the maximum rhamnolipid at 35°C while at 40°C, bacterial growth and biosurfactant production were inhibited.

Table 3. The effect of different pH and Temperature on biosurfactant production

Rhamnolipid Concentration (g/l)			
pH		Temperature	
5.0	1.15±0.11[a]	25^{0}C	0.88±0.23[a]
5.5	1.24±0.13[a]	30^{0}C	2.62±0.17[c]
6.0	2.24±0.13[b]	35^{0}C	3.89±0.11[d]
6.5	3.41±0.23[c]	40^{0}C	3.12±0.04[d]
7.0	3.72±0.12[c]	45^{0}C	2.18±0.04[c]
7.5	3.38±0.04[c]	50^{0}C	1.85±0.11[b]

Table 4. The effect of different carbon and nitrogen source on biosurfactant production

Rhamnolipid Concentration (g/l)			
pH		Temperature	
Corn oil	3.80±0.69 [c]	**Peptone**	2.80±0.25[b]
Coconut oil	3.20±0.19 [c]	**Yeast extract**	3.48±0.37[c]
Sesame oil	1.66±0.10 [a]	**Ammonium chloride**	1.96±0.05 [a]
Sunflower oil	2.41±0.05 [b]	**Sodium nitrate**	3.98±0.58[c]
Kerosene	3.24±0.46 [c]	**Potassium nitrate**	3.34±0.10[c]

Values with the same superscript are not significantly different at P< 0.05 level according to Duncan's multiple range test.

In the present study, the maximum biosurfactant was observed in the corn oil and yeast extract supplemented medium (Table 4). The biosurfactant production depends upon the type of carbon and nitrogen substrate present in the production medium (Davis *et al.*, 1999). Saravanan *et al*., 2011 reported the cheap substrates used corn oil and cassava flour waste shows the maximum biosurfactant production.

In conclusion, the study represented surfactant activity of the bacterial strains isolated from oil contaminated water and sediment from the Ennore. This confirms that environment has an influence on the metabolism of the tested microbes. This study suggests that, *Pseudomonas putida* isolated from oil contaminated water and soil showed biosurfactant producing ability. Further study on the utilization of agro industrial wastes as substrates for the large-scale production of biosurfactants is recommended.

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