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OPTIMIZATION AND CHARACTERIZATION OF BIOSURFACTANT PRODUCED BY PYRENE DEGRADING THERMO-ALKALIPHILIC *BACILLUS SONORENSIS* 4R

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ABSTRACT

Aim: *Main aim of this work was to study the optimization, production, and characterization of the biosurfactant produced by the pyrene utilizing bacterium B. sonorensis 4R under thermo-alkaliphilic conditions.* ***Methods:*** *The strain Bacillus sonorensis 4R was tested for its growth in hydrocarbon containing media and then screened for biosurfactant production ability by oil displacement, emulsification assay and hemolysis activity. Biosurfactant production potential was optimized for its physical and chemical parameters and optimally produced purified biosurfactant was characterized by FTIR analysis.* ***Results:*** *The isolate grew well in presence of anthracene, fluorine and pyrene in Bushnell Hass medium. The biosurfactant production conditions were optimized as pH 8.0, temperature 50°C, 1% trehalose, and 0.5% ammonium nitrate as best carbon and nitrogen sources and 1% olive oil as best substrate for maximum production of biosurfactant. The accumulation of biosurfactant under optimized conditions was characterized to be lipopeptide in nature based on FTIR analysis. The strain 4R degraded pyrene more efficiently under thermo-alkaliphilic conditions with 94.31% pyrene removal at the end of incubation (25 days) and this higher efficiency is expected due to its biosurfactant production ability.* ***Conclusion:*** *The Pyrene degrading Bacillus sonorensis 4R was able to produce biosurfactant. Its physicochemical parameters are easy to be optimized. Hence, strain can be used to remove pyrene pollutants from contaminated sites.*

KEY WORDS

Bacillus sonorensis, biodegradation, biosurfactant, lipopeptide, pyrene.

1.0 INTRODUCTION:

Biosurfactants are chemically active surface compounds synthesized by specific groups of microbes that utilize different substrates like simple sugars, oils, hydrocarbons from contaminated environment. They have the ability to reduce surface and interface tension amongst liquid and solid substances and leads to diffuse them as emulsions in liquids [1]. Compared to chemical surfactants, biosurfactants are eco-friendly, easily degradable, active in any extreme conditions like high salinity/temperature regions and can be produced using cheap organic sources, which facilitates commercialization [2]. Many recent studies report the application of biosurfactant producing microbes in the petroleum contaminated environments to remove hydrocarbon and remediate the environment [3]. Degradation of hydrocarbons is enhanced in the presence of biosurfactant producing microorganisms and many researchers have identified that biosurfactant producing *Bacillus* species are potential biodegrading microbes and widely used in microbial enhanced oil recovery [4], bioremediation purposes [5] and biodegradation [6]. Hence, the biosurfactant plays an important role in bioremediation of hydrocarbon polluted environment.

Bacillus sonorensis used in this study has been shown to have the highest capability to degrade pyrene by synthesizing biosurfactant in the presence of olive oil as carbon source. However, the production of biosurfactants at larger level still represent a challenge, due to the low production level, low activity, and long fermentation conditions. The biosurfactant production should be improved at industrial level, using efficient microbial strains with higher activity. The optimization of production medium with replacement substrates, the improvement of the efficiency of recovery methods and fermentation processes and the development of biosurfactant producing microorganisms, can open the way to their large scale inexpensive production throughout the enlargement of efficient processes [7]. The main purpose of this work was to study the optimization, production, and characterization of the biosurfactant produced by the pyrene utilizing bacterium *B. sonorensis 4R* under thermo-alkaliphilic conditions.

2.0 MATERIALS AND METHODS:

2.1 Microorganism:

The strain *Bacillus sonorensis* 4R (KT368092) used in this study was isolated from soil of Thar dessert Jaisalmer, Rajasthan, India (lat. 27'00N and 71°00E). The organism was selected based on its ability to grow on pyrene, anthracene and fluorine as the sole source of carbon and energy during enrichment studies. The selected isolate was identified on the basis of morphological, biochemical and molecular studies [8].

2.2 Growth on hydrocarbon containing media:

The growth experiments were performed in batch cultures in 500 ml Erlenmeyer flasks containing 100 ml Bushnell-Hass medium [9] pH 8.0. The carbon sources anthracene, pyrene and fluorine (0.1 gm each) were mixed with 10 ml of acetone and the mixture was subjected to evaporation for removal of acetone. After evaporation, 100 ml sterile BHB was added and the experiment was started by inoculation with 5% log phase culture of 4R. The flasks were incubated in dark at 50 °C for 25 days. Respective controls were prepared without addition of bacterial culture. The growth of 4R was observed by measuring absorbance at 600 nm. At the interval of 5days, the culture broths were centrifuged at 10,000 rpm at 4°C for 20 min and supernatants were collected. The percentage degradation of pyrene, anthracene and fluorine was determined spectrophotometrically [10] and at 335 nm, 255 nm and 263 nm respectively.

2.3 Detection of biosurfactant activity:

Biosurfactant production was carried out in 500 ml Erlenmeyer flask containing 200 ml of sterile Minimal salt medium supplemented with 1% (v/v) sterile olive oil. The active culture of *B. sononrensis* 4R was inoculated and incubated at 50ºC for 4days. At the end of incubation, the culture broth was centrifuged at 4 ºC for 20 min at 10,000 rpm and resultant supernatant was used for screening. The biosurfactant production ability determined by oil displacement test [11], hemolytic assay [12], lipase activity [13] and surface tension measurements. All tests were performed in triplicate and by using SDS and distilled water as positive and negative controls respectively.

2.4 Effect of growth conditions and nutritional parameters on biosurfactant production.

Biosurfactant production on 4R was studied under different growth conditions such as pH (4.0-9.0), temperature (30-60°C) and incubation period (24-144 hrs) by considering one variable at a time approach while keeping other conditions constant (pH 8.0, 50°C and 96 hrs). The effects of various concentrations of carbon sources (1 %) and nitrogen sources (0.5 %) on biosurfactant production were also studied.

2.5 Purification and characterization of biosurfactant:

The biosurfactant from culture broth of 4R was extracted by acid precipitation followed by solvent extraction and purification method as per our previous research [13]. Functional group characterization of biosurfactant was carried out by obtaining spectra of biosurfactant on Fourier transformed infrared (FTIR) spectrophotometer (Perkin Elmer Paragon 1000) over the range of 400-4000 cm^{-1}.

3.0 RESULTS AND DISCUSSION:

B. sonorensis 4R isolated from Thar Desert has ability to grow in BHB supplemented with anthracene, fluorine or pyrene as a sole carbon source and determined in terms of residual concentration of PAHs and percent degradation values (Table 1). While the organism grew in presence of all three PAH compounds, the growth was excellent in pyrene containing medium as observed in terms of optical density (Figure 1) at 600 nm. Growth was increased with increasing incubation period. Pyrene is a common PAH obtained from coal tar and frequently used as a chemical intermediate. They were considered

as priority pollutant in the environment because of their toxicity. Due to the hazardous effect of PAH, it is necessary to clear up or lowering the concentration of these substances in the environment. Bacteria have the unique feature in rapidly adapting to toxic environments. The metabolic diversity and plasticity of bacteria allow them to degrade organic pollutants and helps in the mineralization of these compounds [14].

Fig.1: Growth of *B. sonorensis* 4R in presence of pyrene at 50 °C.

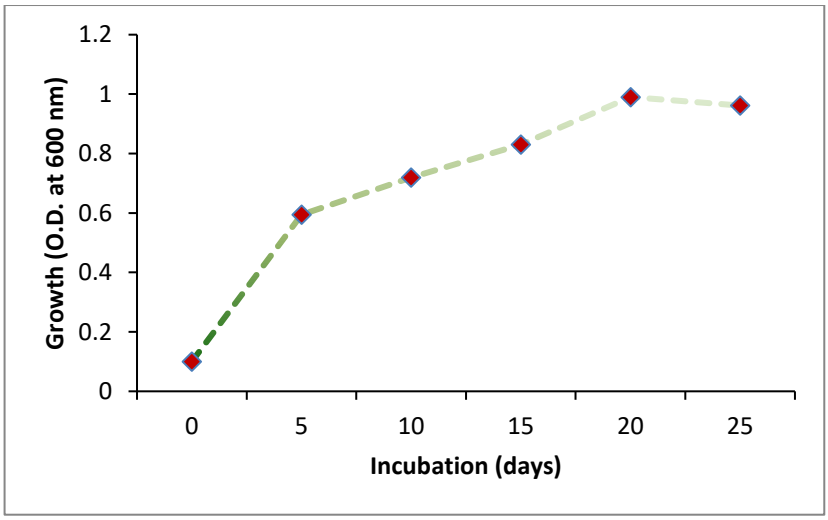


Table 1: Growth of *B. sonorensis* 4R in BHB supplemented with PAH compounds.

PAH compound	OD at λ_{max} in control flask	Days of incubation				
		5	10	15	20	25
Anthracene	1.486	1.353	0.627	0.451	0.235	0.187
Fluorine	0.496	0.379	0.203	0.160	0.126	0.087
Pyrene	2.354	0.879	0.614	0.273	0.165	0.134

The strain 4R showed positive oil displacement (13.19 mm), hemolytic activity (14 mm), emulsification activity (76.19%) (Fig 2), lipase activity (28 mm) as shown in Fig 2 (a, b and c) and reduced the surface tension of water from 72.8 to 61.62 dyne/cm at room temperature (28°C). The growth and metabolic product formation in bacteria are strongly influenced by medium composition and other growth parameters, hence optimization of cultural and growth conditions can be used to attain higher yields [15].

The effects of varying pH, temperature and incubation period on biosurfactant production by *B. sonorensis* 4R are indicated in Fig. 3 (a, b and c) respectively. The strain showed maximum biosurfactant production recorded in terms of oil displacement values at pH 8.0. The production was declined more at lower pH 4.0 compared to pH 9.0 indicating sensitivity of isolate to acidic pH conditions.

The biosurfactant production was reached maximum at 50°C and was absent at temperature 30°C. The optimum biosurfactant production by 4R was attained after 96hrs of incubation at 50°C then decreased gradually. Strain 4R is thermotolerant bacterium, which indicates this strain exhibits higher production levels at higher temperatures (50-60°C).

The biosurfactant production was increased more when production medium was supplemented with trehalose (1%) as a carbon source and ammonium nitrate (0.5%) as nitrogen source (Fig. 4 a, b).

Among the different lipidic substrates incorporated in the medium, presence of olive oil greatly affected biosurfactant production capacity of 4R (Fig. 5). The oil displacement activity was greater when olive oil was used as substrate as compared to tributyrin, petrol, engine oil, soyabean oil and Tween 80.

FTIR analysis of purified biosurfactant showed major characteristic bands of peptides at 3437 cm^{-1} (N-H stretching of CO-N bond). The sharp peaks at 1081-1378 cm^{-1} reflected aliphatic chains (-CH_3-CH_2-) of isolated fraction [16]. These results indicate the presence of aliphatic groups as well as peptide like moiety in the biosurfactant (Fig.6). The lipopeptide surfactant

produced by *Bacillus licheniformis* also shows similar kind of characteristics indicating the biosurfactant produced by *B. sonorensis* is of lipopeptide nature.

Fig.2: a. Hemolytic activity b. Oil displacement test and c. Emulsification activity of biosurfactant produced by *Bacillus sonorensis* 4R

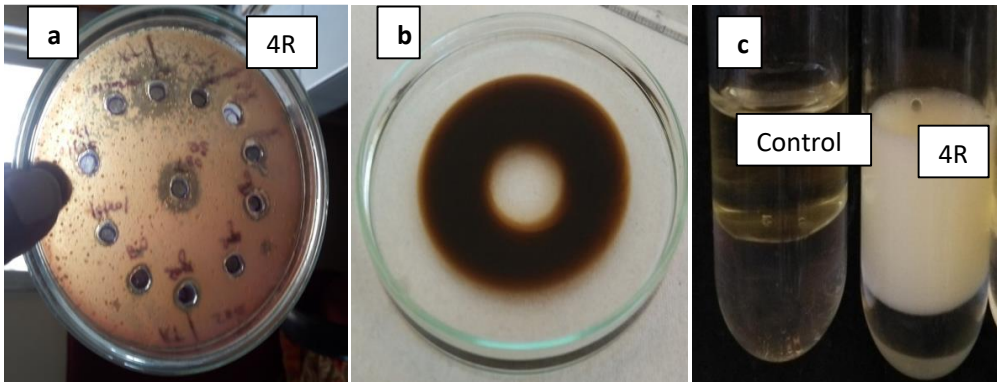
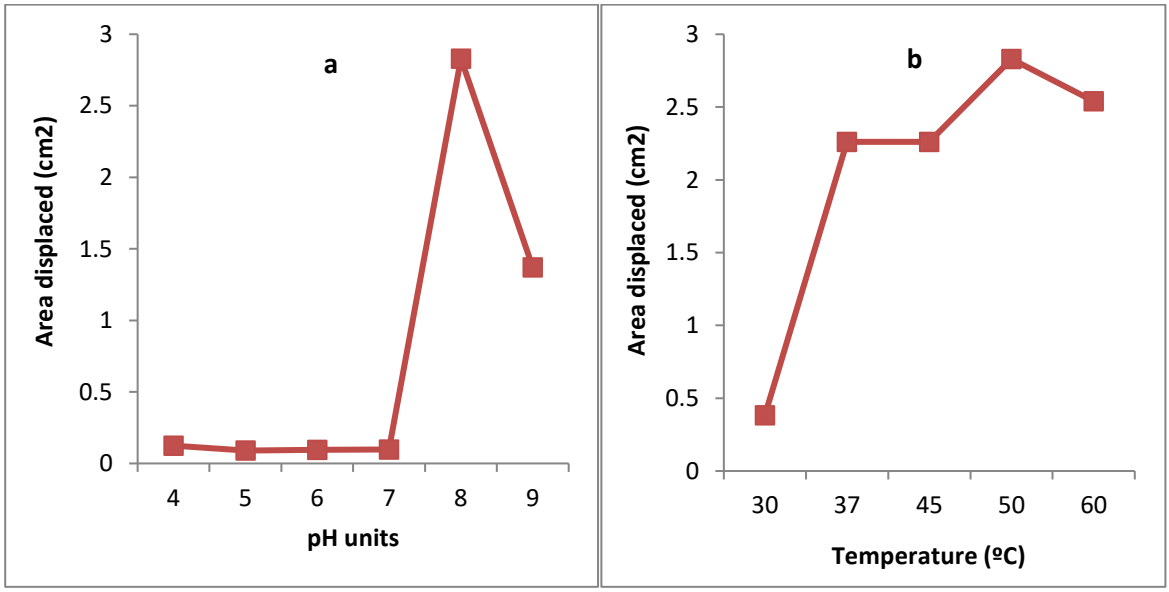


Fig.3: Effect of a. pH, b. Temperature and c. Incubation period on biosurfactant production by *B. sonorensis* 4R



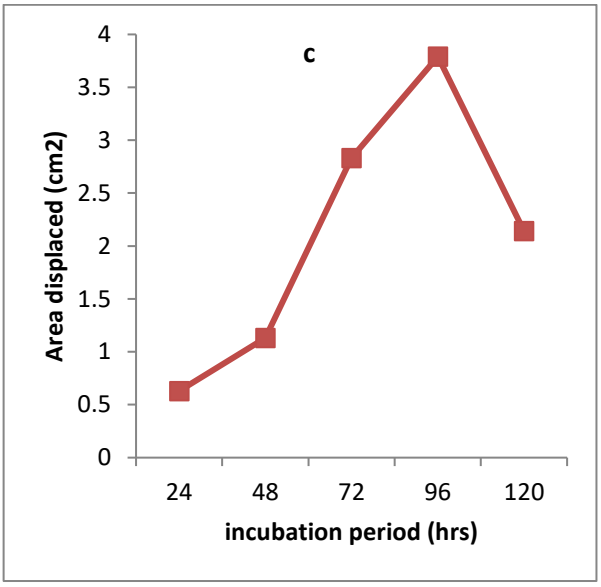


Fig.4: Effect of a. Different carbon sources b. Different nitrogen sources on biosurfactant production by *B. sonorensis* 4R

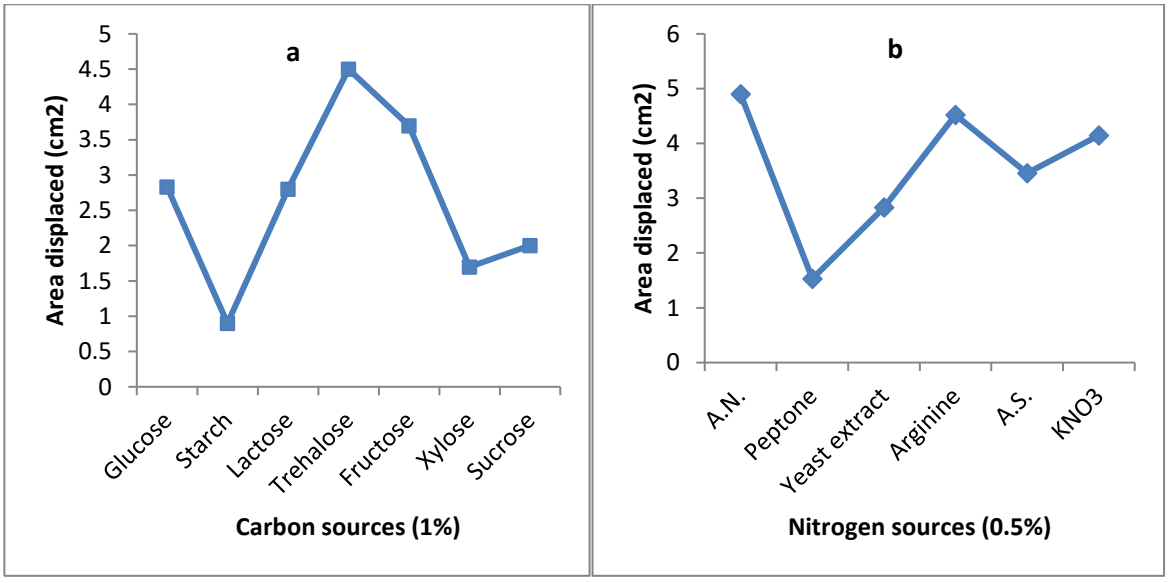


Fig.5: Effect of different substrates on biosurfactant production by *B. sonorensis* 4R

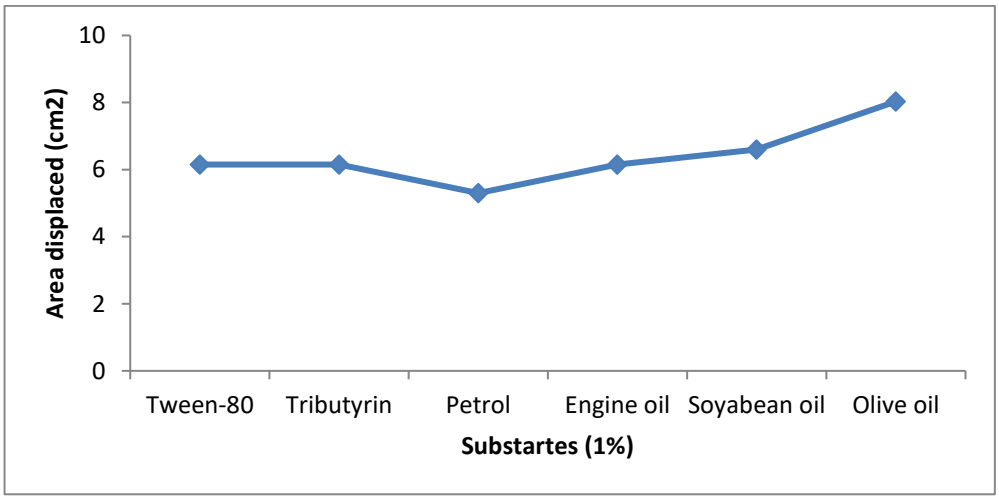
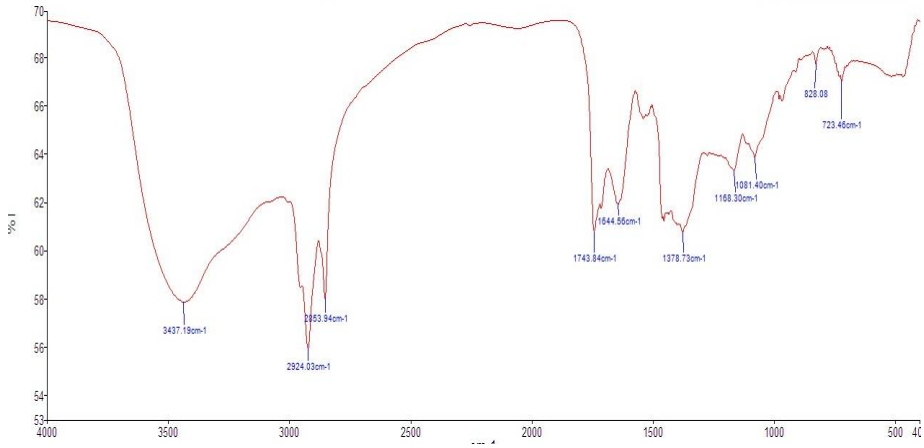


Fig.6: FTIR analysis of biosurfactant produced by *B. sonorensis* 4R



CONCLUSION:

In the present investigation a lipolytic strain of thermo-alkaliphilic *B. sonorensis* 4R is found capable to utilize low as well as high molecular weight PAH compounds at 50°C and pH 8.0. PAH utilization is mediated through a lipopeptide biosurfactant production. The optimal cultural and nutritional factors for biosurfactant production are simple and easy to manage for utilizing this strain in bioremediation of PAH especially pyrene polluted sites.

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