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Screening, Optimization and Production of Biosurfactants by *Candida* Species Isolated from Oil Polluted Soils

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Abstract: Biosurfactants are amphipathic molecules with both hydrophilic and hydrophobic moieties and are surface active agent that are produced extracellularly or as a part of cell membrane by bacteria, yeast and fungi. In the present study, six different microorganisms were isolated from oil contaminated soils. Screening of biosurfactant producing potential strain was done using oil spreading method, oil collapse method; emulsification method and MATH assay method. Using preliminary tests, the potential strain was identified as *Pseudomonas aeruginosa, Candida tropicalis* and *Candida albicans.* In oil spreading method, the organisms were produced maximum zone. High concentration of both the extracellular biosurfactant was produced by *P. aeruginosa* and intracellular biosurfactant was produced by *C. tropicalis* and *C. albicans* using emulsification method. In this present study, the MATH assay and statistical analysis revealed that *C. tropicalis* and *C. albicans* have higher hydrophobicity than *P. aeruginosa.* Optimization studies were carried out for biosurfactant production using *C. tropicalis* and *C. albicans* with different parameters like incubation period, temperature, pH and substrate concentration. The results showed the maximum production in the 6th day, 40°C and 6 respectively for both *C. tropicalis* and *C. albicans,* the substrate concentration was 1.5% for C. *tropicalis* and 2.0% for *C. albicans.* The total quantity of production of biosurfactant was 1.71% for *C. albicans* and 0.91% biosurfactant was produced by *C. tropicalis.*

Key words: Intracellular Biosurfactant · Cell surface hydrophobicity · Optimization · Production

INTRODUCTION

Surface active compounds produced by microorganisms are of two main types, those that reduce surface tension at the air water interface (Biosurfactant) and those that reduce the interfacial tension between immiscible liquids or at the solid-liquid interface (Bioemulsifier). Biosurfactant usually exhibit emulsifying capacity but bioemulsifiers do not necessarily reduce surface tension [1-4].

Microbial biosurfactants are a structurally diverse group of amphipathic surface-active molecules synthesized by a variety of bacteria, yeasts and filamentous fungi [5]. Biosurfactant producing microorganisms were naturally present in the oil environment contain large amount of hydrocarbons. Microorganisms exhibit emulsifying activity by producing biosurfactants and utilize the hydrocarbons as substrate often mineralizing them (or) converting them into harmless products.

The utilization of hydrocarbon will be aided by improved contact cells. The ability to adhere on to hydrocarbon is correlated with cell surface hydrophobicity (CSH). Surfactants can increase the affinity between a microbial cell and an organic compound [6]. The drop collapse method depends on the principle that a drop of a liquid containing a biosurfactant will collapse and spread completely over the surface of oil [7, 8]. This method is easy and can be used to screen large number of samples [9]. The oil spreading technique measures the diameter of clear zones caused when a drop of a biosurfactant- containing is placed on oil-water surface [10].

Biosurfactants occur in nature as chemical entities such as glycolipids, phospholipids and lipopeptides [11]. They have a great variety of applications in medicine, households, agriculture and petroleum industry, including oil extraction and processing; cleaning of vessels from oil fractions, acceleration of well drilling and enhancement of oil recovery; they can also be used for ecosystem

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bioremediation, [12-15]. On the basis molecular mass, biosurfactants isolated from microorganisms are generally classified into two groups: (i) Low molecular mass biosurfactants such as glycolipids, lipopeptides, corynomycolic acids and phospholipids. (ii) High molecular mass molecules such as emulsans, alasan, liposan, polysaccharides and protein complexes [16-18].

The different types of biosurfactants include lipopeptides synthesized by many species of *Bacillus* and other species, glycolipids synthesized by *Pseudomonas sp., & Candida sp.,* phospholipids synthesized by *Thiobacillus thioxidants,* Polysaccharide- lipid complexes synthesized by *Acinetobacter sp.,* or even the microbial cell surface [19].

Biosurfactants have many properties such as soaping, emulsifying, foaming, dispersing etc., they are widely used in industrial and environmental such as microbially enhanced oil recovery (MEOR), oil tanks cleaning and bioremediation of oil polluted water and soil [20]. They are more active and less toxic than chemical surfactants which are difficult to remove or degrade from the environment. They can be used in handling industrial emulsions, control of oil spills, biodegradation and detoxification of industrial effluents and in bioremediation of contaminated soil [21].

The most important advantage of biosurfactant over chemical surfactants is probably their ecological acceptability. Biosurfactants are biodegradable and thus problems of toxicity and accumulation in natural ecosystems are avoided [22, 23]. In the environmental sector, biosurfactants have potential applications in bioremediation and waste treatment because of their inherent degradability. Indeed, microbial populations degrading petroleum hydrocarbons produce biosurfactants to increase substrate bioavailability [24].

MATERIALS AND METHODS

Isolation of Hydrocarbon Producing Organisms: Soil samples were collected from oil polluted soils in the vicinity of petrol bunks near Chidambaram and Coimbatore. Samples were serially diluted and plated on Sabourd's dextrose agar and Nutrient agar using spread plate technique. From this organisms were isolated and identified by using different preliminary techniques.

Screening Methods of Biosurfactant: *Emulsification test:*For emulsification test cultures were grown on Bushnell has broth with diesel as the carbon source, incubated in shaker for 7 days. Broth cultures were centrifuged at 6000 rpm for 20 minutes and emulsification factor was precipitated using chilled acetone and vacuum dried. Then the precipitate was dissolved and diluted with Tris Buffer (pH 8.0) in 30 mL screw capped test tube. 0.1 mL of diesel was added and kept in shaker for 20 minutes at 150 rpm and the mixture was allowed to stand for 20 minutes. Then the values were read at 620 nm in a spectrophotometer. Results were expressed as D610 [25].

Oil Spreading Method: The broth cultures were centrifuged at 6000 rpm for 20 minutes. 50 mL of distilled was added to the petridish followed by the 100 µl of cell free culture broth was dropped on to the surface of the diesel oil. The diameter of clear zone on the oil surface was measured and compared with 10 µl of distilled water as negative control [26].

Drop Collapse Method: [7] developed the drop collapse assay. This assay relies on the destabilization of liquid droplets by surfactants. Therefore, drops of a cell suspension or of culture supernatant are placed on an oil coated, solid surface. If the liquid does not contain surfactants, the polar water molecules are repelled from the hydrophobic surface and the drops remain stable. If the liquid contains surfactants, the drops spread or even collapse because the force or interfacial tension between the liquid drop and the hydrophobic surface is reduced. The stability of drops is dependent on surfactant concentration and correlates with surface and interfacial tension.

Microbial Adhesion to the Hydrocarbon (MATH): Microbial surface hydrophobicity was assessed by microbial adhesion to the hydrocarbon method (MATH) according to [27]. Yeasts cells were harvested from 7 days grown culture by centrifugation at 10,000 x g for 10 min at 4 °C and washed twice with PUM buffer (buffer salt solution (pH 7.0) containing KH2PO4 - 7.26 g l-1, K2HPO4 - 19.7 g l-1, urea - 1.8 g l-1 and MgSO4. 7 H2O - 0.2 g l-1). The cells were again suspended in PUM buffer to fit an optical density of ca.1.0 (A_0). Optical density was measured at 600 nm on UV Visible Spectrophotometer (Shimadzu). Diesel (500µl) was added to 5 ml of microbial suspension and vortexed for 2 min. The optical density of aqueous phase was measured (A_1) after 10 min. The degree of hydrophobicity is calculated as $[1-(A_0-A_1)/A_0.100\%]$. The experiment was repeated thrice.

Optimization Method [28]: To find the optimum conditions for biosurfactant production, the production media were maintained at various incubation periods (3rd, 6th, 9th, 12th and 15th day), temperature (10°C, 20°C, 30°C, 40°C and 50 °C), pH (2, 4, 6, 8 and 10) and the substrate concentration (0%, 0.5%, 1.0%, 1.5%, 2.0% and 2.5%) for both *C. tropicalis* and *C. albicans*. Both the cultures were inoculated in a 50mL of mineral salt medium and incubated. After the incubation period, cultures were centrifuged at 10,000 rpm for 10 minutes, supernatant was discarded and the pellets were taken and mixed with 5 mL of PUM buffer then the OD values were measured at 600nm.

Production of Intracellular Biosurfactant [29]: For the production of biosurfactant 1000mL of mineral salt medium was prepared with optimum pH, then the substrate with optimum concentration was added into the broth with both the cultures of *C. tropicalis* and *C. albicans* and was incubated in an optimum temperature and incubation period. After incubation, the broth cultures were centrifuged at 10,000 rpm for 10 minutes. Then the pellets were washed with PUM buffer and then used for further purification.

RESULTS AND DISCUSSION

Petroleum contaminated soil samples were collected and the organisms *Klebsiella sp., Bacillus sp., Aspergillus niger, Candida tropicalis, Candida albicans* and *Pseudomonas aeruginosa* were isolated from the soil. Then the preliminary tests were done to identify the organisms (Table 1).

The appearance of small tube like filaments projecting from the cell surface confirmed formation of germ tubes. From this the organism confirmed as a *C. albicans*. Lactophenol cotton blue mounting shows Phialides form on top of swollen vesicle at the end of a long conidiophore, from this the organism identified as *Aspergillus niger*. *Candida tropicalis* has appeared as white-cream coloured, puffy growth on solid media.

Among the cell free cultures of selected for in this study, *Pseudomonas aeruginosa* showed the highest production of biosurfactant and in the pellet *Candida tropicalis* and *Candida albicans* showed the highest production of biosurfactant. The results are shown in Table 2. Results showed that more intracellular biosurfactant production, when compared to extracellular by yeast cells. As *C. tropicalis, C. albicans* and P. *aeruginosa* showed similar range in the productivity of biosurfactant in emulsification test. These were further preceded for this study.

Emulsification activity of diesel showed that in each strain of *Bacillus megaterium, Corynebacterium kutscheri* and *Pseudomonas aeruginosa* were produced 0.85, 0.81 & 0.90 respectively, when compared to Triton X-100 is 0.94. These emulsification results showed that, biosurfactant produced from a substrate can emulsify different hydrocarbons to a greater extent which confirmed its applicability against different hydrocarbon pollution [30].

Experimental results on the emulsification activity of biosurfactant obtained from *Bacillus subtilis* HOB2 revealed that the samples were able to form a stable emulsions (E24= 68%) with kerosene for 24 h. Similar results of E24 values were obtained from biosurfactants isolated from 30°C and 45 °C growth culture [31].

Oil spreading and drop collapse methods are used for the screening of biosurfactant. In oil spreading test the three organisms *C. tropicalis, C.albicans* and *P. aeruginosa* were produced clear zone in the maximum level and in the drop collapse test the samples were collapsed (Fig. 1, 2 and 3). So, this is indicated that the three organisms produce biosurfactants.

Table 1: Gram stain and Biochemical test results.

S.No	Gram stain and Biochemical Test	Microorganisms		
		Bacillus	*Klebsiella*	*Pseudomonas aeruginosa*
1.	Gram stain	Gram positive, Rod, Non-motile	Gram Negative, Rod, Non-motile	Gram Negative, Rod, motile
2.	Indole	Negative	Negative	Negative
3.	Methyl Red	Negative	Negative	Negative
4.	Voges proskauer	Positive/Negative	Positive	Negative
5.	Citrate utilization	Negative	Positive	Positive
6.	Oxidase	Negative	Negative	Positive
7.	Urease	Negative	Positive	Negative
8.	H_2S	Negative	Negative	Negative
9.	Catalase	Positive	Positive	Positive

Table 2: Emulsification test values of biosurfactant in Cell free cultures & Pellets.

S.No	Micro organisms	Emulsification values	
		Cell free culture	Intracellular
1	*Klebsiella sp.,*	0.016	0.562
2	*Bacillus sp.,*	0.118	0.280
3	*Aspergillus niger*	0.005	0.512
4	*Candida tropicalis*	0.007	1.560
5	*Candida albicans*	0.015	1.505
6	*Pseudomonas aeruginosa*	0.123	1.070

Table 3: Math Assay

S.No	Organisms	MATH assay (Mean ± SD)
1	*Pseudomonas aeruginosa* (G1)	59.40 ± 20.95
2	*Candida tropicalis* (G2)	92.76 ± 2.61*
3	*Candida albicans* (G3)	89.32 ± 5.14*
	CD ($p<0.05$)	17.28

Values of mean ± SD of 5 samples in each group, Comparison between G1 vs G2, G3 * - Significance at 5% ($p<0.05$)

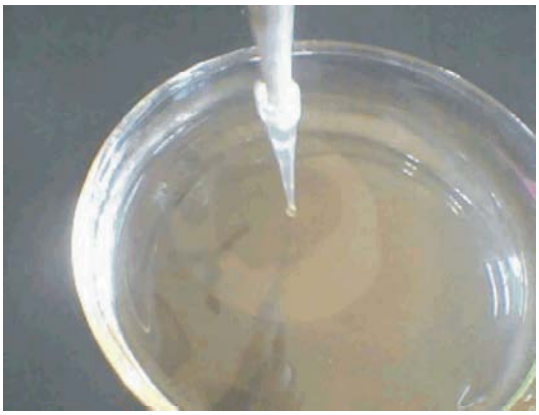

Fig. 1: Zone formation by biosurfactant producing bacteria *Candida tropicalis* in oil spreading technique.

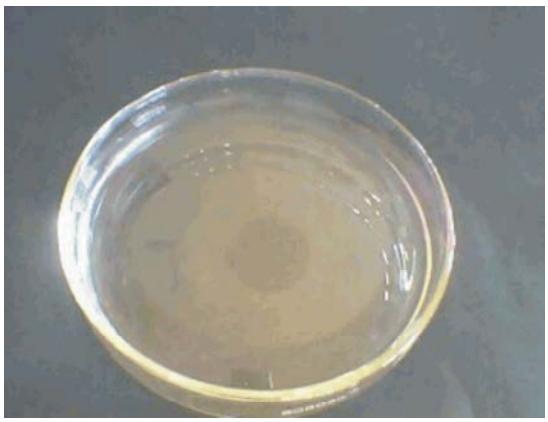

Fig. 2: Zone formation by biosurfactant producing bacteria *Pseudomonas aeruginosa* in oil spreading technique.

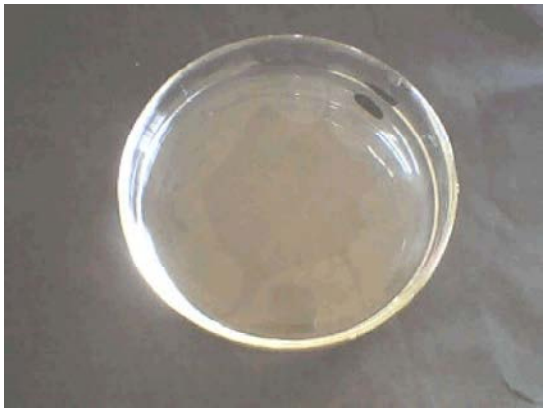

Fig. 3: Zone formations by biosurfactant producing bacteria *Candida albicans* in oil spreading technique.

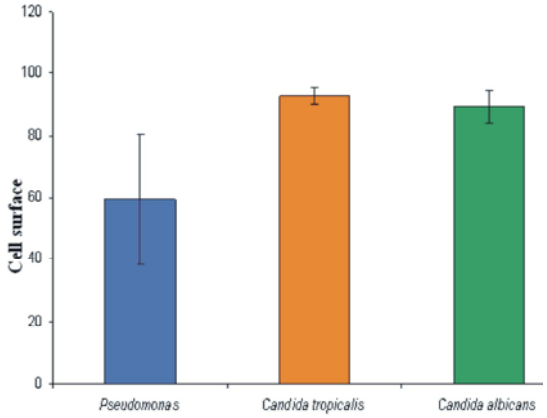

Fig. 4: Math Assay

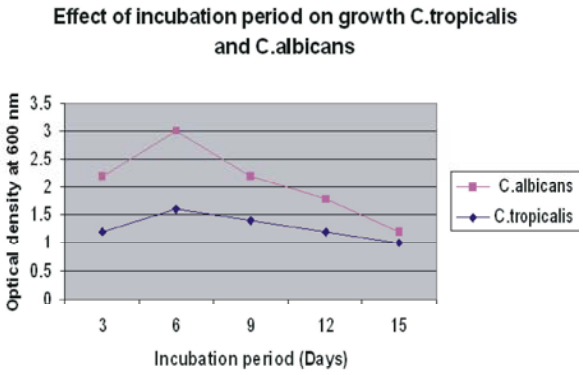

Fig. 5: Effect of incubation period.

In the drop collapsing test a flat drop was observed around the colonies of the isolate, which indicates a biosurfactant activity. Inoculation of the isolate on Tributyrin agar plate produced a clear zone which indicates production of the enzyme, lipase by *Streptomyces* spp. VITDDK3 [32].

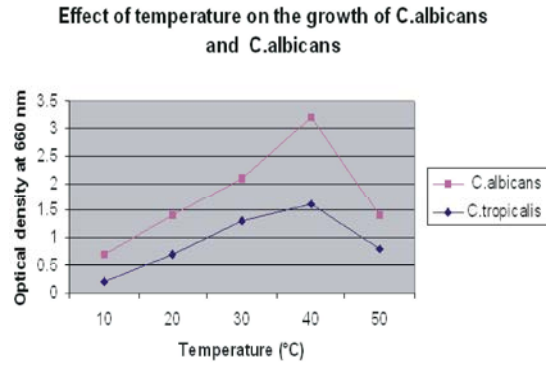

Fig. 6: Effect of temperature.

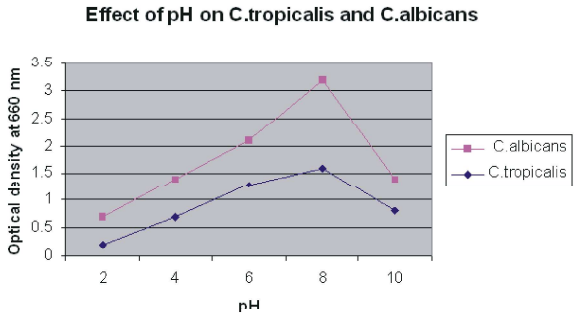

Fig. 7: Effect of pH

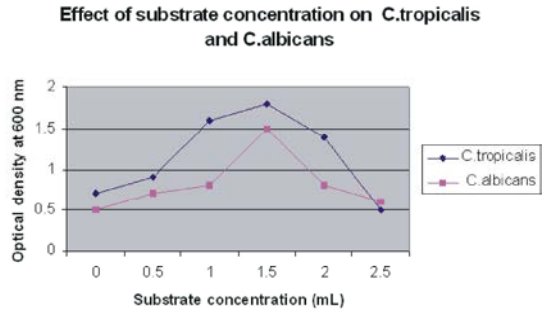

Fig. 8: Effect of substrate concentration.

In this present study, among the three microorganisms isolated *C. tropicalis* and *C. albicans* produce highest values than *P. aeruginosa.* These values are compared to statistical study by ANOVA method. The values are shown in Table 3 and Fig. 4. From this data to determine significant differences in cell surface hydrophobicity in the presence of diesel oil with different concentration while the difference between isolates were analyzed.

In the current study, optimization studies were carried out for the biosurfactant production with different incubation period, temperature, pH and substrate concentration. The maximum production in 6th day, 40°C and 6 respectively for both *C. tropicalis* and *C. albicans*, the substrate concentration was 1.5% for *C. tropicalis* and 2.0% for *C. albicans* (Fig. 5, 6, 7 and 8). The total quantity of production of biosurfactant was 1.71% for *C. albicans* and 0.91% biosurfactant was produced by *C. tropicalis*.

Temperature is one of the critical parameter that greatly affected the culture growth and the biosurfactant production. The result indicates that the optimum temperature for biosurfactant production was found to be between 30°C and 35°C. Another important characteristic of most organisms is their strong dependence on the pH for growth of organism and production of secondary metabolites. The results showed that highest biosurfactant production by *P. aeruginosa* OCD1 was obtained at pH 6 [33].

The pH ranges from 6, 7 and 8, temperature ranges from 35°C, 37 °C, 39 °C and kerosene concentration from 1%, 2% and 5% for optimization of biosurfactant production in *Bacillus* and *Pseudomonas sp*. Both the organisms have maximum growth rate observed at pH 7, temperature of 37°C in 0.3% kerosene concentration. [34].

Optimization studies were carried out for maximum biomass production. Incubation period seems to be the important factor for the biomass production and maximum growth was observed at 72h, growth in different pH values showed maximum at pH 7 and minimum at 10, maximum growth at temperature 35°C and minimum at 25°C, higher growth was observed at 0.3% salinity and minimum at 1.5% and maximum growth at glucose and minimum at glycerol was observed [35].

In the previous study, *Pseudomonas alcaligenes* S22 initial bacterial adhesion to hydrocarbon was only 8.4% and was comparable with the cell surface adhesion in a glucose system. An increase of cell surface adhesion to hydrocarbon was observed when hydrophobic carbon sources were added to the system. In the case when the model mixture of hydrocarbons (dodecane and hexadexane 1:2w/w) was present in the system the adhesion to hydrocarbon was 19.2%; however a higher adhesion to hydrocarbon 31.4% was recorded when diesel oil was in the system [8].

Biosurfactants or microbial surfactants are surface-active biomolecules that are produced by a variety of microorganisms. Biosurfactants have gained importance in the fields of enhanced oil recovery, environmental bioremediation, food processing and pharmaceuticals owing to their unique properties such as higher biodegradability and lower toxicity. Interest in the production of biosurfactants has steadily increased during the past decade. However, large-scale production

of these molecules has not been realized because of low yields in production processes and high recovery and purification costs. In future, due to its potentiality, some practical approaches that have to be adopted to make the biosurfactant production process economically attractive.

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