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ENHANCED AND COST-EFFECTIVE BIOSURFACTANT PRODUCTION FOR MARINE REMEDIATION CONTAMINATED WITH OIL SPILL

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ABSTRACT

Biosurfactant are surface-active agents produced by microbes which are employed in various industrial applications like microbial enhanced oil recovery (MEOR), cleaning oil contaminated sites with oil spillage, heavy metals. The most important property of biosurfactant is effectiveness at extreme conditions of temperature, pH, metal ions, salinity and pressure. An effective production of biosurfactant depends upon the selection of suitable substrate and fermentation medium which has to be cost-competitive to other commercially available chemical surfactants. Fermentation medium represents almost 40% of the total cost incurred for biosurfactant production. Current research focused on biosurfactant production using Candida lipolytica UCP 0988 studied with cost effective medium formulation along with 2% of waste frying oil, 2% corn steep liquor and 5% molasses at 120 h, 30 °C maintained at 180 rpm. Surface activities (ST and IFT) was found to be reduced reasonably (24 mN/m and 11 mN/m respectively). CMC (critical micelle concentration) was determined and found to be 0.32%. Since the produced biosurfactant was has the ability to reduce both ST and IFT, emulsification activity which is considered to be one of the key parameters was determined using motor oil (up to 90%) and vegetable oil (up to 30%). Moreover, the produced biosurfactant was subjected to highly sensitive conditions like temperature (up to 120 °C), pH (2.0 – 10.0) and salinity (10%). Biosurfactant was subjected to test the toxicity against bacteria and filamentous fungi in sea water by incubating for 30 days. Biosurfactant was added to marine conditions simulated degradation of motor oil with the activity of microbes. Results showed that biosurfactant produced by C. lipolytica UCP 0988 was found to be effective in oil recovery. Cell free broth proved to be effective by recovery of oil using displacement method. In a nutshell, the outcomes obtained indicates that produced biosurfactant is capable in oil recover applications and oil spill at marine conditions.

Key word: Biosurfactant, Oil Spillage, Surface Tension, Waste Frying Oil, *Candida lipolytica.*

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1. INTRODUCTION

Biosurfactants are naturally occurring compounds produced by bacteria and yeast with various substrates (sugars and hydrocarbon compounds). Generally various surfactants fall into two categories (i) low molecular weight compounds which includes lipopeptides, glycolypids and peptides. (ii) high molecular weight biosurfactant which includes polysacrrides, proteins and lipopolysaccharides [10]. Production and utilization of biosurfactant is expected to increase more by 2025, due to its industrial application in MEOR, removal of contaminants (oil spillage, heavy metal), cosmetics, food, pharmaceuticals and nanotechnology [7]. Due to the increased interest, bio-based products are produced since they are biodegradable, renewable substrates, less toxicity and biocompatibility at extreme conditions of reservoir like temperature, pH, metal ions and saline [7]. Current scenario, there are various sources of energy available but the need for crude oil had been increased significantly during last few decades. Generally crude oil is extracted using conventional primary method (with native pressure) followed by secondary recovery methods (water or gas injection). Huge amount of crude oil still remains as trapped in the cap rocks which are held by high interfacial tension between oil and water molecules. Approximately 55% of oil can be recovered using enhanced oil recovery (EOR) which is also known as tertiary recovery method. Injection of different agents like thermal, polymers, water alternative gas, chemical surfactants and microbial surfactants are employed to recover crude oil from trapped zone, and these are various methods of enhanced oil recovery m. Nearly 30 – 40% of oil remains trapped in the reservoir. Microbial enhanced oil recovery (MEOR) is one among the few techniques to improve oil recovery rate. Primary role of microbes EOR is utilized (metabolites generated by microbes) helps to recover oil by producing biosurfactant and this technique is known as Microbial EOR [10]. In-order to recover the remaining residual oil, technology like Enhanced Oil Recovery (EOR) is being utilized which is also known as tertiary oil recovery [5].

Microbial EOR considered to be one of the most promising technology for the improvement of residual oil. *Bacillus subtilis* can reduce surface tension from 72 mN/m to 25 Mn/m [1]. Similarly, effect of carbon and nitrogen source helps to improve the production of biosurfactant [3]. Carbon source mainly sucrose helped to produce biosurfactant. Increasing biosurfactant concentrations are highly sensitive to pH compared to concentration in the reservoir, while at larger resident time and water saturation; the microbial and nutrient concentrations were lesser due to enhanced dispersion [3]. Our earlier work on biosurfactant production using *Pseudomonas putida* MTCC 2467 tested with different source of carbon and nitrogen using minimal medium, with initial pH showed that sucrose and ammonium sulphate was best carbon and nitrogen sources [3]. In-spite of several advantages, MEOR at relatively low level because of the following factors: (a) understanding the mechanism on in-situ geo-environmental aspects of bacteria (b) stability of key parameters such as pH and water saturation on fundamental processes of MEOR process [11]. Although studies revealed that *Bacillus subtilis* can grow and produce biosurfactant with presence of different carbon and nitrogen sources under thermophilic conditions 45°C and reduced surface tension to 34 dynes cm^{-1} on 2% sucrose, and

32 dynes cm^{-1} on starch after 96 h of growth. Microbial surfactant activity was found to be unaltered even at elevated temperature of 100°C and wide pH range (3.0 - 11.0). Our previous work was on biosurfactants production from *Pseudomonas aeruginosa* ATCC 9027 at both mesophilic and thermophilic conditions, gave promising result for biosurfactant production with mineral salt medium. Further, reduction of surface tension from 73 mN/m to 34 mN/m and interfacial tension from 41 to 9 mN/m at 37° C which is higher than growth at 55° C. Also, it is highly suitable for MEOR applications where additional recovery rate was 8.5% [2].

2. MATERIALS & EXPERIMENTAL PROCEDURES

2.1. Materials

The waste frying used for our study was obtained from central canteen kitchen of AMET University. Corn steep liquor was purchased from local market.

2.1.1 Microbe

Candida lipolytica UCP 0988 was obtained from Culture Collection of Nucleous of Research in Environmental Sciences, Catholic University of Pernambuco, Brazil. The microorganism was maintained at the anamorph state at 5°C on Yeast Mold Agar (YMA) slants containing (w/v): 0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% glucose and 2% agar.

2.2. Methods

The production medium used for the experiments consisted of the following: 0.1% NH_4NO_3, 0.02% KH_2PO_4 and 0.02% $MgSO_4 \times 7H_2O$. Medium was further supplemented with 2% waste frying oil, 2% corn steep oil and 5% of molasses. The medium was sterilized using autoclaving at 121° C for 20 min. Final pH of the medium was adjusted to 6.2. The inoculum (2% v/v) was introduced in the amount of 10^4 cells/ml to cool medium of yeast [9].

2.2.1. Biosurfactant production

Production of biosurfactant was obtained by fermentation in 500 mL Erlenmeyer flasks with 100 mL working volume. Flasks with substrate were allowed to cool to room temperature (24 °C) before transferring 2% (v/v) inoculum (primary) cell suspension into the production medium. Culture was further incubated by maintaining for 120 h at 30 °C and 180 rpm.

2.2.2. Biosurfactant isolation

The biosurfactant produced was extracted from culture media after centrifugation at 8,000 rpm for 20 min. pH of supernatant was adjusted to 2.0 with 6.0 M HCl at 4 °C, during which precipitate was formed by adding methanol. After 48 h, mixture was again centrifuged and maintained at 30 °C until constant weight was obtained.

2.2.3. Surface, Interfacial and CMC analysis

Surface tension (ST) was determined from supernatants of the culture obtained by centrifuging culture at 8,000 rpm for 20 min using Sigma 700 digital tensiometer using Do Nuoy plate method. Interfacial tension was determined by mixing equal volume against the supernatant using tensiometer. Critical micelle concentration (CMC) was analyzed by measuring the surface tension of dilutions of biosurfactant (isolated) in distilled water until a constant value is obtained. CMC value was obtained from the plot between surface tension and surfactant concentration.

2.2.4. Emulsification index test

Emulsification index (EI) was determined as per the method described [1], where 4 ml of hydrophobic compound (vegetable oil, lube oil and motor oil) was added to 4 ml of the cell free broth in a graduated test tube and vortexed at high speed for 2 min. Emulsion stability was analyzed at 24 h and emulsification index was calculated by dividing the height of emulsion by the mixture of total height x100.

2.2.5. Effect of metal ions and salinity

The influence of metal ions and salinity may affect the activity of biosurfactant. To investigate the effect of metal ions on stability of biosurfactant, 10 g/L of various metal ions Na^+, K^+, Ca^{2+}, Mg^{2+}, Fe^{2+} and Al^{3+} were added to the biosurfactant samples. After 24 h of incubation, the samples were analyzed for surface tension. To determine the effect of salinity on biosurfactant stability, NaCl (10, 20, 30 40 and 50 g/L) was added to each biosurfactant sample and incubated for 48 h. Surface tension was measured to confirm the changes in bio-surfactant activity.

2.2.6. Artemia assay

Toxicity assay was performed by brine shrimp (Artemia salina) as indicator for toxicity. Brine shrimp eggs were utilized from local market. Larvae were used within one day of hatching. Dilutions of biosurfactant solution at 0.5 CMC and at 1.0 CMC with saline water (35 g/L) for concentration of 25%, 50%, 75%, the assays were performed in 10 mL penicillin tubes containing 10 brine shrimp larvae in 5 mL of each concentration of saline water/tube. The brine shrimp larvae in each tube were tested using 5 mL of each concentration of biosurfactant solution. The samples were observed for 24 h for the calculating mortality rate [4]. The toxicity threshold concentration, was expressed as biosurfactant concentration per 100 mL of saline water, the lowest concentration that killed all brine shrimp by 24 h. All the test tubes were run in triplicate and saline water was used as control.

2.2.7. Application of biosurfactant in hydrophilic contaminant spread

Oil displacement assay was done by dropping 10 µL of motor oil to 30 µL layer of distilled water in petri plate, which spreads to surface of water area. Further, 10 µL of cell free broth of surfactant (0.5 CMC) were added. Result were confirmed by taking clear zones of experiment done in triplicate, recorded and percentage were calculated [6].

2.2.8. Application of bio-surfactant in hydrophilic contaminant cleaning test

In order to check the cleaning ability of biosurfactant, inner wall of the beaker was coated with motor oil. The removal of adhered oil, 50 mL of broth (cell free) with isolated biosurfactant (0.5 CMC) and at CMC was added to each beaker followed by vortex for 2 min, allowed to stand for 6 h [5].

2.2.9. Test on bioremediation

In order to evaluate the potential of bioremediation application, methodology was adopted using standard test method for examining both normal water and waste water. 250 mL of Erlenmeyer flasks were filled with 100 mL of fresh sea water obtained from CPCL (Chennai Petroleum Corporation Limited), Chennai. 1% of motor oil, along with biosurfactant at concentration of 0.5 CMC and at 1.0 CMC. Experiment on control was carried out by using sea water (Kanathur beach, Chennai) and motor oil. Flasks were incubated at 30° C and 180 rpm were maintained and sacrificed on day 1, 11, 21 and 30 of incubation and analyzed for number of microbes using most probable number.

3. RESULTS AND DISCUSSION

3.1. Determination of biosurfactant yield, Surface & Interfacial tension and CMC

Biosurfactant yield produced by *Candida lipolytica* was 2.25 g/L and surface activities was reduced from 60 mN/m to 26 mN/m (surface tension) whereas Interfacial tension was reduced from 43 mN/m to 7 mN/m. [11] Similar result were reported for biosurfactant surface and interfacial tension activity. Similarly, the biosurfactant produced by *Candida lipolytica* was compared and in par with our previous work using *Pseudomonas aeruginosa* [2]. Produced biosurfactant were excellently exhibited the ability of ST at CMC of 0.40% (Figure1). Concentration of biosurfactant solution did not cause any reduction further in ST and IFT, which shows that CMC had reached at this concentration. Biosurfactant produced by *C. lipolytica* showed the ability to reduce ST and IFT better than biosurfacant produced by *C. guilliermondii* [9] and in par with the *P. putida*, *B. subtilis* [11] and *P. aeruginosa* [2].

3.2. Effect of environmental factors on biosurfactant activity

Since the diversity of chemical composition and properties, few features are found to be most common on biosurfactant. Features that represents the advantages over the conventional and commercially available surfactants over stability to temperature, pH, metal ions and Salinity [11]. Results (Table 1) shows that biosurfactant was able to resist against different pH values (2.0, 4.0, 6.0, 8.0 and 10.0), demonstrating that stability remains unaffected at extreme pH conditions. As the stability of biosurfactant reveals that the surface and interfacial tension was reduced irrespective of NaCl concentration. Biosurfactant stability was tested by incubating at different temperature ranges (40, 80 and 120° C). Results showed that stability was unaltered at temperature upto 120° C, whereas the temperature above 40 °C surface tension was found to be stable, indicating the possibility of biosurfactant used for control of environment pollution caused by oil compounds at elevated temperatures. Emulsification activity of biosurfactant produced was determined for various immiscible substrates in water under extreme environmental conditions (Table 1). We observed that high emulsion values were obtained from motor oil (>50%) which was tested with 4% NaCl. Stability was found to be neutral for emulsion formed for motor oil independent of pH ranges. Vegetable oil of corn and soybeans showed extremely good stability on emulsification activity (30 – 40%) regardless the amount of NaCl and pH values (Figure 2 & Figure 3).

3.3. Artemia assay

Biosurfactant produced by *C. lipolytica* showed no toxicity when tested against the micro-crustacean Artemia salina irrespective to the concentration (0.5 x CMC, 1.0 x CMC). The toxicity tests also showed that the biosurfactant caused 100 and 50% lethality when tested for concentration of 750 and 500 mg/L respectively.

3.4. Evaluation of the potential of biosurfactant in contaminant spreading

Oil displacement test were determined by gradually dropping 15 µL motor oil on the surface of 50 mL distilled water in petri dish, spread along the boundary of water surface. Further we added 10 µL cell free broth, consist of surfactant 0.5 x CMC and 1.0 x CMC to the oil surface. Experiment was conducted in triplicates and the result were recorded (clear zone) and calculated as % petri dish (Diameter) [6].

3.5. Evaluation the potential for bioremediation

Preformed experiment revealed that the variation in biosurfactant concentration in the production medium where saline water (sea) in presence nor absence, found to be non-toxic to

the microorganism present in the seawater, since there was growth for about 30 days post cultivation.

Table 1: Effect of temperature, pH and NaCl on surface tension, emulsifying activity on cell free broth of *Candida lipolytica* UCP 0988 grown with supplemented medium of waste frying oil, molasses and corn steep liquor

Temperature (° C)	**Surface tension (mN/m)**	**Emulsification Index (%) ***	**Emulsification Index (%) ****
0	48	37	33
40	43	40	37
80	42	48	39
120	42	45	41
pH	**Surface tension (mN/m)**	**Emulsification Index (%) ***	**Emulsification Index (%) ****
2.0	39	44	39
4.0	34	48	43
6.0	36	51	46
8.0	33	47	41
10.0	32	46	38
NaCl (%)	**Surface tension (mN/m)**	**Emulsification Index (%) ***	**Emulsification Index (%) ****
10	41	38	33
20	38	46	37
30	39	52	42
40	40	56	46
50	41	53	48

Emulsification Index (%) * - Motor oil Emulsification Index (%) ** - Vegetable oil

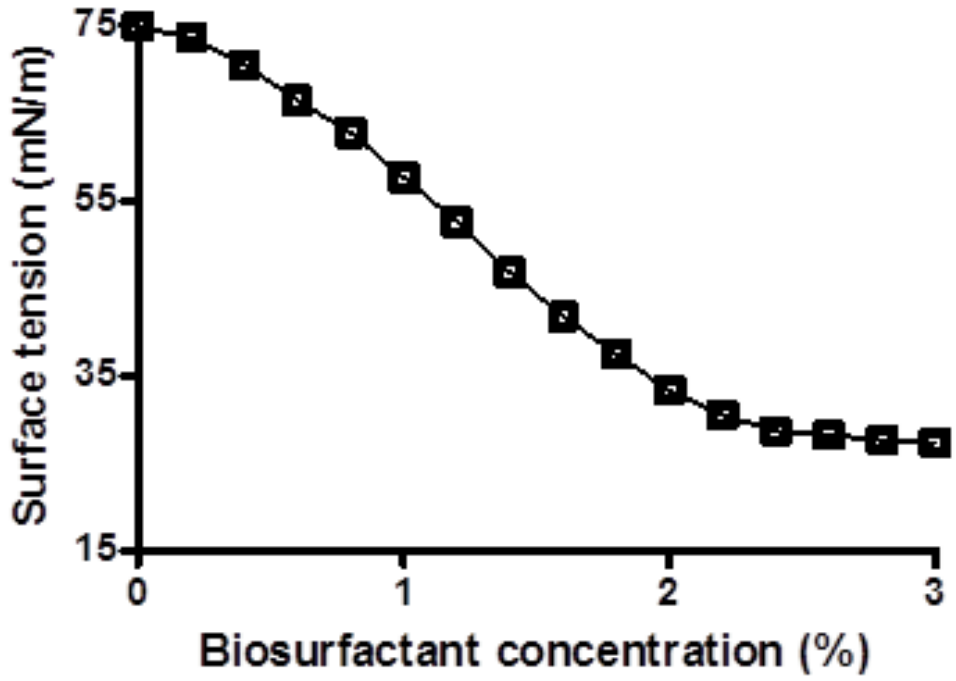

Figure 1: Profile of biosurfactant produced from *Candida lipolytica* UCP 0988 and surface tension

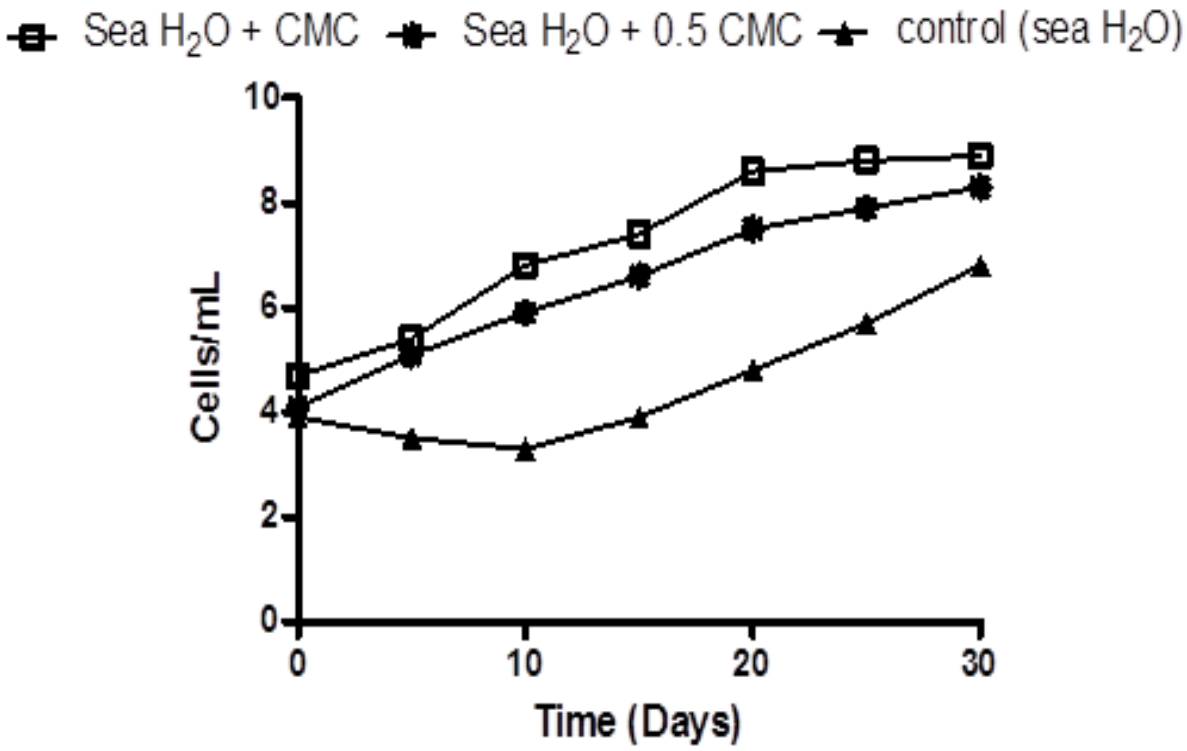

Figure 2: Profile of biosurfactant produced from *Candida lipolytica* UCP 0988 grown in sea water in the absence of petroleum

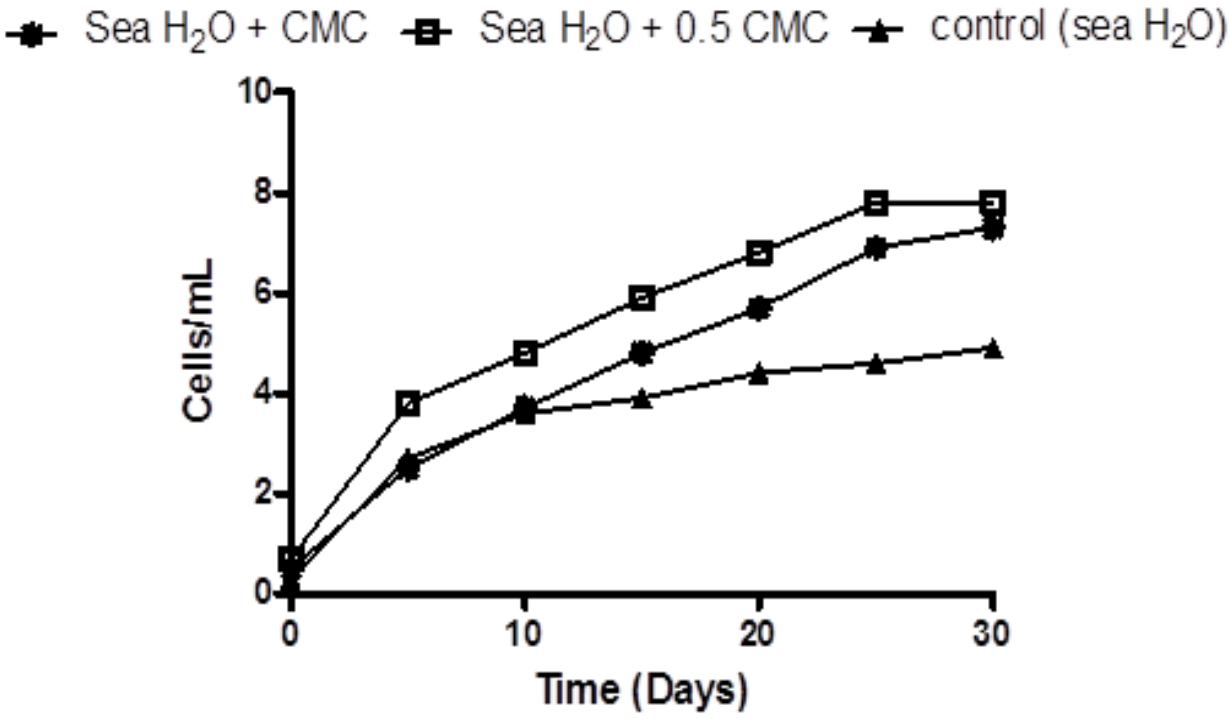

Figure 3: Profile of biosurfactant produced from *Candida lipolytica* UCP 0988 grown in sea water in the presence petroleum

4. CONCLUSION

Current research was mainly focused on biosurfactant production by *Candida lipolytica* in cost effective composition which was constituent with waste frying oil, corn oil and molasses which served as carbon source. The produced biosurfactant was found to be stable at high temperature and pH conditions. Moreover, biosurfactant produced by *C. lipolytica* UCP 0988 was found to be effective in oil recovery with motor oil. Hence it is clear evident from the result that produced biosurfactant is capable for oil recovery applications as well as treating oil spills in ocean.

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