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Production and Characterization of Bio Surfactant Using Renewable Substrates by *Pseudomonas fluorescence* Isolated from Mangrove Ecosystem

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ABSTRACT

This work was aimed to produce biosurfactant by Pseudomonas fluorescence MFS03 isolated from mangrove forest soil, Pitchavaram, Tamilnadu, India using renewable substrates. The maximum biomass (11.73 mg/ml) and biosurfactant production (9.23 mg/ml) was observed with coconut oil cake at 120 and 132 h respectively. Characterization of the biosurfactant revealed that, it is a glycolipid with chemical composition of carbohydrate (48.5 µg 0.1ml^{-1}) and lipid (50.2 µg 0.1ml^{-1}). The biosurfactant shows higher emulsification activity (89%) with crude oil and coconut oil (84%) among the different hydrocarbon tested. FT-IR spectrum revealed that the important adsorption bands at 3466.24 cm^{-1}, 2926.45 cm^{-1}, 1743.47 cm^{-1}, 1407.30 cm^{-1} and 1162.26 cm^{-1}indicate the chemical structure of rhamnolipid. Emulsification activity of the biosurfactant against different hydrocarbons showed its possible application in insecticide cleaning in vegetables. Monocrotophos with initial concentration of 100ppm was washed out with 10ppm concentration of the biosurfactant. From this investigation, biosurfactant production using renewable sources is economically low-cost medium and eco-friendly and the cleaning of insecticide residues in the vegetables leads the bioremediation of pesticides in the environment.

Keywords: Bio surfactant, Renewable sources, Rhamnolipid, Monocrotophos, and Emulsification.

INTRODUCTION

Surfactants is a surface active amphiphilic compounds are widely used in petrol industry, agriculture, pharmaceuticals cosmetics, plastic, textile, food and machinery fields [1]. It was estimated that more than 10 million tonnes of chemical surfactants and microbial biosurfactants were produced every year [2, 3]. Almost all the surfactant are chemically synthesized from non-renewable petroleum products, they are costly and poses potential threats to the environment due to their recalcitrant nature. To overcome with this problem microbial surfactants are the best alternatives in the surfactant production.

As compared with chemical surfactants, biosurfactants are superior with many aspects such as lower toxicity, higher biodegradability and, hence, greater environmental compatibility, better foaming properties (useful in mineral processing), and stable activity at extremes of pH, salinity and temperature [4]. Thus, biosurfactants become alternatives to chemical surfactants in broad range of applications [5,6].

However, the main factor that restricts the widespread use of biosurfactants is their production cost when compared to their synthetic counterparts [7]. One possible strategy for reducing cost is the

utilization of alternative substrates such as agro industrial wastes [8]. In order to solve these problems, many studies have been carried out using low-cost feedstock or agricultural by products as substrates for biosurfactant production. Low-cost carbon sources that have been used for biosurfactant production by microorganisms include sludge palm oil [9], cassava wastewater [10], vegetable oil refinery waste [11], molasses [12] and raw glycerol [13].

Low yield of biosurfactant is a major limitation influencing its commercialization. Hence, in the present study, waste fried vegetable oil and coconut oil cake were tried as cheaper carbon sources as compared to glucose and other petroleum based substrates for biosurfactant production. Considering the importance of biosurfactant, the present investigation was conducted for the production of biosurfactant by *Pseudomonas fluorescence* MFS03using waste fried vegetable oil and coconut oil cake as cheaper carbon sources and the influence of biosurfactant addition on cleaning of insecticide residues in brinjal.

MATERIALS AND METHODS

Microorganism: *Pseudomonas fluorescence* MFS03 was isolated from Mangrove forest soil, Pitchavaram, India using Mineral salt medium supplemented with 0.1% (v/v) of crude oil and the species level is identified by following Bergey's Manual of Determinative Bacteriology [14].

Media and growth conditions: The potential biosurfactant producer was cultured in fermentation medium which contains (g/L^{-1}): 1.0 K_2HPO_4, 0.2 $MgSO_4.7H_2O$, 0.05 $FeSO_47H_2O$, 0.1 $CaCl_2.2H_2O$, 0.01 $Na_2MoO_4.2H_2O$, and 30 NaCl. The culture conditions are as follows- pH 8.0, temperature 38 ° C, salinity 30% (w/v) and 2.0% substrate concentration (Waste fried vegetable oil and coconut oil cake).

Estimation of Growth and Bio surfactant Production : Five ml sample of culture broth were collected at 12h intervals for a period of 168 h. Biomass was estimated gravimetrically, broth culture was filtered through a Millipore filter paper (0.45mm) and dried at 80 ° C in hot air oven and weighed. Dry weight of biomass was quoted in terms of mg ml^{-1}. The culture broth was centrifuged at 12,000 rpm for 10 min at 4 ° C and extracted with equal volume of ethyl acetate. The solvent was removed by rotary evaporation and the residues was partially purified in silica gel (69-120 mesh) column eluted with chloroform and methanol ranging from 20:1 to 2:1 (v/v) in a gradient manner. The fractions were pooled and solvents were evaporated, resulting residues was dialyzed against distilled water and lyophilized. Bio surfactant concentration in the culture broth was estimated according to the procedure described by [15] and the biosurfactant concentration was expressed as mg ml^{-1}

Bio surfactant characterization

Estimation of emulsification activity: TLC purified biosurfactant (2mg) was dissolved in 2ml of Tris buffer (pH 8.0) in a 10 ml test tube. Hydrocarbons like Kerosene, crude oil, xylene, n-hexadecane, heptane, coconut oil, diesel and Benzene were tested for emulsification activity. 2 mg of hydrocarbons was added to the above biosurfactant solution and mixed well in a vortex mixture for 5 min and the mixture was allowed to stand for 30 min. Stability of the emulsion was estimated by the height of emulsified layer, divided by the total height of the liquid layer and multiplied by 100. The emulsification index was expresses as E_{24}%.

Biochemical composition of biosurfactant: Carbohydrate content of the biosurfactant was determined by the method of [16], Protein content was estimated by following the procedure of [17]. The lipid content was determined by the method described by [18].

Fourier Transform infrared spectroscopy: Fourier Transform infrared spectroscopy (FT-IR) is most useful for identifying types of functional groups (chemical bonds), therefore can be used to elucidate some components of an unknown mixture. Freeze-dried biosurfactant (5mg) was ground with 50mg of KBr and

pressed with 7,500 kg for 30 s to obtain translucent pellets through which the beam of the spectrometer can passed. The IR spectra of crude biosurfactant were recorded in a FT-IR spectrometer (Thermo Nicolet, AVATAR 330 FFT-IR system, Madison WI 53711-4495) with the spectral resolution of 4000-400 cm^{-1} respectively. The spectra was recorded and analyzed.

Field experiment of insecticide cleanup with biosurfactant: Bio surfactant as insecticide cleaning agent in brinjal was studied in the present study. The pot culture was conducted at department of microbiology, Annamalai University in November to April (2012) duration. The experiment was conducted in pot culture with two sets; each set containing ten pots. Monocrotophos solution, (100ppm concentration) was sprayed on the 4^{th} month of the vegetation period in two sets of pot culture. Followed by ten days interval biosurfactant with various concentrations (5ppm, 10ppm, 15ppm and 20ppm) were sprayed in one set of the pot culture. After the interval of 10 days brinjal were collected from the two different set of the pot culture and the monocrotophos residues were determined spectrophotometrically by the absorbance at 560nm.

RESULTS AND DISCUSSION

Estimation of growth and biosurfactant production: Physiological and biochemical characteristics of *Pseudomonas fluorescence* MFS03 are illustrated in Table 1. The biosurfactant production was studied using 2.0% waste fried vegetable oil and coconut oil cake. Fig. 1show the time-course of biosurfactant production by *Pseudomonas fluorescence* MFS03 with waste fried vegetable oil as the substrate. Maximum biosurfactant concentration of biosurfactant of 4.21 mg ml^{-1}occurred at 132 h of incubation, when the cells reached their early stationary phase. Maximum biomass was observed at 120 h (6.42 mg ml^{-1}). Bio surfactant with coconut oil cake shows similar result, but with the higher biomass (11.73 mg ml^{-1}) and biosurfactant production (9.23mg/ml) than waste fried vegetable oils (fig.1).

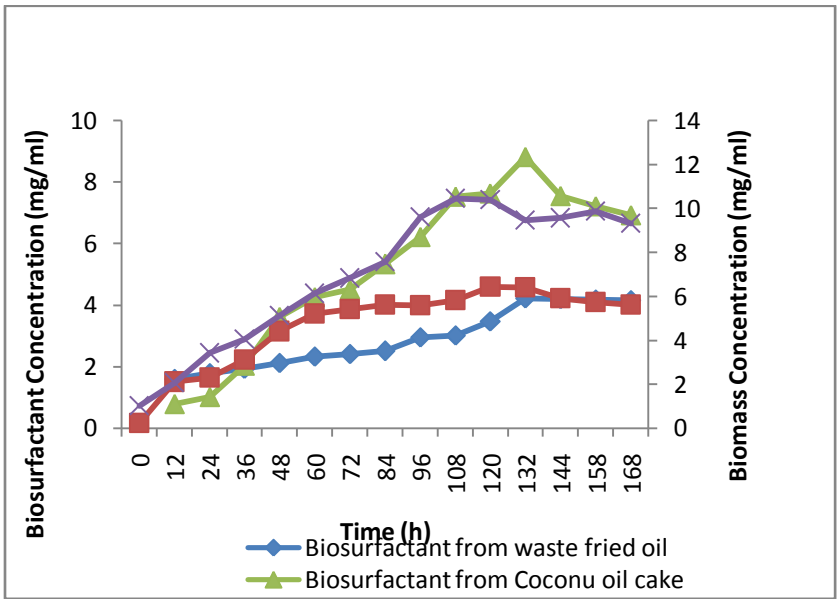


Fig.1 Growth and biosurfactant production by *Pseudomonas fluorescence* MFS03 using waste fried vegetable oil and coconut oil cake.

Table.1 Characterization of *Pseudomonas fluorescence* MFS03

Name of the test	Result
Gram reaction	–
Shape of the cell	Rod
Mobility	+
Spore formation	–
Pigment	Green
IMViC	–
H2S production	–
Utilization of carbohydrate: Glucose	+
Lactose	–
Trehalose	–
Sucrose	+
Xylose	–
Hydrolysis: Strach	–
Gelatin	–
Catalase	+
Oxidase	+
Nitrate reduction	–
Litmus reaction	–

Estimation of Emulsification activity : Bio surfactant isolated from *Pseudomonas fluorescence* MFS03 showed maximum emulsification activity against crude oil. Emulsification activities of the biosurfactant with different hydrocarbons were illustrated in (Fig. 2).The emulsion formed by the biosurfactant against each hydrocarbon was stable for 1 month. Emulsification of the hydrocarbon by the biosurfactant produced by *Pseudomonas fluorescence* MFS03 reflects the possible application in hydrocarbon pollution.

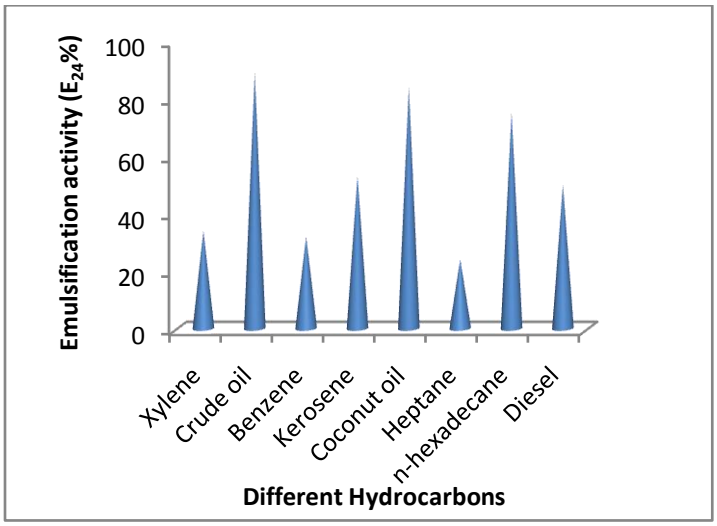


Fig.2 Emulsification activity of different hydrocarbons by biosurfactant produced from *Pseudomonas fluorescence* MFS03.

Characterization of biosurfactant: The biochemical composition of the biosurfactant revealed that, the compound constitutes substantial amount of carbohydrate 48.5 µg 0.1ml^{-1}, protein 18 µg 0.1ml^{-1}and lipid 50.2 µg 0.1ml^{-1}The quantification of compound gave a higher ratio in lipid and carbohydrate compared to protein. Based on the analysis, the compound was partially confirmed as glycolipid. FT-IR analysis of the biosurfactant showed that, the most important adsorption bands located at 3466.24 cm^{-1} (OH bond, typical polysaccharides), 2926.45 cm^{-1} and 2856.23 cm^{-1} (CH band: CH_2-CH_3, hydrocarbon chains), 1743.47 cm^{-1} and1601.26 cm^{-1}(for C=O, C=O ester bond), 1407.30 cm^{-1}(C-N amide groups). The C-O stretching bands at 1162.26-1232.88 cm^{-1} confirm the presence of bonds formed between carbon atoms and hydroxyl groups in the chemical structures of the rhamnose rings and 846.93,652.05(for the CH_2 groups) (Fig.3). Therefore, it can be concluded that the biosurfactant produced by *Pseudomonas fluorescence* MFS03 *is* a rhamnolipid structure. (Fig. 3)

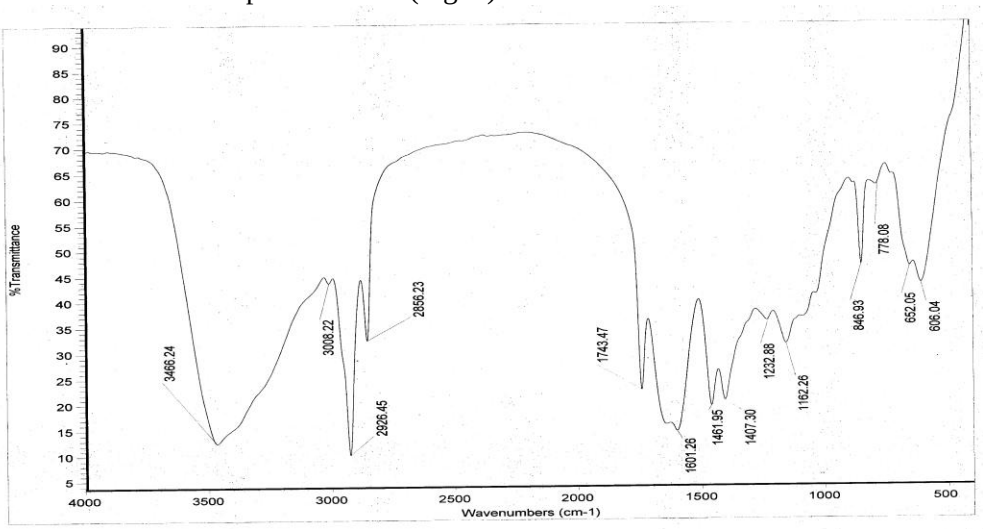
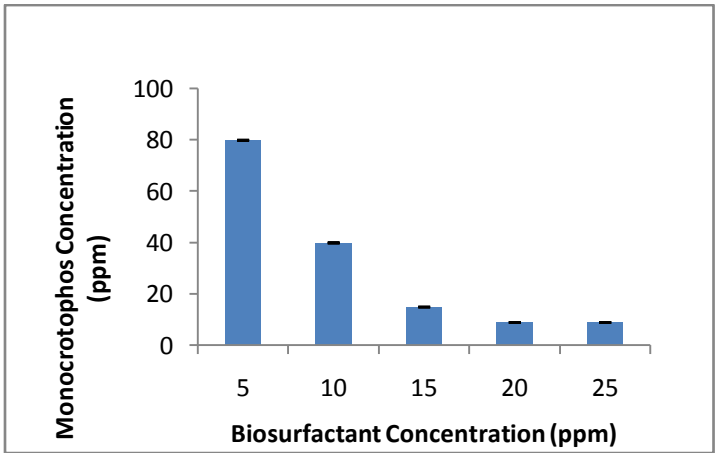


Fig.3 FT-IR spectral analysis of the biosurfactant produced by *Pseudomonas fluorescence* MFS03

Field experiment of insecticide cleanup with biosurfactant: The monocrotophos residues were determined according to the method described by [19] using spectrophotometrically. The residues were

determined by the formation of reddish-violet colour which can be detected quantitatively at 560nm. In the present experimental study, in the first set of the brinjal, monocrotophos residues were detected by the observation of peak at 560nm spectrometrically. Whereas in the second set of brinjal, which has been treated with different concentration of biosurfactant followed by the application of monocrotophos 100ppm no peaks were observed. Monocrotophos solution, with initial concentration of 100ppm was washed out with 10ppm of biosurfactant solution (Fig. 4). After the treatment with biosurfactant the monocrotophos residues were reduced to 2ppm of the safe level.



Percentage of insecticide clean up by biosurfactant

Fig. 4 Effect of Insecticide versus Biosurfactant concentration on washing of insecticide residues in brinjal.

In the present study, the possibility of biosurfactant production using cheaper renewable substrate like waste fried vegetable oil and coconut oil cake as carbon source was reported. The production of biosurfactant using cheaper carbon sources was already reported by earlier studies, plant-derived oils [20],oil wastes [21], starchy substances [22], molasses [23], cashew apple juice[24] and agriculture residues [25] which has been supporting the present study on use of renewable substrates for the biosurfactant production.

The increase in biosurfactant production and biomass were found to be from 16 to 120 h, but the maximum biosurfactant concentration was found at 132 h. Higher production of biosurfactant even after the off set of biomass may be due to the release of cell bound biosurfactant at the early stationary phase (132 h), which leads to rise in extracellular biosurfactant concentration in the medium [26].

In this study, biosurfactant produced by *Pseudomonas fluorescence* MFS03 using waste fried vegetable oil and coconut oil cake showed good emulsification activity against eight different hydrocarbons, whereas Emulsification activity of the biosurfactant was low against kerosene, diesel, xylene, heptanes and benzene. This finding is similar to that of [27] who found that, the biosurfactant obtained from *Rhodococcus* sp. was more effective towards motor oil and corn oil than the shorter-chain alkanes. Soybean oil, diesel oil, gasoline and cyclohexane are good substrates for emulsification by rhamnolipid-type biosurfactant of *Pseudomonas aeroginosa* while n-hexane and fish oil result in poor emulsification was reported by [28]. Further, it encouraged the aim of the present study to produce biosurfactant using *Pseudomonas fluorescence* MFS03 from cheaper renewable substrates as carbon source with high emulsification property. It inferred that, biosurfactant produced with one carbon source like waste fried oil or coconut oil cake could e used to remediate different hydrocarbon pollution. In the present study, the rhamnolipid type biosurfactant from *Pseudomonas fluorescence* MFS03 was confirmed through the use of FT-IR spectral analysis. The allocation conspicuous absorption in the spectra obtained corresponded to the characteristic group absorption of rhamnolipids [29-31]. Monocrotophos (dimethyl-(E)-1-methyl-2-(methylcarbamoyl)vinyl phosphate) is an organophosphorus, nonspecific systemic insecticide used to

control common pests in plants, its water-soluble nature helps it penetrate quickly into plant tissues [32]. Monocrotophos with initial concentration of 100ppm, the amount of biosurfactant that is needed during the treatment to reduce the monocrotophos residues to a safe level below 2ppm is 10ppm of biosurfactant. The determination of monocrotophos residues by spectrophotometrical method was carried out according to [19]. The formation of reddish-violet colour indicated the presence of monocrotophos residues in the test sample, which was occurred by the addition of diazotized p-amino acetophenone (DPAAP) by coupling with N-methyl acetoacetamide, the hydrolytic derivatives of monocrotophos residues [19]. Present finding was similar to that of earlier study reported, cleaning of cypermethrin residues using biosurfactant in leafy vegetables [33]. In the present study, the attempt made on insecticide cleaning in the pot culture experimental set up revealed that the maximum insecticide residues was washed out with the addition of low quantity of biosurfactant. This information obtained by this study may be useful for the bioremediation of pesticides compounds in the environments.

CONCLUSION

The present study is an attempt to find economically cheaper carbon sources for the large scale production of biosurfactant. Results obtained in biosurfactant production using waste fried vegetable oil and coconut oil cake suggested the possibilities of using economically cheaper carbon sources for the industrial level production of biosurfactant. The emulsification activity of the biosurfactant produced from *Pseudomonas fluorescence* MFS03 against different hydrocarbons indicated its diverse applicability against different hydrocarbon pollutions also as a cleaning agent against the insecticide residues in vegetables.

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