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ORIGINAL ARTICLE

BIOSURFACTANT AS A PESTICIDE CLEANING AGENT IN LEAFY VEGETABLES PRODUCED BY PSEUDOMONAS FLUORESCENS ISOLATED FROM MANGROVE ECOSYSTEM

GOVINDAMMAL M AND PARTHASARATHI R

Department of Microbiology, Annamalai University,
Annamalai Nagar, Chidambaram .

Abstract:

In this present investigation, biosurfactant was produced by Pseudomonas fluorescens MFS03 isolated from mangrove forest soil using groundnut oil cake as substrate. Characterization of the biosurfactant revealed that, it is a glycolipid with chemical composition of carbohydrate (0.868 mg/ml) and lipid (1.912 mg/ml). Based on the TLC analysis, carbohydrate and lipid with an Rf value of 0.72 were separated and it was partially identified as rhamnolipid. In FT-IR spectral analysis, important absorption bands at 3466.24, 2926.45, 1743.47, 1407.30 and 1162.26 cm-1 indicates the chemical structure is identical to those of rhamnolipids. Biosurfactant was used for the cleanup of insecticide residues in the spinach varieties Amaranthus tricolor. L generally called as Arakkeerai or Killukeerai. The experiment was carried out under in-vitro conditions. Among the different washing solution (Salt, vinegar KMnO4 and biosurfactant) used for the reduction of pesticide residues, biosurfactant solution shows higher reduction. However the amount of biosurfactant and the contact times shows significant role in reducing cypermethrin residues in spinach. Cypermethrin solution with 100ppm concentration was reduced to below 2 ppm, which is safe level using 25 ppm of biosurfactant solution for 5 minutes. The increase in amount of biosurfactant concentration shows decrease in contact time in reducing cypermethrin residues. From this study we concluded that production of biosurfactant using groundnut oil cake is economically low-cost medium and eco-friendly and it can be used as an effective agent to clean up pesticide residues in vegetables and leafy vegetables.

KEYWORDS:

Biosurfactant, Amaranthus tricolor. L, Pseudomonas fluorescens, cleaning agent, cypermethrin.

INTRODUCTION

In agriculture production, various pesticides have been used for protection against plant diseases and insect pests with the result, these chemicals persist for longer period in fruits and vegetables which causing harm to human beings. Concern over the pesticide residues in fruits and vegetables have led to the development of many clean up and analysis methods. Biosurfactants are amphiphilic compounds produced by microorganisms, which contain both hydrophobic and hydrophilic moieties that confer the ability to accumulate between fluid phases, thus reducing surface and interfacial tension at the surface and interfacial respectively (Cunha et al., 2004).

In comparison to their chemically synthesized surfactants, biosurfactants have many advantages, environmentally friendly, biodegradable, less toxic and non-hazardous. They have better foaming properties and higher selectivity, active at extreme temperature, pH and salinity as well and can be produced from agro-industrial byproducts. This last feature makes cheap production of biosurfactant

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possible and allows utilizing waste substrates and reducing their polluting effect at the same time (Kosaric, 2001; Rahman et al., 2003; Das and Mukherjee, 2007; Das et al., 2008). Because of their potential advantages, biosurfactants are widely used in many industries such as agriculture, food production, chemistry, cosmetic and pharmaceutics (Muthusamy, 2008; Banat et al., 2010).

Despite possessing many commercially attractive properties and clear advantages compared with their synthetic counter parts, the production of microbial surfactants on a commercial scale has not been realized because of their high production costs. The cost can be reduced by the selection of efficient strains, optimizing medium composition and by using the alternative inexpensive substrates for the biosurfactant production. Taking these into account and considering the need of potential biosurfactant producers, economic production processes using agro-industrial wastes and the pesticide clean up in the leafy vegetable. The present study aims to explore the Pseudomonas fluorescens MFS03, an unexplored resource for the production of biosurfactant using groundnut oil cake and explore the possibility of using biosurfactant for the cleanup of insecticide residues (cypermethrin) in the spinach. (Amaranthus tricolor L).

MATERIALS AND METHODS:

Isolation, screening and identification of biosurfactant producing bacteria:

Pseudomonas fluorescens MFS03 was isolated from crude oil enriched Mangrove forest soil, Pichavaram, India. The species level was identified by following Bergey's Manual of Determinative Bacteriology (Buchanan et al., 1974). The isolate was screened for the biosurfactant production which includes, drop collapsing test (Youssef et al., 2004) Oil spreading test (Morikawa et al., 2000), CTAB plate assay (Siegmund and Wagner 1991), Haemolytic activity (Carrillo et al., 1996) was detected as the occurrence of a define clear zone around the colony. Lipase activity was measured using the tributyrin agar plates as described by (Kiran et al., 2010). Emulsification activity was performed according to (Cooper and Goldenberg, 1987). The entire assays were performed in triplicates.

MEDIA AND GROWTH CONDITIONS

The potential biosurfactant producer was cultured in fermentation medium which contains (g/L-1): NaNO3 (1.28), K2HPO4 (0.87), MgSO4.7H2O (0.1), NaCl (0.1), KCl (0.2), Tris (hydroxymethyl) aminomethane (6.5), glucose (20); mineral salt solution (5 ml). The mineral salt solution contained the following ingredients (gL-1): FeSO4 (NH4)2SO4.6H2O, H3BO3, COCl2.6H2O, CuSO4.5H2O, MnSO4.H2O, (NH4)6Mo7O24.4H2O, ZnSO4.7H2O. The pH of medium was initially adjusted to 6.7 by 1.0 M HCl. In groundnut oil cake media, glucose in the above medium was replaced by groundnut oil cake (2%). All the media prepared were autoclaved under standard conditions and used in the experimentation.

EXTRACTION OF BIOSURFACTANTS:

The cells were removed from the culture broth by centrifugation at 10,000 x for 20 min and the supernatant was filtered through whattmans filter paper No.1. The cell-free supernatant was acidified with 2 N HCl to pH 2 and extracted with ethyl acetate (1:1 ratio, 5 times). The extracted fractions were combined and evaporated to dryness under reduced pressure in a rotary vacuum evaporator (Rotavapor R-205; Buchi, Bern, Switzerland). Rhamnolipids from the extract were purified on a silica gel (100-200 mesh, 30 × 2 cm) column washed with chloroform and then eluted with 3% methanol in chloroform to remove traces of contaminants. The active fractions were analyzed by thin-layer chromatography (TLC) on silica gel 60 plates (F254; Merck, Darmstadt, Germany) in a mobile phase consisting of 20% methanol in chloroform. The plates were developed by spraying with phosphomolybdicacid reagent followed by heating at 100° C for 5 min. The pure rhamnolipids were obtained after vacuum evaporation of the solvents and lyophilized (Cameotra, 1995).

FTIR SPECTRAL ANALYSIS OF BIOSURFACTANT

The FT-IR spectra was recorded in a Thermo Niocolet, AVATAR 330 FT-IR system, Madison WI 53711-4495, in the spectral region of 4000-400 cm-1 using potassium bromide (KBr) solid cells. The analysis was done in the Department of Chemistry, Annamalai University, India. The spectra were recorded and analyzed using the standard methods described by the previous authors (Lin et al., 1994; Yin et al., 2008).

ANTIMICROBIAL ACTIVITY OF THE BIOSURFACTANT:

Extracted active compounds were tested against human pathogens such as Candida albicans, E. coli, Proteus mirabilis, Staphylococcus epidermidis, hemolytic Streptococcus, Enterococcus faecalis, Klebsielle pneumonia and Staphylococcus aureus using well-diffusion method. Mueller Hinton agar plates were prepared and swabbed with pathogens. Using cork borer well was made and 50 µl of those extracted compound and the standard (10 µl streptomycin) was added in wells, incubated at 30 °C for 24 h. After incubation, the clear zone was measured and calculated.

Biosurfactant as cleaning agent for insecticide residues on spinach

Effect of biosurfactant on washing insecticide residues:

The experiment was carried out under invitro conditions. Cypermethrin solution with 100ppm concentration (200µl of cypermethrin, 10% in 200 ml water) was prepared and the spinach was treated with the prepared solution. Different washing solutions were prepared which includes, Salt (dissolve 0.2 g of salt in 200 ml of water), Vinegar (2ml in 200ml of water), KMnO4 (dissolve 0.375 g of KMnO4 in 200 ml of water) and biosurfactant solution (2µl of biosurfactant in 200 ml of water i.e. 10ppm) Cypermethrin treated spinach were washed with the different washing solution for 25 minute. Few drops of ninhydrin were added and heat for the colour change was observed by measuring the absorbance at 485 to 590nm spectrophotometrically.

Effect of concentration biosurfactant in reducing cypermethrin residues:

Cypermethrin solution with 100ppm concentration was prepared and applied to the spinach. Biosurfactant solution with various concentrations (5, 10, 15, 20, 25 ppm by adding 1, 2, 3, 4, 5 µl of biosurfactant in 200 ml of water).The spinach treated with cypermethrin residues were washed with the biosurfactant solution for 25 minutes. Few drops of ninhydrin were added and heat for the colour change. The pesticide residues in the solution were determined by measuring absorbance at 490nm by spectrophotometrically.

Effect of concentration of biosurfactant and contact time in reducing cypermethrin residues:

Cypermethrin solution with 100ppm concentration was prepared and applied to the spinach. Biosurfactant solution with various concentrations (5, 10, 15, 20, 25 ppm by adding 1, 2, 3, 4, 5 µl of biosurfactant in 200 ml of water).The spinach treated with cypermethrin solution were washed with various concentration of biosurfactant solutions and the spinach was taken out at different time interval (5, 10, 15, 20, 25 min). Few drops of ninhydrin were added and heat for the colour change. The pesticide residues in the solution were determined by measuring absorbance at 490nm by spectrophotometrically.

RESULTS:

Isolation screening, identification of biosurfactant producer:

The morphological and biochemical characters of the isolate were studied and the species level i.e. Pseudomonas fluorescens MFS03 was identified by following Bergey's Manual of Determinative Bacteriology (Buchanan et al., 1974). The screening tests performed on Pseudomonas fluorescens shows positive results, which includes haemolytic activity (8.6mm) (Fig-1), Oil spreading test (7.6 mm), lipase activity(71U/mg) positive for drop collapsing test and formation of dark blue colour zone on blue agar plates indicates positive for CTAB (Fig-1) plate assay and Emulsification activity (82%) (Fig-1). Based on the screening tests performed the pseudomonas fluorescencs was identified as biosurfactant producing bacteria.

Chemical characterization of biosurfactant:

The concentrated extract of Pseudomonas fluorescens MFS03 contained 0.125 mg/ml protein, 0.868 mg/ml carbohydrate, 1.912 mg/ml lipid. Based on the TLC analysis, carbohydrate and lipid with an Rf value of 0.72 were separated. From the above result it was confirmed as rhamnolipid. The column eluted

fraction shows high emulsification active (83%) with kerosene that active fraction was subjected to FT-IR analysis. Fourier Transform Infrared (FT-IR) spectrum revealed that, the most important adsorption bands located at 3466.24 (OH bond, typical polysaccharides), 2926.45 and 2856.23(CH band: CH2-CH3, hydrocarbon chains), 1743.47 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 CH2 groups) (Fig-2).

Antimicrobial activity of biosurfactant

The purified biosurfactant compound showed highest antimicrobial activity against human pathogens (Fig-3).The compound showed highest activity against Entercoccus faecalis and Staphylococcus epidermidis, it also showed activity against the pathogenic yeast C. albicans. However, it does not show activity against E. coli and Klebsielle pneumoniae.

Biosurfactant as pesticide cleaning agents:

The cleaning process was carried out with four different solutions, salt, vinegar, KMnO4 and biosurfactant to determine the effectiveness in reducing pesticides from spinach. The pesticide contaminated spinach was washed with the four sets of solution for 25 minutes The amount of cypermethrin residues were detected at OD 485nm to 590nm (Fig-4).

Effect of concentration of biosurfactant and contact time in washing cypermethrin residues:

The cypermethrin concentration 100ppm was significantly reduced to the safe level below 2ppm (1.24 ppm) when washed with biosurfactant concentration at 20 ppm. Biosurfactant concentration and contact time have a significant effect in reducing cypermethrin residues. Using 20ppm of biosurfactant, the cypermethrin residues was reduced to safe level in 5 minutes, and the use of 15 ppm of biosurfactant resulted in 15 minutes, while 10ppm of biosurfactant required 25 minutes respectively (Fig-5).

DISCUSSION:

In the present study, the possibility of biosurfactant production using groundnut oil cake as substrate and the use of biosurfactant as a potential pesticide cleaning agent in leafy vegetables was reported. The production of biosurfactant using cheaper carbon sources was already reported by earlier studies, molasses (Makker and Cameotra, 1999), plant-derived oils (Oliveira et al., 2009) starchy substances (Fox et al., 2000), agriculture residues (Moldes et al., 2009), cashew apple juice (Parthasarathi et al., 2009) and oil wastes (Thavasi et al., 2008b) which has been supporting the present study on use of renewable substrates for the biosurfactant production.

In this study, Pseudomonas fluorescens MFS03 showed positive results for the screening tests carried out. Biosurfactant producing microorganisms can be screened using different assays. Hemolysis was included in this study since it is widely used to screen biosurfactant production and in some cases, it is the sole method used (Yonebayashi et al., 2000). The reduction in surface tension in the liquid- liquid interface leads to complete spreading of liquid drop over the surface of oil (Youssef et al., 2004). So the drop collapsing and oil displacement tests are the easiest and effective method to screen biosurfactant producers. The biosurfactant producing ability, to form clear halos in methylene blue / cetyltrimethylammonium bromide (CTAB) plate and N-cethylpyridinium chloridemethylene blue agar plate (Lin et al., 1998).FT-IR spectrum results revealed that, the presence of (OH bond, typical polysaccharides), (CH band: CH2-CH3, hydrocarbon chains),(C=O, C=O ester bond) and (C-N amide groups) confirm the chemicals group is rhamnolipid type. The results obtained in the present study on the biosurfactant production was corroborates with the findings of (Rodrigues et al., 2006; Pornsunthorntawee et al., 2008).

The standard method in detecting cypermethrin residues is gas chromatography (Gonzalez-Rodriguez et al., 2008). In the present study; we follow the method of detecting cypermethrin residues from the work of Drochioiu et al., 2004 using spectrophotometric to determine the trace element of cyanide with ninhydrin. Among the different washing solution biosurfactant shows high reduction of cypermethrine residues when compared to others. For 100 ppm concentration of cypermethrin residues, the amount of biosurfactant used for the washing to reduce the cypermethrine residues to safe level below 2 ppm is 25ppm of biosurfactant for 5 minutes, the concentration and the contact time plays a main role in reducing pesticide residues from the spinach. The increase in the concentration of biosurfactant shows decrease in the contact

time in washing the pesticide residues. From this study, we reported that biosurfactant can be used as an effective agent to clean up insecticide on vegetables and leafy vegetables.

CONCLUSION:

The biosurfactant produced by Pseudomonas fluorescens MFS03 using groundnut oil cake as a substrate was studied in this work. From the above studies, it can be concluded that production of biosurfactant using groundnut oil cake is an alternative economically low cost carbon source for the biosurfactant production. Biosurfactant increase the bioavailability of hydrocarbon resulting in enhanced growth and degradation of contaminants by hydrocarbon-degrading bacteria. This feature leads biosurfactant as a cleaning agent for insecticide residues on leafy vegetables. So, in the present study, it was concluded that the groundnut can serve as a low cost carbon source for biosurfactant production, which can make the process economical and the biosurfactant can also be used against the biodegradation of pesticide contamination in the environment.

LIST OF FIGURES:

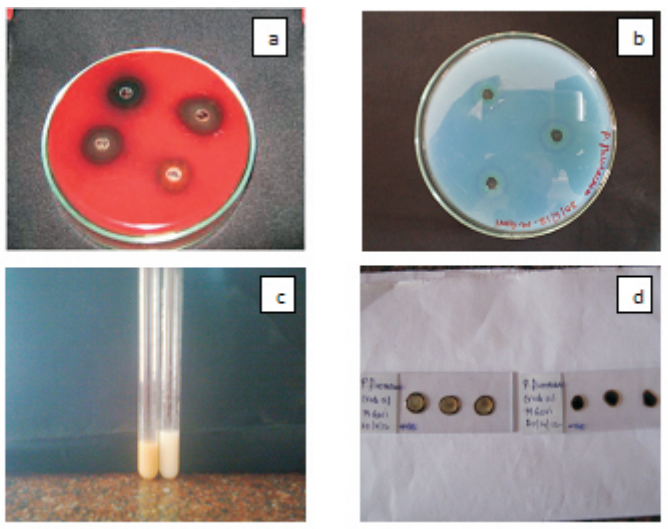

Fig. 1. Screening results of the Pseudomonas flurosecence MFS 16. A. Blood agar lysis. B. CTAB plate assay shows clear blue zone. C. Emulsification activity. d. Oil spreading test.

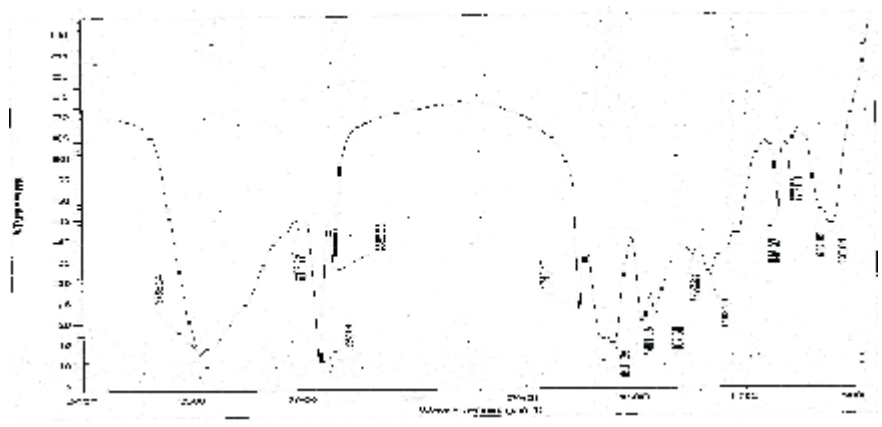

Fig.2. Fourier transform infrared (FTIR spectra) of rhamnolipid produced by Pseudomonas fluorescence MFS03.

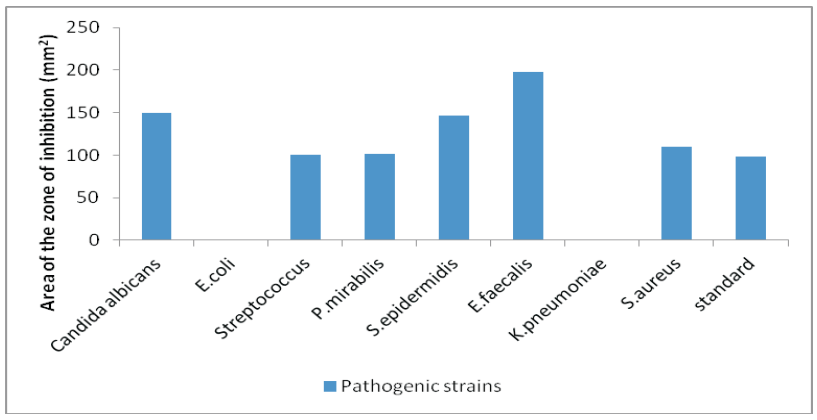

Fig. 3 Antimicrobial activity was tested against established human pathogens using well-diffusion method. The activity index was calculated as mm2 area based on the diameter halo produced by the biosurfactant.

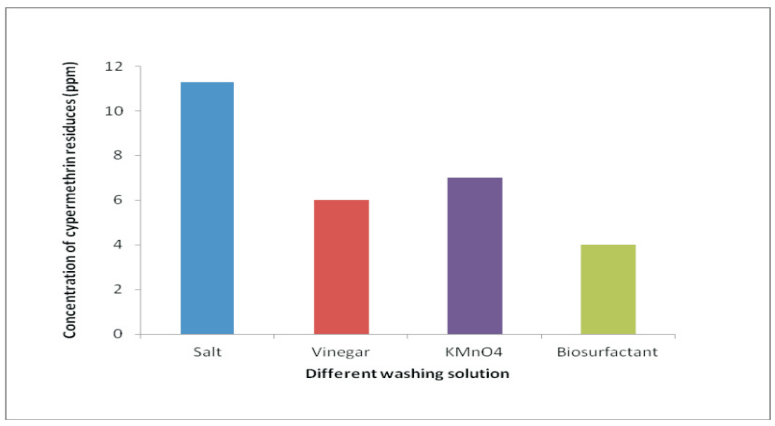

Fig.4. Effect of salt, vinegar, KMnO4 and biosurfactant in reducing cypermethrin residues.

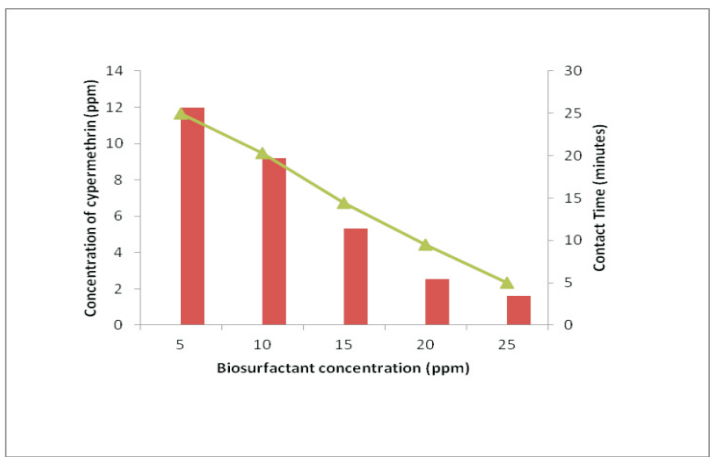

Fig.5. Effect of different concentration of biosurfactant and contact time in reducing cypermethrin residues.

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