Solid-State Fermentation of Rhamnolipid Biosurfactant Production from Serratia Rubideae SNAU02 and Its Action on Egg Plant Fusarium Wilt

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***Abstract*—**This experiment was conducted to find rhamnolipid biosurfactant production under solid state fermentation using a substrate mahua oil cake and to evaluate its action against Fusarium wilt of eggplant. The combination of Response Surface Methodology (RSM) and Central Composite Design (CCD) was employed to optimize higher biosurfactant production. The four factors viz,. Substrate concentration, inoculum size, pH and temperature were selected for optimization of rhamnolipid production. The results revealed that the optimum condition for reduction of surface tension were as follows- mahua oil cake 8.48g, 2.2 ml inoculum size (1×108 cells/ml), pH 7.2 and 32ºC temperature. To evaluate the biocontrol efficacy the application of rhamnolipid at various concentrations (0,100,250 and 500 µg/ml) by soil and foliar application was employed in the pot culture assay. In vitro study indicated that rhamnolipid producing strain SNAU02 was the most effective antagonist against Fusarium oxysporum f. sp. melongenae and used for pot culture study. On the basis of economic analysis, treatment T9 (Fusarium oxysporum f. sp. Melongenae (×106 spores/ml) + 50 ml of 250µg biosurfactant /ml to soil + foliar spraying of biosurfactant (250µg/ml) ranked among the efficacious treatments and was just as effective as a synthetic fungicide. In control treatment, occurrence of disease severity and disease incidence was observed from early stage of crop growth until at harvest stage. The pot experiment results indicated that SNAU02 rhamnolipid could be a promising agent in the biocontrol of Fusarium wilt in eggplant, which might help to minimize the yield loss of eggplant.

***Keywords*—** Rhamnolipid, Fusarium wilt and Biocontrol

I. INTRODUCTION

WELCOME Biosurfactants are amphiphilic molecules, capable of reducing surface and interfacial tension between immiscible fluids . Biosurfactants are generally categorized based on their chemical composition and microbial origin. They include lipopeptides, glycolipids, polysaccharide–protein complexes, lipopolysaccharides, protein-like substances, neutral lipids and phospholipids, fatty acids[1]. In recent years, chemical surfactants are extensively used in food, agriculture, cosmetics, industrial and pharmaceutical applications. The surfactants are mostly chemically synthesized which cause environmental hazards. Therefore, increasing environmental awareness has led to the isolation of biosurfactant due to their unique properties including lower toxicity, lower Critical Micelle Concentration, higher biodegradability and ecofriendly. Most of the literature has reported the biosurfactant, effective against plant fungal pathogens [2,3]. Using Mahua oil cake, in present study, we adopt RSM to optimize the biosurfactant production by *Serratia rubideae* SNAU02 employing solid state fermentation. Furthermore, to evaluate the rhamnolipid biosurfactant efficacy to control *Fusarium* wilt disease on eggplant. Also, the effect of rhamnolipids biosurfactant on growth parameters and yield attributes were evaluated under pot culture conditions.

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II. MATERIALS AND METHODS

A. Microorganism

The strain *S. rubidaea* SNAU02 (Accession number KC560769) was used in the present study[3] . The strain was grown in nutrient agar (NA), sub-cultured each month and stored at 4ºC.The fungal strain *Fusarium oxysporum* f. sp. *melongenae* was procured from Department of Plant Pathology, Faculty of Agriculture, Annamalai University, India. The fungal culture was maintained in Potato Dextrose Agar (PDA) slants and Potato Dextrose Broth (PDB). The biocontrol strain *Pseudomonas aeruginosa* MTCC 2581 obtained from the Microbial Type Culture Collection (MTCC), Chandigarh, India was used as a reference strain.The chemical fungicide Carbendazim 50% WP (Carzim-50, Cheminova India Limited) was procured from the local market of Chidambaram, Tamilnadu, India was used for the pot culture study.

B. Solid State Fermentation

All For the development of SSF, seven agro-industrial by-products were obtained from local market in Chidambaram *viz.* coconut oil cake, groundnut oil cake, castor oil cake, gingelly oil cake, sunflower oil cake, palm oil cake and mahua oil cake. All the aforementioned substrates were dried at 60 ˚C for 48 h, blended to fine powder and stored in air-tight polythene bags

till use. All the solid substrates were directly used in the fermentation media without any pretreatment. To develop the solid state fermentation, 5.0 g substrate was transferred to 250 ml Erlenmeyer flask and to this a salt solution was added to maintain the final moisture level at 65 percent. The composition of the salt solution was as follows (%): NH_4NO_3 0.5, NaCl 0.9, $MgSO_4$ $7H_2O$ 0.1, pH 7.0. The fermentation media was sterilized at 121˚C for 15 min, cooled and inoculated with 2.0 ml inoculum of *S. rubidaea* SNAU02 and incubated at 30˚C for seven days. All the experiments were performed in triplicates.

C. Response surface methodology (RSM) for the optimization of Biosurfactant Production Under SSF

The experiment was performed using Central Composite Design (CCD) for which a total of 30 treatment combinations were generated using designer expert 7.0 software (Stat-Ease Inc. Minneapolis, USA)[5]. From the experimental data according to this design, a second-order polynomial regression model equation was derived as below:

$$Y= \beta_0+ \beta_1A+ \beta_2B+ \beta_3C+ \beta_4D+ \beta_{11}A^2+ \beta_{22}B^2+ \beta_{33}C^2+ \beta_{44}D^2+ \beta_{12}AB+ \beta_{13}AC+ \beta_{14}AD+ \beta_{23}BC+ \beta_{24}BD+ \beta_{34}CD.$$

where Y: predicted response (Surface tension (mN/m), ST), β_0: intercept , A: Substrate concentration, B: Inoculum size, C: pH, D: temperature, β_1, β_2, β_3 and β_4 are the linear coefficients; β_{11}, β_{22}, β_{33}, and β_{44} are the squared coefficients; β_{13}, β_{12}, β_{14}, β_{23}, β_{24}, β_{34} are the interaction coefficients; A^2, B^2, C^2, D^2, AB, AC, AD, BC, BD, CD are the interaction between the variables as significant terms.

D. Determination of the Biosurfactant Production

For extraction of biosurfactant, 100ml of distilled water was added to each SSF flask and contents were agitated at 200rpm at 30°C for 1h on an orbital shaker. Then the contents were filtered using a cheese cloth, filtrate was pooled and then centrifuged at 2822G-force for10min for 10 min. Surface tension was measured according to Velioglu and Urek [6]. The surface tension of extracted supernatant obtained from SSF process was measured by Du Nuoy ring method using Krüss-K6 tensiometer.

E. In vitro screening for potential antagonism

The antagonist activity of the selected SNAU02 strain was evaluated in Petri dishes by dual-culture as described by Tian et al [7].

F. Efficacy of Rhamnolipid Biosurfactant against Fusarium Wilt in Pot Assay

The experiments was conducted on eggplant (variety-Magadh long (Mahyco seeds) during 2013(June-august) at the Department of Microbiology, Faculty of Agriculture, Annamalai university, Annamalai nagar , India. The pot assay was carried out to explore the efficacy of rhamnolipid biosurfactant at a concentration of 0, 100, 250 and 500µg/ml (w/v in distilled water) to biocontrol the *Fusarium wilt o*n eggplant by soil drenching and foliar application.

The eggplant seedlings were grown on plastic tray for two weeks and then transplanted to pots containing Annamalai Nagar soils, Sand, Red earth and FYM in equal parts. The nursery bed was prepared by mixing equal parts of FYM, sand and red earth and beds were formed to a height of 12cm. The eggplant seeds were sown in lines at an interval of 5.0 cm between lines and covered with fine sand and soil mixtures. Prior to the treatment with *Fusarium* spore and purified rhamnolipid biosurfactant solution, the egg plant were maintained under normal condition for three weeks. The fungal culture *Fusarium oxysporum f. sp. melongenae was* grown in PDA plates and incubated at 25ºC for four days. The *Fusarium* spores were harvested according to Prasad et al. [8] and 10^6ml spores were adjusted with distilled water using hemocytometer.

The pot culture study was performed with three replicates for each control plant and purified biosurfactant treatments respectively. The treatments were arranged in CRBD design (Complete Randomized Block Design) and 50ml spore suspension of *Fusarium oxysporum f. sp. melongenae* was sprayed at a time over all the treatments. The control treatment only received water spray. Carbendazim concentration (0.2%) was applied at once in soil. The soil without addition of Carbendazim served as control. Three replicates were maintained for all the treatments. Foliar application of Carbendazim at 0.1% on 30, 60 and 90 days after transplanting (DAT). Carbendazim was sprayed during the early morning or late evening to avoid scorching effect. The partially purified biosurfactant produced from *Serratia rubidaea* SNAU02 were applied in pot at various concentrations namely 100, 250 and 500 µg/ml respectively. Whereas, foliar sprays of biosurfactant was applied at the rate 50ml of 100, 250 and 500 µg/ml on eggplants were undertaken on 30 60 and 90 Days After Transplanting (DAT). The pot trial experiment treatment details as follows: T_1-Control (Distilled water), T_2- *Fusarium oxysporum* f. sp. *melongenae*, T_3-*Fusarium oxysporum* f. sp. *melongenae* + *P. areuginosa* MTCC 2581 (Reference strain) + Soil drenching of 50ml ($x10^6$cells/ml) + 50ml foliar spray ($\times10^6$ cells/ml) on 30, 60 and 90th DAT, T_4-*Fusarium oxysporum* f. sp. *Melongenae* + Carbendazim at 0.2% soil drenching + foliar spraying at 0.1% on 30th and 60th DAT, T_5-*Fusarium oxysporum* f. sp. *melongenae*($\times10^6$ spores/ml) + 50 ml of 100µg biosurfactant /ml to soil, T_6-*Fusarium*oxysporum f. sp. *melongenae*($\times10^6$ spores/ml) + 50ml of 250µg biosurfactant/ml to soil, T_7-*Fusarium oxysporum* f. sp. *melongenae*($\times10^6$ spores/ml)+50 ml of 500µg biosurfactant/ml to soil, T_8-*Fusarium oxysporum* f. sp. *melongenae* ($\times10^6$ spores/ml) +50 ml of 100µg biosurfactant/ml to soil + foliar spraying of biosurfactant (100µg/ml) on 30,60 and 90th DAT, T_9-*Fusarium oxysporum* f. sp. *melongenae*($\times10^6$ spores/ml) +50 ml of 250µg biosurfactant/ml to soil + foliar spraying of biosurfactant (250µg/ml) on 30,60 and 90th DAT, T_{10}-*Fusarium oxysporum* f. sp. *melongenae*($\times10^6$ spores/ml) +50ml of 500µg biosurfactant/ml to soil + foliar spraying of biosurfactant (500µg/ml) on 30,60 and 90th DAT.

III. Results and Discussion

Mahua oil cake was chosen as potent substrate for biosurfactant production by S. rubidaea SNAU02 under SSF based on our pervious findings.RSM is a statistical technique, employed to assess the optimal conditions of the experiment using different input factors. Considering the advantages of the solid-state fermentation, four parameters, substrate concentration, inoculum size, pH and temperature were used to study the biosurfactant production. In the current study, four variables (substrate concentration, inoculum size, pH and temperature) were assessed for the biosurfactant production using a central composite design (CCD). The above mentioned parameters strongly influence the biosurfactant production. Response methodology can be an excellent statistical tool to study the best correlation among the parameters used in experimental model. The response surface methodology is employed as a statistical design to model biosurfactant production in terms of surface tension reduction.

A. In vitro Assay for Screening Potential Antagonism

The potent rhamnolipid producer *Serratia rubidaea* SNAU02 was found to be the most effective antagonist in vitro against *Fusarium oxysporum* f.sp. *melongenae*. For fungal pathogens, the zone of inhibition on solid media has been traditionally used for culture based studies of potential biocontrol agent and as an indicator of competitive ability. In the present study, strain SNAU02 was effective antagonist producing a distinct and wide area (ranging from 9.8 to 13.75mm) of pathogen mycelium inhibition. Based on the result, strain SNAU02 was chosen for the pot culture study.

B. Evaluation of Antagonism of Crude Biosurfactant in Pot Assay

The result indicated that, significantly higher plant growth of eggplant at 45, 75 and 105 DAT on the application of biosurfactant at various concentrations were recorded under all the treatment over control during both the season 2013 . Among the various concentrations used, the higher plant height was registered in T_{10}. The effect of biosurfactant on plant height, Leaf Area Index, fruit weight and fruit yield, as revealed in the present study showed that application of 250µg/ml rhamnolipid by soil drenching and foliar spray significantly improved the biometric and yield attributes. This may be due to mode of action of biosurfactants in biological control involves the formation of channels in the cell wall and perturbations of the cell surface of the pathogen

IV. Conclusion

It can be concluded that rhamnolipid biosurfactant produced by *Serratia rubidaea* SNAU02 utilized mahua oil cake as best substrate under SSF. The use of empirical method, combination of RSM-CCD enhanced higher biosurfactant production. The results also demonstrated rhamnolipids biosurfactant could potentially be used as biocontrol agent against *Fusarium* wilt disease. The application of 250 ug/ml of SNAU02 rhamnolipid biosurfactant was effective against *Fusarium oxysporum* f. sp.*melongenae (Fomg)* and completely inhibit the disease severity in eggplant. In addition, biosurfactants have the potential to be an alternative of replacing the chemical surfactant as they are low toxicity, higher biodegradability, environmental friendly and which aid in reducing the use of agro chemicals.

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