Larvicidal activity of rhamnolipid biosurfactant produced by *Stenotrophomonas maltophilia*.

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Abstract— Mosquitoes pose a serious health problem throughout the world as they serve vectors for spreading human diseases like malaria, dengue fever, filariasis. Conventional control strategy involves application of broad-spectrum chemicals and pesticides which produce undesirable effects in environment and human health. Thus, bioactive compounds produced by bacteria like rhamnolipid biosurfactant exhibiting larvicidal property could be an effective and eco-friendly solution. For this purpose, 84 isolates obtained from oil contaminated samples and screened. One potent biosurfactant producer isolate selected, identified to be *Stenotrophomonas maltophilia* was used for biosurfact production. The crude biosurfactant produced was characterized as rhamnolipid and used further to test its larvicidal properties. The mosquito larvae in similar stage of lifecycle were subjected to different concentrations (1-10 mg%) of crude biosurfactant with distilled water as control. The larvae were observed for a period of 72 hours post application and LC50 and LC100 of the biosurfactant were calculated. Results suggested that mortality rate increased along the increase in time and concentrations. LC100 was exhibited by 1 mg% and 2 mg% biosurfactant concentrations by 72 hrs. These findings infer that biosurfactant can be used for curbing the mosquito larvae at lower concentrations.

Index Terms— Biosurfactant, Larvicidal, LC100, Oil contaminated soils, Rhamnolipid, *Stenotrophomonas maltophilia*, Surface tension.

1 INTRODUCTION

MOSQUITOES act as vectors for spreading human diseases such as malaria, dengue fever, yellow fever, encephalitis, West Nile fever, lymphatic filariasis and continue to pose a serious public health problem throughout the world. Conventional arthropod control strategy involves application of broad-spectrum chemicals and pesticides, which often produce undesirable effects on the environment [1]. As an alternative to this, various microbial pesticides produced from *Bacillus thuringiensis* subsp. *israelensis* and *B. sphaericus* are being advocated for the control of mosquitoes [2][3]. However, reports indicate development of resistance by certain species of mosquitoes to certain strains of *B. sphaericus*. Though resistance development has not been a major problem encountered with *B.thuringiensis* till date, the stability, solubility and insecticidal activity of the crystal toxins of *B. thuringiensis* are known to be affected by pH of the habitat and by exposure to sunlight [4][5]. These drawbacks call for the development of new mosquitocidal bacterial agents which can overcome the above limitations.

Biosurfactants are surface active substances synthesized by living cells having the properties of reducing surface tension, stabilizing emulsions, promoting foaming and are generally non-toxic and biodegradable [4]. Interest in microbial surfactants has been steadily increasing in recent years due to their diversity, environmentally friendly nature, possibility of large-scale production, selectivity, performance under extreme conditions and potential applications in environmental protection [6].

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In the present study, a potent bacterial strain identified as *Stenotrophomonas maltophilia* was selected after screening of 84 isolates obtained from oil contaminated soil and used for biosurfactant (BS) production. Application of this produced biosurfactant was then evaluated as a larvicide.

2 METHODOLOGY

2.1 SAMPLING OF CONTAMINATED SOIL:

Oil contaminated soils were collected from four different areas viz. (i) Automobile Servicing center, Nasik road, (ii) Rickshaw center, Narayan Bapu nagar, Nasik road, (iii) Petrol contaminated soil, Nasik, (iv) Crude oil contaminated soil, Mumbai. Crude oil, waste oil, petrol, diesel were obtained from Nasik road area. All the above samples were maintained at 4°C until further processing.

2.2 ISOLATION OF BIOSURFACTANT PRODUCING BACTERIA:

Mineral Salts Medium (MSM) containing, in (gm/L) Glucose: 10 gm;$(NH_4)_2SO_4$: 1gm; $NaHPO_4$: 4 gm; Yeast Extract: 5 gm; KH_2PO_4: 3gm; NaCl: 2.7 gm; $MgSO_4$: 0.6 gm; and 5ml/L trace element solution (mg/L) containing $FeSO_4$: 5mg; $ZnSO_4.7H_2O$: 3.34mg; $MnSO_4.7H_2O$: 1.56 mg; $CoCl_2.2H_2O$: 2mg (1L Distilled water) [7] with 5% of petrol, diesel, crude oil, waste oil, glycerol was used for enrichment and isolation of biosurfactant producers [2]. Primary Screening of BS producing bacteria was done by Oil Spread method (OSM) [8], Emulsification index (EI-24) [9], Drop Collapse Method (DCM) [10], Titled glass slide test (TGST) and Hydrocarbon Overlay Method [7]. On the basis of primary screening, two bacterial isolates which showed positive results out of 84 screened isolates were selected for secondary screening like Blood Haemolysis (BH) [2] and Surface Tension using Tensiometer (JENCON, India) based on du Nouy ring method for measuring the ability to reduce the surface tension of liquids [11].

2.3 BIOSURFACTANT PRODUCTION:

Out of the two potential isolates, most potent biosurfactant producer giving highest reduction in surface tension was selected for BS production (named as IWDS). Furthermore media optimization was done for biosurfactant production by Plackett Burman method [12] and the produced biosurfactant was then characterized as well as identified [13]. Physical properties of BS like surface tension, critical micelle concentration (CMC)[14] and emulsification activity were also examined [15].

2.4 LARVICIDAL ACTIVITY OF BIOSURFACTANT:

The mosquito larvae containing water was collected from water logged area and then kept in an open earthen pot until most of the larvae were in similar developmental stages of their lifecycle. Crude biosurfactant was used to prepare different concentrations (1 - 10mg %) in distilled water and larvae were transferred to individual glass tubes and incubated at room temperature (~23^{0}C) for 24–72 h, followed by counting the time taken for the death of larvae and number of dead larvae in each tube. Distilled water served as control. The lethal concentrations 100% (LC100) and 50% (LC50) of the biosurfactant were calculated [16].

Stability testing of the biosurfactant was also tested by exposing BS to varying temperature (50 to 100°C) for a period of one hour and then determining the OSM activity and effect of pH change was determined by adjusting the pH of biosurfactant with the help of buffers in the range of 2.0 to 12 at RT for an hour. The stability was determined by determining the OSM. Storage stability of the biosurfactant verified by storing it at 4°C in the refrigerator and room temperature (25-27°C) then with activity by OSM test [16].

3 RESULTS AND DISCUSSION

In this study, oil/ petrol contaminated soil samples and the water sample were enriched for a period of 21 days for isolation of potential biosurfactant producing microorganism. Out of the 84 isolates obtained, screened and isolate, IWDS was selected as it showed zone of clearance on Blood hemolysis (Fig.1) and also maximum reduction in surface of the medium (68 to 47.5 dyne/ cm^2). IWDS- a gram negative rod was identified by VITEK 2 SYSTEM VERSION 6.0 as *Stenotrophomonas maltophilia.* Other studies describing strains of *Pseudomonas aeuraginosa, Bacillus subtilis, Bacillus spp.* isolated from petroleum contaminated soil [1][3][8][17] and Marine *Actinobacteria* isolated from Nicobar Marine Sediment have been reported [18].

In this study, the media was then optimized for BS production by *S.maltophilia* and yield of biosurfactant was 0.2 g/ 100ml of the medium on extraction. On characterization, biosurfactant showed rhamnolipids presence [Fig 2 and 3] which was further confirmed by FTIR analysis [Fig. 4]. The reduction in the surface tension of water from 72.2 dyne/cm^2 to 38.2 dyne/cm^2 was observed on the addition of biosurfactant and the CMC, concentration of biosurfactant for effective micelle formation was found to be 70 mg/L. The emulsification index was determined of the aqueous solution of biosurfactant (10 mg%) against the different emulsified substrates. Stable emulsion was formed which was stable for a longer period of time with EI-24 values more than 70%.

Fig 1. Zone of clearance IWDS isolate

Fig 2. Characterization of BS by CTAB

Fig 3. Characterization of BS by TLC

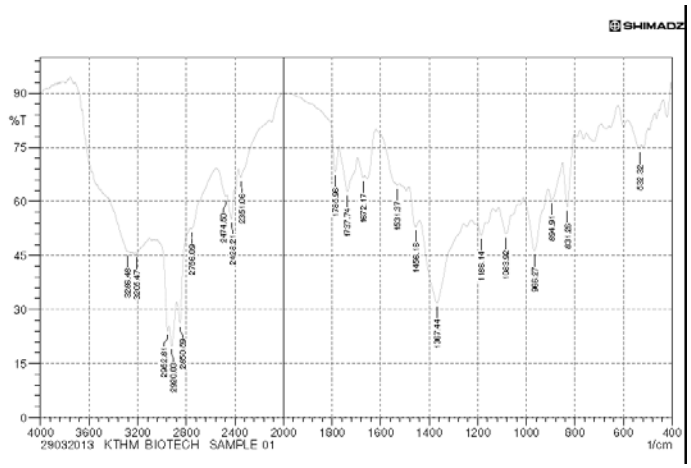

Fig 4.FTIR analysis

Larvicidal activity: The effect of biosurfactant at the concentrations ranged from 1mg% to 10mg% on mosquito larvae is represented in Table 1. Two hours of exposure had no mortality at the concentration 1mg% to 4mg % , however mortality observed at high concentration of biosurfactant (9 and 10 mg%) in which 40-50% deaths were observed. On 24 hours exposure, LC50 recorded at 3 mg% concentration. The mortality percentage at and above concentration of 6 mg % after 24 hrs was 100%. These results are similar to the study done by Das et al., (2005) LC50 and the LC 100 mosquito larvicidal potency of cyclic lipopeptides (CLPs) secreted by two *Bacillus subtilis* strains were determined. LC50 of the crude CLPs secreted by *B. subtilis* DM-03 and DM-04 strains against third instar larvae of *Culex quinquefasciatus* was found to be 12.0±0.5 and 30.0±0.8 mg% respectively post 24 h of treatment [1].

At 72 hours exposure, LC100 was recorded for the lowest concentrations i.e. 1 and 2mg%. Studies done in present work showed heavy mortality during the duration of 48 to 72 hours. At these time exposure the low concentration of crude biosurfactant proved to be lethal for the larvae. 100% mortality was observed for the 10 mg % concentration for 12 hours, however as the time period increased the same mortality was for 1 mg% concentration for 72 hours. Thus, as the period of exposure increased, the rate of mortality increased (Table 1). Biosurfactant hence can prove to be toxic to the larvae even at lower concentrations. The tests done for stability of BS (pH, temperature) hardly influenced the larvicidal potency of BS. Therefore the present study provides the evidence that *Stenotrophomonas maltophilia* rhamnolipid can be exploited for the formulation of a safer, biopesticide for effective control of mosquito larvae.

Table 1. Larvicidal activity of Biosurfactant

Crude Bio-surfactant (mg %)	Mortality at different times exposures (%)				
	2 hours	12 hours	24 hours	48 hours	72 hours
Control	0	0	0	0	0
1	0	0	10	20	100
2	0	0	20	30	100
3	0	30	50	100	
4	0	40	60	100	
5	10	40	60	100	
6	20	50	100		
7	40	70	100		
8	40	70	100		
9	40	90			
10	50	100			
LC 50	**10 mg%**	**6 mg%**	**3 mg%**	**-**	**-**
LC 100	**-**	**10 mg%**	**6 mg%**	**3 mg%**	**1 mg%**

4 Conclusion

Stenotrophomonas maltophilia-a potent biosurfactant producing bacterium was selected on screening 84 isolates from oil contaminated samples and used for production of biosurfactant. The produced crude biosurfactant was characterized as rhamnolipid and tested for its larvicidal properties. The mortality rate of mosquito larvae increased along the increase in time or concentrations of biosurfactant. With exposure time of 72 hrs. effective mortality (LC100) was obtained even at low BS concentration of 1mg% inferring that the produced biosurfactant exhibit an efficient and eco-friendly larvicidal activities.

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