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PRODUCTION OF BIOSURFACTANT FROM *BACILLUS* SP. AND ITS LARVICIDAL ACTIVITY

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Keywords:

Biosurfactant, *B. subtilis*, Petroleum-contaminated soil, Lipopeptide, Larvicidal activity

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ABSTRACT: Mosquitoes are becoming a serious threat to the humans causing infectious diseases like dengue, malaria, chikungunya, yellow fever *etc*. They multiply in large numbers in a polluted environment and stagnant water bodies. The control measures include the use of pesticides and chemical compounds which further causes harmful effects. Thus, an alternative solution is to use a biologically active compound which has larvicidal property and is eco-friendly. Biosurfactants are such compounds. In this present study, 75 isolates obtained from petroleum contaminated soil samples were screened and one potent isolate was selected and identified. The biosurfactant was produced from *Bacillus subtilis* B50. The crude compound was characterized as lipopeptide. Its larvicidal activity was tested using the similar stage of mosquito larvae at different concentration of crude biosurfactant (1 - 10 mg %). The LC_{50} and LC_{100} were calculated after observing the larvae for 72 h. The obtained results showed that as the time and concentration increases the mortality also increases. The maximum number of larvae was killed at a concentration of 1 - 4 mg for 72 h. The observations suggest the application of biosurfactant as an eco-friendly product for the eradication of mosquitoes.

INTRODUCTION: Human diseases like dengue, malaria, chikungunya, yellow fever, West Nile fever, elephantiasis, encephalitis and other deadly diseases are spread by mosquitoes which act as vectors. Thus several measures are taken to control mosquito which includes the elimination of breeding places, bio-controls with parasites like nematodes and fungi [1] or predators such as fish and lizards [2].

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Another way is the application of pesticides and broad-spectrum chemicals which are harmful to the environment as well as for living beings [3].

Temephos and Fenthion are the chemical agents which are most frequently used for inhibition of larval population [4]. Diethylmetatoluamide (DEET), pyrethrum, methoprene, briquet, and malathion are the poisonous chemicals which are present in commercial mosquito repellent sprays and coils [5]. The overuse of these chemicals causes several negative impacts such as the development of resistance to the mosquito, the effect on non-target insects and contamination of drinking water sources. The increasing number of resistant mosquitoes due to genetic variation results in the ineffectiveness of insecticides [6].

On the other hand, the microbial insecticides have selective toxicity and easily decomposed in the ecosystem. Unlike the risks involved in the production of the chemical insecticides, the manufacture of the microbial insecticides is contained, safe and less contaminated [7].

Wolbachia, bacteria which is pathogenic to insect was found to minimize the susceptibility of *Aedes* to dengue virus [8]. The metabolites produced by *Bacillus thurengiensis* and serratia has been found to inhibit the larvae of *Aedes*, *Culex* and anopheles mosquitoes [9]. *Bacillus thurengiensis* subsp. Israelensis and *B. sphaericus* produce various microbial pesticides which are administered as an alternative to mosquito control [10, 11].

However, some reports indicate the development of resistant mosquito species to *B. sphaericus*. Even though the development of resistance has not yet become a serious problem with *B. thurengiensis*, the insecticidal property, stability and the solubility of the toxin crystals produced by them are susceptible to change in the pH and excessive exposure to the sunlight [12, 13]. To overcome such limitations, new bacterial agents are developed [14]. One such compound is biosurfactant.

Biosurfactants, as the name suggests, are the surface active agents synthesized by living organisms. They have various properties such as surface tension reduction, emulsification, foaming activity and as basically non-toxic and eco-friendly [12]. Most of the researchers are interested in the study of biosurfactants due to its unique properties which also includes selectivity, tolerance to extreme conditions, biodegradability, and possibility for a wide range of application in different fields [15].

In the present research work, one potent biosurfactant producing bacterial strain of *Bacillus subtilis* B50 was selected after the screening of 75 different isolates obtained from various petroleum contaminated soil samples and the larvicidal activity of BS was evaluated.

MATERIALS AND METHODS:

Sampling of Petroleum - Contaminated Soil: Petroleum-contaminated soil samples were taken from different areas *viz*. (i) Venkat Bajaj, Coimbatore, (ii) Bajaj three wheeler workshop, Koduvayur, (iii) Aravind workshop, Palakkad (iv) Arumughan Engineering works, Palakkad (v) Petrol pump, Palakkad. 2-stroke servo engine oil was obtained from Palakkad. The samples were maintained at 4 °C until further processing.

Isolation of Bacteria Producing Biosurfactant: The soil samples were enriched in mineral salt medium (MSM) which consists of 0.1% NH_4NO_3, 0.1% KH_2PO_4, 0.1% K_2HPO_4, 0.02% $MgSO_4$, 0.02% $CaCl_2$, 0.005% $FeCl_3.6H_2O$, 1% Dextrose [16]. 2% of engine oil was used as carbon source to enhance the growth of biosurfactant producer. The sample was incubated at room temperature for 72 h. The organisms were isolated by serial dilution method and screened for the production.

The primary screening of BS producing cultures was done by oil spreading technique [17], emulsification index (E24) [18], drop collapse test [19] and Foaming activity test [20]. From the results obtained for primary screening two isolates which showed positive results were selected for secondary screening *i.e.* blood hemolysis [21].

Identification of isolate by 16S rRNA Sequencing: The selected isolate was identified by 16S rRNA sequencing. This was done at Yaazh Xenomix Laboratory, Coimbatore. The identified strain sequence was deposited in GenBank and accession number was given.

Biosurfactant Production and Extraction: The positive isolate was inoculated in the production medium (MSM) and incubated at 30°C for 48-96 h. The supernatant was collected following the incubation by centrifugation at 10,000 rpm at 4 °C for 30 min. The cell pellet was used for the determination of biomass. It was washed with petroleum ether and Acetone at the ratio of 1:3 and centrifuged at 3000 rpm for 20 min to remove the oil from the cell debris. This process was repeated thrice. Further, it was washed with distilled water and dried at 60 °C and weighed. 6 N HCl was used to adjust the pH of the supernatant to pH 2.0 and kept in the refrigerator for 24 h. The biosurfactant was extracted by mixing chilled Chloroform: Methanol (2:1) to the supernatant in equal volume. An organic layer form at the top which contains the biosurfactant was pooled and evaporated. The dry weight of the crude extract was recorded [22].

FTIR Analysis of Biosurfactant: FTIR spectroscopy (Shimadzu) was employed to explore the chemical bonds and functional groups present in the extracted biosurfactant. IR spectra of the sample were recorded over the range of 4000- 400 cm^{-1} spectral region.

Larvicidal Activity of Biosurfactant: The mosquito larvae were collected from waterlogged area and kept in an open earthen pot to attain a similar developmental stage of the larval lifecycle. Different concentrations (1 - 10 mg %) of the extracted biosurfactant was prepared in distilled water in test tubes. To them, an equal number of larvae at same stage were transferred and incubated for 24 - 72 h at room temperature. The number and the time taken for the larval death in each tube were noted. Distilled water was kept as control. The lethal concentrations LC_{50} and LC_{100} for the sample were calculated [23].

RESULTS AND DISCUSSION:

Isolation of Bacteria Producing Biosurfactant: In this study, petroleum contaminated soil samples were enriched in MSM medium containing 2% engine oil as a carbon source. From the samples, 75 different isolates were obtained and screened for biosurfactant producing properties **Table 1**. Among them, two cultures which showed positive results for all the screening tests were selected for a combination study **Fig. 1, Table 2**.

TABLE 1: SCREENING RESULTS FOR BIOSURFACTANT PRODUCTION

Isolates	Blood Hemolysis	Drop collapse test	Oil spreading test	Emulsification index (E_{24}%)	Foaming Activity
B1	β	+ ve	10 mm	35	+ ve
B14	β	+ ve	20 mm	32	+ ve
B16	β	+ ve	15 mm	38	+ ve
B25	β	+ ve	15 mm	39	+ ve
B50	β	+ ve	25 mm	60	+ ve
B55	β	+ ve	25 mm	63	+ ve

TABLE 2: SELECTION OF BIOSURFACTANT PRODUCER BASED ON BIOMASS AND BIOSURFACTANT YIELD

Isolates	Biomass (g/100 ml)	Biosurfactant (g/100 ml)
B1	0.50	0.010
B14	1.20	0.055
B16	1.34	0.062
B25	0.39	0.021
B50	1.32	0.689
B55	1.5	0.15

FIG. 1: *BACILLUS SUBTILIS* B50

Identification of isolate by 16S rRNA sequencing: The culture was identified as Bacillus subtilis B50 **Fig. 2** by16S rRNA sequencing and the accession number given by GenBank is MF521625.

Many other reports describe the isolation and production of biosurfactant by *Pseudomonas aeruginosa* [24], *Stenotrophomonas maltophilia* [25], *Bacillus licheniformis* [26], Mycobacterium [27], Rhodococcus [28, 29].

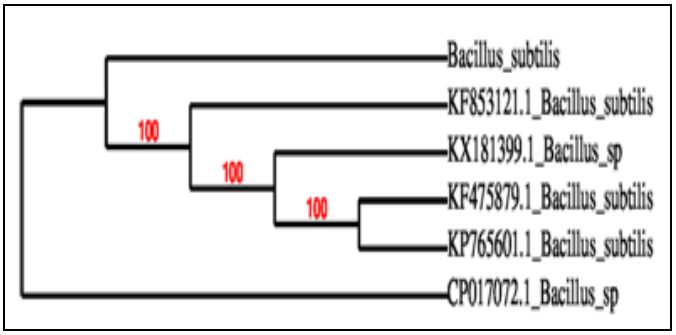

FIG. 2: PHYLOGENETIC TREE OF *BACILLUS SUBTILIS*

Biosurfactant Production and Extraction: The chloroform: methanol extraction **Fig. 3** gave the yield of about 0.689 gm / 100 ml of the production medium **Fig.4**.

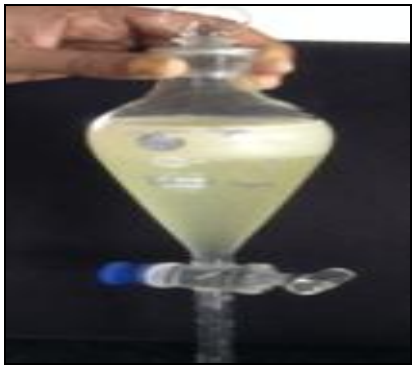

FIG. 3: SOLVENT EXTRACTION

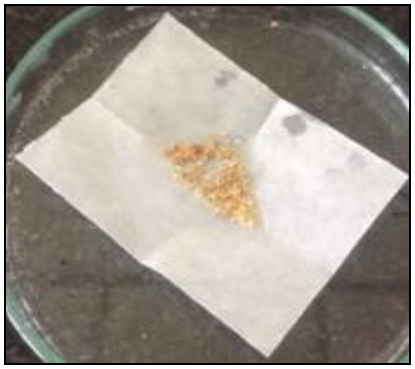

FIG. 4: CRUDE BIOSURFACTANT

FTIR Analysis of Biosurfactant: The spectrum in the FTIR analysis infers the characteristics of lipopeptide **Fig. 5**.

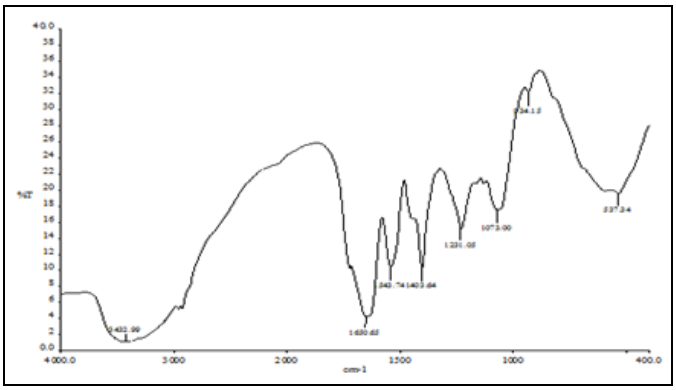

FIG. 5: FTIR SPECTRUM OF BIOSURFACTANT

Larvicidal Activity of BS: The larvicidal activity at different concentrations from 1 mg % - 10 mg % is given in **Table 3**, **Fig. 6**. Following incubation with crude biosurfactant, mortality was observed at high concentration after exposure for 12 h. LC_{50} was recorded at 5 mg % and LC_{100} at 7 mg % on 24 h of exposure. A similar study was done by Das *et al*., 2005 in which larvicidal activity of lipopeptide secreted by *B. subtilis* was determined [3]. LC_{100} was recorded for 1 - 2 mg % for 72 h exposure. This present work shows that the maximum larval death is observed at low concentrations when exposed to the biosurfactant for a longer period of incubation *i.e.* 48 - 72 h. Thus it is found to have the toxic effect on mosquito larvae.

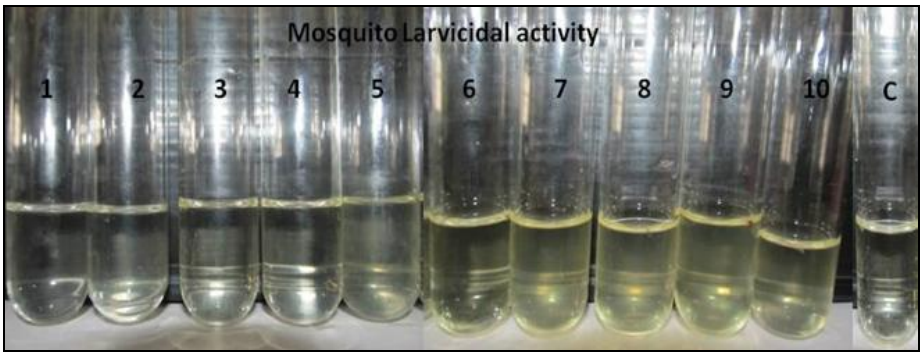

FIG. 6: LARVICIDAL ACTIVITY OF BIOSURFACTANT

TABLE 3: LARVICIDAL ACTIVITY OF BIOSURFACTANT

Crude Biosurfactant (mg %)	Mortality at different time intervals (%)				
	2 hrs	12 hrs	24 hrs	48 hrs	72 hrs
Control	0	0	0	0	0
1	0	0	10	20	100
2	0	0	10	20	100
3	0	0	20	40	100
4	0	0	30	50	100
5	0	20	50	100	
6	0	50	80	100	
7	30	60	100		
8	30	70	100		
9	40	90			
10	50	100			
LC_{50}	10 mg %	6 mg %	5 mg %	4 mg %	-
LC_{100}	-	10 mg %	7 mg %	5 mg %	1 mg %

CONCLUSION: *Bacillus subtilis* B50, a potent producer of biosurfactant was isolated and identified from among 75 isolates obtained from petroleum contaminated soil samples. The crude biosurfactant produced was characterized as lipopeptide and its larvicidal activity was determined. It was found that the mortality increases even at lower concentration when incubated for a longer period of time. LC_{100} was obtained at 1 mg % on 72 h of exposure. Thus it is suggested that biosurfactant can be used as a biopesticide to control the mosquito larvae.

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