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

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Keywords:

Biosurfactant, *B. subtilis*, *B. cereus*, 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. 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 two potent isolates selected and identified. The biosurfactant was produced from a consortium of two strains of *Bacillus i.e. B. subtilis* and *B. cereus* at the ratio of 1:2. 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 show that the as 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]. Another way is the application of pesticides and broad-spectrum chemicals which are harmful to the environment as well as for living beings [3].

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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 chemical 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 the 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, two potent bacterial strains of *Bacillus subtilis* and *Bacillus cereus* were selected after the screening of 75 different isolates obtained from various petroleum contaminated soil. Biosurfactant was produced from a consortium of these two strains and its larvicidal activity 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 Isolates by 16S rRNA Sequencing: The selected isolates were identified by 16S rRNA sequencing. This was done at Yaazh Xenomix Laboratory, Coimbatore. The identified strains were given accession number by GenBank.

Culture Preparation for Combination Study: The two selected strains were maintained in nutrient broth which was then inoculated in the MSM medium at different ratios *i.e.* (1:1), (2:1), (1:2), (3:1) and (1:3), followed by incubation at 37°C for 78 h in shaker. They were then screened for its activities and the best ratio was selected.

Biosurfactant Production and Extraction: The selected ratio of strains was inoculated in the production medium at its optimized conditions. 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 are 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, Fig. 2, 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	0.089
B55	1.5	0.15

FIG. 1: *BACILLUS SUBTILIS* B50

FIG. 2: *BACILLUS CEREUS* B55

Identification of Isolates by 16S rRNA Sequencing: They were identified as *Bacillus subtilis* **Fig. 3** and *Bacillus cereus* **Fig. 4** by 16S rRNA sequencing. The accession number for these organisms is MF521625 and MF684782 respectively.

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].

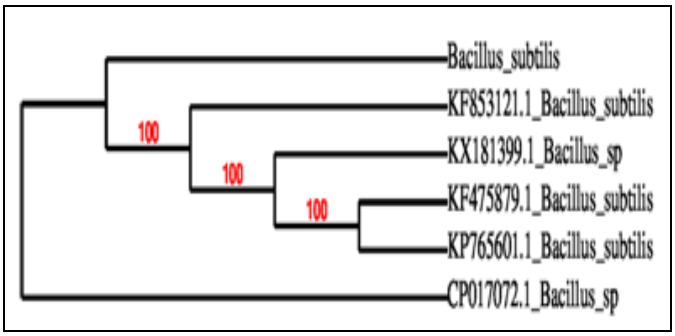

FIG. 3: PHYLOGENETIC TREE OF *BACILLUS SUBTILIS*

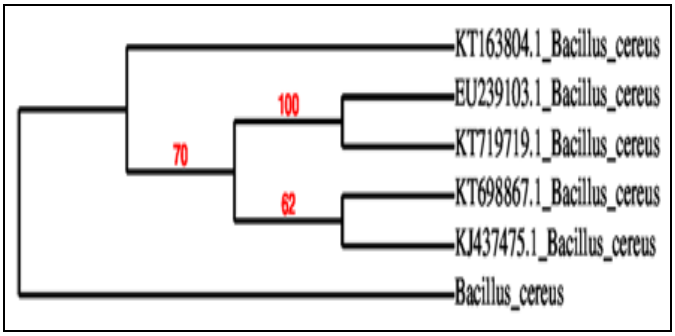

FIG. 4: PHYLOGENETIC TREE OF *BACILLUS CEREUS*

Culture Preparation for Combination Study: In this research work, biosurfactant production was carried out with a consortium of *B. subtilis* and *B. cereus* in an optimized media **Fig. 5**. The ratio 1:2 of cultures was found to show better emulsification activity and production of biosurfactant [30]. Thus it was used for further study.

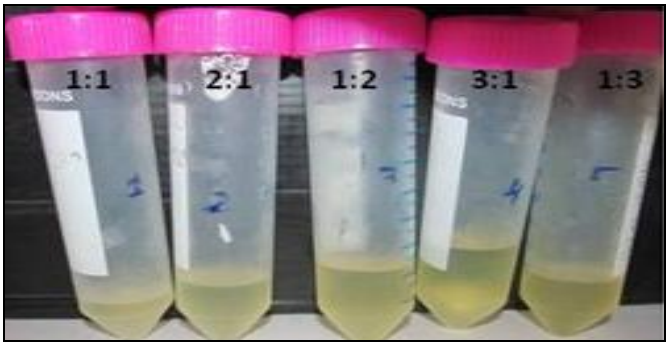

FIG. 5: OPTIMIZED CULTURE RATIOS

Biosurfactant Production and Extraction: The chloroform: methanol extraction **Fig. 6** gave the yield of about 0.24 gm / 100 ml of the production medium which was higher than individual yields obtained **Fig. 7**.

FIG. 6: SOLVENT EXTRACTION

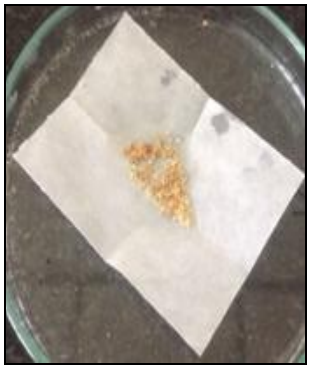

FIG. 7: CRUDE BIOSURFACTANT

FTIR Analysis of Biosurfactant: The spectrum in the FTIR analysis infers the characteristics of lipopeptide **Fig. 8**.

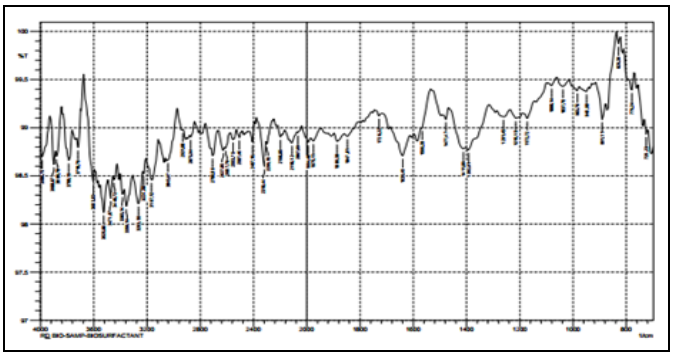

FIG. 8: FTIR SPECTRUM OF BIOSURFACTANT

Larvicidal Activity of BS: The larvicidal activity at different concentrations from 1 mg % - 10 mg % is given in **Table 3**, **Fig. 9**. 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.

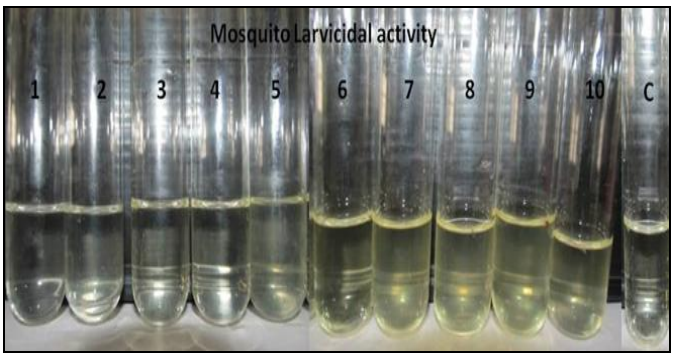

FIG. 9: LARVICIDAL ACTIVITY OF BIOSURFACTANT

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* and *B. cereus*, potent producers of biosurfactant were isolated and identified from 75 isolates 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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CONFLICT OF INTEREST: The authors declare no conflicts of interest.

REFERENCES:

1. Deepali K, Sneha P and Sucheta P: Larvicidal activity of rhamnolipid biosurfactant produced by Stenotrophomonas maltophilia. International Journal of Scientific and Engineering Research 2014; 5(4): 60-63.
2. Louis KA: Reproduction in the Western Mosquitofish, *Gambusia affinis* (Baird & Girard), and its use in mosquito control. Ecological Monographs 1948; 18(1):1-43.
3. Das K and Mukherjee AK: Assessment of mosquito larvicidal potency of cyclic lipopeptides produced by *Bacillus subtilis* strains. Acta Tropica 2005; 97: 168-173.
4. Apperson C and Waldvogel M: Mosquito control around the home & in communities. Insect Note, - ENT/rsc-6 2004.
5. Katayama: Recombinant bacteria for mosquito control. Department of Entomology, University of California Riverside, Riverside, CA 92521, USA vector control 2008.
6. Chevillon C, Raymond M, Guillemaud T, Lenormand T and Pasteur N: Population genetics of insecticide resistance in the mosquito Culex pipiens. Biological Journal of the Linnean Society 1999; 68: 147-157.
7. Syed Z and Leal WS: Mosquitoes smell and avoid the insect repellent DEET. Proceedings of the National Academy of Sciences 2008; 105: 13598-13603.
8. Frentiu FD, Robinson J, Young PR, McGraw EA and O'Neill SL: Wolbachia-mediated resistance to dengue virus infection and death at the cellular level. PloS one, 2010; 5(10): e13398.
9. Patil CD: Insecticidal potency of bacterial species *Bacillus thuringiensis* SV2 and *Serratia nematodiphila* SV6 against larvae of mosquito species *Aedes aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus*. Parasitology research 2012; 110: 1841-1847.
10. Satpute SK, Bhawsar BD, Dhakephalkar PK and Chopade BA: Assessment of different methods for biosurfactant producing marine bacteria. Indian J Marine Science 2008; 37(3): 243-250.
11. Nasr S, Mehrnia MR and Sarrafzadeh MH: Characterization of novel biosurfactant producing strains of *Bacillus sp*. isolated from petroleum contaminated soil. Iranian Journal Microbiology 2009; 1(2): 54-61.
12. Urum K and Pekdemir T: Evaluation of biosurfactants from crude oil contaminated soil washing. Chemosphere 2004; 57: 1139-1150.
13. Mukherjee S, Das P and Sen R: Towards commercial production of microbial surfactants. Trends in Biotechnology 2006; 24(11).
14. Marcelino PRF, Silva VLD, Philippini RR, Zuben CJV, Contiero J, Santos JCD and Silva SSD: Biosurfactants produced by *Scheffersomyces stipitis* cultured in sugarcane bagasse hydrolysate as new green larvicides for the control of Aedes aegypti, a vector of neglected tropical diseases. PLoS one 2017; 12(11): e0187125.
15. Kalyani R, Bishwambhar M and Vuppu S: Recent potential usage of surfactant from microbial origin in Pharmaceutical and Biomedical Arena: A perspective. International Research Journal of pharmacy 2011; 11-15.
16. Parthipan P, Preetham E, Machuca LL, Pattanathu KS, Rahman M, Murugan K and Rajasekar A: Biosurfactant and degradative enzymes mediated crude oil degradation by bacterium *Bacillus subtilis* A1. Frontiers in Microbiology 2017; 8: 193.
17. Morikawa M, Hirata Y and Imanaka T: A study on the structure-function relationship of lipopeptide biosurfactant, Biochem Biophy Acta 2000; 1488: 211-218.
18. Cooper DG and Goldenberg BG: Surface active compounds from two Bacillus species. Appl. Environ. Microbiol. 1987; 53: 224-229.
19. Jain DK, Collins-Thompson DL and Lee H: A drop collapsing test for screening biosurfactant-producing microorganisms. Journal of Microbiological Methods 1991; 13: 271-279.

20. Abouseoud M, Maachi R, Amranec A, Boudergua S and Nabi A: Evaluation of different carbon and nitrogen sources in the production of biosurfactant by *Pseudomonas fluorescens*. Desalination 2008; 223: 143-151.
21. De Franca IWL, Lima AP, Lemos JAM, Lemos CGF, Melo VMM and De Santana HB: Production of a biosurfactant by *Bacillus subtilis* ICA56 aiming bioremediation of impacted soils. Catal. Today 2015; 255: 10-15.
22. Izard J and Limberger RJ: Rapid screening method for quantification of bacterial cell lipids from whole cells. Journal of Microbiology Methods. 2003; 2: 411-418.
23. Haddad Namir IA, Wang JI and Bozhong M: Identification of a biosurfactant producing Strain: *Bacillus subtilis* HOB_2. Protein and Peptide Letters 2009; 16(1): 7-13.
24. Akintunde TA, Abioye OP, Oyeleke SB, Boboye BE and Ijah UJJ: Remediation of iron using rhamnolipid-surfactant produced by *Pseudomonas aeruginosa*. Res. J. Environ. Sci 2015; 9: 169-177.
25. Deepali K, Sneha P and Patil S: Larvicidal activity of rhamnolipid biosurfactant produced by *Stenotrophomonas maltophilia*. IJSER 2014; 5(4): 60-63.
26. Chandankere R, Yao J, Cai M, Masakorala K, Jain AK and Choig MMF: Properties and characterization of biosurfactant in crude oil biodegradation by bacterium Bacillus methylotrophicus USTBa. Fuel 2014; 122: 140-148.
27. Cooper DG, Liss SN, Longay R and Zajic JE: Surface activities of Mycobacterium and Pseudomonas. Journal of Fermentation Technology 1989; 59: 97-101.
28. Peng F, Wang Y, Sun F, Liu Z, Lai Q, and Shao Z: A novel lipopeptide produced by a Pacific Ocean deep-sea bacterium *Rhodococcus sp*. TW53. J. Appl. Microbiol 2008; 105: 698-705.
29. Pirog TP, Shevchuk TA and Klimenko LA: Intensification of surfactant synthesis in *Rhodococcus erythropolis* EK-1 cultivated on hexadecane. Appl. Biochem. Microbiol 2010; 46: 599-606.
30. Patowary K, Patowary R, Kalita MC and Deka S: Development of an efficient bacterial consortium for the potential remediation of hydrocarbons from contaminated sites. Front. Microbiol 2016; 7: 1092.

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