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Research Article

BIOSURFUCTANT PRODUCTION BY LOCALLY ISOLATED *BACILLUS CEREUS*

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ABSTRACT

Oil pollution is found to be serious environmental issues and various techniques are being studied for their removal. In this context biosurfactants have attracted interest in recent years as a potential tool in bioremediation due to their emulsifying abilities. However, biosurfactant manufacture on a commercial scale has high production cost. Low yield of biosurfactant is a major limitation influencing its commercialization. The present study focused on production of biosurfactant by *Bacillus cereus* for different substrates and trace metal elements given. A modified orcinol method was used to assess the amount of glycolipids in the sample followed by absorbance measurement using UV-VIS spectrophotometer.

Keywords: Bioremediation, *Bacillus cereus*, Biosurfactant, Rhamnolipid and Emulsification.

1. INTRODUCTION

Surfactants (surface active agents) are molecules with the capability of reduces surface tension between two liquids, a gas or a liquid or a liquid and a solid. It accumulates at air water interface and break down oily materials from a media by micelle formation (FakruddinMd*et al*., 2012). Biosurfactants are produced by living cells that grows in water immiscible substrates (Desai JD, Banat IM, 1997), especially at microbial cell surfaces or secreted extracellularly. It enables the organism to utilize substrates in adverse environmental conditions. Biosurfactants are produced by many microorganisms like *Acinobactersp, Bacillus sp, Candida sp, Pseudomonas aeruginosa*etc. (Dimple Pardhi*et al*.,2018). Biosurfactant production regulate attachment and detachment of microbes for biofilm establishment on the surfaces they grow. Biosurfactants are classified according to their microbial original and chemical composition. They can be phospholipids, glycolipids, lipoproteins, lipopeptides, lipopolysaccharides, fatty acids, neutral lipids or polymeric in nature and have amphiphilic properties (Karanth*et al*., 1999). Biosurfactants have extensive uses in industries like oil recovery, oil spill cleanup, textiles, pharmaceuticals, oleo chemicals, due to their superior detergency, foaming, dispersing traits, broad substrate availability, wetting, emulsification, thickening, microbial growth enhancement, metal sequestering, and resource recovering properties. They are also used to make stable emulsions in food and cosmetics industry (Cirigliano and Carman, 1985; Makkar RS, Rockne KJ,2003; Das K, Mukherjee AK *et al.*, 2007). They are environment friendly, biological products with good biodegradability and lower toxicity and high ecological acceptability (M Nitschke ,2004).

They produce anionic surfactants like rhamnolipids that emulsify and make possible diffusion of the C_xH_y substrate present in the media into their cells (Burger *et al*., 1963; Guerra-Santos *et al*., 1986).*Bacillus cereus* is mostly an environmental spore forming organism,ubiquitously distributed in nature. Their sources include soil,decaying organic matter,fresh and marine waters and the invertebrate gut. The properties of biosurfactant suggest its potential use as a low

cost, ecofriendly tool for bioremediation. The following study was performed with the objective of estimating biosurfactant production by *Bacillus cereus* cultures under optimized conditions.

2. MATERIALS AND METHODS

2.1. SAMPLING SITE

Oil contaminated soil sample was collected from Balachandra Oil Industry, Sreekaryam, Thiruvanamthapuram, Kerala, India. The sample was collected in a sterile polythene bag, transported to the laboratory aseptically and refrigerated until isolation procedures.

2.2. ISOLATION OF *BACILLUS CEREUS*

The soil sample was serially diluted to 10^{-5} and the diluted sample was plated on nutrient agar. Isolated colony from mixed population on nutrient agar plate was characterized and identified based on colony characteristics and biochemical analysis (Bergey's manual). Further confirmation was done using molecular approach. The sequence obtained was compared using BLAST search program. Pure culture was made on nutrient agar plates and incubated overnight. The bacterial species was identified by BLAST analysis.

2.3. SCREENING OF THE BACTERIA FOR BS PRODUCTION

Seigmund-Wagner (SW) medium was used, a semi quantitative agar plate medium,developed by Inka Siegmund and Fritz Wagner. Also known as CTAB(cetyltrimethyl ammonium bromide) plates or blue agar plates, it is used to detect the presence of glycolipids like rhamnolipids secreted by bacteria.it was prepared by adding 0.2mg/ml CTAB and 0.005mg/ml of methylene blue and 15g/L of agar to 1L of mineral salt media supplemented with sucrose.

Rhamnolipid production by the colonies could be identified by visible blue halos around the colonies on a light blue plate background.

2.4. MEDIA AND GROWTH CONDITION FOR BS PRODUCTION

Biosurfactant production was carried out in 250ml conical flasks. The strain was cultured in mineral salt medium (Table 7.1.1).

Initial pH value of the BSproduction medium was adjusted to 7.0 before sterilization (autoclaved) by means of 1N HCl and 1N NaOH. Inoculation was carried out followed by incubation under the best parameters already known. Jacques *et al.*, (1999) noticed that an optimum temperature for high- surfactant production by *B. subtilis* S 499 was 30^0C, when it was grown aerobically with sucrose as a carbon source. Then BS assay was carried out.

2.5. OPTIMIZATION

The following parameters were carried out to optimize the productivity of the biosurfactant substance. After determining each parameter, the best result was applied in the subsequent parameters.

I. Substrate

Mineral salt medium was supplemented with one of the following substrates:

- ✓ Control (no substrate)
- ✓ Diesel
- ✓ Petrol
- ✓ Kerosene
- ✓ Glycerol
- ✓ Glucose

900ml of the mineral salt medium was transferred 150 ml each to 6 conical flasks with one as a Control. Each substrate was added to corresponding conical flask at concentration of 2.0% after filter sterilization and then inoculated with 3 ml of supernatant bacterial broth. it was incubated in the shaker at a temperature of 30˚C. Readings were taken from the 0^{th} hour to 72^{th} hour. From this, the ideal substrate was found for BS production.

II. Trace elements

Each trace elements (Table 7.1.2) were weighed and dissolved in 250mL of distilled water. And 5 mL of each solution was added to corresponding conical flasks containing 150 mL of Mineral salt media.

III. Effect of different incubation periods:

According to the previously performed optimization for the biosurfactants production, flasks containing the optimum medium were incubated in a shaker incubator (100 rpm) at 30ºC for 24, 48, 72, and 72 hrs. Biosurfactant production was evaluated every 24 hrs depending on emulsification activity measurement.

2.6. EXTRACTION OF THE BS

Rhamnolipid estimation (orcinol assay)

Rhamnolipids were extracted from the cell free culture broth y acid precipitation followed by liquid-liquid extraction. a modified orcinol method was used to assess the amount of glycolipids in the sample. Samples at different time intervals of incubation were centrifuged at 10000 rpm for 10 minutes to obtain the cell free culture broth. Biosurfactant (Rhamnolipids) in cell free supernatant obtained after centrifugation (10,000 rpm, 10 minutes) of the culture broth was precipitated by adjusting the pH to 2.0 by using 1N HCl and

1N NaOH and the extraction was done 2-3 times using ethyl acetate at room temperature (28) ºC. Rhamnolipid product was concentrated using rotary vacuum evaporator. The dried extract was dissolved in distilled water to determine rhamnolipids by the standard orcinol assay. The colour was measured at 421 nm using UV-VIS Spectrophotometer.

2.7. ASSAY OF BS PRODUCTIVITY

Emulsification Assay (Cooper and Goldenberg, 1987)

The emulsifying ability of the biosurfactant produced by *B. cereus* was evaluated by an Emulsification index (E24). The E24 of culture samples were determined by adding 2 ml of kerosene and 2 ml of the cell-free broth in a test tube, the cell-free broth obtained after incubation period and centrifuge at 4000 rpm for 4 min, after that vortex the mixture at high speed for 2 min and allowed to stand for 24h. The E24 index is the percentage of the ratio of height of emulsified layer (cm) to the total height of the liquid column (cm) in the test tube. The percentage of emulsification index calculated by using the following equation:

$$E_{24} = \frac{\text{Height of emulsion layer} \times 100}{\text{Total height}}$$

3. RESULTS AND DISCUSSIONS

3.1. ISOLATION OF BACTERIA

The bacteria isolated from the soil sample was found to be *Bacillus cereus* according to tests conducted suitable for identification of *bacillus* genus(Claus and Berkeley, 1986).

3.2. BIOCHEMICAL CHARACTERIZATION

In the present study, the bacteria were gram positive and showed positive for Catalase test, Citrate test, VP test. And negative for Oxidase and could utilize glucose and glycerol.

3.3. SCREENING OF BIOSURFACTANT PRODUCING BACTERIA

In the present study, biosurfactant producing bacteria were identified by culturing them on Sigmund Wagner medium and the one showing a halo around the colonies were chosen (Figure 7.2.1).

3.4. OPTIMIZATION

Optimization for various parameters were performed and maximum activity was found when glycerol was used as a substrate, and CuSO4.5H2O as trace element. Maximum biosurfactant production for *B.cereus* was observed after 72 hours of incubation.

I. Substrate

Out of different substrates like Petrol, Diesel, Kerosene, Glycerol and Glucose; maximum BS production was observed when Petrol was used as a sole carbon source (substrate). High emulsification activity coincided with this result. These results were comparable with a previous study, Tuleva*et al.*, (2005) found that the *B. cereus* grew well and produced effective biosurfactants in the presence of n-alkanes, naphthalene, crude oil and vegetable oils. Tables 7.1.3, 7.1.4, 7.1.5 and Graphs 7.3.1, 7.3.2, 7.3.3 shows the results of Spectrophotometric Analysis.

II. Trace elements

The production media containing petrol was supplemented with different trace elements. Results from the figure showed that CuSO4.5H2O enhanced the ability of *B. cereus* in biosurfactant production. (Table 7.1.6 and Graph 7.3.4).

III. Incubation period

The optimum period required for biosurfactant production from the locally isolated B. cereus under the previously monitored culture conditions was determined as 72 hours. The measurements were made within constant intervals (Graph 7.3.5).

3.5. EMULSIFICATION ASSAY

The emulsification index of all the substrates during each incubation period clearly states that 48hrs is the best incubation period, where maximum biosurfactant activity is observed (Table 7.1.7. and Graph 7.3.6).

4. CONCLUSION

Very few literatures were available, indicating little research has happened on biosurfactant production by bacillus sp. the present study was performed to assay the biosurfactant production by *B. cereus* under certain parameters like substrate, trace elements, incubation time etc. Maximum BS production was shown at the following optimal conditions:- 30^{o}C, pH 7.0, h incubation at shaken conditions (180 rpm), 2.0% inoculum, 2.0% (V/V) substrate in mineral salt media as the sole carbon source, $NaNO_3$ as the Nitrogen source and 0.0025g of CuSO4.5H2Oas trace element. The other tested substrates and trace elements did not show higher productivity. The BS was extracted using ethyl acetate at pH 2.0. Among all the above results, maximum BS was found to be 538.693 mg/ml.

Enhanced research in this field is required to bring to light new and efficient types of surface-active compounds from less studied microorganisms. Finding the optimum

conditions for maximum biosurfactant production from easily available strains is vital for overcoming high cost of production. fields of application of biosurfactant producing bacteria are phytoremediation of hydrocarbon polluted soil. Careful use of biosurfactants can replace the use of detergents and prove to be an eco-friendly tool for magnified clean-up of the toxic environmental pollutants and provide us with a clean environment.

5. ACKNOWLEDGEMENTS

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7.1: TABLES

Table 7.1.1:

COMPONENTS	g/L
NaNO3	15.0
KCl	1.1
NaCl	1.1
FeSO4.7H2O	1.1
KH2PO4	3.4
K2HPO4	4.4
MgSO4.7H2O	0.5
Yeast extract	0.5
Distilled Water	1L

Table 7.1.2:

TRACE ELEMENTS	g/250ml
ZnSO4.7H2O	0.0029
CaCl2.4H2O	0.0024
CuSO4.5H2O	0.0025
MnSO4.7H2O	0.0017

Table 7.1.3:
24 hrs incubation

Sample	Concentration	Absorbance
Control	0	0
Kerosene	0	0
Petrol	20.616	4.491
Diesel	61.797	4.929
Glycerol	24.726	4.535
Glucose	0.639	4.279

Table 7.1.4:
48 hrs incubation

Sample	Concentration	Absorbance
Control	7.096	4.348
Kerosene	13.257	4.413
Petrol	277.445	8.397
Diesel	48.804	4.791
Glycerol	162.503	6
Glucose	13.257	4.413

Table 7.1.5:
72 hrs incubation

sample	Concentration	Absorbance
control	9.484	4.373
kerosene	125.59	5.08
petrol	538.693	10
diesel	97.092	5.304
glycerol	161.051	5.985
glucose	12.932	4.41

Table 7.1.6:

Trace Elements	E24 (24hrs)	E24 (48hrs)	E24 (72hrs)
ZnSO4.7H2O	7.962	40	43.478
CaCl2.4H2O	13.043	45.833	30.769
CuSO4.5H2O	27.272	51.851	66.667
MnSO4.7H2O	20.833	36	26.086

Table 7.1.7

Sample	E24 (24hrs)	E24 (48hrs)	E24 (72hrs)
Control	0	4.761	0
Kerosene	0	32	24
Petrol	29.166	70.833	62.5
Diesel	32	33.333	39.13
Glycerol	20.833	13.043	15.217
Glucose	0	3.703	0

7.2 : FIGURES

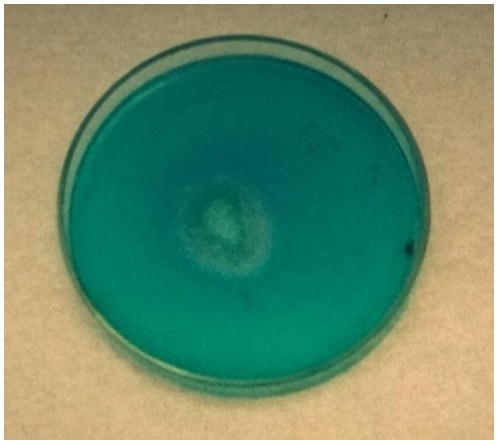
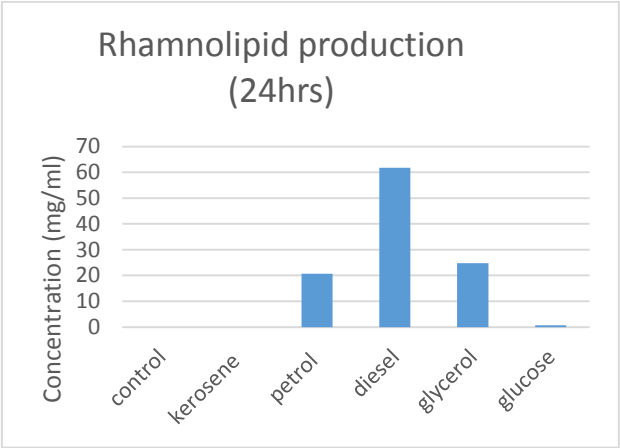
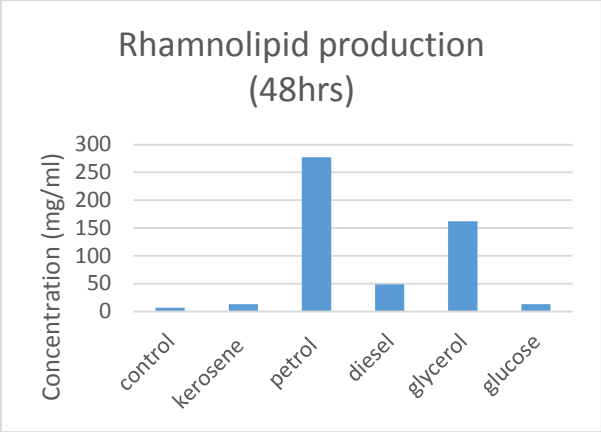


Fig. 7.2.1:

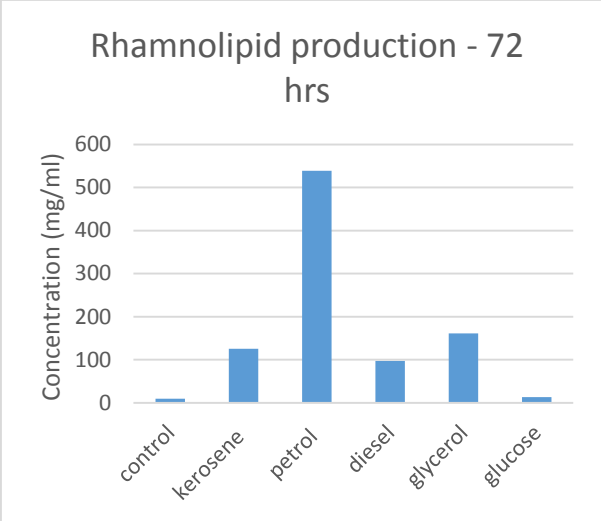
7.3. GRAPHS



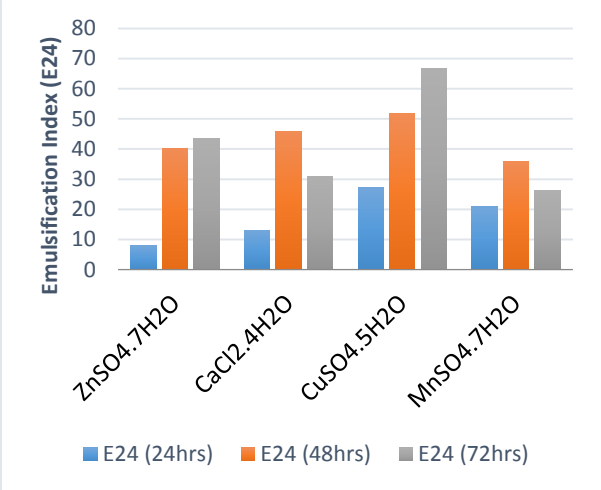
Graph. 7.3.1:



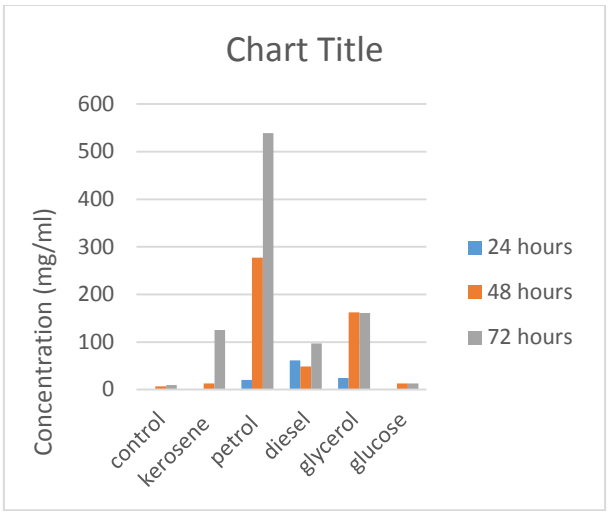
Graph. 7.3.2:



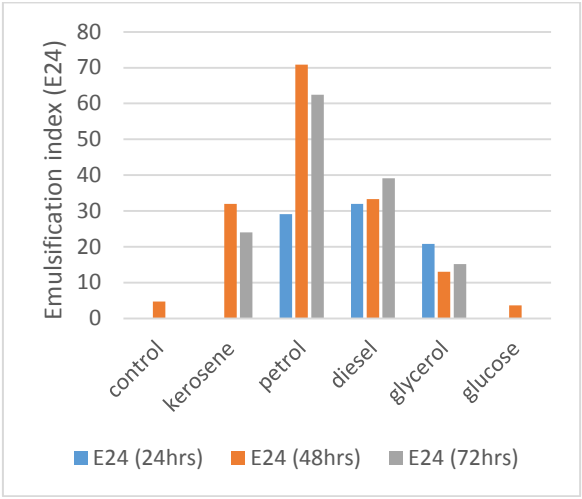
Graph. 7.3.3:



Graph. 7.3.4:



Graph. 7.3.5:



Graph. 7.3.6:

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