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Optimization of Environmental Parameters for Biosurfactant Production by *Bacillus thuringiensis*

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Biosurfactants are biological compounds produced by microorganisms such as bacteria, yeasts and fungi etc. on the cell surface or extracellularly. Biosurfactants are used in agriculture, and industry such as detergent, cosmetics, paper etc., and it has enlarged its significance for its large production. In the present study, *Bacillus thuringiensis* (JB) was used to optimize at different environmental parameters such as pH (6 to 9), NaCl concentrations (1 to 4 %) and water soluble fraction (WSF 1 to 4%) concentrations to maximize the production of biosurfactant. Culture samples were drawn and assayed for biomass, emulsification activity and biosurfactant production for 5 days. It showed maximum activity and yield at pH 7 and 3 and 4 % of NaCl. 3% WSF showed good activity whereas 4% WSF provided good yield of biosurfactant. *Bacillus thuringiensis* showed good growth, activity and yield during the optimized environmental conditions of the bacteria.

Key words: Biosurfactant, *Bacillus thuringiensis,* biodegradation, optimization, diesel, water soluble fraction.

Biosurfactants are biological compounds produced by microorganisms such as bacteria, yeasts and fungi etc. on the cell surface or extracellularly. Biosurfactants have property to reduce surface and interfacial tension due to the presence of both hydrophobic and hydrophilic groups in their structure. This property has made a wide spread applications in industrial processes such as emulsification, defoaming, wetting, dispersing, and solubilisation[1]. They also enhance bioavailability, biodegradability by increasing the poorly soluble organic compounds such as polycyclic aromatics hydrocarbons (PAH)[2]. Applications of biosurfactant in agriculture, detergent industry, cosmetics, and paper industry[3] including bioremediation have grown up the significance and need for its large production.

The high potential of the bacterial strain in showing good surface activity led to the optimization of environmental parameters for large production of the biosurfactant. This will favourably facilitate in the reduction of cost in the bioremediation process. The main objectives for the study were (i) to optimize the different environmental parameters such as pH, salt concentration and diesel concentration (WSF) in an effort for large production of biosurfactant and (ii) to compare the growth, activity and the yield of biosurfactant during the different environmental parameters.

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MATERIALS AND METHODS

Media preparation

Nutrient broth medium which was used for the studies, composed as follows (g per 1000 ml of distilled water); peptone (10); meat extract (10). Medium pH was 7.2 ± 0.2 and the medium was autoclaved at 121^{0}C for 20 min at 15 lbs pressure.

Water soluble fraction (WSF) of diesel concentration was prepared by using 10% of diesel in the autoclaved double distilled water (v/v). It was kept in a rotary shaker at 100 rpm and the soluble part of the diesel fraction was used for the experiment.

Microorganism

The microorganism used in this study *Bacillus thuringiensis* had already been isolated and the sequence data were submitted in Genbank with accession number KJ372208. This strain has been qualitatively analyzed for surface active properties.

Inoculum

The strain was maintained in agar slants and *B. thuringiensis* was inoculated in a 10 ml of nutrient broth. After 24 hours of growth, 1 ml was transferred to the experimental flasks.

Optimization of environmental parameters

To determine the optimum environmental conditions for the maximum production of biosurfactant by the isolate, experiments were conducted. The different parameters such as pH (6.0 – 9.0), NaCl concentration (1- 4 %), diesel concentration (1- 4%) of the water soluble fraction of diesel were used. Nutrient broth was used as medium and water soluble fraction of diesel was used as additional carbon source. 1% of WSF was added in each of the experimental flask other than WSF experiment. 10 ml of the medium was withdrawn from the culture flask at a regular interval for 5 days and analyzed for biomass, emulsification activity and biosurfactant yield.

Biomass estimation

Growth of the culture was measured by turbidity method using calorimeter (Systronics 9130, Ahmedabad, India) at 600 nm.

Emulsification assay (E_{24})

The *B.thuringiensis* was evaluated for emulsion forming capacity, according to the method proposed by Das et al. (4). 0.5 ml of the cell-free supernatant was added with 1 ml kerosene in a test tube. This mixture was homogenized and vortex at high speed for 2 min. After 24 hrs, relative emulsion volume (E_{24} %) was calculated using the following equation:

$$E_{24}\,(\%) = \text{Emulsion height (cm)} / \text{Total liquid volume} \times 100$$

Biosurfactant extraction

Bacterial culture broth was centrifuged at 10,000 rpm, for 20 min at 4°C. The pellet was discarded and the cell-free broth was adjusted to pH 2 using 1N H_2SO_4[5]. It was kept at 4°C overnight, and then centrifuged at 10,000 rpm for 10 min. Thus formed precipitate was called as acid precipitate. After drying it, the weight was expressed as mg/ml (w/v).

Statistical analysis

Mean and standard deviation were calculated. ANOVA was used for testing significance using Graph Pad Prism version 6.

RESULTS AND DISCUSSION

Optimization of environmental parameters

Environmental parameters such as pH, salt concentration, and concentration of water soluble fraction were optimized for the growth, emulsification activity and yield of the acid precipitate from *B.thuringiensis* for a period of five days. The experimental medium for pH and salt was added with 1% water soluble fraction as additional carbon source.

pH evaluation

There was a gradual increase in the growth but did not show much difference in the pattern of growth among the pH values tested. The growth of the organism reached to 0.4 O.D at pH 6 and 7 which was equivalent to control flask and 0.52 O.D at 70 hrs in pH 9 (Fig.1b). Since the organism was able to grow in a similar pattern in all the pH tested, it could be inferred that this bacterial strain could tolerate a range of pH from 6 to 9. The emulsification activity was not detected on the first day of incubation (Fig. 2a and b), which might be due to the fact that during the initial period, the biosurfactant might be attached to the cell membrane of the producing microorganism as suggested by Agatha 1998 [6].

The emulsification activity of *B.thuringiensis* was observed from the second day and there was a gradual increase in the activity

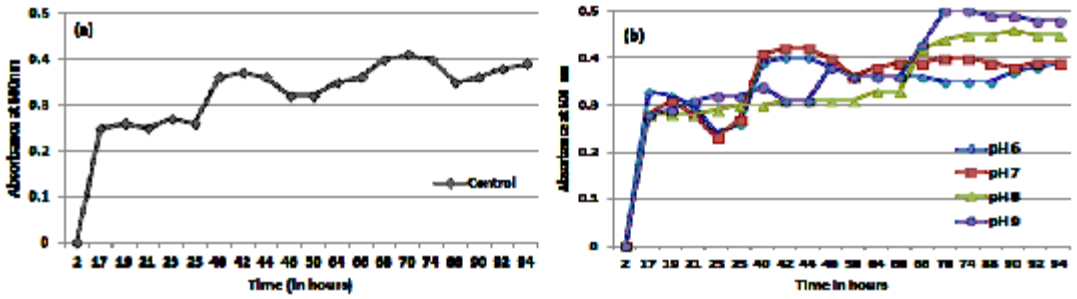

Fig. 1. Growth of *Bacillus sp.* at control (a) different pH (b)

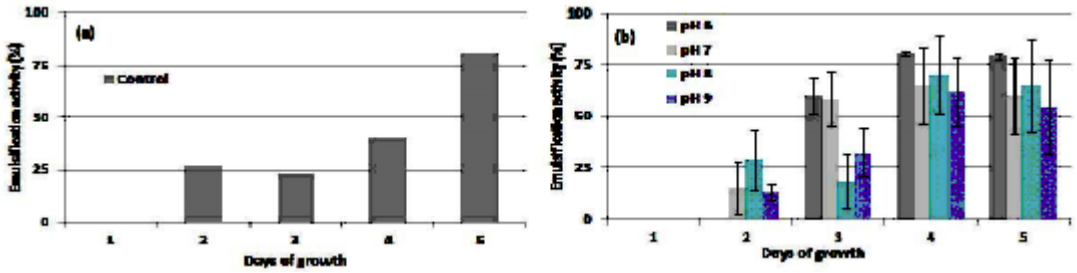

Fig. 2. Emulsification activity of *B.thuringiensis* in control (a) and different pH (b).

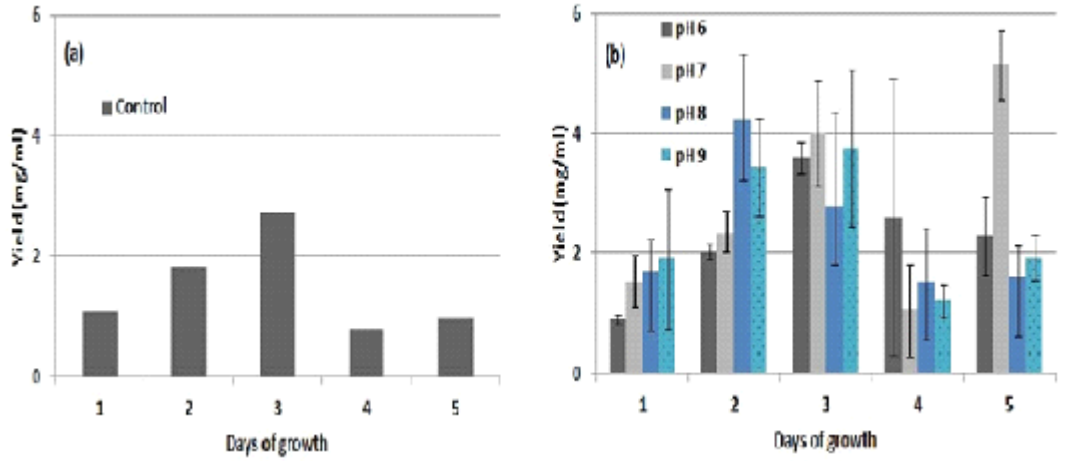

Fig. 3. Biosurfactant yield of *B.thuringiensis* in control (a) and different pH (b).

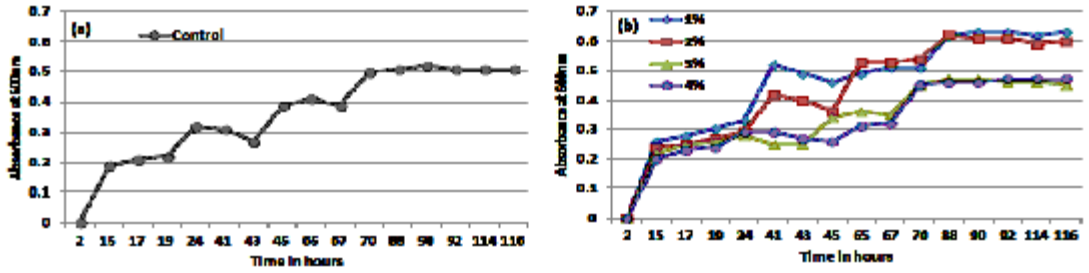

Fig. 4. Growth of *B.thuringiensis* in control (a) different NaCl concentrations (b).

until last day of the experiment. Since biosurfactant was not required for the growth of the organism, it might be released into the ambient medium as opined by Agatha 1998[6]. This may be a plausible reason for the maximum biosurfactant production that was seen during the end of the experiment. It was reported that stationary growth phase of the cells may contain high levels of biosurfactant[7]. More than 50% activity of *B.thuringiensis* was seen at all the pH values on 4th and 5th day (Fig. 2b). Emulsion stability may increase with increase pH as suggested by Bernard *et al*., 2006[8] but in the

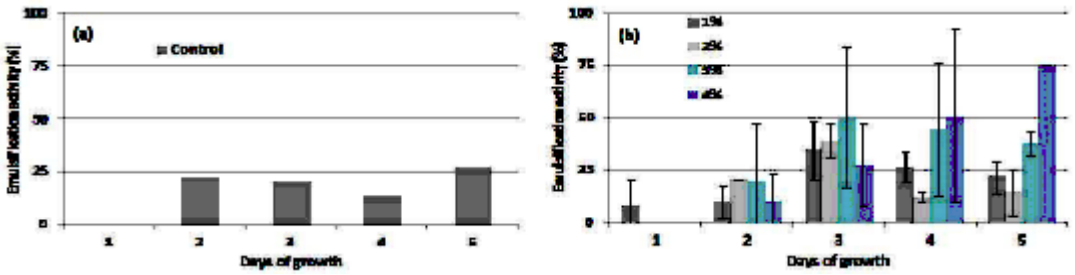

Fig.5. Emulsification activity of *B.thuringiensis* in control (a) and different NaCl concentrations (b).

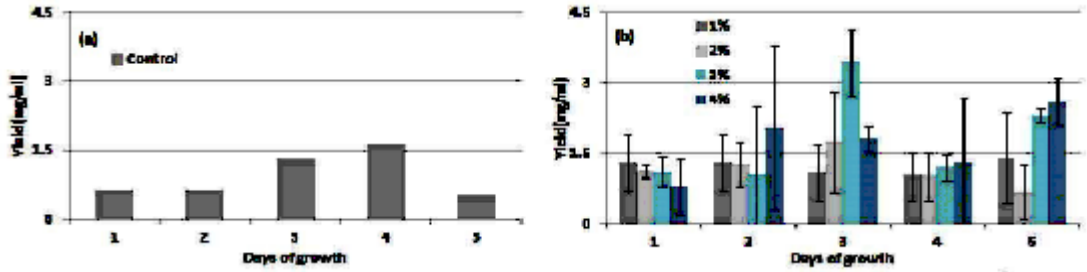

Fig. 6. Biosurfactant yield of *B.thuringiensis* in control (a) and different NaCl concentrations (b).

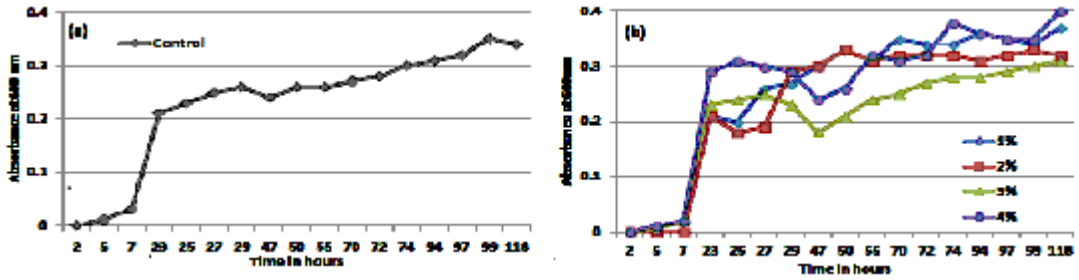

Fig. 7. Growth of *B.thuringiensis* at control (a) different WSF concentrations (b)

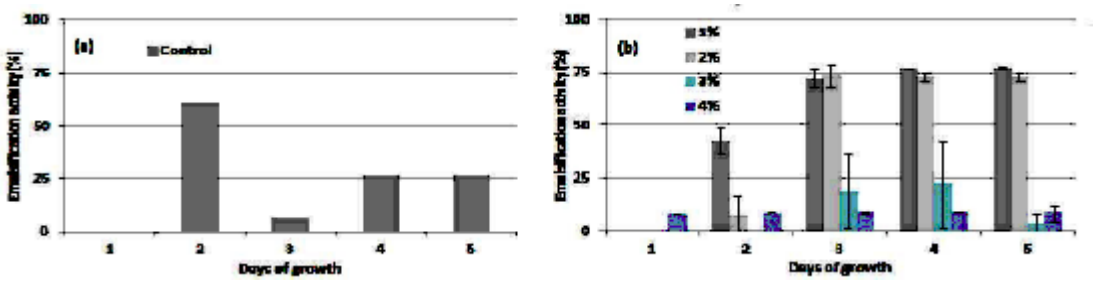

Fig.8. Emulsification activity of *B.thuringiensis* in control (a) and different WSF concentrations (b).

present study production of emulsifying compounds did not show a similar trend.

The biosurfactant yield was seen maximum on the 3rd day having 2.8 mg/ml in control (Fig. 3a) and 5.5 mg/ml in test at pH 7 on 5th day (Fig. 3b) which may be due to the addition of 1% WSF that had stimulated the production of biosurfactant to two times of the control. But the amount of biosurfactant produced at each consecutive day (Fig. 3) did not correlate with (Fig. 2) emulsification activity. Ana et al., 1997 (9) reported that when the concentration of biosurfactant was increased above the critical micellization concentration (CMC), micelles were formed, and thus emulsion became stable. The maximum production of biosurfactant was seen on 2nd and 3rd day and then it showed a decreasing trend. It could be understood from the results that

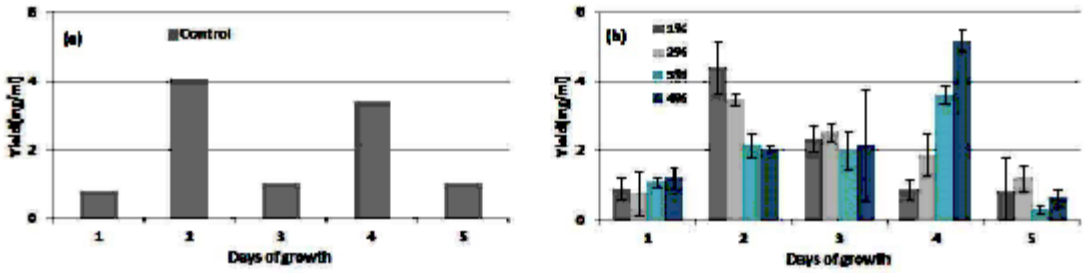

Fig.9. Biosurfactant yield of *B.thuringiensis* in control (a) and different WSF concentrations (b).

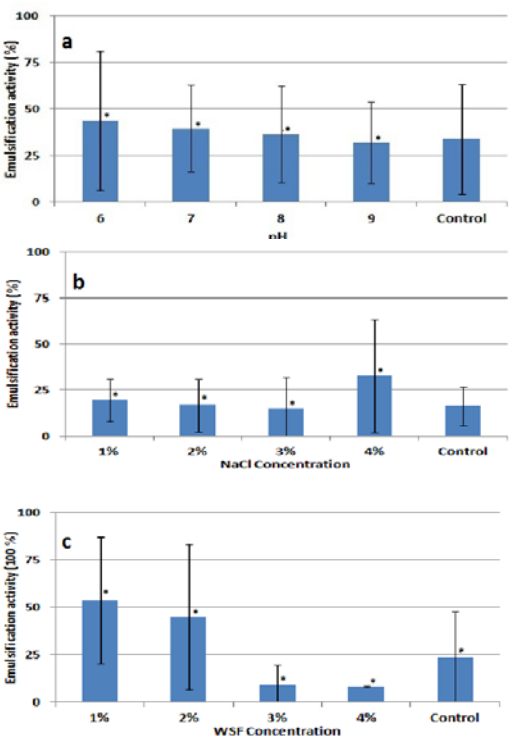

Fig. 10. Mean and standard deviation of emulsification activity of different (a) pH, (b) NaCl, (c) WSF concentration and control during the experimental period.
(**Note** * significant P<0.05; ** significant P<0.0001)

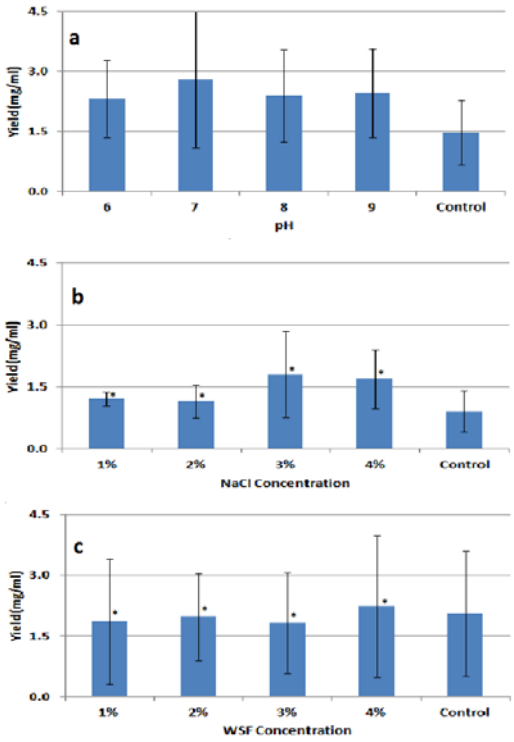

Fig. 11. Mean and standard deviation of biosurfactant yield of different (a) pH, (b) NaCl, (c) WSF concentration and control during the experimental period.
(**Note** * significant P<0.05; ** significant P<0.0001)

production of biosurfactant was higher during stationary phase which may be noticed during the growth of the bacteria (Fig. 1).

Salt concentration

The growth of the organism at NaCl concentration of 1 and 2% was found to be above 0.6 O.D but the growth at 3 and 4% was lower than the 1 and 2% NaCl concentration (Fig.4b). There was an increase in the growth of the organism compared to control which may be due to the addition of 1% WSF as carbon source. However at 3% and 4%, the growth was at a moderate level due to the high concentration of salt. The relation between growth and emulsification activity was not proportioned at 1% and 2% of NaCl concentration, i.e. increase in biomass had not increased the emulsification activity (Fig. 5a and b). Maximum activity of control was seen having 25% (Fig.5a) but a test with 75% activity was noticed at 4% NaCl concentration (Fig. 5b) on the 5th day of the experiment. This was similar to as Kiran et al., 2010 in which the biosurfactant produced by the marine actinobacterium was stable at higher NaCl levels[10] and similar increase in biosurfactant production was seen at high percentage of NaCl at 3% on the 3rd day[11].

The maximum yields of biosurfactant in control and test were 1.8 mg/ml and 3.9 mg/ml respectively as shown in Fig. 6a and b. It could be suggested that the high production of biosurfactant could be obtained at 3% NaCl concentration. However, the yield of biosurfactant was relatively lower at different salt concentrations than that were obtained for pH (5.5 mg/ml for pH 7).

Diesel concentration

The growth patterns of the organism at different water soluble fraction concentrations were similar during the experiment. 1% and 2% WSF concentration showed maximum emulsification activity compared to 3% and 4% of WSF (Fig 8). The activity might have diminished due to the increase in hydrocarbon concentration. The organism had produced biosurfactant to combat the increased concentration of the hydrocarbon (Fig. 9). The biosurfactant production was found to be higher at 4% of WSF concentration (5.2 mg/ml) compared to 1% WSF concentration (4.2 mg/ml). It could be inferred from these results that the high concentration of WSF was able to induce the higher levels of biosurfactant production, even though the observed emulsification activity was not proportional to the biosurfactant production in 3% and 4% as shown in Fig. 8b and 9b.

The mean and standard deviation values were calculated during the experiment period and they were presented in Fig. 10-11. Good emulsification activity was noticed at pH 6 than the other pH levels (Fig. 10a) and showed significant difference between different pH values but the control did not show significant differences statistically. This may be due to the absence of additional carbon source in control. Figure 11a showed the mean biosurfactant yields at different pH values. There was no significant difference among the pH values was, but the maximum yield was (2.8 mg/ml) was observed at pH 7.

Figure 10b showed high emulsification activity at 4 % NaCl concentration (32%) of the *B. thuringiensis.* This showed that the organism was capable of showing good emulsification activity. The avareage activity was less than 25% in all NaCl concentration except 4% and the activity was lower that the activity observed in pH (Fig 10a and Fig 10b). There was significant difference in the activity among the experimental salt concentration. Figure 11b showed high biosurfactant yield in 3% NaCl concentration which was 1.8 mg/ml when compared to other NaCl concentrations. From the mean values it could be inferred that this bacterium was capable of producing high levels of biosurfactant in various pH than salt concentrations (Fig. 11a and 11b).

Mean emulsification activity of the WSF concentration showed more than 50% activity at 1% WSF and 45% activity at 2% WSF (Fig. 10c). When WSF concentration activity was compared with pH and NaCl, WSF gave the maximum emulsification activity. In fig. 11c showed the mean biosurfactant yield in different WSF concentrations was always above 1.5 mg/ml with the maximum of about 2 mg/ml at 4% but these yields were less than the values of pH (2.8 mg/ml) and higher than NaCl concentration (1.8 mg/ml).

This bacterium showed good activity at different pH and WSF concentrations but comparatively low activity in NaCl concentration. It should be noticed that this bacterim showed slow growth in salt concentration.

CONCLUSION

To understand the potential of *B.thuringiensis*, it has been subjected to the different environmental parameters such as pH, NaCl concentrations and WSF diesel concentrations. From the activity and biosurfactants yields of this bacterium, it could be inferred that this bacterium can be a potential candidate for future applications.

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