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BIOSURFACTANT PRODUCTION BY HALOPHILIC BACTERIA

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ABSTRACT

Biosurfactants that are synthesized by microorganisms are capable of reducing surface and interfacial tension with low toxicity and high specificity. In this study, two bacterial strains JS05 and JS06 were isolated from the soil near to a fishery storage plant containing high salt content. Molecular analysis of these strains revealed that they were *Brevibacterium lutescens* (JSO5) and *Bacillus sp.* (JSO6). Both of them were screened for the production of biosurfactant by oil spreading test, emulsification activity and blood hemolytic test. Both the strains showed the capability to produce biosurfactant, but *Brevibacterium lutescens* (JS05) showed better activity when compared to *Bacillus sp.* (JS06) giving a oil displacement of about 4cm and emulsification index of 68%. The results of this study suggested that these two bacterial strains could be considered as potential candidates for biosurfactant production, but needs to be studied further for field application.

KEYWORDS: Biosurfactant, hemolytic method, soil, halophilic, bacteria

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INTRODUCTION

Biosurfactants are a diverse group of surface-active agents produced by many microorganisms[1]. These amphiphilic compounds contain a hydrophobic and a hydrophilic moiety, and have the ability to reduce interfacial tension between two or more different fluid phases. Biosurfactants are known to occur in a variety of chemical structures, such as glycolipids, lipopeptides and lipoproteins, fatty acids, neutral lipids, phospholipids, and polymeric and particulate structures[2]. Surfactants produced from chemically based materials are known as synthetic surfactants like sodium dodecyl sulphate and those from biologically based materials are biosurfactants like rhamnolipids. Biosurfactants can be synthesized by many different microorganisms such as bacteria, fungi and yeasts[3]. Biosurfactants display excellent surface activities despite their bulky molecular structure in comparison to synthetic ones. As they originate from living organisms, biosurfactants have advantages of eco-friendly, biodegradability, easily produced using renewable resources, possible regeneration, high specificity and less toxicity[4]. The use of biosurfactants eliminates the need for surfactant removal from effluents because of their innocuous nature. The biosurfactants show high activity at extreme temperatures, pH, and salinity conditions and thus they are expected to be more effective than synthetic surfactants[3]. Due to their physiochemical characteristics, biosurfactants are thus better suited to environmental applications than synthetic surfactants[1,5]. Some microorganisms can survive and grow over a wide range of salt concentrations. In aquatic environments the conditions range from freshwater (containing less than 0.05% w/v dissolved salts), seawater with total salinities of 3.2–3.8% (w/v) to saturated salt solutions up to 30% (w/v) and above[6]. It has been stated that improving the method of biosurfactant production and characterizing their major properties are highly important in the commercial application of biosurfactant. In this study, isolation and identification of microorganisms from salt dumped soil near fishery storage plant have been carried out. The potential of these isolates in producing biosurfactant was examined by measuring the oil displacement and emulsification index during growth so as to know the extent of production of biosurfactant during different growth phase.

MATERIALS AND METHODS

Sampling Area

For isolation of biosurfactant producing bacteria, the surface sample was collected from salt dumped soil near a fishery storage plant. The samples were collected in sterile polythene bags and were transported to Molecular and Microbiology Research Laboratory and stored at 4°C until further analysis.

Media Preparation

To isolate bacteria from the soil sample, nutrient agar medium was used. The composition of medium was as follows (gram per 1000 ml of distilled water); peptone (10); meat extract (10); agar (15). Medium pH was 7.2 ± 0.2 and the medium was autoclaved at 121^0C for 15 min at 15 lbs pressure. Medium was incorporated with 3% of sodium chloride.

Isolation of Microorganisms

One gram of soil sample was serially diluted up to 10^{-5} and 10^{-6}, then plated by spread plate method on nutrient agar plates and incubated under aerobic conditions at 37°C for 24 hours. The colonies were randomly selected for further study. The strains isolated were cultured by streak plate method and the slants were prepared for storage of the strains at 4°C for future use.

Morphological and Biochemical Characterization

Gram staining was performed by heat-fixing as described by Dussault[7]. Spore and capsule staining was done and other biochemical tests were carried out based on the standard protocols

using kit method using Bergey's manual of Determinative Bacteriology[8].

Identification of the Strain

The bacterial strains JSO5 and JSO6 were identified using 16S rRNA sequencing and the PCR analysis was performed as follows: universal 16S rRNA primers 518F and 800R were used for amplification of 16S rRNA. The 16S rRNA gene sequence obtained from the isolate JSO5 and JSO6 was compared with other bacterial sequences by using NCBI BLASTN (http://blast.ncbi.nlm.nih.gov/Blast. cgi) for their pairwise identities. Multiple alignments of these sequences were carried out by Clustal W2 (www.ebi.ac.uk/clustalw). Phylogenetic tree was constructed in Jalview software using neighbour joining with distance calculation. The 16S rRNA sequence of JSO5 and JSO6 was deposited in NCBI with accession numbers of KC351488 and KC351489.

SCREENING FOR BIOSURFACTANT-PRODUCING ISOLATES

Growth Studies

The strains JSO5 and JSO6 were inoculated in 250 ml side arm flask having 150 ml nutrient broth containing 3% NaCl both in control and test. One ml of water soluble diesel was added to the experimental flask. Both were incubated at 35°C in an orbital shaker at 100 rpm. The culture was grown for 80 hours, and the samples were collected at regular interval of every 6 hrs or so for the measurement of growth at 600nm. The cell-free suspensions were prepared by centrifuging at 10,000 rpm for 10 min and tested for the presence of biosurfactant by using the emulsification activity (E_{24}) and oil displacement methods.

Oil Displacement Measurement

Every 6 hrs, the cells were removed and centrifuged at 10,000 rpm for 10 min. The supernatant was taken to perform oil displacement test. Qualitative oil displacement test was performed in petri plates. 25 ml of distilled water was taken in a petri plate and 20 µl of hydrocarbon source kerosene was added making a thin layer of oil on the surface of water. Then, 10µl aliquot of supernatant was delivered onto the oil. Distilled water was used for negative control and displacement of oil was considered as positive and it was measured in cm.

Emulsification Activity Assay

The two isolates were also evaluated for emulsion-forming capacity, according to the method proposed by Das et al.[9]. 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 (EV, %) was measured using the following equations :

EV (%) = Emulsion height (cm) / Total liquid volume X 100

Emulsions formed by the isolates were compared with positive control such as SDS and CTAB and negative as distilled water.

Hemolytic Activity

Strains JSO5 and JSO6 were streaked ~~into~~ onto blood agar plate and incubated for 24 hours at 37°C. The plates were visually observed for the zone of clearness around the colony. The concentration of biosurfactant depends on the diameter of the clear zone[10].

Biosurfactant Extraction

Bacterial culture was centrifuged at 10,000 rpm, 4°C for 20 minutes. The supernatant was was adjusted to pH 2, using 1N H_2SO_4[11]. Biosurfactant was extracted using equal volume of chloroform:methanol (2:1). The solvent from the residue was separated by using rotary evaporator and the residue collected was crude biosurfactant.

Characterization of Biosurfactant

The method used for biosurfactant characterization was achieved by preparative silica gel thin-layer chromatography (TLC) of the extract. The extract was spotted onto preparative silica gel TLC plates with a solvent system of chloroform/methanol (2:1, v/v). Reagent like ninhydrin and Iodine vapour was used for detecting the presence of protein and carbohydrate respectively.

RESULTS AND DISCUSSION

Based on the sequence analysis of the bacterial isolates by BLASTN for JSO5 and JSO6 were identified as *Brevibacterium lutescens* and *Bacillus sp.* respectively. The phylogenetic tree of the results was shown in Fig. 1 and the neighbouring distance between *Brevibacterium lutescens* (JSO5) and *Bacillus sp.* (JSO6) was found to be 8.33 and 3.33 respectively.

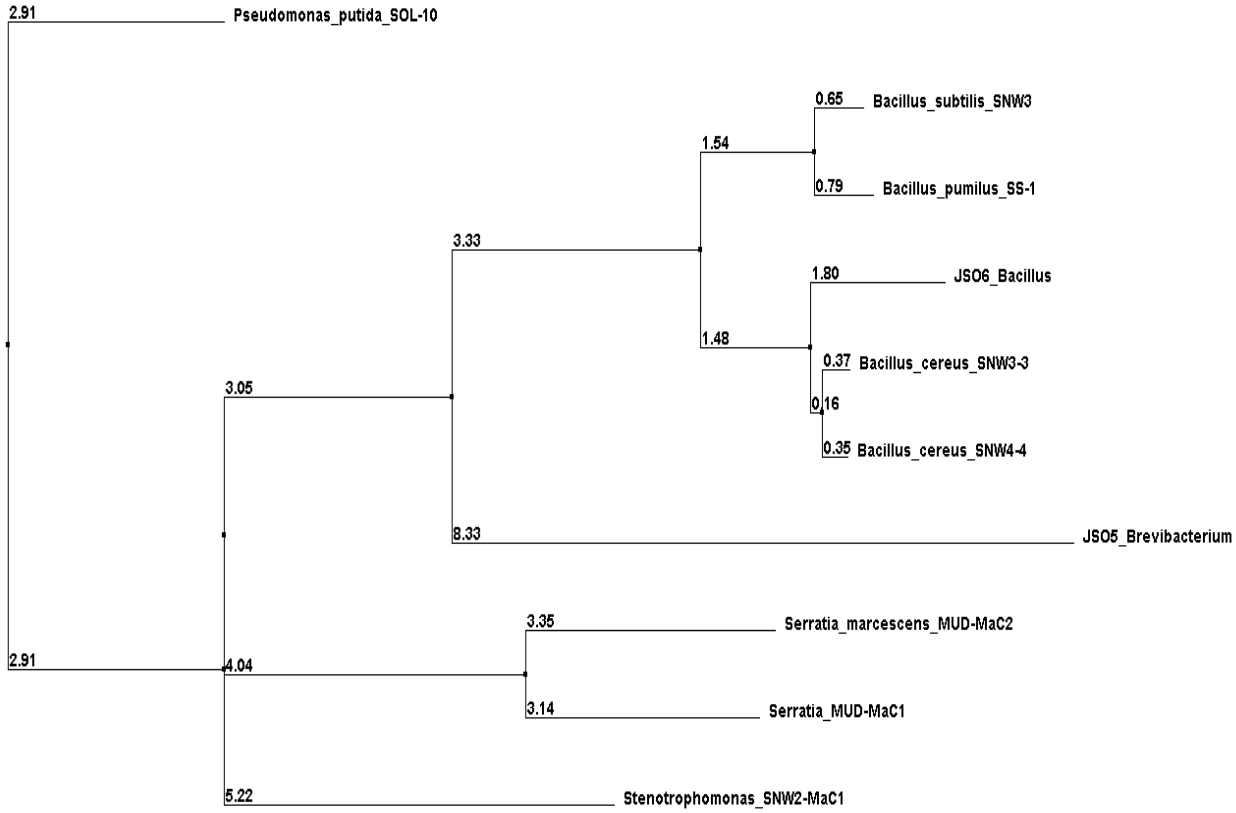

Figure 1
Phylogenetic tree representing Brevibacterium lutescens (JSO5) and Bacillus sp. (JSO6) with neighbour joining distance.

Morphological and Biochemical Characterization

The isolated bacteria were both Gram-positive and produced endospores and capsule. *Brevibacterium lutescens* (JS05) strain was found to be cocci and in clusters having short and long chains. *Bacillus sp.* (JS06) was found to be rod shaped. Results of the biochemical and morphological tests done according to the Bergey's manual of Determinative Bacteriology were presented in Table 1.

Table 1
Biochemical Test

Test	*Brevibacterium Lutescens*	*Bacillus sp.*
Gram staining	+	+
Spore	+	+
Capsule	+	+
Indole	-	-
Methyl red	+	+
Voges proskauer	-	-
Citrate	+	+
Glucose	-	-
Adonitol	-	-
Arabinose	-	-
Lactose	+	+
Sorbitol	-	-
Mannitol	+	-
Rhamnose	+	-
Sucrose	-	-

Biosurfactant Studies of the Isolates
I. Brevibacterium lutescens
Growth Studies

The growth was observed for both the control and test (broth+1ml water soluble fraction of diesel). The growth was monitored at different time intervals and optical density was measured at 600nm. The growth of the bacteria, in the presence of water soluble fraction of diesel, showed a similar growth patterns to the control, suggesting that water soluble fraction of diesel had been used as sole carbon sources. Visible change in growth was observed at 12 hr and the maximum OD values of 0.9 – 1.0 was noticed during 34 – 45 hr (Fig.2).

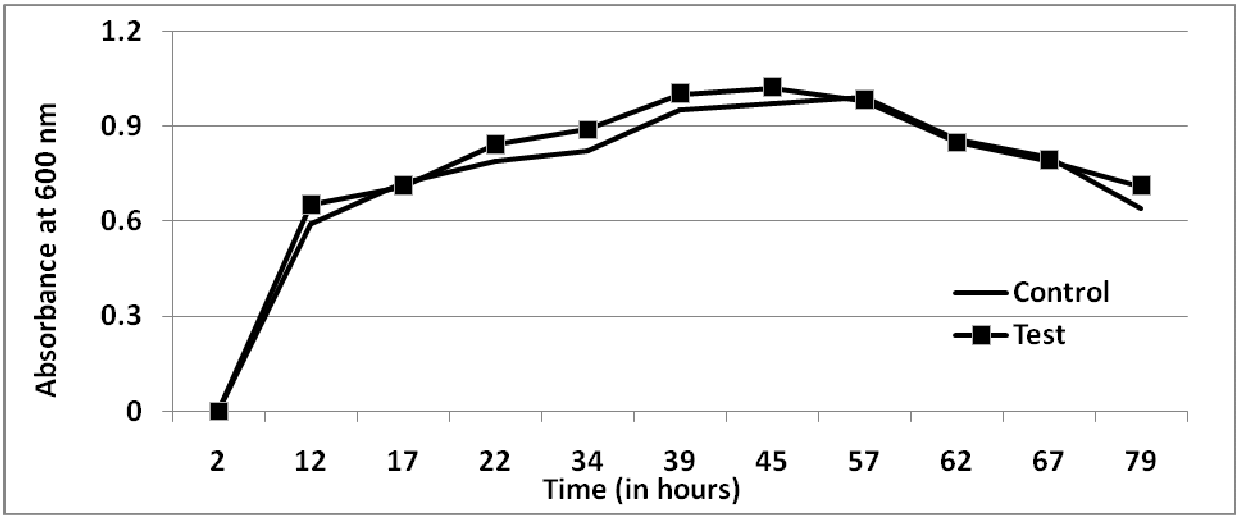

Figure 2
Growth curve of Brevibacterium lutescens.

Oil Displacement Activity

It can be clearly understood that this organism produced good results for oil displacement in test compared to the control (Fig.3). The higher activity of 4.2 cm was noticed at 22 hrs. *B. lutescens* showed more activity during the exponential phase of growth, i.e till 34 hrs. The oil displacement activity was found to be very low after 45 hrs in both control and test.

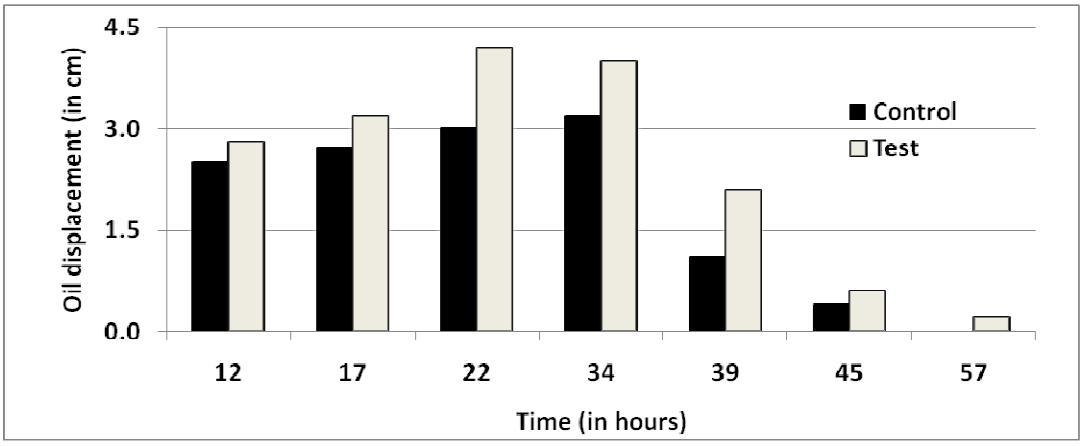

Figure 3
Oil displacement activity of Brevibacterium lutescens.

Emulsification Activity

The emulsification activity was observed from 22 hr of growth of *B. lutescens*. Highest emulsification activity of 68% by *B. lutescens* was noticed at 62 hr. As seen for oil displacement activity, emulsification activity was also found to be higher in test than that observed in control (Fig. 4).

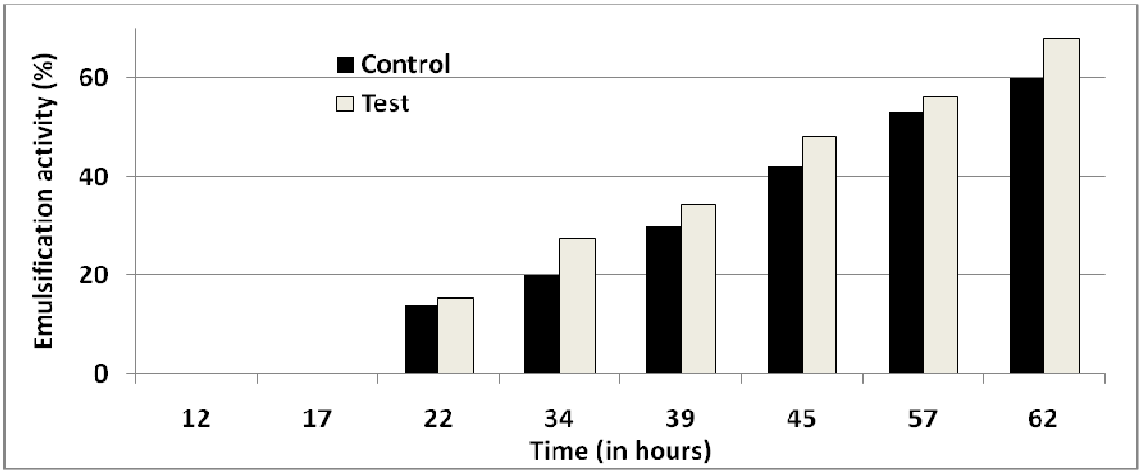

Figure 4
Emulsification activity of Brevibacterium lutescens.

Hemolytic Activity

The plates streaked with the *B. lutescens* showed the β – hemolytic activity having an inhibiting zone of 33 mm in length and 14 mm in width.

II. Bacillus sp.

The growth of the other isolated strain, *Bacillus sp.* was carried out for both the control and test (broth + 1 ml water soluble fraction of diesel). The growth of the bacteria in the test was slightly

higher when compared to the control indicating that diesel was utilized as carbon source as observed for *B. lutescens*. Growth was slow till 12 hr and then it reached the maximum of 1.1 OD at 45 hr (Fig. 5).

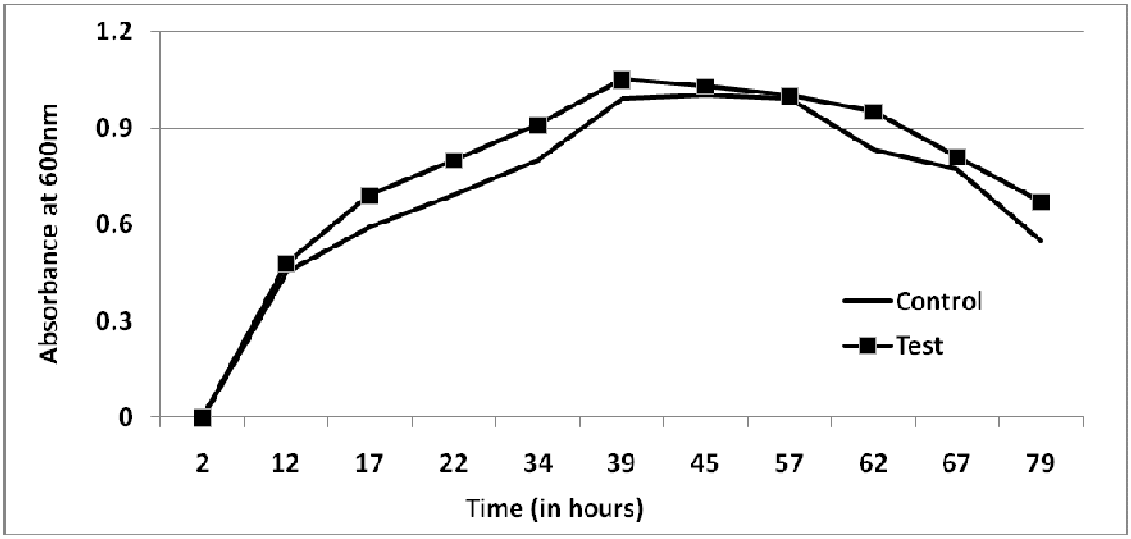

Figure 5
Growth curve of Bacillus sp.

Oil Displacement Activity

From results shown in the Fig. 6, it can be clearly noticed that this organism produced good results for oil displacement in test when compared to the control. *Bacillus sp.* showed the maximum displacement of 3.0 cm during the exponential phase of growth (39th hour) and the oil displacement activity was observed till 45th hour. As noticed for *B. lutescens*, the oil displacement activity was poor in both control and test after 57th hour. The zones were smaller in *Bacillus sp.* than that observed in *B. lutescens.*

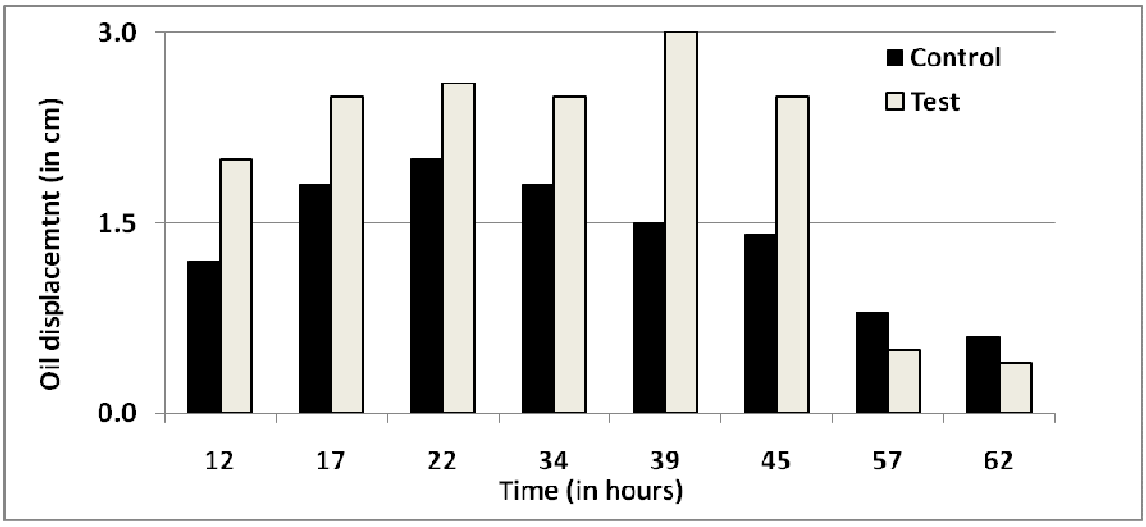

Figure 6
Oil displacement activity of Bacillus sp.

Emulsification Activity

Emulsification activity of *Bacillus sp.* was presented in Fig. 7. From 34th hour, emulsification activity was observed and it showed an ascending trend till the end of the experiment and the emulsification activity was always higher in tests than the control. As observed for *B. lutescens*, a similar trend was seen, but the values were lower than that observed for B. *lutescens.*

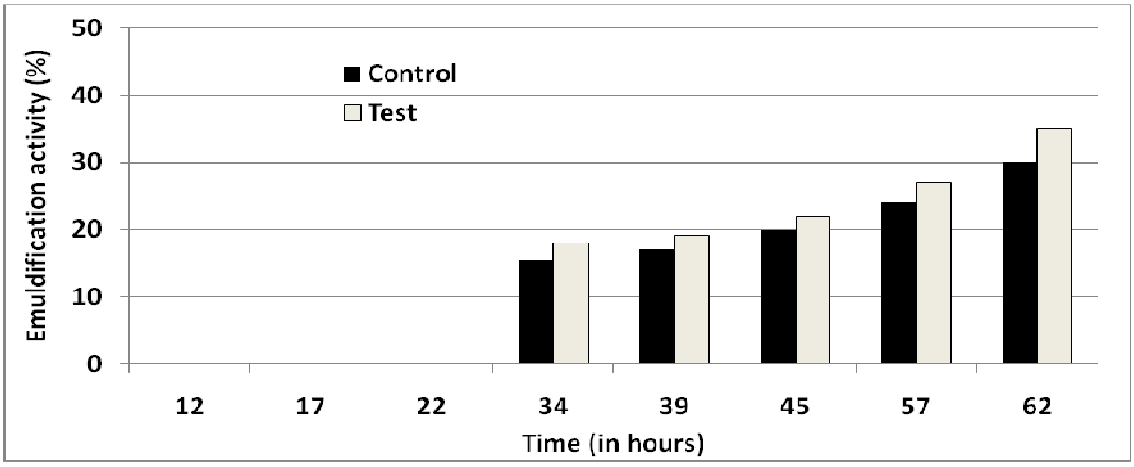

Figure 7
Emulsification activity of Bacillus sp.

Hemolytic Activity

Bacillus sp. showed the α – hemolytic activity having inhibiting zone of 25 mm in length and 15 mm width which was comparable to the other bacterial strain.

Characterization of Biosurfactant

The biosurfactant produced was characterized by using TLC plates. The crude biosurfactant showed positive for carbohydrate and protein components.

DISCUSSION

In this study, *Brevibacterium lutescens* and *Bacillus* sp. that were isolated from soil showed to possess the biosurfactant producing capabilities. The sequenced data were submitted in the genbank.and they found to belong to the genera *Bacillus*. Phylogenetic tree was established between the two bacterial strains and other organisms which had the capability to produce biosurfactant at different environmental conditions. Growth results did not show a distinct difference in the growth pattern of both the organisms in control and test. The possible reason may be that the hydrocarbon concentration supplemented with the medium was not sufficient to bring out marked difference in the growth of both organisms. The quantity of surfactant increase after 24 hours was assumed to be biomass related because of the production of biosurfactants by the bacteria as secondary metabolites as indicated in earlier studies[12]. The emulsification activity showed that the stability of the emulsion directly proportional to the quantity of the biosurfactant present in the medium. The strain JS05 showed β haemolytic activity on blood agar plate. The culture producing beta haemolysis was able to produce more biosurfactants as suggested in the study of Anandaraj and Thivakaran[3] who used blood hemolysis test for screening biosurfactant producing organisms. The two strains JS05 and JS06, inoculated in nutrient broth medium with oil produced biosurfactants maximum at exponential growth phase. The earlier workers[13] used the similar method for the extraction. The biosurfactants extracted was characterized by using TLC.

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