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A glycolipid biosurfactant from a native halophilic *Bacillus* species of Porto Novo, India and its potential microbially enhanced oil recovery properties

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Abstract

A potential biosurfactant producing halophilic bacterium was isolated from the saltpan environment of Porto Novo, Tamilnadu, India. The potential bacterium showed 69.9 % emulsification activity, 56.7 units/ml lipase activity and 22 mm oil displacement in the biosurfactant multiple screening methods. This halophilic bacterium was identified as *Bacillus* species using 16S rRNA molecular sequencing method. This strain showed growth dependent biosurfactant production, which was partially purified and weighed at 0.945 ±0.021 g/L on dry weight basis. The isolated biosurfactant was biochemically identified as glycolipid using biochemical methods and FT-IR functional group analysis. Further, the glycolipid surfactant showed consistent oil recovery efficiency than the tested synthetic surfactant at the temperature up to 50°C and pH ranged from 5 to 8. Based on these observations, the isolated biosurfactant using this halophilic bacterium revealed promising microbially enhanced oil recovery and bioremediation purposes at a wide range of environmental conditions.

Keywords: Multiple screening methods; Halophilic *Bacillus*; Glycolipid biosurfactant; Saltpan environment; Porto Novo; Microbially enhanced oil recovery.

1. Introduction

Chemical surfactants are used over few decades for the bioremediation of hydrocarbon compounds, almost all chemical surfactants being currently produced are derived from petroleum derived compounds (Banat, 1995). However, these compounds are usually toxic themselves and hardly degraded by microorganisms. They are therefore, a potential source of pollution and damage to the environment (Urum and Pekdemir, 2004). With the increasingly strict regulation on the use of environmentally compatible products, the use of biosurfactants in place of chemical surfactants is increasing. Like chemical surfactants, biosurfactants exhibit high surface-active properties, emulsification activities (Li and Chen, 2009) and they are stable under extreme physicochemical conditions (Ruggeri et al., 2009). Due to their good biocompatibility with the cell membrane, they are less toxic to microorganisms than chemicals (Sotirova et al., 2008), therefore, they considered to be good alternatives to chemical counterparts.

Biosurfactants are amphipathic molecules with both hydrophilic and hydrophobic moieties that partition preferentially at the interface between fluid phases that have different degrees of polarity and hydrogen bonding, such as oil and water or air and water

interfaces. This property explains their broad use in many industrial and environmental applications (Singh and Cameotra, 2004). Biosurfactants have different localizations viz., intracellular, cell surface bound and extracellular, and they play divergent physiological roles in the various producing microorganisms (Makkar and Cameotra, 2002). It comprises a wide range of biochemical structures, such as glycolipids, lipopeptides, polysaccharide–protein complexes, phospholipids, fatty acids and neutral lipid (Angelova and Schmauder, 1999). Usually, different microbial genera synthesize structurally different classes of biosurfactants whereas strains belonging to a same species produce structurally similar biosurfactants. However, small differences in the molecular structures can have major impact on the functions and potential industrial applications of biosurfactants (Bodour et al., 2003).

The best-studied biosurfactants are glycolipids, such as rhamnolipids produced by *Pseudomonas* (Benincasa et al., 2004), mannosylerythritol lipids from *Candida antarctica* (Arutchelvi et al., 2008) and a variety of structurally different lipopeptides produced by several *Bacillus* species (Van Hamme et al., 2006). They are also easy to produce cheaper and renewable feedstock. One more striking advantages of biosurfactants over chemically synthesized surface active compounds include their broad range of novel structural characteristics and their physico-chemical properties (Kiran et al., 2009).

Many studies have been carried out with different microbes to explore the biosurfactant production and its activity delivering the industrial and environmental significance of these compounds. However, a very few literatures has been solely reported about the halophilic bacterial isolates for biosurfactant production. In considering the lack of information, the present study undertook an investigation for the isolation and characterization of a potential halophilic biosurfactant producer from a saltpan location of Porto Novo, Cuddalore District, Tamil nadu, India. The collected samples were investigated for the potent biosurfactant producer and further detailed the potential strain with time course on biosurfactant production, biochemical characterization and its oil recovery and

2. Materials and Methods

2.1. Isolation of halophilic biosurfactant producing bacteria from saltpan environment

Sediment samples were collected using sterile spatula from the saltpan environments of Porto Novo, Cuddalore district, Tamilnadu, India. Possible aseptic techniques were applied while sampling to avoid contaminations and collected samples were transferred to pre-sterilized bottle containers which were kept in an icebox maintained at 4˚C while transfer. One gram of sample was serially diluted using 100ppt of sea water prepared using synthetic sea salt (Himedia) and spread plated on 100ppt sea water prepared Bushnell Haas agar (BHA) plates supplemented with 0.1% crude oil, a selective medium for biosurfactant producers which was incubated at 30ºC for 7 days (Atlas, 1946). After incubation period, individual colonies were selected based on the difference in colony morphology. Pure culture of colonies obtained in 100ppt sea water prepared nutrient agar plates and stored nutrient agar slant under 4°C refrigeration conditions.

2.2. Screening of potential halophilic biosurfactant producing bacteria

All the axenic cultures were inoculated into individual Bushnell Haas Broth (BHB) for biosurfactant screening under same salinity conditions. All the fermentations were carried out using 30ml crew cap tubes with 10ml working volume and were incubated in a rotary incubator at 30ºC for 7days. The centrifuged supernatants of the cultured broth were screened for the most potential biosurfactant producer using the following multiple screening methods includes emulsification activity (Cooper and Goldenberg, 1987), lipase activity (Kiran et al., 2009) and oil displacement test (Youssef et al., 2004). Based on the promising results from these screening methods, the potential strain was selected and further studies were carried out using the potential bacterium.

2.3. Molecular identification of the potential bacterium

Molecular identification of the potential strain was referred with 16S rRNA gene sequence analysis using the bacterial universal primer set of Eubac 27F (5′-AGAG TTTG ATCM TGGC TCAG -3′) and 1492R (5′- GGTT ACCT TGTT ACGA CTT -3′). The PCR was done on a thermal cycler (Eppendorf) with 50µl reaction mix. The reaction mix contained 10×

amplification buffer (5µl), 1.5mM $MgCl_2$ (5µl), 1µl of each forward and reverse primer, 1µl dNTP and 0.25µl Taq polymerase. After an initial denaturation at 95°C for 1min, amplification was carried out with 35 cycles of 35s at 94 ◦C, 40s at 55°C, 2 min at 72°C followed by a final extension for 8min at 72°C. The PCR product was purified using the Qiagen PCR purification kit and then sequenced on an ABI Prism 377 automatic sequencer (Applied Biosystems, CA, USA). The 16S rRNA gene sequence obtained from the potential bacterium was compared with other bacterial sequences by using NCBI megaBLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for their pairwise identities. The evolutionary distances were computed using the maximum-composite-likelihood method (Tamura et al., 2004) and the evolutionary analyses was conducted using MEGA7 software (Kumar et al., 2016).

2.5. Time course on biosurfactant production

For time course on biosurfactant production, the optimized medium was monitored for 120 h with an interval of 6 h starting from the lag phase to stationary phase under batch culture conditions during which the production peak time was determined. The time course on production was plotted with reference to biosurfactant production and bacterial growth. The biosurfactant activity of this potential bacterium was estimated using oil displacement test (Youssef et al., 2004) and bacterial growth was determined using hot air over (50°C) dried weight of centrifuged cell biomass at 3000 rpm for 15 min. This experiment were conducted in 250ml Erlenmeyer flask using Bushnell Haas broth as the basal medium supplemented with 1% crude oil under 100ppt salinity. Two percentage of inoculum was used which was prepared with the exponential phase culture of the potential strain using the same production conditions where the optical density (OD 620nm) of the inoculum culture was adjusted to 0.1 as per the procedure of McFarland turbidity 0.5 standard which was equivalents to the bacterial concentration of 1×10^8 CFU/ml

2.6. Extraction of biosurfactant

After fermentation and centrifugation of cultured broth, the cell free supernatant was subjected to the acid precipitation method (Nitschke and Pastore, 2006) for recovering the crude biosurfactant and the acidified biosurfactant was neutralized using phosphate buffer (pH 7) solution. The resultant biosurfactant was again extracted with chloroform/methanol mixture added in the ratio of 2:1 following the method of Kuyukina et al., (2001). The extraction was preceded kept for a period of whole night; the organic phase was separated and was evaporated using rotary evaporator. After evaporation of the solvents, the dry weight of the extracted product was estimated and assayed for recovery of biosurfactant using oil displacement test (Youssef et al., 2004).

2.7. Biochemical characterization and functional group analysis through FT-IR

The partially purified biosurfactant was biochemically determined its total content of protein, carbohydrate and lipid using Lowry's method (Lowry et al., 1951), phenol sulphuric acid method (Dubois et al., 1956) and total free fatty acids (Folch et al., 1956) respectively. FT-IR spectroscopy can be used to elucidate the chemical structures of some components in an unknown mixture by identifying the types of chemical bonds or the functional groups present in their chemical structures (Rodrigues et al., 2006). One milligram of freeze-dried partially purified biosurfactant was ground with 100 mg of KBr. Infrared absorption spectra were recorded on a Thermo Niocolet, AVATAR 330 FTIR system with a spectral resolution and wave number accuracy of 4 and 0.01 cm^{-1}, respectively. All measurements consisted of 500 scans, and a KBr pellet was used as background reference.

2.8. Microbially enhanced oil recovery (MEOR) assay

The potential application of this partially purified biosurfactant in microbially enhanced oil recovery (MEOR) was evaluated using the modified 'sand pack column' technique described by Suthar et al. (2008). A vertical glass column of 20 mm×25 mm×85 mm dimensions with a sieve (100 µm pore size) and cap fixed at the bottom was packed with 150 g sand of 140 µm particle size. The column was then saturated with heavy duty gear oil (density 0.918 kg/l) (50 ml). The potential of the isolated surfactant for oil recovery was estimated by pouring 50 ml of aqueous solution of biosurfactant in the column. The amount of oil released was measured. The experiment was carried out at different temperature (20-80°C) and pH (4-9) conditions to assess the influence of temperature on biosurfactant-induced oil recovery and all the parameters were compared with sodium lauryl sulphate, a notable synthetic surfactant with board industrial applications. Further, control without added any surfactant was simultaneously done on all the different environmental parameters.

2.9 Statistical analysis

All the experiments were carried out in triplicate times and the values were expressed as mean ± standard deviation (S.D).

3. Results

3.1. Isolation and screening of most potential biosurfactant producing halophilic bacterium

Halophilic bacteria were isolated from the sediment samples of saltpan environments from Porto Novo, Cuddalore District, Tamil Nadu, India. After incubation period, the Bushnell Haas agar plates were visualized for distinct morphological colonies which were isolated and pure cultured on nutrient agar plates. Totally, 57 axenic halophilic strains were isolated during the collection. For convenience, the isolated axenic strains were named by SP which represents "Saltpan" followed by first authors initials MS followed by the strains number specified by Arabic numerals (eg., SPMS1 to SPMS57). These isolates were screened for the most potential biosurfactant producer using multiple screening tests. Only 7 to 21% of the isolates showed potential activity in the individual screening examinations viz., 11% of strains showed emulsification activity followed by 17% and 21% of halophilic strains showed potential activity in lipase activity and oil displacement test. Among these isolates, only seven strains showed considerable activities on the multiple screening tests and only one bacterium SPMS3 showed appreciable activity in the entire screening examinations than the rest of all isolates which showed 69.9 % emulsification activity, 56.7 units/ml lipase activity and 22 mm oil displacement (Table 1). This strain was chosen for the further characterization studies.

Table 1: Screening results of potential biosurfactant bacteria isolated from saltpan environment, Porto Novo. Bold letters in the resulted values indicates the most potential strain of this study.

S. No	Strain name	Emulsification activity (%)	Lipase activity (Units/ml)	Oil displacement test (cm)
1.	**SPMS3**	**69.9**	**56.7**	**2.2**
2.	SPMS11	51.3	38.9	1..2
3.	SPMS15	41.3	40.1	2.0
4.	SPMS28	40.1	38.1	1.6
5.	SPMS33	42.1	45.2	1.9
6.	SPMS41	51.8	47.1	1.7
7.	SPMS57	49.9	41.8	1.1

3.2. Molecular identification of the potential halophilic bacterium

The identification of the potential strain was referred using 16S rRNA molecular sequence method. The phylogenetic position of the strain was examined by BLAST analysis and the results revealed that the strain belongs to the genus *Bacillus* of the family Bacillaceae. Based on the sequence homology, the strain SPMS3 showed 100% similarity with *Bacillus* sp. WR-12 (KU159251.1). Following these comparisons, the strain SPMS3 was identified as *Bacillus* sp. and the 16S rRNA sequence was deposited in GenBank with the accession number KX827605.1. Phylogenetic tree of *Bacillus* sp. SPMS3 was plotted with their closest NCBI strains using the Neighbor joining tree based on the Maximum Composite Likelihood model using MEGA7 (Fig. 1).

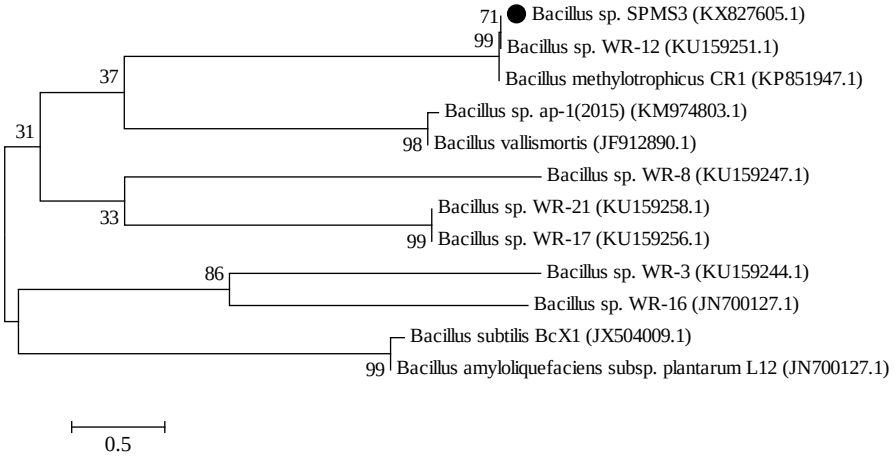

Fig. 1: Phylogenetic analysis was performed for the potential strain *Bacillus* sp. SPMS3 with their closest NCBI (BLASTn) strains based on the 16S rRNA gene sequences. This analysis was done using the Neighbor joining tree based on the Maximum Composite Likelihood model with MEGA 7.0. Numbers at nodes indicate levels of bootstrap values (expressed as percentages of 1000 replications) and the black dot indicates the potential strain of this study.

3.3. Time course on biosurfactant production

The time course on biosurfactant production predicted that the biosurfactant secretion was evidenced at the early logarithmic phase of bacterial growth. Further, the bacterium showed growth associated biosurfactant production which revealed its peak biosurfactant production (2.2 cm oil displacement) during the initiation of the stationary growth phase of the bacterium (84^{th} hr) with 8.13 g/L cell biomass concentration and it was maintained up to 120^{th} hr on prediction (Fig. 2). The production of biosurfactant was examined in function of time with its biomass concentration revealed that it is a primary metabolite.

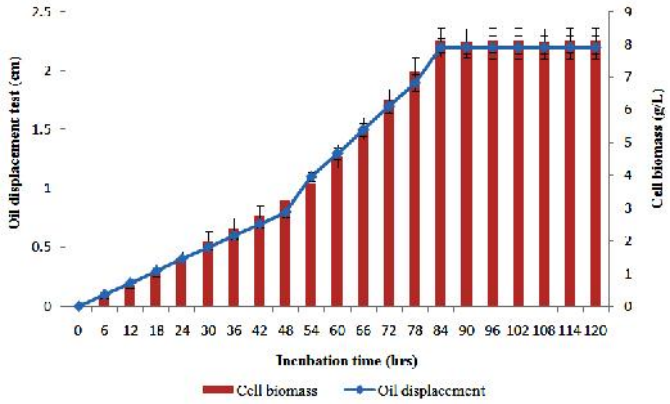

Fig. 2: Time course on biosurfactant production using the halophilic *Bacillus* sp. SPMS3

3.4. Biochemical characterization of partially purified biosurfactant

The biosurfactant was partially purified using acid precipitation and methanol and chloroform solvent extraction method which was weighed at 0.945 ±0.021 g/L on dry weight basis. Further, the isolated biosurfactant was qualitative estimated for its biochemical composition using biochemical estimating methods which determined its total carbohydrate content and fatty acids which was found to be 35% and 65%. The results clearly stated that the major portion of the biosurfactant was fatty acid and carbohydrate content and protein was found to be absent which also proved that it could be a glycolipid molecule in nature. The isolated biosurfactant was further analyzed for its functional groups using FT-IR spectrum (Fig. 3). The characteristic functional groups of aliphatic long fatty acid chain which was evidenced from the important vibrations of 1377 cm^{-1}, 1458cm^{-1}, 2854 cm^{-1}, 2870 cm^{-1}, 2924 cm^{-1} and 2953 cm^{-1}. Further, the intense stretch vibration band located at 3401 cm^{-1} predicted the characteristics hydroxyl groups in the biosurfactant structure.

Moreover, the most important bands located at the 1105 cm^{-1}, 1628 cm^{-1}, 1652 cm^{-1} and 1733 cm^{-1} vibrations indicated the presence of alkene (C=C) and carbonyl group (C=O) in the monosaccharide structures of carbohydrates. From the above results, the presence of long aliphatic fatty acid chain and monosaccharide functional groups represented that it is a glycolipid surfactant.

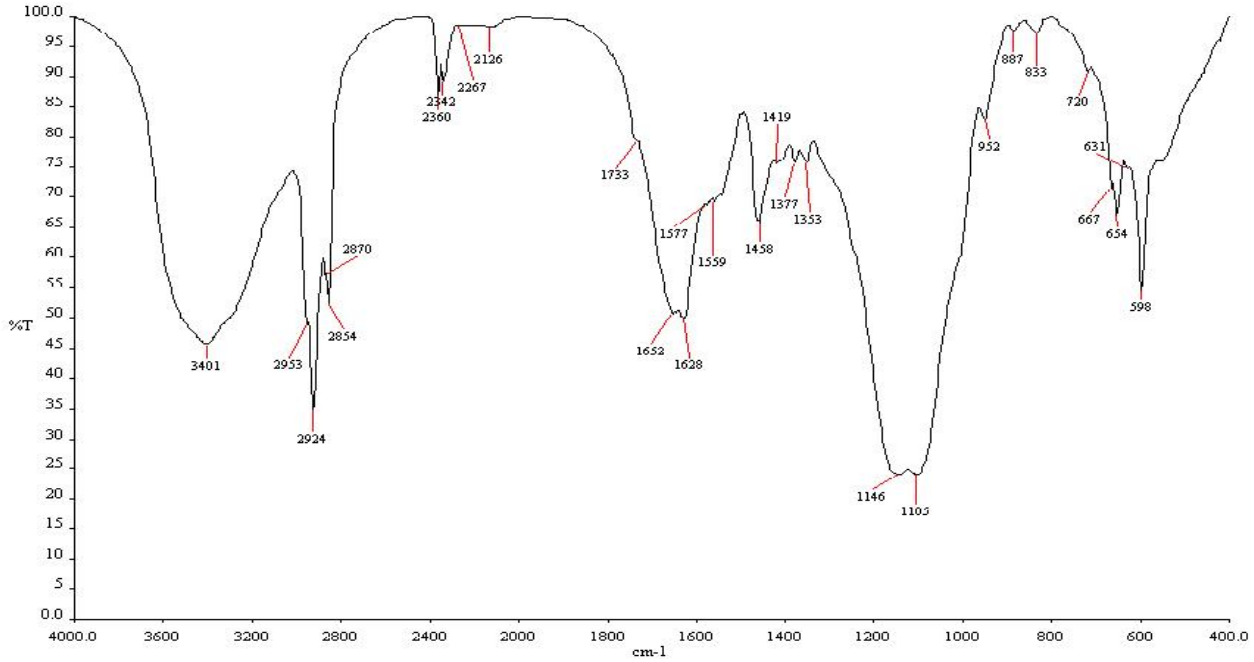

Fig. 3: FT-IR spectrum of the isolated glycolipid surfactant from halophilic *Bacillus* sp. SPMS3

3.5. MEOR assay

In the present study, the isolated biosurfactant was examined for enhanced gear oil recovery rate using MEOR assay. The biosurfactant revealed a consistent and appreciable oil recovery rate on all the different parameters tested than the synthetic surfactant, SLS used for comparison in this study. In case of biosurfactant, the peak recovery of gear oil was achieved between the temperature conditions of 30 - 50°C within the range of 80.5 -74.5% recovery rate, but in case of synthetic surfactant it was 59.5 - 54.2% and when compared to all the temperature conditions, the extracted biosurfactant showed the most appreciable results (Fig. 4). The control without any added surfactant recovered only 6.5 - 7.3% of gear oil. Regarding pH conditions, the maximum recovery was observed within the pH conditions of 5 - 8 with 76.4 – 76.3% in case of biosurfactant and in case of synthetic surfactant, recovered only 40.7 – 52.3% (Fig. 5). The control recovered only 6 - 6.5% of gear oil in different pH conditions. This comparative examination clearly depicted the consistent gear oil recovering efficiency of this biosurfactant even at different physic-chemical conditions than the synthetic surfactant tested in this study. From these studies, the isolated biosurfactant proved that it could be an ideal product for the promising oil recovery and for bioremediation purposes.

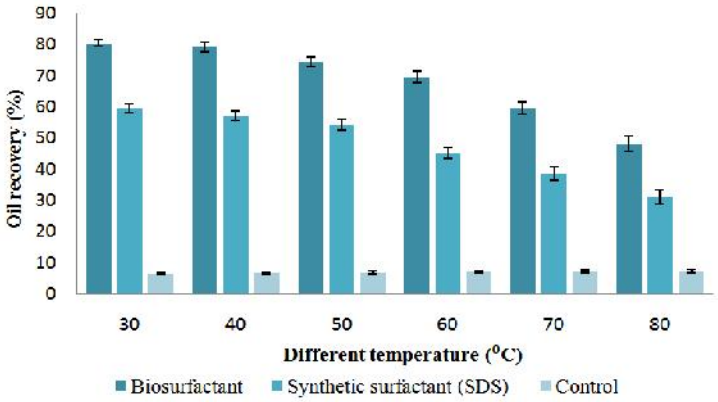

Fig.4: Stability of glycolipid biosurfactant from halophilic *Bacillus* sp. SPMS3 to different temperature conditions

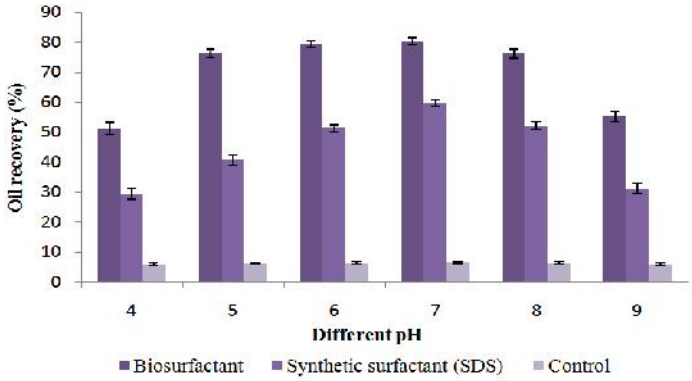

Fig.5: Stability of glycolipid biosurfactant from halophilic *Bacillus* sp. SPMS3 to various pH conditions

4. Discussion

In the present study, saltpan environment was concentrated for the isolation of biosurfactant producing halophilic bacteria which was less explored till date. This study utilized three different biosurfactant screening methods, based on the potential activities on the entire screening examination, the strain *Bacillus* sp. SPMS3 was chosen for further characterization studies. Many researchers have been previously reported about the significance of multiple screening tests for the isolation of most potential biosurfactant producers and chosen methods were standard techniques for the effective isolation of biosurfactant producer (Mani et al., 2016a; Khopade et al. 2012; Kiran et al. 2009). Similarly, Walter *et al.*, 2010 described that the combination of different methods is appropriate for a successful screening of biosurfactant producing bacteria.

Cell growth and production of metabolites plays a significant role in standardizing biosurfactant production (Sen, 1997). The study demonstrating that the accumulated biosurfactant was growth associated, because a good correlation was observed between biosurfactant production and bacterial cell growth which showed maximum biosurfactant production between 84hrs – 120hrs during the stationary growth phase of the bacterium. A study has agreed with this present observation that it has no significant difference in the yield of biosurfactant was noticed between 48 h and 96 h of bacterial stationery growth phase. It was suggesting that the produced lipopeptide biosurfactant by *B. subtilis* DM-03 and DM-04 strains are growth dependent production (Das and Mukherjee, 2007).

In the present study, the biochemical nature of the partially purified biosurfactant was identified as glycolipd using biochemical methods and FT-IR spectroscopy. The presence of characteristic adsorption bands at 1377 cm^{-1}, 1458cm^{-1}, 2854 cm^{-1}, 2870 cm^{-1}, 2924 cm^{-1} and 2953 demonstrated the presence of aliphatic long hydrocarbon chain which was endorsed by Rahman et al. (2010). The strong absorbance at 3401 cm^{-1} was considered as the characteristic peak of glycolipid biosurfactants (Li et al., 2002) which was assigned to the C–H stretching vibrations of the hydrocarbon chain positions. Peaks observed in the regions, 1628 cm^{-1}, 1652 cm^{-1} and 1733 cm^{-1} indicated the presence of monosaccharides (Pornsunthorntawee et al., 2008). Both the results concluded that it was a glycolipid surfactant in nature. Till date, most of the studied biosurfactant from *Bacillus* sp. showed lipopeptide surfactant but this study revealed a different biochemical nature belongs to the same species. It shows the biomolecular uniqueness of the bacterium isolated from the saltpan environment.

Petroleum hydrocarbon compounds bind to soil components and are difficult to remove and degrade. Biosurfactants can emulsify hydrocarbons enhancing their water solubility, decreasing surface tension and increasing the displacement of oil substances from soil particles (Banat, 2000). Mostly, biosurfactants are preferred nowadays for oil recovery because of their activity and ecological safety perspective than synthetic counterpart (Desai and Banat, 1997). In this study, the isolated biosurfactant showed appreciable results on all the temperature and pH conditions applied and comparison with the synthetic surfactant proved its efficiency than it. Similar to this investigation, a recent study on lipopeptide surfactant from a marine *Bacillus simplex* SBN 19 showed efficient crude oil recovery even at 30% salinity (Mani et al., 2016b).

The study revealed a different biochemical nature of biosurfactant from the native strains of *Bacillus* sp. isolated from a saltpan environment. Further, the results of MEOR assay hold as the baseline data for the promising application of this isolated biosurfactant in petroleum industries for oil recovery and cleaning of oil contaminants, reducing oil viscosity during crude oil transportation as well as for bioremediation, moreover, this biosurfactant can act effectively even at wide range of environmental condition.

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