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Research Article

Production and characterization of biosurfactant by *Bacillus* and its applicability in enhanced oil recovery

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Abstract

In this study, biosurfactant producing bacteria were isolated from soil samples collected from oil spills around oil refineries. Isolates were screened for biosurfactant production using Cetyl Tri Ammonium Bromide (CTAB)-Methylene blue agar selection medium, oil spreading technique, blood haemolysis, emulsification capacity and rapid drop collapse activity. Biochemical characteristics of the isolate, B7, identified the bacterium as *Bacillus* sp. Different carbon and nitrogen sources were evaluated for their effect on biosurfactant production. The biosurfactant showed better emulsifying activity on glycerol among the carbon sources and on ammonium chloride among nitrogen sources. The emulsification activity was quite stable at temperature 100^0 C, pH 12 and 10% NaCl. The extracted biosurfactant was characterized by thin layer chromatography. Structural determination was carried out by FT-IR spectroscopy and GC-MS, revealed the chemical structure of the crude biosurfactant as lipopeptide. The application of the biosurfactant in oil recovery showed that, biosurfactant have better oil recovery efficiency, thus being more attractive to be applied in Microbial Enhanced Oil Recovery.

Keywords: *Bacillus*, biosurfactant, emulsification, MEOR

Introduction

Surfactants are indispensable components of daily life. They are amphiphilic compounds that reduce the free energy of the system by utilizing the bulk molecules of higher energy at an interface. Some surfactants, known as biosurfactants, are biologically produced by yeast or bacteria from various substrates including sugars, oils, alkanes and wastes Lin (1996). Biosurfactants attracted attention as hydrocarbon dissolution agents in the 1960's and their applications have been greatly extended in the past five decades as an improved alternative to chemical surfactants because of their biodegradability, low toxicity, ecological acceptability and ability to be produced from renewable and cheaper substrates Makkar and Cameotra (1999). Biosurfactants are grouped as glycolipids, lipopeptides, phospholipids fatty acids, and neutral lipids, polymeric and particulate compounds Bierman *et al.* (1987). Most biosurfactants derived from diverse microbial sources are either anionic or neutral and hydrophobic moiety is based on long chain fatty acid or derivatives, whereas the hydrophilic portion can be a carbohydrate, aminoacid, phosphate or cyclic peptide Nitschke and Coast (2007). Lipopeptides represent a class of microbial surfactants, which has attained increasing scientific, therapeutic and biotechnological interest Cameotra and Makkar (2004). In addition to surfactin produced by *Bacillus subtilis*, other lipopeptide biosurfactants include lichenysin A , produced by *Bacillus licheniformis* BAS-50, Yakimov *et al*. (1995) and the surfactant BL-86 produced by *B. licheniformis* Horowitz *et al*. (1990). Biosurfactant producing microorganisms

belong to different genera including: *Arthrobacter* spp., *Bacillus* spp., *Candida* spp., *Clostridium* spp., *Corynebacterium* spp., *Nocardia* spp., *Pseudomonas* spp., *Rhodococcus* spp. and more other genera have been reviewed Nasr (2009), Christofi and Ivshina (2002). Among the many classes of biosurfactants, lipopeptides from *Bacillus subtilis* are particularly interesting because of their high surface activity and therapeutical potential Besson and Michel (1992), Sandrin *et al*. (1990).

All the surface-active lipopeptides consist of several amino acids covalently bound with the carboxy and hydroxy groups of β-hydroxy fatty acids. They vary in amino acid composition, position of the lactone ring and lipid portion. This wide range of structural diversity results in broad spectrum of potential industrial applications including production of food, cosmetics, pharmaceuticals, agriculture, mining, enhanced oil recovery, transportation of crude oil, cleaning oil storage tanks and pipelines and soil remediation Gautam and Tyagi (2006), Shoeb *et al*.(2013).

Several biosurfactant are produced by a diversity of microorganisms in order to survive in an oil-rich environment, and this adaptation process selected for surfactants with highly adaptable physical and chemical properties. Biosurfactants are therefore, very suitable for applications in the oil industry and this is reflected in the market, where the large majority of biosurfactants produced are in petroleum-related applications Bognola (1999). The applications are, in general, in oil recovery, oil spill management, microbial enhanced oil recovery (MEOR) and as oil dispersants and emulsifiers Singh *et al*. (2008). MEOR processes employ the use of microbial metabolites, such as biosurfactants, to lower interfacial tension Li (2002). Several biosurfactants, in particular the lipopeptides made by *Bacillus* species, generate the low interfacial tensions between the hydrocarbon and the aqueous phases required to mobilize residual hydrocarbon Lin *et al.* (1994). MEOR has several advantages compared to other enhanced oil recovery processes in that it does not consume large amounts of energy, as do thermal processes, nor does it depend on the price of crude oil, as do many chemical processes McInerney (2002), Banat (1995). The present study was focused on screening of biosurfactant production from *Bacillus* species isolated from oil spills, to optimize the nutrient parameters, to characterize the biosurfactant produced and to determine the efficacy of biosurfactant towards oil recovery.

Materials and Methods

Sample collection

Soil samples were collected in sterile polythene bags from oil refineries in and around Chennai. The sample was brought to the laboratory and processed for the isolation of biosurfactant producing bacteria.

Isolation of Biosurfactant producing bacteria

Serial dilution of the soil samples were carried out using Ringer's solution and inoculated on nutrient agar plates and incubated for 24 h at 37^0 C. Morphologically distinct colonies were identified and purified by transferring to fresh agar plates thrice to obtain pure cultures. The chosen isolates were further screened for the production of biosurfactants using multiple screening methods.

Screening for biosurfactant production

The potential biosurfactant producer was initially screened by growing the isolates on Cetyl Tri Ammonium Bromide (CTAB)-Methylene blue agar medium. The colonies showing halo on the selective CTAB-Methylene blue agar medium were further screened for haemolytic activity, drop collapse method, oil spreading test and emulsification index.

Identification of the isolated potential strain

Isolated potential strain was subjected to identification on the basis of colony morphology, Gram's staining and motility. Further identification by biochemical test was done based on the standard methods of Bergey's Manual of Systematic Bacteriology. The isolated bacterial culture was maintained on nutrient agar medium and stored at 4^0C.

Inoculum and Culture conditions

The isolated potential strain was streaked in a nutrient agar slant and incubated for 24 h at 30^0C. Two loop full of culture was inoculated in 20 mL of nutrient broth in a 50 mL of Erlenmeyer flask and incubated in a rotary shaker at 120 rpm at 30^0 C for 8- 12 h until cell numbers reach 10^8 CFU/ mL. An aliquot of 5 mL

of inoculum was transferred to 250 ml Erlenmeyer flasks containing 100 mL of Mineral salt medium (g/L): Potassium dihydrogen phosphate 0.7; Sodium hydrogen phosphate 0.9; Sodium nitrite 2.0; Magnesium sulfate hepahydrate 0.4; Calcium chloride 0.1; Ferrous sulphate hepahydrate 2.0; Manganese sulphate hepahydrate 1.5; at pH 7.0. The flasks were incubated at 30^0C on a shaker incubator at 120 rpm for 7 days.

Extraction of Biosurfactant

To isolate the biosurfactant, the bacterial cells were removed from surfactant containing culture broth by centrifugation at 10,000 rpm for 10 min. The cell free supernatant was subjected to acid precipitation by adjusting the pH to 2.0 using 6N HCl and left overnight at 4^0 C. The precipitate thus obtained was pelleted by centrifugation at 8000 rpm for 20 minutes, dried and weighed. For further purification, the crude surfactant was dissolved in distilled water at pH 7.0 and dried at 60^0 C. The dry product was extracted with Chloroform: Methanol (65:15), filtered and the solvent, evaporated. The product thus obtained was used for further analysis.

Optimization of Nutrient parameters

Optimization of biosurfactant production was done by utilizing different carbon and nitrogen sources. The production medium was supplemented with glucose, molasses, olive oil and mannitol as carbon sources (1%). The nitrogen source (1%) in the production medium was replaced by sodium nitrite, ammonium nitrite, magnesium sulphate and ammonium chloride for optimization and surfactant production was estimated.

Assay of Emulsification Activity

Three ml of hydrocarbon (crude oil) was added to 2.0 ml of the biosurfactant solution in a graduated test tube and vortexed at high speed for 2 min, and distilled water was used as control. The emulsion stability was determined after 24 h, and the emulsification index (E_{24}) was calculated by dividing the measured height of emulsion layer by the mixture's total height and multiplying by 100.

Stability Studies

pH Stability

Stability studies were carried out using 0.1% (w/v) biosurfactant solution in 0.1M-phosphate buffer, pH 7.0. The effect of pH on the biosurfactant activity was performed by introducing the biosurfactant solution into test tubes and the pH adjusted to various values (2 - 12) using HCl and NaOH solution and kept at room temperature.

Heat Stability

The heat stability study of the biosurfactant was carried out by incubating the biosurfactant solution in water bath at different temperatures (30-100^0 C) for 30 min after which they were cooled to room temperature.

Effect of sodium chloride

The effect of sodium chloride concentration on biosurfactant activity was determined by adding different concentrations (0 - 25%, w/v) of NaCl to the biosurfactant solution and allowed to stand for 30 min. The emulsification indexes of each treatment were determined at the end of each period.

Chemical characterization of biosurfactant

Thin layer chromatography

The components of the extract were separated on silica gel plates (Silicagel 60-F-254) using solvent system Chloroform : Methanol : water (65:15:1). The components were detected by spraying the plate with distilled water and heating at 110^0C for 5 min.

Fourier transform infrared spectroscopy

The purified product was subjected to Fourier Transform Infrared Spectroscopy (FTIR) in JASCO FT/IR 4100 series instrument, recorded from 4000 to 400 per cm; for the determination of functional groups present in the purified product in the Department of Sophisticated Analytical Instrument Facility (SAIF), Indian Institute of Technology (IIT), Chennai.

Gas chromatography – Mass spectroscopy

Gas chromatography – Mass spectroscopy analysis was done in Hewlett Pac gas chromatograph (GC) JEOL GC mate II model equipped with a capillary inlet and set to scan from *m/z* 50 to *m/z* 600 at a scan rate of 1.2 scans per second at the Department of Sophisticated Analytical Instrument Facility (SAIF), Indian Institute of Technology (IIT), Chennai.

Soil Column Study

Glass columns (40.0 x 2.0 cm), packed with 80.0 g of sandy loam soil, and were saturated with 25 ml of engine oil and 22 ml of kerosene. The efficiency of the biosurfactant solution in releasing the oil from the soil was tested by adding 100 ml of aqueous solution of 0.5% of the purified biosurfactant solution to the column. Distilled water was used as control. Efficiency of oil recovery was estimated by measuring the volume of engine oil and kerosene oil released.

Results and Discussion

Biosurfactants are amphiphilic molecules with great diversity, environmental acceptability and broad spectrum of functions and industrial applications which make them interesting bio-products.

Isolation and screening of biosurfactant producing organism

Biosurfactant producing bacteria was isolated from soil sample collected from oil refineries around Chennai. Eleven different bacteria were isolated. Based on the Biosurfactant screening tests, four isolates showed halos around the colonies on CTAB Methylene blue agar medium. Among these, one of the bacterial isolates, designated as B7, was grouped as potential biosurfactant producer, since it showed rapid drop collapsing reaction, β haemolytic activity and higher zone formation in oil spreading test and excellent emulsification activity. In the present study, we report the biosurfactant production potential of the isolate B7.

Identification of the bacterium

Morphological and biochemical identification tests were performed following directions in Bergey's Manual of Systematic Bacteriology. Based on the morphological and biochemical characteristics, the bacterial isolate B7 was identified as *Bacillus* sp. The strain *Bacillus* sp. was motile, Gram-positive spore-forming and rod-shaped. Colonies of *Bacillus* sp.formed filamentous margins that appeared cream-white in colour.

Production and Optimization of biosurfactant

Effect of carbon source on biosurfactant production

Carbon is the major component of the cell and the rate at which a carbon source is metabolized can often influence the production of metabolites. The quality and quantity of biosurfactant production are affected and influenced by the nature of the carbon substrate Raza *et al*. (2007). The production of biosurfactant was studied by supplementing with carbon sources (1%) such as glucose, molasses, olive oil, glycerol and mannitol .The maximum biosurfactant production was occurring only with glycerol, mannitol, and olive oil. The emulsifying activity was 55% with glycerol. The use of glycerol as carbon source was found to be optimum for biosurfactant production Zhang *et al*. (2005). According to Sandrin *et al*. (1990) glucose, fructose and sucrose were the best carbon substrates for the synthesis of surfactin.

Effect of nitrogen source on biosurfactant production

The effect of nitrogen source influence the biosurfactant production. The maximum emulsifying activity reached in media supplemented with ammonium chloride (52%) and sodium nitrite (50%). A similar result was reported in biosurfactant isolated from *Pseudomonas aeruginosa* by Hamzah *et al*. (2013), Aparna *et al*. (2012). These results are also in agreement report of Fagade *et al*. (2009), in which ammonium salt was observed as a preferable nitrogen source for the biosurfactant production by *Arthrobacter paraffinus*.

Stability study

The applicability of biosurfactants in several fields depends on their stability at different temperatures and pH values.

Temperature Stability

The stability of biosurfactant was tested over a wide range of temperature. The biosurfactant produced by *Bacillus* species in the present study was shown to be thermostable. Heating of the biosurfactant to 100^0 C caused no significant effect on the biosurfactant performance. The emulsification activity was quite stable at temperature 100^0 C (Fig-1). Biosurfactant produced by *Bacillus methylotrophicus* is thermostable and saved its activity even when exposed to high temperature (120^0 C for 15 min) Moussa and Abdel Azeiz (2013). Biosurfactant exhibited high thermal stability since only small variations in the surface activity could be observed with *Bacillus subtilis* Vaz *et al*. (2012).

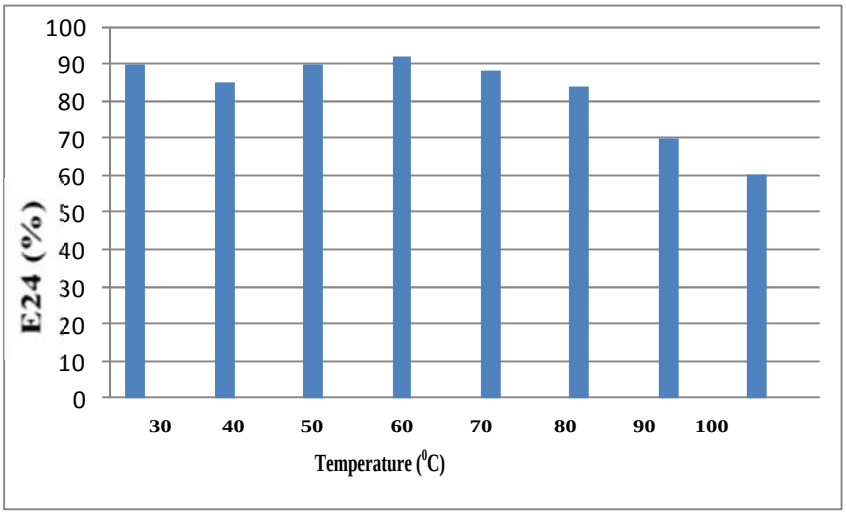

Fig-1. Stability of biosurfactant on temperature

pH Stability

The emulsification activity of the crude biosurfactant at pH 12 showed almost 50% activity, whereas below pH 8 the activity decreased up 34% showing higher stability at alkaline than acidic conditions. The biosurfactant showed tolerance of up to 12% sodium chloride (Fig-2). This result indicates pH increase has a positive effect on emulsification activity. A study conducted by Khopade *et al*. (2012) on biosurfactant production from *Nocardiopsis* showed 66% emulsification at pH 12. Abdel-Mawgoud *et al*. (2008) reported surfactin was found to be soluble in aqueous solutions at pH values higher than 5.0.

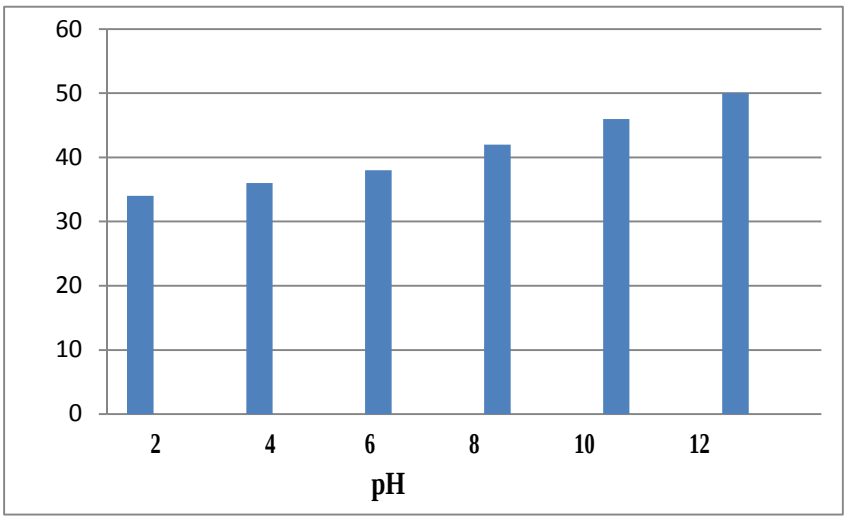

Fig-2. Stability of biosurfactant on pH

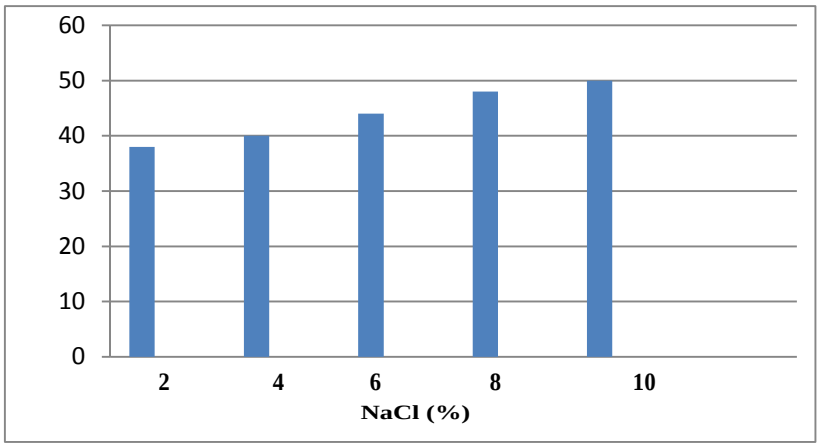

Fig-3. Stability of biosurfactant on temperature

Salinity Stability

The effect of sodium chloride addition on biosurfactant produced from *Bacillus* species was studied (Fig-5). At higher concentration of sodium chloride the biosurfactant retained 50% of the emulsification activity. Stability of emulsification in the presence of salt has been reported as one of the properties of the biosurfactant produced by *Bacillus licheniformis* strain JF-2 Ilori *et al.* (2005). The emulsification index (E_{24}) decreased to 64% at 20% saline level Venkatesan and Ramachandra Murty, (2012). The literature study in the past also confirms that many biosurfactants have stable surface activity even at high levels of salinity Wang *et al*. (2011), Al-Bahry *et al*. (2011).

Chemical Characterization

Preliminary characterization using thin layer chromatography revealed red colour spots on silica gel plate. Similar results have been observed by Priya and Usharani (2009) during lipopeptide biosurfactant production by *Bacillus subtilis.*

Fourier-Transform Spectroscopy

It can be clearly observed characteristic absorbance band of peptides at 3411 cm^{-1}, 1652 cm^{-1} and 1545 cm^{-1}. These bands resulted from the stretching mode of N-H, stretching mode of the C=O, and the deformation mode of the N–H bond combined with C–N stretching mode. In addition, the bands 2924 cm^{-1}, and 1243 cm^{-1} reflect aliphatic chains (-CH3, -CH2) of the sample. These results indicate that the product contains peptide-like moiety as well as aliphatic hydrocarbons. The results obtained are comparable with the reports of several authors Dehghan-Noudeh *et al*. (2005), Nalini *et al*. (2013), Pereira *et al*. (2013). The exposure of a large number of carboxylic groups on the surface due to β-sheet organization may contribute to the special behaviours of biosurfactant such as the ease of surface β-sheet micelles and the ease of surface adsorption.

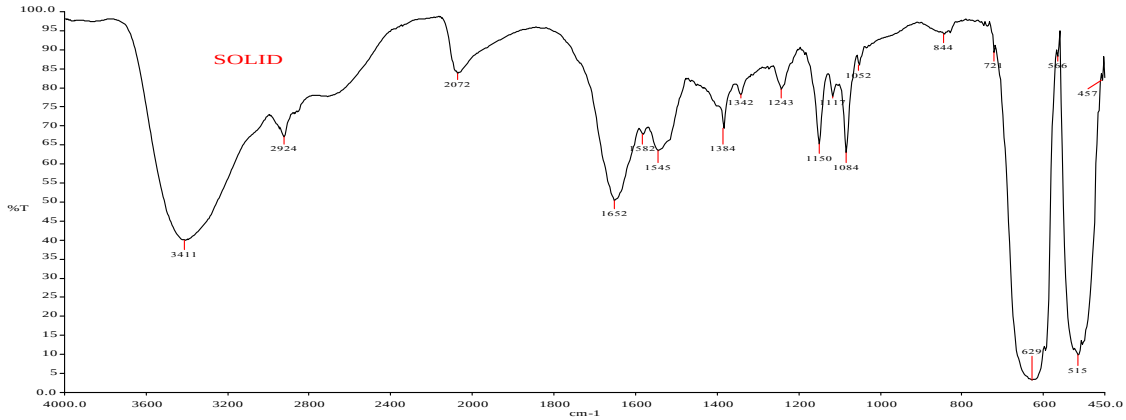

Fig. 4. FT-IR of biosurfactant produced by *Bacillus* sp.

Gas Chromatography

The GC-MS analysis showed that the compound produced by *Bacillus* species was a lipopeptide derivative. The hydrophobic moiety envisaged to be an octadecanoic acid methyl ester. The constituents present in the extract were phenol, 2,4-bis (1-1 dimethyl) (RT 12.51), 1,2-Benzenedicarboxylic acid and butyl 2-ethylhexyl ester (RT 16.6), Hexadecanoic acid, methyl ester (RT 17.13), 9- Octadecenoic acid (Z)-methyl ester (RT 18.84) and Trioxoclane-2-octanoic acid 5 octyl, methyl ester (RT 19.09) . The result showed that the biosurfactant activity of the major chemical constituent of chloroform: methanol extracts of *Bacillus* sp., was identified due to octadecanoic acid. The result is in accordance with Seghal Kiran *et al*. (2010), Moussa and Abdel Azeiz (2013).There is considerable structural diversity in the lipopeptides produced by *Bacillus* species, as a consequence of differences in the nature of the fatty acid component, for example in chain-length (C6-C18) and often the presence of hydroxyl groups and/or *iso*- or *anteiso*-methyl branches, as well as in the type, number and configuration of the amino acids in the peptide chain Christie (2014).

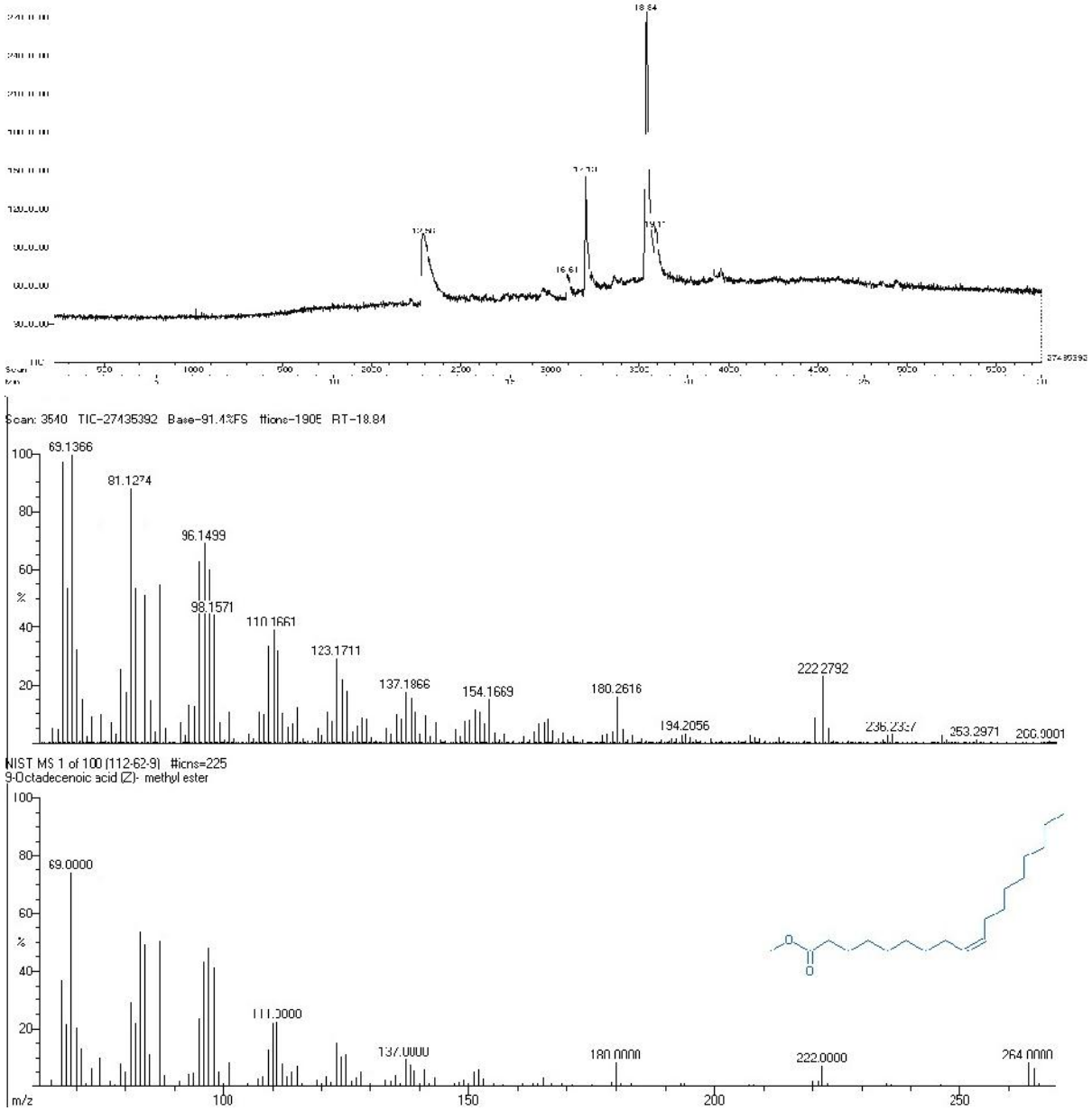

Fig.5. GC-MS of biosurfactant produced by *Bacillus* sp.

Soil column study

In soil column study the competence of the crude extracts in oil recovery was observed. biosurfactant produced by *Bacillus* species accounted for 58% and 56% of petrol and kerosene respectively. The factors responsible for decreasing the oil viscosity and making its recovery easier is due to gas and acid production, oil viscosity, plugging by biomass accumulation, reduction in interfacial tension by biosurfactant and degradation by large organic molecules on both environment and oil (Jack ,1988).The oil recovery efficiency reported by Makkar and Cameotra, 1997 ranged between 56 % and 62% from biosurfactant produced by two strains, *B.subtilis* MTCC 1427 and *B.subtilis* MTCC 2423 respectively. The potential of *Serratia marcescens* NSk-1 biosurfactant removed 60% and 51% of engine oil and kerosene respectively Anyanwu *et al*. (2011). The efficiency of biosurfactant from *B.subtilis* PT2 and *Pseudomonas aeruginosa* SP4 in removing oil was investigated in which they exhibited 68 % and 57% respectively Jain *et al*. (2012). Several authors Simpson *et al*. (2011), Varadavenkatesan and Murty (2013), Gudina (2013) have evaluated and reported the efficiency of biosurfactant produced from *Bacillus* species in oil recovery.

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