Original Article

DEGRADATION OF TWO HYDROCARBONS BY A MARINE *STREPTOCOCCUS* SP. ISOLATED FROM GULF OF MANNAR, SOUTHEAST COAST OF INDIA

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ABSTRACT:

Aim of the study: *Gulf of Mannar and Palk Bay regions of the Southeastern coast of India have been reported to experience hydrocarbon related pollution. The present study was thus carried out to screen hydrocarbon degrading bacteria from these locations with a notion that these polluted areas may harbor some oil-utilizing bacteria.*

Methodology: *A total of 123 bacteria were isolated and those which were screened as lipase producers were selected for biosurfactant production. The lipase [+] isolates were tested for oil spread, tilted glass slide and modified drop collapse assays in determining the efficacy of biosurfactants. The isolate/s which has least MEC for these assays was chosen for tributyrin degradation and analyzed on GC-MS. The potent isolate was identified up to the genus level using standard tests.*

Results: *Out of all the bacteria, isolate K from Gulf of Mannar that produced biosurfactant had least MEC of 150, 500 and 250 µg/ mL for oil spread, tilted glass slide and modified drop collapse assays respectively. It was observed that tributyrin was degraded to more than 96% by the bacterium in a GC-MS spectrum.*

Conclusions: *The potent isolate was identified as a Streptococcus sp and to our knowledge this is the first report of marine Streptococcus which possessed biosurfactant properties. We propose that this study may pave a way to identify biosurfactant producing bacteria from marine realm too.*

Keywords: *Biosurfactants, Gas Chromatography-Mass Spectrometry (GC-MS), marine, Streptococcus sp, tributyrin.*

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INTRODUCTION

Oil pollution in the marine environs is considered as most devastating than any other problems related to pollution because of the deleterious effects on marine life. This may be because of two factors: i) from the oil itself and ii) effects associated with clean-up operations. Gulf of Mannar and Palk Bay areas located on the South eastern parts of India are considered one of the highly biodiversified areas which support a huge group of organisms at various levels. Unfortunately these two areas are categorized as sensitive because there have been reports on oil-pollution due to shipping, by and large. Though the shipping lines are far away from the gulf area, oil spills occurring from ships/tankers moving toward Colombo which cross Gulf of Mannar, would result in oil pollution.[1] Apart from this, oil pollution also occurs from terrestrial sources along the shorelines in areas as cited in literature pertinent.[2, 3] Immediate to an oil spill, when all the organisms are categorized to be stressed, a few groups of bacterial population emerge, survive and grow well in oil polluted areas. It is widely documented that bacteria thriving in oil-polluted areas secrete extracellular enzymes like lipases to utilize hydrocarbons especially with a triglyceride base. Some bacteria, in addition, may also produce biosurfactants for accelerated lipid recovery.[4-7] Biosurfactants are categorized as surface-active substances synthesized by living cells, mainly bacteria and are believed to reduce surface tension and stabilize emulsions. There have been many studies checking biosurfactant-producing marine bacteria.[8-11] However, only a very few studies have been done on identification of isolates of Gulf of Mannar and Palk Bay. Hence, with a notion that Gulf of Mannar and Palk Bay may harbor oil-degrading bacteria, the present investigation is centered to screen bacteria from these areas for lipase and/ or biosurfactant production and to check their bioactivities to degrade hydrocarbon sources, with reference to olive oil and tributyrin. Also, this study attempts to determine the extent of tributyrin degradation using Gas Chromatography-Mass Spectrometry [GC-MS] analysis and to preliminary identify the potent isolate/s using biochemical tests.

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MATERIALS AND METHODS

Collection and processing of samples:

Sub-surface marine water samples (2-4 m deep) were collected from Gulf of Mannar (lat. 9° 16' long. 79° 7') and Palk Bay (lat. 9° 17' long. 79° 7') of Rameswaram coast of Tamil Nadu, India, in sterile screw-capped bottles and were brought immediately to the laboratory for microbiological processing. Bacteria of both the stations were briefly extracted by shaking 10 mL of the seawater samples vigorously in 90 mL of filtered and autoclaved

seawater. Thereafter dilutions up to of 10^4 were spread on ZoBell Marine Agar (ZMA) (5 g peptone, 1 g yeast extract and 15 g bacteriological agar in 1L filtered and autoclaved seawater) supplemented with 10% tributyrin (HiMedia) in duplicates. Colonies with varying morphotypes were quadrant streaked to obtain pure cultures and each of them were checked for lipase activity. Only the colonies showing considerable zones of clearance [a minimum of 10 mm diameter] from each station were furthered for testing biosurfactant activities.

Extraction of biosurfactants:

Extraction of crude biosurfactant precipitates was done for all lipase [+] isolates as per method of Jenneman et al., (1983).[12] Loopful of bacterial cultures were inoculated in Erlenmeyer flasks containing 100 mL of ZoBell Marine Broth (ZMB) and were shaken at 250 rpm at 37°C for 3 days. Thereafter cultures [1 OD_{660}] were briefly spun at 10,000 x g at 4º C for 10 min and pelleted down. The supernatants were acidified using 6 N hydrochloric acid to pH 2.0 and incubated overnight at 4ºC. A precipitate containing biosurfactant was collected by spinning the solution at 15,000 x g for 20 min. Following this, the precipitate was dissolved in distilled water and the extract yield for each of the isolates was noted and expressed as mg/100 mL. To check activities, a known concentration of biosurfactant (5 mg/mL) was prepared as a stock solution and concentrations were standardized for each of the assays.

Assays to determine biosurfactant properties:

Oil spread, Tilted glass slide, Modified drop collapse:

Petridishes were filled with 50 mL of distilled water and to this, 20 µL of olive oil was spread uniformly. Further, 100, 120, 150, 160, 170, 180, 190 and 200 µg/ 10 µL concentration ranges of biosurfactant precipitates of each of the isolates were added on top of the oil to observe its disintegration. Disruption of oil as a clear zone indicated biosurfactant action.[13] In the case of tilted glass slide method, biosurfactant preparation of different concentrations such as, 100, 200, 300, 400, 500 and 600 µg/ 10µL were placed at the one end of the slide coated uniformly with olive oil. Dripping of the biosurfactant precipitate along the oil coating indicates positive for surfactant action.[14] Whereas for the case of modified drop collapse method, 10µL of olive oil was placed on the slide and biosurfactant preparation in different concentrations of 100, 200, 300, 400, 500, 600 and 700 µg/ 10 µL were added at the centre of oil drop. Biosurfactant producers were detected as collapsing oil droplet within a min as described by Bodour and Miller-Maier (1998).[15] All these experiments were performed in triplicates and the mean values ±SD is noted. A positive control such as Sodium Dodecyl Sulfate (SDS) and controls without biosurfactant preparation/ SDS [1 µg/ 10 µL] were also run simultaneously. Minimum Effective Concentrations (MECs) were noted for each of the tests which denote the least concentration of surfactant to elicit a visible oil droplet spread/ drip/ collapse.

Evaluation of other properties of the isolates (Hemolytic activity and growth on Rhodamine B Agar plates)

All the isolates showing positive for lipase production were tested for hemolytic properties as per Abouseouda et al., (2008)[16] and Gandhimathi et al., (2009).[17] Briefly the cells were grown at 37° C for 48 hrs on blood agar plates to evaluate possible cell lysis. To check growth in Rhodamine Agar, lipodial solution of tributyrin and ZMA were prepared and mixed together with Rhodamine B solution and set on sterilized petriplates. The isolates were patch-inoculated and incubated at 37° C for 30 hrs. Cultures were examined on a transilluminator for fluorescence which indicated positive for degradation of hydrocarbons and release of free fatty acids.[18]

Gas chromatogram-Mass Spectrometry analysis of tributyrin degradation and identification of the potent isolate.

Isolate, K which exhibited potent tributyrin as well as olive oil degrading activities was cultured in ZMB amended with 10% (v/v) tributyrin and after 5 days of incubation at 37° C the culture broth was submitted to GC-MS (GC-FID, VARIAN-CP-3800) to find out whether or not there has been a degradation of tributyrin. Standard tributyrin and a control [uninoculated with K cultures] were also run simultaneously in GC-MS for comparison. Isolate K was identified up to the genus levels using the protocols outlined in Bergey's Manual of Systematic Bacteriology. These included observation of morphology [pigmentation, Gram staining, shape, endospore staining], growth on Mac Conkey Agar, IMViC, gas and acid production from various sugars, casein and gelatin hydrolysis, catalase test and growth with Tween20 and Tween 80.

RESULTS AND DISCUSSION

The Total Heterotrophic bacterial count from Gulf of Mannar was 15.7 x 10^2 and that of Palk Bay was 14.5x10^2 CFU/ 100µL of seawater. A total of 123 isolates were screened out of which only 7 from Gulf of Mannar and 9 from Palk Bay recorded positive for the production of extracellular enzyme, lipase (Fig. 1). Occurrence of lesser percentage of oil degrading bacteria of the total Bacterial Load is not uncommon because not many bacteria utilize hydrocarbons as their sole source of energy.[19, 20] Not only that, when microorganisms grow in such oil-rich environs, they must acquire many adaptations to utilize chained fatty acids which ultimately becomes a prerequisite for their growth.[21-24] In the present work, a maximum zone of clearance of 33 and 30 mm diameter was conferred by the isolates O and K (Gulf of Mannar), whereas, the isolates of Palk Bay did not exhibit more zones of clearance on tributyrin agar plates. In general, a maximum zone of only 20 mm was elicited by isolate 9 at the Palk Bay regions.

Preliminary lipase screening experiments were used as base for selection of isolates for mass production of biosurfactants based on literature pertinent because lipase secreting bacteria are by and large considered to secrete biosurfactants too.[25-28, 5] Based on zones of clearance on tributyrin supplied medium, only isolates, I, K, M, N, O, Q and R from Gulf of Mannar station and 1, 2, 7, 9, 10, 14, 15, 16 and 19 from Palk Bay were selected for production of biosurfactants.. In Gulf of Mannar region, most of the isolates categorized as lipase [+] yielded good amount of biosurfactants. The order of biosurfactant production is as follows: N>O>I>K>Q in Gulf of Mannar and 19>1>7 in Palk Bay with maximal yields from N (32mg/ 100mL) and 19 (20mg/ 100mL) (Fig. 1). As indicated before here again, only a very small population of the total heterotrophic load is categorized as biosurfactant producers in general. In fact, a study showcases seawater-derived biosurfactant producers contributed only 0.5% of the total heterotrophic bacterial load[29] and the current study recorded 6.5%.

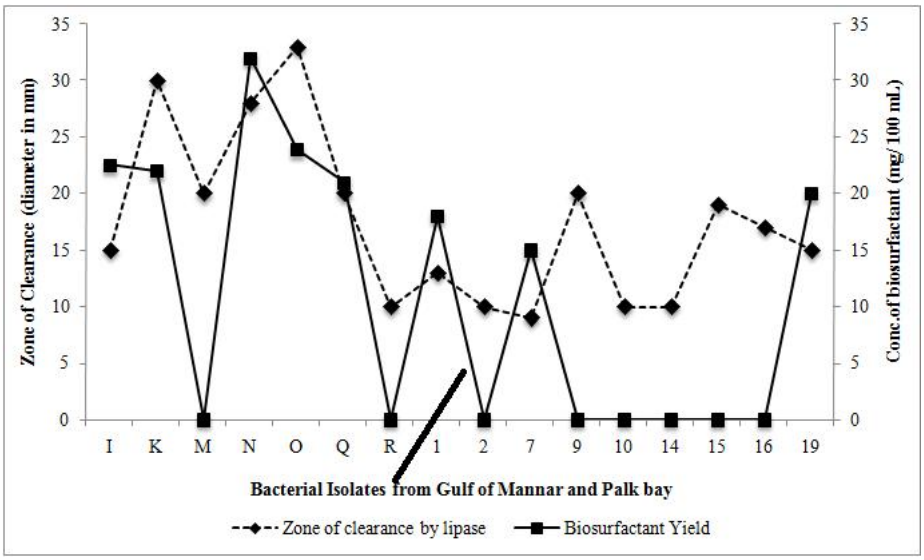

Fig. 1. Crude biosurfactant yield of bacterial isolates from waters of Gulf of Mannar and Palk bay

There was an overall strong positive correlation between the potent lipase producers with biosurfactant yield (r=+0.54). However, it was also found that isolates, M and R from Gulf of Mannar and 2, 9, 10, 14, 15 and 16 isolated from Palk Bay did not produce any biosurfactants though recorded positive for lipase production, indicating quite a few of lipase [+] isolates may not produce biosurfactants too. This indicates that biosurfactant may enhance the degradation of hydrocarbons together with extracellular lipases.[5-7]

Isolates K, O and Q had olive oil spreading capabilities by displacing them vigorously on the surface of water, at MECs of 120, 110 and 102 µg/ 10 µL respectively (Fig. 2). For tilted glass slide assay, strains K and O showed activities with MECs of 500 and 230 µg/ 10 µL respectively. For the modified drop collapse method, which is a tough test to analyze the efficacy of biosurfactants [owing to the piercing biosurfactant into a drop and breaking], isolate K had MEC of 250 µg/ 10 µL (Fig. 3). A few studies have only qualified isolates for biosurfactant activities by using a known volume of bacterial extracellular fluid than the extracted biosurfactant rather.[15,20] In general, is always justified to use drop collapse as a primary method to detect biosurfactant producers and thereafter countercheck by oil spread to determine biosurfactant concentration.[30]

It is in general said all the three tests correlate positively, ie., when a biosurfactant is able to spread oil, it might also drip and collapse it as well.[31, 20] We indicate that all the three tests showed good correlation among each other, ie., between oil spread and drop collapse methods (r=+0.67), tilted glass slide and drop collapse (r=+0.20). Similar conclusions with positive correlation between all the three tests were reported in a few previous investigations[31,20] as well. Hence we suggest that all the three tests which show good positive correlation be used as a tool to evaluate biosurfactant properties. The supernatants when assayed for biosurfactant activity (data not shown) did not prove any positive results indicating that the biosurfactants were pelleted down. All the bacterial strains which were potent lipase producers were found to be Gram positive [60% being cocci and 40% rods] (data not shown).

Evaluation of other properties of the isolates

Hemolysis has been always been looked upon as a screening method for the isolation of biosurfactant producing strains.[32, 33] In the present study, all the isolates which possessed biosurfactant properties have shown hemolysis as blood agar plates as well showing a fair positive correlation (r= +0.54) as given in Fig. 4. However, there are also reports to mention that hemolytic activity is not always associated with biosurfactant property.[30] This could be true due to the presence of virulence factors of the hemocytes and weak infusibility of certain

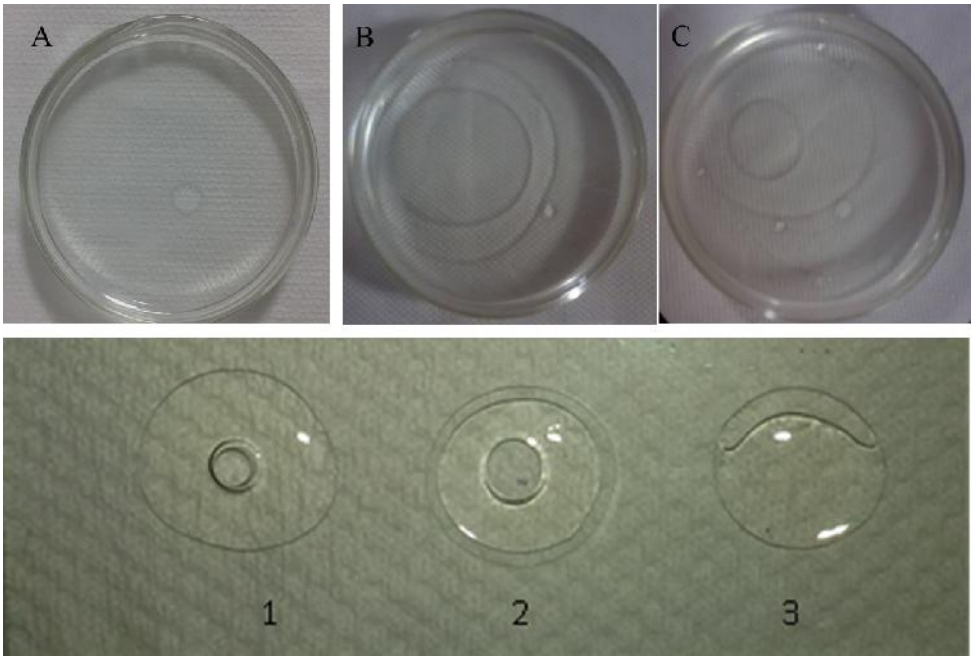

Fig.2. Displacement of olive oil by biosurfactants of strain K in oil spread method. (a) Control-oil droplet in water (b) Positive control - SDS (1 µg) and (c) Biosurfactants of isolate K (120 µg). Disrupting the oil droplet on water by the biosurfactant of strain K (1)Water (2) SDS (2 µg) (3) Strain K (250 µg). (*correlation between oil spread and drop collapse *r*=+0.67 and between tilted glass slide and drop collapse- *r*=+0.20)

biosurfactants. Thus, it is not clear whether or not blood agar lysis could be used as a screening methodology to identify biosurfactant (+) isolates. However, such screening could only be used as rapid methods, in which isolates with a positive result should be tested for other tests like oil spread, titled glass and drop collapse methods to confirm surfactant properties.

It is essential to find out whether or not the potent biosurfactant producers grow on Rhodamine B agar. Rhodamine B forms colored complexes with many acidic materials, especially when uranyl ion is present. According to Feigl (1956)[34], uranyl ions form complexes with fatty acids (breakdown product of fats and hydrocarbons) and completely ionize them. Thus protons released from fatty

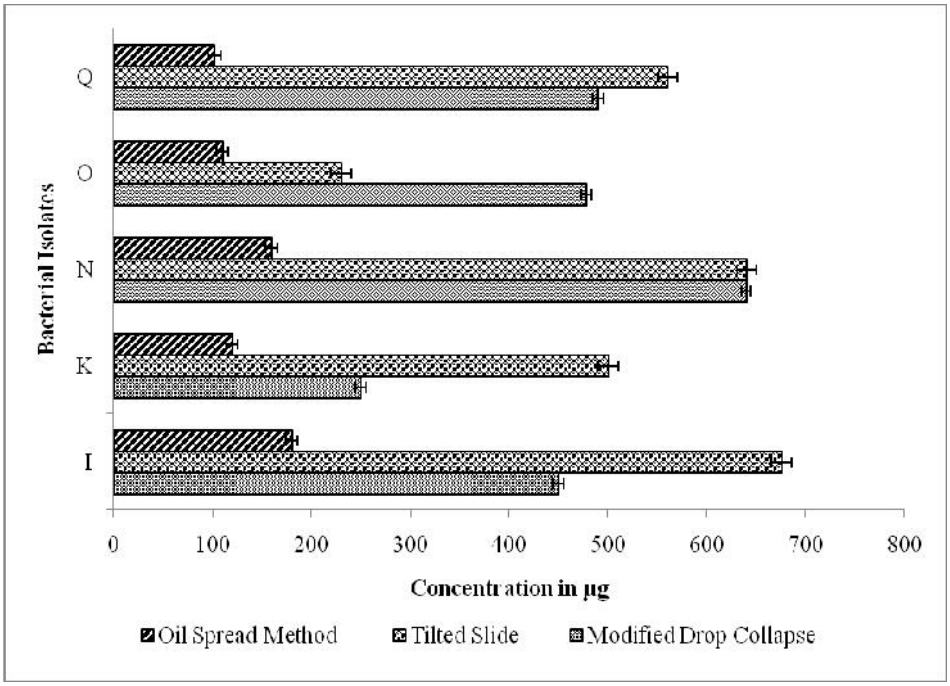

Fig. 3. Minimum Effective Concentration (MEC) of the crude precipitate showing bioactivities

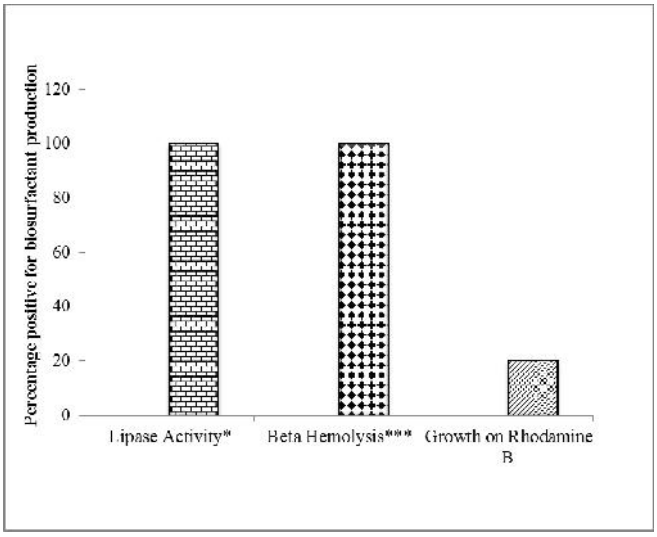

Fig. 4. Percentage of lipase, hemolytic and Rhodamine B positive strains possessing biosurfactant properties. (*r* values: *+0.54; *+0.89)**

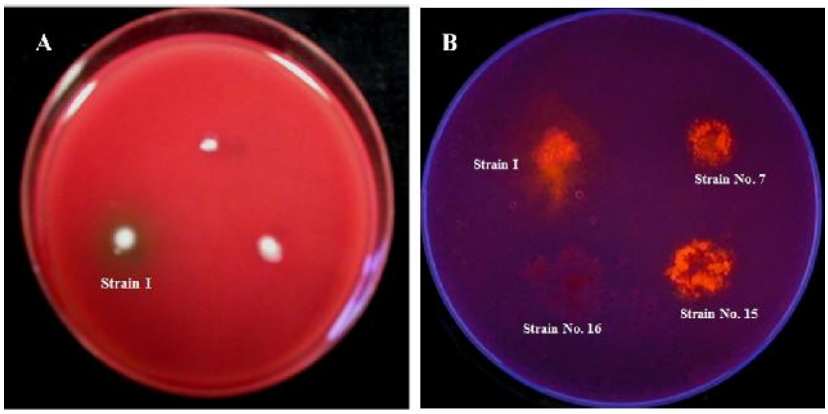

Fig. 5. Blood Agar plates showing ß Hemolysis (A) and fluorescence in Rhodamine B agar (B).

acids changes Rhodamine B into cationic form, which complexes with uranyl fatty acid ions which is an orange colored fluorescent compound. Thus many of the bacterial species which do not utilize tributyrin accumulates rhodamine B and only forms pink colored colonies, but do not fluoresce upon UV irradiation[18] and those which utilize hydrocarbons appear as fluorescent orange colored colonies. The current study indicates that isolate I (Gulf of Mannar) and 7, 15, 16 (Palk Bay) showed positive result for the Rhodamine test (Fig. 5) and put together, only 20% of the biosurfactant [+] isolates fluoresced when grown on Rhodamine B. These results contradict with a few other studies which suggest the usage of Rhodamine B agar for selective isolation of biosurfactant producing bacteria.[35-37] However this contradiction should be substantiated with further tests.

Gas chromatogram- Mass Spectrometry analysis of tributyrin degradation and identification of the potent isolate.

Based on the zones on tributyrin agar, biosurfactant yield, results of oil spread, tilted slide and modified drop collapse methods, isolate K was chosen for tributyrin degradation studies. Degradation of tributyrin was witnessed by culture K at 5 days of incubation. GC-MS analysis suggested that the isolate K degraded almost all the hydrocarbon present in tributyrin (Fig. 6). Our preliminary results [on olive oil] for biosurfactant assays are confirmed as indicated by the degradation of this hydrocarbon too by the isolate K, as proved by decrease in peak heights. In comparison to the control, the produced surfactant reduced tributyrin to the tune of 96% only after 5 days of incubation. Based on the morphological and biochemical tests as listed in materials and methods [data not shown], isolate K is identified as a species belonging to the genus, *Streptococcus.*

To our knowledge this is the first work on biosurfactants from marine *Streptococcus* sp. Biosurfactants sourced from *Streptococcus* species are previously reported only from terrestrial sources.[38,39] Surfactants of *Streptococcus thermophilus* were mainly reported to be rich in glycolipid fractions. However marine Streptococcal species may secrete a variety of surfactants

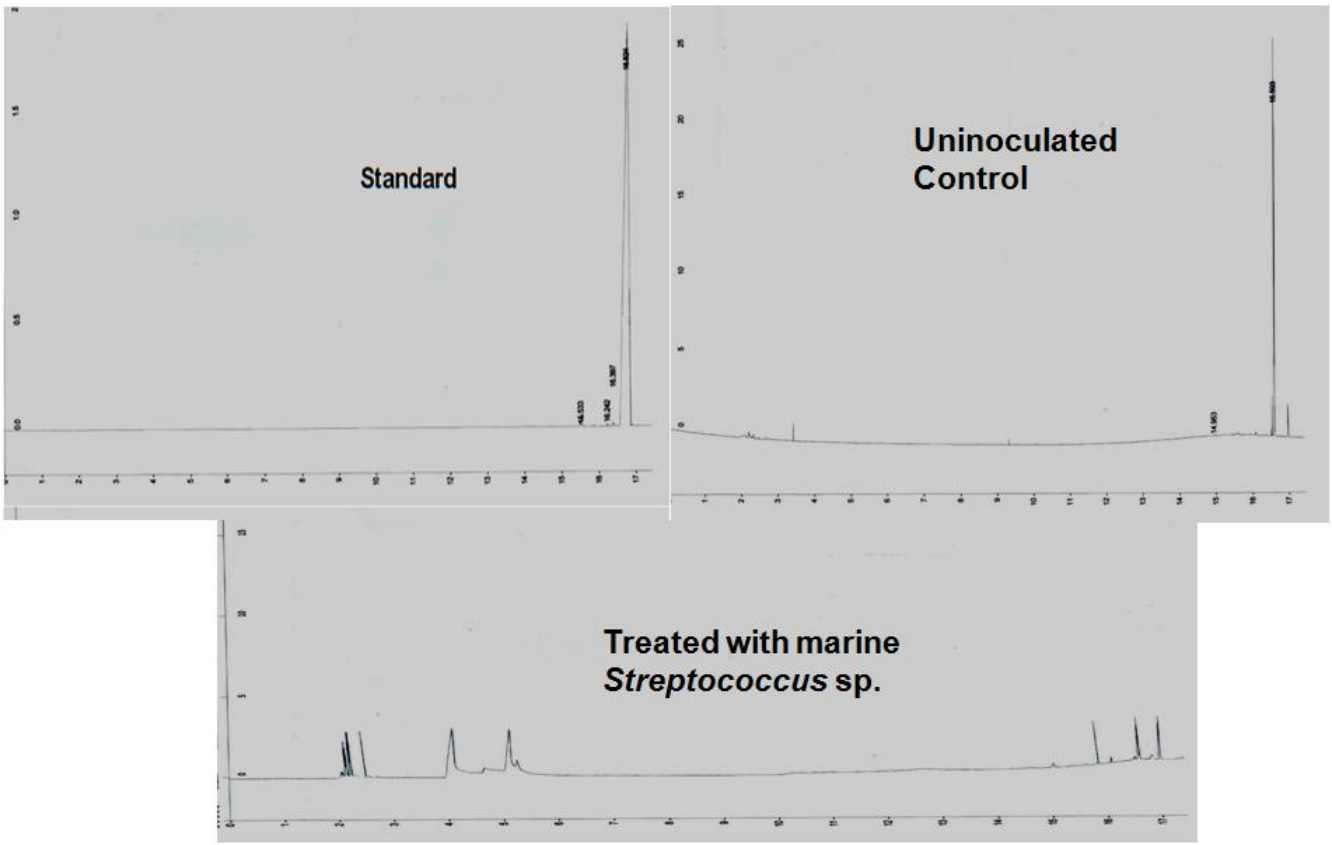

Fig. 6. Degraded tributyrin on Gas Chromatography- Mass Spectrum (GC-MS)

and could be looked upon as a new platform for exploitation of potent surfactants. Hence we conclude that more studies on the species level identification, pathogenicity of the isolate, analysis and purification of surfactant from this strain may offer a potent and possibly a novel surfactant. This study makes evident that hydrocarbon degrading bacteria are extant in the Gulf of Mannar regions which can be used to ameliorate oil pollution.

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