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Isolation and characterization of biosurfactant - producing marine bacteria, isolated from the Chinna Muttam fishing harbor, Kanyakumari District.

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ABSTRACT

Biosurfactants produced by some marine microorganisms have been paid more attention in the recent times, particularly for the bioremediation of the sea polluted by crude oil. The goal this study is the isolation and characterization of biosurfactants - producing bacteria from the water sample of Chinna muttam harbor. Four morphologically different organisms, namely SMWC (Sample Muttam White Colony), SMC (sample Muttam cloudy colony), SMWZ (sample Muttam white zone) and SMSC (sample Muttam striped colony) were isolated. Among four strains SMSC gave positive results for biosurfactants production. The primary screening test followed by 16S RNA sequencing as well as biochemical characterization for the identification of bacteria. In addition to this, antibacterial activity of the extracted biosurfactants was also performed.

Keywords: Biosurfactants, marine microorganism, hemolytic activity, antibacterial activity.

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INTRODUCTION

Oil spills can cause a wide range of impacts in the marine environment and are often portrayed as 'environmental disasters' with dire consequences predicted for the survival of marine flora and fauna[1]. In a major incident the short-term environmental impact can be severe, causing serious distress to ecosystems and to the people living near the contaminated coastline, affecting their livelihoods and impairing their quality of life. Contamination of an ocean by oil spills is a widespread environmental problem that often requires cleaning up of the contaminated sites. The annual release of crude oil in the oceans is estimated to be around 1.7 to 1.8 metric tons and the impact of this pollution can be severe environmental imbalance [2]. Crude oil is a highly hydrophobic material with most of its components having low water solubility [3]. When oil spread in the environment, most of the oil hydrocarbons remain on the water surface /or adhered to soil particles due to their low solubility [4]. To increase the bioavailability or hydrocarbon pollutants, surface-active agents (surfactant) may be used, allowing desorption and solubilization of petroleum hydrocarbon and thus facilitating their assimilation by microbial cell [3,4]. In recent years the interest in surface-active agents has increased [5]. Microbial compounds, which exhibit pronounced surface activity, are classified as Biosurfactant [6] . Biosurfactants are amphiphilic compounds produced by bacteria, fungi and yeast. They belong to various classes, including glycolipids, lipopeptides, fatty acids, phospholipids, neutral lipids and lipopolysaccharides[7]. The properties and applications of biosurfactants includes excellent detergency, emulsification, foaming, dispersing traits, wetting, penetrating, thickening, microbial growth enhancement, metal sequestering and resource recovering (oil) which allows biosurfactants an ability to replace some of the most versatile process chemicals. In addition biosurfactants are promising natural surfactants that offer several advantages over chemically synthesized surfactants, such as lower toxicity, biodegradability and ecological acceptability[8]. Hence, the present study focuses on the isolation, screening and characterization of biosurfactant (-) producing marine bacteria from the Chinna Muttam harbor, which is a special grade village, which handles about 300 boats in Kanyakumari District in the state of Tamil Nadu in India. Leakage of crude oil from tankers in harbors and other anthropogenic activities suspect the occurrence of biosurfactant - producing bacteria at this site. There have been very few studies so for that evaluated the presence of natural biosurfactant producing microbes in this region.

MATERIALS AND METHODS

Collection of Soil Samples

Water sample was collected in a sterile conical flask, from Chinna muttam fishing Harbor, Kanyakumari . The sample was then investigated for microbial screening[9].

Isolation of Bacteria

One ml of water sample was serially diluted to 10^{-6} and used as a source of inoculum for the isolation of biosurfactants producing bacteria. 100 µl inoculum was then plated in nutrient agar and incubated at 37 °C for 24 hrs. The pure cultures made by a streak plate technique were then subjected to screening for biosurfactant production [10].

Screening and identification of biosurfactants - producing microorganism

In order to scrutinize the ability to produce biosurfactants, the isolates were subjected to following activity such as Hemolytic activity test [11], methylene blue agar plate method [11], drop collapse assay [12], fossil fuel degradation[13], emulsification and stability test [13] and dye degradation test[14]

The isolate which generate positive results from the above tests were confirmed by 16S RNA sequencing as well as biochemical characterization.

Extraction of biosurfactants

Cells are harvested by centrifugation (8000 rpm , 4 °C for 10 min), then the precipitation reaction of the harvested cells were carried out with 5M Hcl. Finally the biosurfactants was extracted with the mixture of a solvent such as chloroform: methanol (3:1) which was further analyzed by antimicrobial studies.

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Analysis of antibacterial activity of biosurfactants

The extracted biosurfactants has an ability to produce antibacterial activity which was further confirmed by clear zone test [15].

RESULT AND DISCUSSION

The water samples were subjected to serial dilution to isolate oil degrading microorganisms. From that four morphologically different colonies were screened, which were named as SMWC (sample Muttam white colony), SMC (sample Muttam cloudy colony), SMWZ (sample Muttam white zone), SMSC (sample Muttam striped colony) respectively (Table 1).

Table 1: Bacterial isolates of Chinna Muttam Fishing Harbor

S. No.	Morphologically different colony	Sample codes
1	White Colony	SMWC
2	Cloudy	SMC
3	White zone	SMWZ
4	Striped colony	SMSC

The isolated colonies were obtained in pure cultures and tested for their biosurfactants production by blood hemolytic method and methylene blue agar plate method. The blood agar method is often used for a preliminary screening of microorganisms for the ability to produce biosurfactants on hydrophilic media[16]. Hemolytic test of the bacterial isolates revealed that SMSC showed positive result. But the other three organisms were reported negative for biosurfactant production. Hence the further study was carried out with hemolytic positive SMSC (Table 2). The above results were further confirmed by fossil fuel degradation, emulsification stability test and dye degradation assay. The Fossil fuel degradation assay revealed high activity with crude oil when compare with kerosene , petrol and palm oil The Organism SMSC showed 33.33% stability in emulsification test. From the dye degradation assay methylene blue was highly decolorized by SMSC when compared with safranin and malachite green **(Table 3)**.

Table 2: Hemolytic activity of the bacterial isolates in Blood Agar and Methylene blue plate agar method

S. No.	Isolated strains	Blood Agar method	Methylene Blue Plate Agar Method
1	SMWC	Negative	Negative
2	SMC	Negative	Negative
3	SMWZ	Negative	Negative
4	SMSC	Positive	Positive

Table 3 : Screening of Biosurfactants Producing Bacteria

S. No.	Isolated Strain	performed Assay	Effect of Biosurfactants producing microorganism on various oil			
1	SMSC	**Fossil Fuel Degradation**	Crude oil	Kerosene	Petrol	Palm oil
			1.5 (High)	-----	Mild	1 (Moderate)
2		**Emulsification Stability Test**	33.33%			
3		**Dye Degradation**	**Percentage of decolorization (%)**			
		Safranin	2.94			
		Methylene Blue	82.35			
		Malachite Green	74.07			

The results of screening tests proved that the strain SMSC have the ability to produce biosurfactants which was further subjected to biochemical as well as molecular characterization. Various biochemical tests (Table 4) were carried out followed by 16 S rRNA sequencing. From the BLAST result it was proved that the isolated marine organism was *Chromobacterium violaceum.* (Figure 1-2).

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Table 4: Biochemical Characterization of bacterial isolate

S.No.	Tests	SMSC
1.	Motility test	Positive
2.	Oxidase test	Positive
3.	Indole test	Negative
4.	Methyl red test	Negative
5.	Voges Proskauer test	Negative
6.	Catalase test	Negative
7.	Urease test	Negative
8.	Starch hydrolysis test	Negative
	Chromobacterium violaceum	

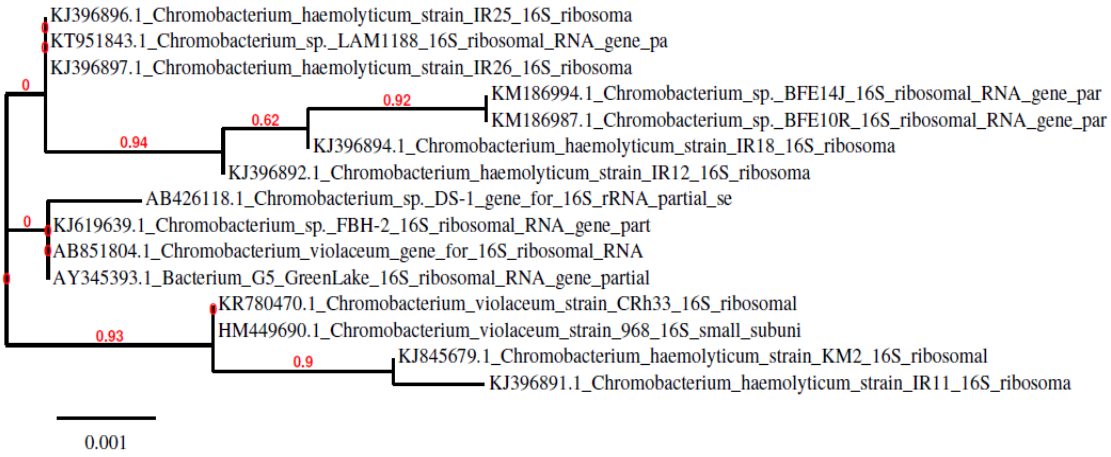

Figure 1: Phylogenetic analysis of Chromobacterium violaceum

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Chromobacterium sp. FBH-2 16S ribosomal RNA gene, partial sequence	2322	2322	100%	0.0	100%	KJ619639.1
Chromobacterium violaceum gene for 16S ribosomal RNA, partial sequence	2322	2322	100%	0.0	100%	AB851804.1
Chromobacterium sp. LAM1188 16S ribosomal RNA gene, partial sequence	2316	2316	100%	0.0	99%	KT951843.1
Chromobacterium haemolyticum strain IR26 16S ribosomal RNA gene, partial sequence	2316	2316	100%	0.0	99%	KJ396897.1
Chromobacterium haemolyticum strain IR25 16S ribosomal RNA gene, partial sequence	2316	2316	100%	0.0	99%	KJ396896.1
Bacterium G5 GreenLake 16S ribosomal RNA gene, partial sequence	2316	2316	100%	0.0	99%	AY345393.1
Chromobacterium violaceum strain CRh33 16S ribosomal RNA gene, partial sequence	2311	2311	100%	0.0	99%	KR780470.1
Chromobacterium violaceum strain 968 16S small subunit ribosomal RNA gene, partial sequence	2311	2311	100%	0.0	99%	HM449690.1
Chromobacterium sp. DS-1 gene for 16S rRNA, partial sequence	2309	2309	100%	0.0	99%	AB426118.1
Chromobacterium haemolyticum strain IR12 16S ribosomal RNA gene, partial sequence	2305	2305	100%	0.0	99%	KJ396892.1
Chromobacterium haemolyticum strain IR18 16S ribosomal RNA gene, partial sequence	2300	2300	100%	0.0	99%	KJ396894.1
Chromobacterium sp. BFE14J 16S ribosomal RNA gene, partial sequence	2300	2300	100%	0.0	99%	KM186994.1
Chromobacterium sp. BFE10R 16S ribosomal RNA gene, partial sequence	2300	2300	100%	0.0	99%	KM186987.1
Chromobacterium haemolyticum strain KM2 16S ribosomal RNA gene, partial sequence	2300	2300	100%	0.0	99%	KJ845679.1
Chromobacterium haemolyticum strain IR11 16S ribosomal RNA gene, partial sequence	2294	2294	100%	0.0	99%	KJ396891.1
Chromobacterium haemolyticum strain EAPL14 16S ribosomal RNA gene, partial sequence	2294	2294	100%	0.0	99%	JX500185.1
Chromobacterium haemolyticum strain MDA0585 16S ribosomal RNA gene, complete sequence	2294	2294	100%	0.0	99%	NR_043957.1
Chromobacterium sp. PRE11F 16S ribosomal RNA gene, partial sequence	2278	2278	100%	0.0	99%	KM187571.1
Chromobacterium aquaticum partial 16S rRNA gene, strain R-50671	2266	2266	100%	0.0	99%	LN995680.1
Uncultured Neisseriaceae bacterium clone LHE098 16S ribosomal RNA gene, partial sequence	2266	2266	100%	0.0	99%	JX093173.1

Figure 2: Sequences Producing Significant alignment

One useful property of many biosurfactant that has not been reviewed extensively is their antimicrobial activity. Other medical relevant uses of biosurfactants include their role as anti-adhesive agents to pathogens, making them useful for treating many diseases and as therapeutic agents. The biosurfactant produced by *Chromobacterium violaceum* exhibited interesting antimicrobial activities and the results were listed in Table 5 which revealed that the higher antimicrobial activity was observed against *Staphylococcus*. The antimicrobial effect of biosurfactants is the adhering property of biosurfactants to cell surfaces caused deterioration in the integrity of cell membranes and also breakdown in the nutrition cycle.

Table 5 : Antibacterial activity of isolated biosurfactants against various bacteria

S.No.	Extracted biosurfactants	Bacterial Pathogens	Activity
1	SMSC	*Streptococcus*	mild
2		*Klebsiella*	mild
3		*Staphylococcus*	High
4		*E.coli*	mild

CONCLUSION

The production of novel biosurfactants is increasing day by day due to the interest upon it. Since it is highly used for bioremediation, much research has been undertaken. Both organic and inorganic contaminants can be removed through physicochemical and biological processes in which biosurfactants are involved. The commercial success of such technologies is still limited by their high production cost. Even though biosurfactants have many positive aspects, it has certain hazardous nature too towards the environment. Hence, careful and controlled use of this interesting surface active molecule will help to provide a clean and sophistic atmosphere.

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