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Isolation and Primary Screening of Biosurfactant Producing Marine Bacteria From Hydrocarbon Contaminated Soil and Water Samples of Sikka Coastal Area, Gujarat, India

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Abstract - The present study describes isolation and primary screening of Biosurfactant producing marine bacteria isolated from Jamnagar coastal area of Gujarat, India. Water and soil samples were inoculated in nutrient broth and special media containing olive oil as a source hydrocarbon for enrichment and isolation of Biosurfactant producers. Primary screening was done by using various methods like Emulsion index, Rhamnose test and Oil spreading method. Salt tolerance of the samples shows turbidity up to 10% NaCl in nutrient broth. Total 24 isolates were found to produce Biosurfactant out of these 24 isolates 20% were found to be gram positive while remaining were gram negative. While emulsion activity shows that out of 24 isolates only 2 had less than 40% of emulsion index while remaining all had more than 40% of emulsification index. Highest emulsification index were observed for SKW_3, SSK-23, SSL-2, WB_1P-1 and WJ-11 and that was 50% for all. Lowest emulsification index was 36.66% for WJ-22. Emulsification index was measured after 24 hrs which shows stability of emulsion formed in tubes.

Key Words: Biosurfactant, Emulsification index, Hydrocarbon

I. INTRODUCTION

The pollution of soil and water by industrial chemicals is a serious problem afflicting the modern world. Petroleum hydrocarbons are the most frequently occurring environmental contaminants because of their extensive use, in both aquatic and terrestrial ecosystems[3]. Effective petroleum hydrocarbon remediation is challenging because petroleum is a complex mixture of aliphatic and aromatic hydrocarbons. Oil spills, leakage and inadequate disposal of petroleum and petroleum products pose a serious threat to both aquatic and terrestrial ecosystems , and effective remediation methods are needed. One of the most effective methods to treat oil-related contamination is the use of surfactants that disperse the oil and accelerate its mineralization. Synthetic surfactants have applications in the degradation processes of petroleum hydrocarbons, but they are environmentally hazardous[9]. While Biosurfactants have several advantages over chemical surfactants such as lower toxicity, higher biodegradability and effectiveness at extreme temperatures or pH values. Biosurfactants, produced by microorganisms [5], are amphipathic surface active molecules containing hydrophilic and hydrophobic moieties that act by emulsifying hydrocarbons, increasing their solubilisation and subsequently rendering them available for microbial degradation[1]. Biosurfactant or bioemulsans are surface active compounds that do not necessarily reduce surface tension but form stable emulsions between liquid hydrocarbons and water mixtures and are hence also often referred to as biosurfactants. Apart from being used in bioremediaton these biological products have potential uses in agriculture and the cosmetic, pharmaceutical, detergent, food, textile, paper and paint industries [2]. Gujarat has highest coastal area of 1600 km in India and coastal area of Jamnagar have high risk of contamination of petroleum because of ports as well as petroleum refineries like reliance situated near to the Jamnagar. The present paper reports an isolation and primary screening of Biosurfactant producing bacteria from marine soil and water sample of Jamnagar coastal area, Gujarat, India.

II. MATERIALS AND METHODS

SAMPLE COLLECTION

Soil and water samples were collected in polythene bags and sterile bottles respectively from oil contaminated area of Sikka, Salaya and Jodiya in the month of September, 2013. Sikka, Salaya and Jodiya are ports situated in coastal area of Jamnagar.

PHYSICAL AND CHEMICAL ANALYSIS OF SAMPLES

Samples were analyzed for color, turbidity and pH. pH of the samples were checked by using pH meter(CL 54+ Toshcon Industries PVT. LTD.) And pH strips (HiMedia lab, Mumbai).

ENRICHMENT AND ISOLATION OF CULTURES

Enrichment of samples was carried out by using Nutrient broth (HiMedia lab, Mumbai). Samples were inoculated in sterile Nutrient broth and incubated at 37˚C on rotary shaker at 100 rpm for 24 hrs. After 24 hrs of incubation turbid nutrient broth was further inoculated on nutrient agar (HiMedia lab, Mumbai). Plates were incubated at 37˚C for 24 hrs in incubator. Viscous Colonies were selected on the basis of morphological characteristics.

STUDY OF SALT TOLERANCE OF SAMPLES

Salt tolerance of samples was studied by using nutrient broth with graded amount of NaCl from 0.5% to 12%. Media were inoculated with enriched culture from nutrient broth and incubated at 37˚C for 24 hrs on rotary shaker at 100 rpm.

ENRICHMENT IN SPECIAL MEDIA

Selected colonies were inoculated in selective medium containing congo red and supplemented with hydrocarbons. Medium was prepared in three parts separately (Part-A Congo red 0.008%, NaCl 0.05% and olive oil 0.2% as a hydrocarbon, part-B sucrose 1%, Part-C Urea 0.01%). After inoculation broths were incubated at 37˚C on rotary shaker at 100 rpm for 4-5 days. After 4-5 days white, sticky, gummy product found to be stick on internal surface of flask.

STAINING OF CULTURES

Monochrome staining, Gram staining and capsule staining (Hiss method) were performed for morphological characteristics of organisms.

PRIMARY SCREENING OF BIOSURFACTANT PRODUCING BACTERIA

EXTRACTION OF BIOSURFACTANT

Biosurfactant from broth was extracted by using acetone precipitation method. In this method broth was centrifuged at 10000 rpm for 10 minutes to remove cells (REMI centrifuge was used). After centrifugation supernatant was extracted with 3 volumes of chilled acetone (HiMedia lab, Mumbai). After addition of acetone tubes were incubated for 24 hrs in refrigerator. Next day precipitates were collected by centrifuging tubes at 10000 rpm for 10 minutes. Precipitates were dissolved in mili-Q water.

RHAMNO TEST FOR QUALITATIVE ANALYSIS OF SAMPLES

After centrifugation of broth at 10000 rpm for 10 minutes supernatant was checked for the presence of Rhamno compounds.0.5 ml of broth was mixed with 0.5 ml of 5% phenol (HiMedia lab, Mumbai) and 2.5 ml of concentrated sulphuric acid (HiMedia lab, Mumbai) and incubated for 15 minutes at room temperature. After 15 minutes color was checked for the presence of Rhamno compounds.

OIL SPREADING TECHNIQUE (PLATE ASSAY)

Precipitates dissolved in mili-Q water were used for oil spreading technique. 50 ml of distilled water was added in petriplate of 150 mm diameter(Glassco) followed by addition of 1 ml of olive oil(HiMedia lab, Mumbai) on surface of distilled water and 0.5 ml of precipitates dissolved in mili-Q water on a drop of olive oil. For visualization of oil drops congo red is mixed with olive oil.

EMULSIFICATION INDEX (E_{24})

Precipitates dissolved in mili-Q water were analyzed for emulsification index and stability was checked for 24 hrs. 2 ml of olive oil and 2 ml of precipitates dissolved in mili-Q were mixed in test tube and vortexed for 5 minutes then tubes were put for 24 hrs. Next day height of emulsion layer was measured and emulsion index was calculated by using following equation.

E_{24}= Height of emulsion layer/Height of total mixture × 100

III. RESULTS AND DISCUSSION

ANALYSIS OF SAMPLE

Following results were found by measuring color, turbidity and pH of samples

Table 1. Physical and chemical analysis of samples

Sample	pH	Color	Turbidity
WSK 1	6.3	Light brown	Slight turbid
WSK 2	7.5	Light brown	Turbid
WSL	6.5	Light brown	Slight turbid
WJ	8	Brown	Highly turbid
SSK 1	6	Black	-
SSK 2	6.2	Black	-
SSL	6	Brownish black	-
SJ	6.1	Brownish black	-

The samples were given following codes which are as follows

SSK-1 – Soil sample 1 of Sikka

SSK-2 – Soil sample 2 of Sikka

SSL - Soil sample of Salaya

SJ - Soil sample of Jodiya

WSK-1 – Water sample 1 of Sikka

WSK-2 – Water sample 2 of Sikka

WSL – Water sample of Salaya

WJ – Water sample of Jodiya

The chemical analysis of the samples listed in the table – 1shows the pH values of the collected samples. As can be seen from the table, most of the samples have pH values in the range of 6.0 to 6.5. Only two samples have pH above 7 indicating that the marine samples collected have slightly acidic to neutral pH. All water samples were found to be turbid and color of samples were found to be brown to black.

SALT TOLERANCE OF SAMPLES

Turbidity was found in all flasks for all samples containing nutrient broth with graded amount of NaCl viz. 1% to 10%. Sticky products were also found to stick on internal surface of all flasks.

Fig. 3.1 Salt tolerance of samples

ISOLATION AND CHARACTERIZATION OF ISOLATES

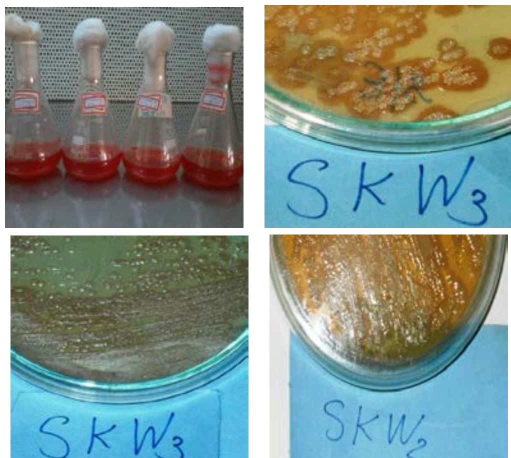

Fig. 3.2 Biosurfactant producers in hydrocarbon containing liquid nad solid media

Total 24 bacterial isolates were isolated from water and soil samples collected from ports.

Table 2. Colony characterization of isolates

Isolates	Shape	Size	Margin	Elevation
SA_2P	Irregular	Small	Undulate	Flat
SJ 21	Irregular	Big	Undulate	Raised
SJ 23	Irregular	Big	Undulate	Raised
SKW_3	Round	Small	Entire	Flat
SSK 112	Regular	Small	Entire	Flat
SSK 121	Regular	Small	Entire	Slightly raised
SSK 13	Round	Small	Entire	Flat
SSK 21	Irregular	Big	Entire	Slightly Raised
SSK 22	Round	Big	Entire	Slightly Raised
SSK 23	Regular	Medium	Entire	Slightly raised
SSL 12	Iregular	Big	Entire	Flat
SSL 2	Round	Big	Entire	Slightly Raised
WB_1P 1	Regular	Big	Entire	Flat
WB_1P 2	Irregular	Big	Entire	Flat
WJ 11	Irregular	Medium	Entire	Flat
WJ 12	Regular	Small	Entire	Flat
WJ 13	Irregular	Big	Entire	Flat
WJ 21	Regular	Medium	Entire	Flat
WJ 22	Irregular	Medium	Entire	Flat
WSK 11	Round	Small	Entire	Slightly Raised
WSK 12	Irregular	Big	Entire	Slightly Raised
WSL 1	Irregular	Big	Entire	Slightly Raised
WSL 21	Irregular	Big	Undulate	Raised
WSL 22	Irregular	Big	Undulate	Raised

Table 3. Colony characterization of isolates

Isolates	Texture	Consistency	Opacity
SA_2P	Smooth	Gummy	Translucent
SJ 21	Smooth	Gummy	Opaque
SJ 23	Smooth	Gummy	Opaque
SKW_3	Smooth	Gummy	Translucent
SSK 112	Smooth	Gummy	Translucent
SSK 121	Smooth	Gummy	Opaque
SSK 13	Smooth	Gummy	Translucent
SSK 21	Smooth	Gummy	Translucent
SSK 22	Smooth	Gummy	Translucent
SSK 23	Smooth	Gummy	Opaque
SSL 12	Smooth	Gummy	Translucent
SSL 2	Smooth	Gummy	Translucent
WB_1P 1	Smooth	Gummy	Tanslucent
WB_1P 2	Smooth	Gumm	Translucent
WJ 11	Smooth	Gummy	Translucent
WJ 12	Smooth	Gummy	Translucent
WJ 13	Smooth	Gummy	Translucent
WJ 21	Smooth	Gummy	Opaque
WJ 22	Smooth	Gummy	Translucent
WSK 11	Smooth	Gummy	Translucent
WSK 12	Smooth	Gummy	Translucent
WSL 1	Smooth	Gummy	Translucent
WSL 21	Smooth	Gummy	Opaque
WSL 22	Smooth	Gummy	Opaque

As shown in above table 2 & 3, 52% of all isolates had big colony and irregular shape while rest of had small and regular shape colonies. 100% of all isolates produced gummy colonies with smooth texture. 7 isolates out of 24

had produced pigments of green colored while rest of were non- pigmented.

STAINING OF ISOLATES

Cell morphology of all isolates were checked by using monochrome staining, gram staining and capsule staining and the following table shows results of staining.

Table 4. Cell morphology of isolates

Isolates	Capsule staining
SA_2P	Light violet colored capsule was observed
SJ 21	Light violet colored capsule was observed
SJ 23	Light violet colored capsule was observed
SKW_3	Light violet colored capsule was observed
SSK 112	Light violet colored capsule was observed
SSK 121	Light violet colored capsule was observed
SSK 13	Light violet colored capsule was observed
SSK 21	Light violet colored capsule was observed
SSK 22	Light violet colored capsule was observed
SSK 23	Light violet colored capsule was observed
SSL 12	Light violet colored capsule was observed
SSL 2	Light violet colored capsule was observed
WB_1P 1	Light violet colored capsule was observed
WB_1P 2	Light violet colored capsule was observed
WJ 11	Light violet colored capsule was observed
WJ 12	Light violet colored capsule was observed
WJ 13	Light violet colored capsule was observed
WJ 21	Light violet colored capsule was observed
WJ 22	Light violet colored capsule was observed
WSK 11	Light violet colored capsule was observed
WSK 12	Light violet colored capsule was observed
WSL 1	Light violet colored capsule was observed
WSL 21	Light violet colored capsule was observed
WSL 22	Light violet colored capsule was observed

Table 5. Cell morphology of isolates

Isolates	Gram staining	Monochrome staining
SA_2P	Gram negative short rod	Short rods
SJ 21	Gram positive coccobacillus	Coccobacillus
SJ 23	Gram positive rod	Rods
SKW_3	Gram negative coccobacillus	Coccobacillus
SSK 112	Gram negative cocci	Coccobacillus
SSK 121	Gram positive cocci	cocci
SSK 13	Gram negative short rod	Short rods
SSK 21	Gram negative short rod	Coccobacillus
SSK 22	Gram negative coccobacillus	Coccobacillus
SSK 23	Gram positive coccobacillus	Coccobacillus
SSL 12	Gram negative coccobacillus	Coccobacillus
SSL 2	Gram negative cocci	Cocci
WB_1P 1	Gram negative coccobacillus	Coccobacillus
WB_1P 2	Gram negative short rod	Coccobacillus
WJ 11	Gram negative coccobacillus	Coccobacillus
WJ 12	Gram negative coccobacillus	Coccobacillus
WJ 13	Gram negative coccobacillus	Short rods
WJ 21	Gram positive short rods	Coccobacillus
WJ 22	Gram negative cocci	Cocci
WSK 11	Gram negative coccobacillus	Coccobacillus`
WSK 12	Gram negative short rods	Coccobacillus
WSL 1	Gram negative rods	Rods
WSL 21	Gram positive cocci	Cocci
WSL 22	Gram positive coccobacillus	Coccobacillus

As shown in above table 4 & 5 all isolates was capsulated and light violet colored capsules were observed. Out of 24 isolates 4 isolates were rod shaped while rest of were coccobacillus in shape. Gram staining of isolates shows 20% of all isolates were gram positive while remaining 80% were gram negative.

SCREENING OF BIOSURFACTANT PRODUCING BACTERIA

OIL SPREADING TECHNIQUE (PLATE ASSAY)

All isolates had given oil spreading test positive which shows the presence of Biosurfactant in the broth. Control plate had spreading of oil is more on the surface of water while in test samples spreading is less compare to control plate. Biosurfactant reduces surface tension so spreading is less in test samples. Following pictures shows positive plate
assay.

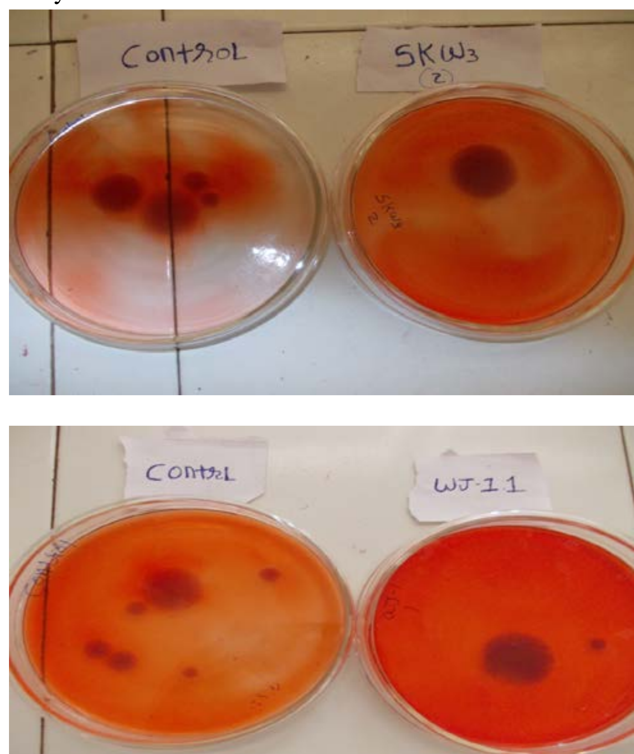

Fig. 3.3 Plates showing positive plate assay

EMULSIFICATION INDEX (E_{24} ACTIVITY)

E_{24} activity was checked for all isolates and the following graphs shows emulsification index of isolates.

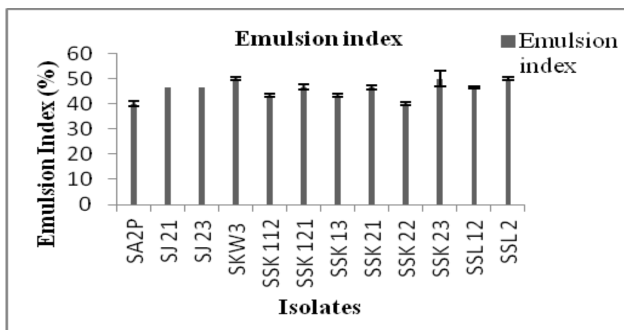

Fig. 3.4 Emulsion Index of isolates

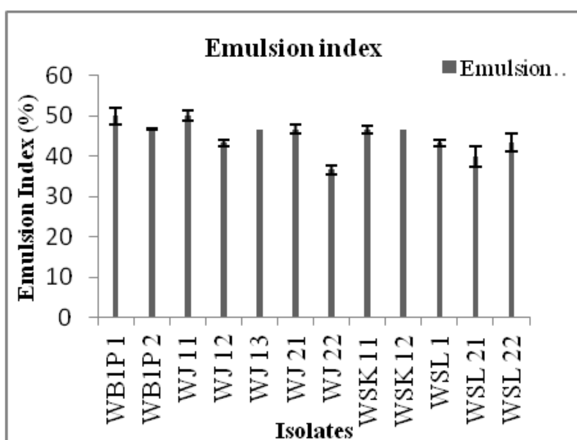

Fig. 3.5 Emulsion Index of isolates

As shown in above graphs out of 24 isolates only 2 had less than 40% of emulsion index while remaining all had more than 40% of emulsification index. Highest emulsification index were observed for SKW_3, SSK-23, SSL-2, WB_1P-1 and WJ-11 and that was 50% for all. Lowest emulsification index was 36.66% for WJ-22. Emulsification index was measured after 24 hrs which shows stability of emulsion formed in tubes.

IV. CONCLUSION

Total 24 isolates were isolated from soil and water samples for Biosurfactant production and isolates from water had more potential for Biosurfactant production in compare to isolates from soil. SKW_3, SSK-23, SSL-2, WB_1P-1 and WJ-11 had more potential in compare to other isolates. All isolates had given positive test for Rhamno presence and oil spreading technique and also all isolates were found to be producer of capsule. Most of isolates had coccobacillus shape and most of them were gram negative. Further analysis will be carried out by optimization of cultural condition for production of Biosurfactant and identification of chemical structure of Biosurfactant.

V. ACKNOWLEDGEMENT

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VI. FUTURE SCOPE

Future scope of this particular study includes optimization of growth parameter for the better production of Biosurfactant, characterization of Biosurfactant using TLC and HPLC, comparison of chemical emulsifier with Biosurfactant and biochemical characterization of Biosurfactant producing marine bacteria.

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