ORIGINAL RESEARCH PAPER

Microbiology

ISOLATION OF BIOSURFACTANT PRODUCING *PSEUDOMONAS AERUGINOSA* VB MCC 4751 FROM SEA WATER NEAR URAN BEACH

KEY WORDS: Biosurfactants, marine bacteria, screening, 16S rRNA identification, extraction

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ABSTRACT

Biosurfactants are structurally diverse group of bioactive compounds produced by variety of marine microorganisms surviving at extremes of environmental conditions. Due to wide variety of industrial applications of biosurfactants, researchers are focussing on synthesizing these natural products due to their high biodegradability, low toxicity and activity at very low critical micelle concentration as compared to the chemical surfactants. In the present study biosurfactant producing bacteria are isolated from sea water from variety of locations such as Mumbai, Chennai, Orissa, Goa and Uran beach near ONGC plant. About 33 morphologically different bacterial isolates obtained which were primarily screened for biosurfactant production by variety of tests such as drop collapse test, emulsification assay, emulsification index, oil displacement test ,hydrocarbon overlay agar method and blue agar plate method. Potential biosurfactant producer was confirmed by surface tension measurement studies using Optical Contact Angle Goniometer by pendant drop technique. Isolate UW1 isolated from sea water near Uran beach showed highest surface tension reduction of the growth medium from 69.91 (mN/m) to 26.18 (mN/m) i.e 43.73(mN/m) when grown in a mineral salt medium supplemented with trace element solution. 16S rRNA studies revealed closest phylogenetic affiliation of UW1 to *Pseudomonas aeruginosa* with 99.45% identity. Acid precipitation of cell free broth followed by solvent extraction using equal quantity of Ethyl acetate: Methanol in 4:1 ratio yield yellowish brown coloured viscous crude biosurfactant. In future purification using column chromatography and physicochemical characterization using TLC, FTIR, NMR and LC-MS will be carried out for elucidation of structure of biosurfactant.

INTRODUCTION

Biosurfactants are surface active compounds produced mainly by microorganisms. They are amphiphilic molecules with hydrophilic and hydrophobic moieties that reduce surface and interfacial tensions by accumulating at the interface between two immiscible fluids like oil and water (Satpute et al., 2017; Sumathi et al. ,2016). Due to their low toxicity and biodegradability, these are environmental friendly alternative to synthetic chemical surfactants. Biosurfactants are widely used in different industries such as food processing, pharmaceutical, agricultural, cosmetic and petroleum due to which these are considered as 'Multifunctional Biomolecules of the 21st Century'. Biosurfactants are categorized into different types based on their chemical nature such as lipoproteins, glycolipids, phospholipids, polymeric and particulate surfactants. They have a very low critical micelle concentrations (CMC) (Santos et al. ,2016; Chopra et al., 2020).

Majority of marine microorganisms capable of producing variety of bioactive compounds are yet to be discovered. Biosurfactants are one such functional molecules produced by marine microorganisms and can be used protect marine environment by their ability to degrade crude oil, a major cause of sea water pollution (Maneerat, S.,2005). Marine microorganisms are receiving much more attention due to their adaptability to survive in an extreme of environmental conditions. They grow over wide range of environmental conditions such as pH, temperature, pressure and nutrient conditions during which they produce novel bioactive compounds (Floris et al.,2018).

In the present study, focus is given on the isolation, screening and identification of potential biosurfactant producing bacteria from different sea water samples and preliminary detection of biosurfactant.

MATERIAL AND METHODS

Sea water samples were collected from various coastal areas of India. locations such as Calangute beach (Goa), Juhu chaupati (Mumbai), Gate way of India (Mumbai), Gopalpur (Orissa), Elliot's beach (Chennai). Sea water sample and sediment fromUran beach near ONGC plant (Navi Mumbai), The samples were collected in sterile bottles and stored in refrigerator for further use.

Enrichment, Isolation of Marine Bacteria

Sea water samples and sediment sample were inoculated in sterile Zobell marine broth for enrichment of bacteria. Flasks were kept for 24-72 hours at 28°C on shaker incubator (150 rpm). Enriched media was then serially diluted and plated on Zobell marine agar plates. Plates were incubated for 24-48 hours at 28°C. Isolated bacterial colonies showing morphologically different characteristics were individually picked and streaked onto fresh Zobell marine agar plates to maintain pure culture. Screening of these isolates were carried out for testing biosurfactant production (Satpute et al., 2008).

Screening of biosurfacatnt producing bacteria

33 bacteria were isolated on Zobell Marine agar plates. Various screening methods were performed for isolation of efficient biosurfactant producers. Bacterial isolates were inoculated in Zobell marine broth and incubated at 28 °C for 48 hours. Culture broth was centrifuged at 10,000 rpm for 15 minutes at 4°C. Cell free broth was used for some of the screening methods and culture suspension was used other screening tests.

Drop collapse test

2 µl of engine oil was applied to the well regions delimited on the covers of 96 – well microplate. Plates were equilibrated for 24 hours. 5 µl of cell free broth was transferred to the oil coated well regions and drop size was observed 1 minute later. The biosurfactant producers were detected from the increase in drop size and drop collapsing within a minute from the oil coated well. Distilled water was used as negative control and 0.1%SDS was used as a positive control (Thavasi et al., 2011).

Further the test was confirmed by placing 30 µl of cell free supernatant on parafilm M. After one minute the shape of the

drop was observed. Positive test was confirmed by the drop spread or collapse. If the drop remained beaded then result was recorded as negative. Distilled water was used as negative control and 0.1%SDS was used as a positive control.

Oil Displacement Test

20 µl of engine oil was added to a Petridish containing 20 ml of distilled water. On the oil surface ,10 µl of cell free culture broth was added. Displacement of oil with an oil free clearing zone confirmed the presence of the surface-active agent in the cell free broth. No displacement or clear zone was observed with distilled water which was used as a negative control. 0.1% SDS was used as a positive control (Vanavil et al.,2013).

Emulsification index

Emulsification activity was measured according to the method of Cooper and Goldenberg (1987) with a slight modification. To 2 ml of culture supernatant 2 ml of engine oil were added and vortexed at high speed for 2 min. The mixture was allowed to stand for 24 hours. the emulsification index (E24) was calculated by the total height of the emulsion layer divided by the total height and expressed as percentage (Maneerat, S. et al., 2007; Santos et al. 2017).

Emulsification assay

Bacterial isolates were grown in ZMB for 24 hours at 28°C in shaker incubator at 150 rpm. Culture broth was then centrifuged at 10,000 rpm for 15 minutes at room temperature and cell free supernatant was collected. 3 ml of cell free supernatant was mixed with 0.5 ml of engine oil and mixed vigorously for 2 minutes using vortex mixer. This mixture was kept undisturbed at 28°C for 1 hour for separating aqueous and oil phase. Further aqueous phase was collected carefully using 1 ml micropipette and absorption was measured at wavelength of 400 nm. Uninoculated broth was taken as a blank. Emulsification activity per ml (EU/ml) was calculated by using the formula,

1 Emulsification unit = 0.01O.D. × Dilution factor.
(Satpute S. *et al*. 2008).

Hydrocarbon overlay agar method

40 µl of engine oil was coated on Zobell marine agar plates. Broth culture of bacterial isolates were spot inoculated on these coated plates. Plates were incubated at 28°C for 7-10 days. Biosurfactant production was indicated by an emulsified halo surrounding the bacterial growth and was considered as positive test (Satpute et al., 2008).

Blue agar plate method

Blue agar plates were prepared by using mineral salts medium MSM, (2 g Glucose,0.7 g KH_2PO_4, 0.9 g Na_2HPO_4, 2 g $NaNO_3$, 0.4 g $MgSO_4$. $7H_2O$, 0.1 g $CaCl_2$. $2H_2O$, 1% NaCl per litre. Filter sterilized 2 ml of trace elements [per liter,2 g $FeSO_4$. $7H_2O$, 1.5 g $MnSO_4$. H_2O, 0.6 g $(NH_4)_6Mo_7O_{24}$. $4H_2O$] is added separately) containing of 0.2 g cetyltrimethylammonium bromide (CTAB) and 0.005 g methylene blue, and 1.5% agar. 24 hours grown bacterial cultures were spot inoculated and incubated at 28°C for up to 7 days. Extracellular glycolipids specifically rhamnolipids and other anionic surfactants are detected by this method. A dark blue halo around the bacterial growth was considered as a positive test (Jamal, et al., 2012; Satpute et al., 2008; Seigmud et al., 1991).

Cultures showing maximum tests positive for biosurfactant production were selected for further studies. Out of 33 bacterial isolates, 3 isolates were selected which were showing immediate drop collapse. These isolates were further screened on the basis of surface tension measurement studies.

Production of biosurfactant and Surface tension measurement

For biosurfactant production, mineral salt medium was used (MSM). 3 bacterial cultures showing maximum tests positive for biosurfactant production were streaked on mineral salt medium agar plates. Plates were incubated for 48-72 hours at 28° C. Isolated colony of each culture was inoculated in a mineral salt medium and incubated at 28 °C for 48 hours. 6% inoculum of each culture was inoculated in 150 ml of mineral salt medium and kept in shaker incubator at 28 °C for 7 days at 150 rpm (Gunther et al., 2005; Siegmund, et al., 1991; Chopra et al., 2020). Culture broth was centrifuged at 10,000 rpm for 15 minutes.

Cell free supernatant was used for surface tension measurement studies. Surface tension measurement was carried out using Optical Contact Angle Goniometer (OCA 15+, Data Physics Instruments GmbH, Germany) by pendant drop technique. In this method, the equilibrium shape of the pendent drop in the gravitational force was captured using charge-coupled device (CCD) camera and analyzed in real time using SCA 20 software (Thavasi et al., 2011).

Identification of efficient biosurfactant producing culture Uw1

Culture UW1 showing significant reduction in surface tension was selected for further studies.

Identification of culture was carried out by biochemical characterization using Bergey's Manual of Systematic Bacteriology.

Genetic analysis was carried out using 16s r RNA gene sequencing method(partial) using Sophisticated Analytical Instrumentation Facility at Agharkar research institute, Pune (Zhang et al.,2012; Chopra et al., 2020).

The cultural sample was processed for identification in the following manner –

1. Genomic DNA was isolated from the culture by using Sigma's,''GenElute Bacterial Genomic DNA'' Kit.
2. PCR was carried out using the following combination of primers –

27F- 1492R (universal primers for 1.5 kb fragment amplification for eubacteria)

The PCR was set using the following Mix –
ddH2O - 12.0ul
10 X PCR buffer – 2.0ul
dNTPs – 2.0ul
Forward primer - 0.4ul
Reverse primer - 0.4ul
Taq DNA Pol. - 0.2ul
Template DNA - 3.0ul
Total volume - 20.0ul
Template DNA – Genomic DNA

3. The PCR products obtained from above reactions were then processed for Cycle sequencing reaction (PCR performed using only one primer – 27F).
4. Following the above reaction, the samples were cleaned up and loaded on the sequencer (Avant 3100 Gene Analyzer).

Protocol:

Total genomic DNA was isolated using GeneElute Genomic DNA isolation kit (Sigma, USA) as per the manufacturer's instructions and used as template for PCR. Each reaction mixture contained approximately 10 ng of DNA; 2.5 mM MgCl2; 1x PCR buffer (Bangalore Genei, Bangalore, India);

200 µM each dCTP, dGTP, dATP, and dTTP; 2 pmol of each, forward and reverse primer; and 1 U of Taq DNA polymerase (Bangalore Genei, Bangalore, India) in a final volume of 20 µl. 27F and 1492R primers were used to amplify almost entire 16S rRNA gene, as described previously (Rawlings 1995). The PCR

was performed using the Eppendorf Gradient Mastercycler system with a cycle of 94 °C for 3 min; 32 cycles of 94 °C for 45 sec, 51 °C for 1 min and 72 °C for 1.30 min and final extension at 72° C for 10mins, and the mixture was held at 4°C. The PCR product was precipitated using polyethylene glycol (PEG 6000, 8.5%) washed thrice using 70% ethanol and dissolved in Tris HCL (10mM, pH 8.0).

The ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, Calif.) was used for the sequencing of the PCR product. A combination of universal primers was chosen to sequence the nearly complete gene (Rawlings 1995; Muyzer et al. 1993).

The sequencing reaction and template preparation were performed and purified in accordance with the directions of the manufacturer (Applied Biosystems). Samples were run on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The sequencing output was analysed using the accompanying DNA Analyzer computer software (Applied Biosystems). The sequence was compared with EZTAXON for sequence identity.

Extraction of biosurfactant

Culture was grown in MSM broth at 28 °C for 7 days in shaker incubator at 150 rpm. Broth was centrifuged at 10,000 rpm for 15 minutes at 4 ° C. Cell free supernatant was used for extraction of biosurfactant.

Cell free supernatant was acidified to pH 2 with 6N HCl and then kept in the refrigerator overnight for precipitation of biosurfactant. Biosurfactant was extracted using equal volume of the acidified cell free supernatant and the solvent system, Ethyl acetate and methanol in 4:1 ratio at room temperature. After extraction ,the mixture was kept stationary to allow separation of phases. Organic phase was collected and kept at 40 ° C for evaporation of the solvent. Yellowish brown colored viscous crude biosurfactant was obtained and stored in the refrigerator for further studies. (Patowary et al. 2017;Chopra et al.,2020 ;Satpute et al., 2010; Ceresa et al., 2020)

RESULTS AND DISCUSSION

Screening of biosurfactant producing bacteria

33 morphologically distinct bacterial isolates were obtained from various sea water samples. As indicated in Table 1. All isolates were examined for biosurfactant production by various screening methods. Out of 33 bacterial isolates 16 isolates showed positive drop collapse test and 9 isolates showed significant increase in drop size on parafilm M. As Parafilm M is hydrophobic in nature drop collapse indicates ability of biosurfactant to wet hydrophobic surface (Chopra et al., 2020). Out of 16 isolates showing positive drop collapse test only 3 isolates GO3, CHE5, GWI1 showed good emulsification activity measured by emulsification assay. Good emulsification activity (E24) by measuring emulsification index was exhibited by 10 isolates. Maximum emulsification activity was shown by culture UW1which was 56.66% followed by GWI3 and GWI1(50%). These cultures were also showing positive drop collapse test. Around 11 isolates showed emulsified halo around bacterial growth in the hydrocarbon overlay agar method .10 isolates showed positive results for oil spread method. Blue agar plate method using CTAB which is used to detect extracellular glycolipids and anionic biosurfactants was found to be positive for 7 bacterial isolates. A dark blue halo around the growth was indicative of anionic surfactants /glycolipids secreted by the bacteria that form a dark blue, insoluble ion pair with cetyltrimethylammonium bromide and methylene blue (Sumathi et al.,2016; Satpute et al., 2008; Seigmud et al., 1991).

Table 1: Results of various screening tests.

Bacterial isolate	Drop collapse test	Emulsification assay (EU/ml)	Emulsification Index (%)	Hydrocarbon overlay agar method	Oil spreading assay	Blue agar plate method
GO1	+	10	Nil	-	+	-
GO2	-	18	Nil	-	+	-
GO3	-	56	Nil	+	-	-
GO4	-	23.8	20	+	-	-
GO5	-	25.0	33.33	+	-	-
GO6	-	33	10	-	-	-
BOM1	-	21	20	+	-	-
BOM2	+	26	33.33	-	+	-
BOM3	+	13	33.33	-	-	-
BOM4	-	Nil	16.66	+	-	-
BOM5	+	19	20	-	-	-
CHE1	-	Nil	30	-	+	-
CHE2	-	27	Nil	-	-	-
CHE3	++	25	50	-	-	+
CHE4	++	24	26.66	+	-	-
CHE5	++	46	46.66	-	-	+
ORI1	-	Nil	16.66	-	-	-
ORI2	-	11	46.66	-	-	-
ORI3	-	Nil	Nil	-	-	-
ORI4	++	1	46.66	-	+	-
ORI5	-	Nil	Nil	+	-	-
UW1	++	26	56.66	+	+	+
UW2	++	14	50	-	+	+
UW3	++	7	50	+	-	+
GWI1	++	62	50	+	+	+
GWI2	+	24	33	-	-	+
GWI3	++	28	50	+	+	-
GWI4	+	8	Nil	+	-	-
GWI5	+	1	Nil	-	-	-
GWI6	-	10	20	-	+	-
GWI7	-	26	10	-	-	-
US1	-	18	46.66	+	+	-
US2	-	4	23.33	-	-	-

GO = Goa, BOM= Juhu chaupati, Mumbai, CHE = Chennai, ORI =Orissa, UW = Uran beach water, Navi Mumbai, GWI = Gate way of India, Mumbai, US = Uran beach sediment, Navi Mumbai. + indicates positive test for drop collapse in one minute, ++ indicates immediate flattening of the drop with considerable increase in size.

Fig.1 Drop collapse test

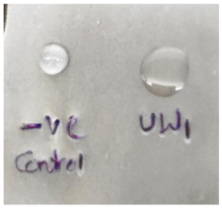


Fig.2 Emulsification index

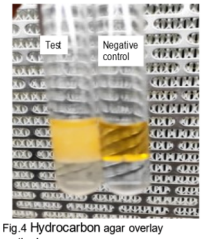


Fig.3 Blue agar plate

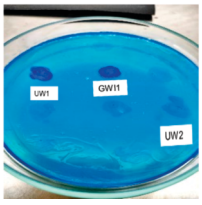
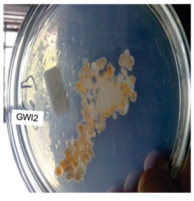


Fig.4 Hydrocarbon agar overlay method



Production of biosurfactant and Surface tension measurement
Production of biosurfactant was carried out using mineral salt medium. After 7 days of incubation at 28°C cell free supernatant was used for surface tension measurement studies. Foam formation in the medium is positive indicator of biosurfactant production as shown in Fig.5

Surface tension measurement studies were carried using Optical Contact Angle Goniometer (OCA 15+, Data Physics Instruments GmbH, Germany) by pendant drop technique. 3 bacterial isolates (UW1, GWI1 and GWI3) showing maximum screening tests positive were selected for surface tension measurement studies. Cell free supernatants of these cultures grown in mineral salt medium were used for determining surface tension. Distilled water was used initially for calibration of the instrument. Uninoculated mineral salt medium was used as a control (Santhini et al.,2014; Chopra et al.,2020). Maximum surface tension reduction was observed in CFS of UW1 bacterial isolate i.e.43.73 mN/m .

Table 2: results of surface tension measurement

Bacterial isolate	Surface tension readings(mN/m)	Surface tension reduction(mN/m)
UW1	26.18	43.73
GWI1	39.97	29.94
GWI3	35.11	34.8

Distilled water -72.1, Uninoculated media control – 69.91
As significant reduction in surface reduction was observed by cell free supernatant of UW1 culture which was isolated from sea water near Uran beach, further studies such as identification of culture using 16S rRNA method and extraction using solvent were carried out using this culture.

Extraction of biosurfactant
Acid precipitation of cell free supernatant followed by extraction with equal volume of Ethyl acetate : Methanol (4:1) was carried out. Yellowish brown colored crude extract of biosurfactant was obtained after drying at 40o C as indicated in Fig.6. (Patowary et al. 2017;Chopra et al. ,2020; Satpute et al.,2010;Ceresa et al.,2020).

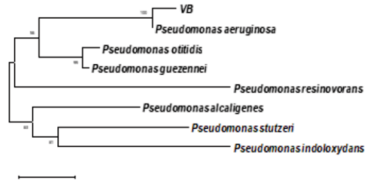
Fig.5 Growth of biosurfactant producer in MSM

Fig.6.Crude biosurfactant after solvent extraction

Identification of bacterial isolate Uw1
The identification of using 16S rRNA gene sequencing (partial) revealed its Closest Phylogenetic affiliation to *Pseudomonas aeruginosa* (Figure 1B) with 99.45% identity.

Identified culture was deposited in National centre for Microbial resource (NCMR, Pune and was allocated the accession number MCC 4751.

Identification report by 16SrRNA gene sequencing approach revealed following results.



Identification report by 16SrRNA gene sequencing approach

VB

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GTAAACGATGTCGACTAGCCGTTGGGATCCTTGAGATCT
TAGTGGCGCAGCTAACGCGATAAGTCGACCGCCTGGG
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GGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCG
AAGCAACGCGAAGAACCTTACCTGGCCTTGACATGCTG
AGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCA
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GAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTT
GTCCTTAGTTACCAGCACCTCGGGTGGGCACTCTAAGG
AAACTGCCGGTGACAAACCGGAGGAAGGGGGGATGAC
GTCAAGTCATCATGCCTTACGGCAGGGCTACACGTGCTA
CATGGTCGGTACAAGGTTG

Results of biochemical tests revealed the isolate UW1 showing positive results for Oxidase test and Citrate utilization test.

CONCLUSION

Biosurfacatnt producing marine bacteria *Pseudomonas aeruginosa* VB isolated from sea water near Uran beach close to ONGC plant will find potential applications in variety of fields including bioremediation of oil spills which is a major concern in the marine environment. Many biosurfactants acts as an antimicrobial agent that can treat multidrug resistant bacteria. Marine microorganisms and their enzymes are preferred as these are capable of adapting to fluctuations in the environmental conditions.

Further studies will be carried out on purification of crude biosurfactant using column chromatography and physicochemical characterization of purified biosurfactant using TLC, FTIR, NMR and LC-MS for structural analysis of the biosurfactant.

Acknowledgement
First author would like to thank the Department of Physics, SPPU, for providing facility for surface tension measurement studies.

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