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Isolation and screening of microorganisms producing biosurfactants

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Short communication

Abstract

Oil spills can be hazardous for marine life and can cause physiological as well as biochemical damage. Biosurfactants are eco-friendly surface-active compounds, which reduce surface tension at the interface between oil and water and exhibit emulsifying activity. Microorganisms producing biosurfactants are omnipresent and can also be extremophiles. Bushnell and Haas medium is suitable for isolating biosurfactant producers as it allows the selection of carbon sources. From six different oil-contaminated sites, eight morphologically different isolates were generated. These isolates were screened using various assays namely, Haemolysis assay, Phenol: Sulphuric acid assay, Microtitre plate assay, oil displacement assay, emulsification index assay and drop collapse assay. The isolate CD03A was selected as a potent isolate and was identified as *Alcaligenes faecalis* by 16s-rRNA sequencing. The biosurfactant produced by the organism was subjected to FTIR and results indicated that it might contain alcohol and nitro group along with N-H bend.

Keywords: *Biosurfactant, Emulsification Index, Drop collapse assay, oil spill, FTIR*

Introduction

In India, around 84,361,000 metric tons of crude oil are processed by oil refineries (Petroleum Planning and Analysis Cell, 2021). The net import of crude oil and its products in India per year is around 68,708,000 metric tons (Petroleum Planning and Analysis cell, 2021). Most of this trade is carried out through ships via sea. An oil spill is defined as the dissemination of liquid petroleum hydrocarbons in the surroundings due to human activity or natural disasters. In the last decade, around 164,000 tonnes of oil were spilt (ITOPF, 2021). Oil can have detrimental physiological and or biochemical effects on the living organisms in the sea. Absorption, ingestion or inhalation of oil can handicap marine organisms. Polyaromatic hydrocarbons (PAHs) can cause sub-lethal biochemical injury or even death of fish eggs and larvae (Langangen *et al.*, 2017). Another aspect of damage is that some of the polynuclear compounds of petroleum are carcinogenic and are difficult to undergo bioremediation. Such compounds can come up the marine food chain and affect fishes that can be used as feed or food (Charan and Patel, 2017). Biosurfactants are ecological, biodegradable, less toxic, and active at a wide range of pH, temperature and salinity. Whereas the chemical agents used for the treatment of oil spills are perilous to the environment (Pacwa-Płociniczak *et al.*, 2011). Biosurfactants are surface-active compounds produced by microorganisms for hydrocarbon consumption. (Uzoigwe *et al.*, 2015). Biosurfactant reduces surface tension at the interface between air and water and exhibits emulsifying activity (Joshi and Shekhawat, 2014). Thus, the main aim of this research was to isolate biosurfactant-producing microorganisms and screen them for their biosurfactant-producing ability.

Material and methods

Chemicals and media

For enrichment and isolation purposes, Bushnell and Haas (BH) broth was purchased from HiMedia Laboratories Pvt Ltd. The blood agar base used to check for the haemolysis pattern of the isolate was also purchased from HiMedia Laboratories Pvt. Ltd. and human blood for overlaying the media was obtained from Central Hospital Blood Bank, Ulhasnagar. All the chemicals required were of analytical grade and were purchased from Loba Chemie Pvt Ltd.

Sample collection and maintenance

Oil-contaminated soil and water samples were taken from six different sites, including creeks in and around thane and different petrol pumps, before the rainy season, as oil-

contaminated water could wash off due to rain. A total of five oil-contaminated soil samples were collected, out of which one was collected from a creek in Dombivli along with a water sample and the remaining samples were collected from petrol pumps. Samples were maintained at 4 °C till further use (Soltanighias *et al.*, 2019). The geographical locations of sampling sites are listed in Table 1.

Table 1. Details of sampling sites

No.	Sampling site name	Geographical Co-ordinates	Sample
1	Retibunder, Dombivli	19° 13′ 40.1″ N 73° 04′ 03.9″ E	Water
2	Retibunder, Dombivli	19°13′ 40.7″ N 73° 04′ 04.2″ E	Soil
3	HP Petrol pump, Kalyan	19°14′ 18.24″ N 73° 7′ 35.4″ E	Soil
4	Bharat Petroleum, Dombivli	19°13′ 2.208″ N 73° 7′ 7.356″ E	Soil
5	Techno Petrol pump, Dombivli	19°12′ 41.112″ N 73°6′46.224″ E	Soil
6	Bharat Petroleum, Dombivli	19°11′12.12″ N 73°5′31.632″ E	Soil

Enrichment and isolation

Enrichment and isolation were executed as described by Ezebuiro *et al.* (2019) with slight modification. One gram of soil samples and 5.0 ml of water samples were used for enrichment purposes. Enrichment was carried out in 3 stages using Bushnell and Haas broth. In the first stage, samples were enriched for one week on an orbital shaker with 0.5% phenol as a carbon source and 3% NaCl at room temperature. From each enriched sample, 1.0 ml was taken as inoculum for second-stage enrichment. It was carried out in Bushnell and Haas Broth supplemented with 1.0% phenol and 3.0% NaCl, on an orbital shaker for one week at room temperature. For the last stage of enrichment, 1.0 ml from the second enriched batch was used as inoculum. The last stage enrichment was carried out in Bushnell and Haas broth containing 1% ship crude oil and 3% NaCl for one week on an orbital shaker at room temperature.

For isolation, a loop full of tertiary enrichment was streaked on Bushnell and Haas agar (BHA) containing 1.0% crude oil and 3.0% NaCl, which was used to selectively isolate halotolerant organisms as sea salinity ranges from 3.2 - 3.7 % (Veerlan *et al.*, 2021). The plates were incubated at room temperature for 48-72 hours.

Extraction of cell-free supernatant (CFS)

The isolated pure colonies were inoculated in fresh sterile Bushnell and Haas broth containing 1.0% crude oil and 3.0% NaCl and were incubated at room temperature on an orbital shaker for 72 hours. The CFS was procured by centrifuging the contents of the flask at 3000 rpm at 4 °C for 15 minutes (Abbasi *et al.*, 2012).

Haemolysis assay

For this assay, culture was grown in Bushnell and Haas broth for 3.0 days and was streaked on a Blood agar plate followed by incubation at RT for 24 hours (Saravanan and Vijayakumar, 2012).

Phenol: Sulphuric confirmatory assay

It is a confirmatory assay for the production of biosurfactants. The CFS was added to 1.0 ml 5.0% phenol after which 5.0 ml of con. H_2SO_4 was added in a drop-wise manner. The formation of orange colour indicated a confirmatory positive test (Aziz *et al.*, 2014).

Micro-titre plate assay

This assay is based on the fact that surfactants present in the aqueous phase cause optical distortion. For this assay, cell-free supernatant was taken and 100 μl of it was added to the well. A sheet of paper with a grid on it was placed below the Microtitre plate. The image of graph paper was observed for distortion (Walter *et al.*, 2010).

Drop collapse assay

In this assay, a 96-well microtiter plate was coated with 2.0 μl of crude oil and was allowed to stand for 24 hours for stabilizing. Then 5.0 μl of CFS was added to these wells. Then after 1.0 minute the diameter of the drop was measured using a magnifying glass. Test for production of biosurfactant is considered positive if the diameter produced is 1.0 mm larger than the negative control which was distilled water. A positive standard was placed by preparing 1.0 mg/ml solution of Triton X-100 (Thavasi *et al.*, 2011).

Oil displacement assay

In this assay, an empty Petri plate was filled with 20 ml of water. To it, a few drops of oil were over-layered. Then a drop of CFS was added to its surface and the formation of a clear zone was observed (Ndibe *et al.*, 2018). Negative control was set by using a drop of distilled water and positive control was set by using 1.0 mg/ml solution of Triton X-100.

Emulsification index assay (E24)

This assay was performed to find out the emulsifying capacity of the produced biosurfactant. In this assay, 2.0 ml of CFS was added along with 2.0 ml of oil in a tube. The mixture was strenuously vortexed for 2.0 minutes and then kept standstill for 24 hours. Emulsifying capacity was calculated in percentage.

The height of the emulsified layer (cm) was divided by the total height of the liquid column (cm) (Rajesh *et al.*, 2013). The DW was used as negative control and 1.0 mg/ml solution of Triton X-100 as a positive control. E_{24} = (Height of emulsion formed/ Total height of solution) x 100

Biochemical identification of the potent isolate

Gram staining of the most potent isolate was performed with 100x optical zoom. It was followed by standard biochemical tests. Results procured were cumulatively assessed to find out bacterial genus in conformity with Bergey's Manual of Systematic Bacteriology (Singh *et al.*, 2016).

16s rRNA identification

Identification of potent isolate showing promising results was done using 16s rRNA sequencing method. Isolation of DNA was performed using EXpure Microbial DNA isolation kit manufactured by Bogar Bio Bee stores Pvt Ltd. Polymerase Chain Reaction (PCR) of isolated DNA was carried out using two primers: F (5' AGAGTTTGATCTGGCTCAG 3') and R (5' TACGGTACCTTGTTACGACTT 3') (Edwards, 1989). ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems) were used for sequencing reactions. The NCBI blast similarity search tool was used to blast the 16s rRNA sequence. For multiple alignments of sequences program MUSCLE 3.7 was used (Edgar, 2004). The Program Gblocks 0.91b was used to cure the resulting aligned sequences. The poorly aligned positions and divergent regions are removed by Gblocks (Talavera and Castresana, 2007). Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as a Substitution model. For tree rendering, the Tree Dyn program was used (Dereeper *et al.*, 2008).

Extraction of crude biosurfactant

CD03A was inoculated in growth media, Bushnell and Haas broth having 1% ship spent oil and 3% NaCl and was incubated for 7 days at room temperature on an orbital shaker. After incubation, the entire content was centrifuged at 12,500 rpm at 4^{o}C for 30 minutes. Then the CFS was collected, and by using 6N HCl it was acidified up to pH 2 and then it was kept at 4^{o}C for 24 hours to facilitate precipitation. After 24 hours of incubation, it was centrifuged for 20 min at 4^{o}C and 12,500 rpm. A crude biosurfactant was obtained in the form of a pellet, which was resuspended in water. pH was raised to 7.5 using 1N NaOH solution. This suspension was lyophilized and then subjected to methanol extraction. The methanol soluble fragment was transferred to a beaker and was kept at 37^{o} C till complete evaporation of the solvent. It was again subjected to methanol extraction and finally partially purified biosurfactant was obtained (Das *et al.*, 2008).

Fourier Transform Infrared Spectroscopy

FTIR analysis of partially purified biosurfactant was employed to determine the presence of different functional groups. FTIR analysis was carried out in IRPrestige-21 (Shimadzu). The background noise was cancelled out using KBr absorbance in a dry atmosphere and further absorbance of the sample was taken (Almansoory *et al.*, 2019).

Results

A total of eight physiologically different isolates were obtained from six different sites screened for the production of biosurfactants (Table 2). Out of eight isolates, three showed beta hemolysis, while others showed alpha hemolysis. However, other than biosurfactants, hemolysis can occur due to different reasons like the production of hemolysin. Hence, other tests were also conducted to confirm biosurfactant production. Phenol: Sulfuric acid test is a confirmatory test for the production of biosurfactants. Production of biosurfactant was confirmed by the orange colour which was formed in every sample. However, the intensity of this colour varied in samples, which might be due to variation in concentration of biosurfactant. Micro-titer plate assay is based on the fact that biosurfactant causes wetting of edges which leads to distortion of the image. All 8 isolates showed a distorted image of graph paper placed below the plate. If a biosurfactant is present in the sample then it reduces the surface tension between the water and oil layer and enables the drop to spread more, thus it is called Drop collapse assay. That is its diameter increases. Positive control of Triton X-100 (1 mg/ml) showed a diameter of 4 mm and negative control which was distilled water showed a diameter of 1.0 mm. Out of the eight isolates, two isolates showed a diameter of 3.0 mm whereas CD04A showed a diameter of 2.5 mm and the rest showed diameters of 2.0 mm. The displacement of oil due to the reduction in surface tension was scored as shown in Table 2. Isolates CD03A showed significant oil displacement as compared to other isolates. The emulsification of oil means combining it with water so that it can be taken up by the cell. More is the emulsifying capacity, better the uptake of oil by organisms. And thus emulsifying capacity was measured using the method mentioned above. The emulsification index of positive control, Triton X-100 (1 mg/ml) was found to be 38.46%. Isolate CD03A showed the highest per cent of emulsification which was 44.75%. Isolate CD03A was followed by CD01A and CD05A showing an

Table 2. Biosurfactant Screening Assays

Isolate	Hemolysis assay	Phenol: Sulphuric Acid test	Microtitre plate assay	Drop collapse test	Oil displacement test	Emulsification assay
CD01A	β	POS	Distortion	3 mm	+ + +	42.42 %
CD02A	β	POS	Distortion	2 mm	+ +	39.39 %
CD03A	β	POS	Distortion	3 mm	+ + + +	44.75 %
CD03B	β	POS	Distortion	2 mm	+ +	41.17 %
CD04A	β	POS	Distortion	2.5 mm	+ + +	41.93 %
CD05A	β	POS	Distortion	2 mm	+	42.42 %
CD06A	β	POS	Distortion	2 mm	+	41.93 %
CD07A	β	POS	Distortion	2 mm	+	38.70 %
Negative control	-	-	No Distortion	1 mm	-	0.0 %
Positive control	-	POS	Distortion	4 mm	+ + + + +	38.46 %

+: ok, ++: good, +++: very good, ++++: excellent, +++++: Impressive; POS: formation of orange red colour indicating presence of biosurfactant.

TTable 3. Results of the biochemical test

Test	Gram Staining	Motility	Indole	MR	VP	Citrate	Nitrate reduction	Catalase	Oxidase	Urease	Lysine decarboxylase	Phenylalanine deaminase
Result	Gram-negative	+	-	-	+	+	-	+	+	-	-	+

emulsification index of 42.42%. CD02A and CD07A showed comparatively less emulsification percentage as compared to other isolates, yet it was a bit higher than that obtained from the positive control.

Isolate CD03A was chosen as a potent organism as it showed the best results among all isolates, and was further selected for biochemical analysis followed by identification using the 16s rRNA gene sequencing method. By comparing the results of biochemical tests with Bergey's Manual of Systematic Bacteriology-Vol. 2. It was confirmed that the organism CD03A belongs to the genus *Alcaligenes*. Results of biochemical tests are given in Tables 3 and 4.

The base pair length of the PCR product was around 1400 bp. NCBI BLAST was used to compare the 16s rRNA sequence of CD03A with a sequence database. Isolate CD03A was identified as *A. faecalis* strain TUTXS-06 with 100% similarity (Fig. 1). The sequence was submitted in NCBI GenBank and accession number OL587517 was allotted.

The FTIR analysis was performed to find out functional groups present in biosurfactants extracted from CD03A. Biosurfactant obtained from CD03A (Fig. 2) showed a broad peak within the range 3000-3500 cm^{-1}, which indicates the presence of the alcohol (-OH) group. The presence of two sharp peaks at 2953 cm and 2926 cm indicated the presence of alkyl chains. A sharp peak at 1587 cm^{-1}, indicates the presence of an N-H bend which may appear due to the presence of amine or amide. Two sharp peaks between 1300 to 1390 cm^{-1} may appear due to the presence of a nitro group (-NO_2).

Table 4. Results of Sugar Fermentation test

Sugars	Glucose	Fructose	Sucrose	Lactose	Xylose	Mannitol
Results	-	-	-	+/-	-	-

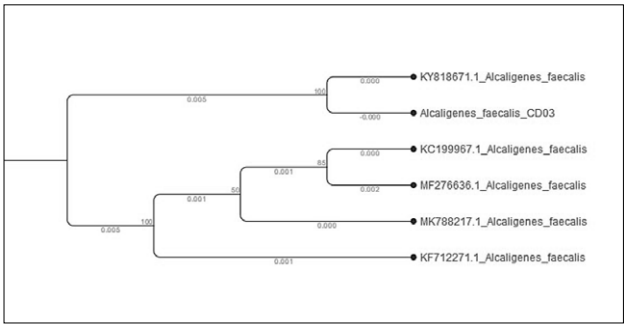

Fig. 1. Phylogenetic tree for Isolate CD03A identified as *Alcaligenes faecalis* with 100% similarity

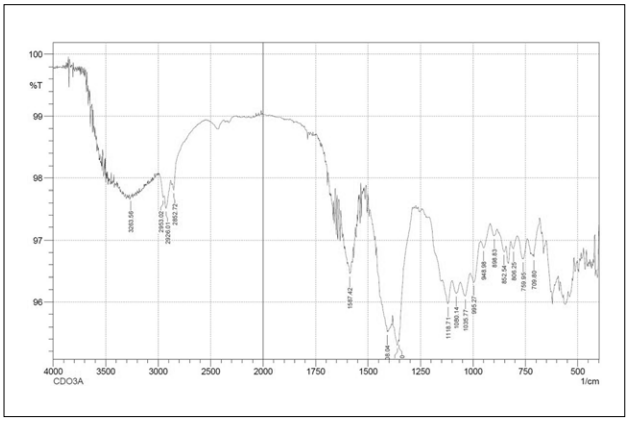

Fig. 2. The FTIR analysis of biosurfactant produced by CD03A

Discussion

The capability of biosurfactants to enhance the growth of microbes by increasing the uptake of hydrophobic substrates makes it a leading light in the field of hydrocarbon bioremediation (Das and Chandran, 2010). The biodegradable, least toxic and eco-friendly nature of biosurfactants gives them an upper edge over chemical biosurfactants (Banat *et al*., 2000). The main aim of this research was to isolate hydrocarbonoclastic microorganisms that could degrade ship oil and thus can be used for bioremediation of oil spills. Powthong and Suntornthiticharoen (2018) isolated biosurfactant-producing bacteria from an agricultural area in Thailand. Three different isolates FWS41 (2), RSS7 (1) and VSNY5 (9) were identified as *A. faecalis* sub sp. *faecalis*. They showed different values for the emulsification index with diesel oil, 22.2±5.6%, 6.3±3.6% and 1.9±3.2% respectively. They also showed promising antimicrobial results against plant pathogens. Adeleye *et al.* (2020), used the co-culture of *Pseudomonas aeruginosa* and *A. faecalis* to check how the rate of bioremediation is affected in presence of a co-culture system and biostimulants. Uba *et al.* (2018), checked the bioremediation activity of *A. faecalis* isolated from the contaminated marine environment of the Niger Delta. A notable correlation was found between biofilm formation and emulsification activity. Biofilm of *A. faecalis* gave an emulsification value of 89.5% in a mixture of xylene, anthracene and pyrene aromatic hydrocarbons. Adebajo *et al.* (2019), isolated *A. faecalis* from the rhizosphere of sawdust biochar amended soil and studied the effect of different extraction mediums on the yield obtained. *A. faecalis* showed a 65.9% of emulsification index after 24 hours of incubation in media containing crude oil. The chloroform/methanol (2/1) system yielded a maximum amount of dried biosurfactant followed by acetone, acid preparation and dichloromethane. Lalevic *et al.* (2015), also demonstrated the capability of *A. faecalis* isolated from oil-polluted groundwater to degrade crude oil. Results obtained from FTIR analysis somewhat resembled the results procured by Bharali *et al.* (2011), who found alcohol, alkyl, and carbonyl group in the biosurfactant. Vibrations for the alkyl chain, -OH group and carbonyl group were also obtained by Tsipa *et al.* (2021). Datta *et al.* (2018), characterized biosurfactants by FTIR and found a peak representing the Nitro group.

There have been several works done in the field of oil bioremediation using *A. faecalis*. Yet a lot of work is to be done in terms of practical on-field application. Here we presented how we can isolate biosurfactant-producing bacteria from different sampling stations and also how to screen them. Identification of the organism was carried out and its gene sequence was submitted to NCBI GenBank followed by FTIR analysis to characterize the functional groups present in the molecule. The prospect of this research is to carry out a detailed investigation of biosurfactant molecules and to find out techniques by which it can be applied on-site for the bioremediation of oil spilt.

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