



ORIGINAL ARTICLE

# Characterization of furnace oil bioremediation potential of hydrocarbonoclastic bacteria isolated from petroleum contaminated sites of the Sundarbans, Bangladesh



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## KEYWORDS

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The Sundarbans

**Abstract** Spillage of furnace oil is a more frequent event in recent times. In this study, environmental samples from furnace oil spillage sites of the Shela River, the Sundarbans, Bangladesh, were collected after three weeks of spillage. Serial dilution was applied and total seven bacterial isolates were separated as pure cultures. The oil-degrading potentiality of all seven isolates was further assessed, confirmed and compared with the growth pattern in furnace oil supplemented media, 2, 6-dichlorophenolindophenol test, and gravimetric analysis. After 7 days of incubation, isolates SS<sub>3</sub>, RW<sub>2</sub>, and SB degraded 56%, 43%, and 52% of supplemented furnace oil, respectively. The top three hydrocarbonoclastic bacterial isolates were selected as potential and identified as *Pseudomonas aeruginosa* (SS<sub>3</sub>), *Bacillus* sp. (RW<sub>2</sub>), and *Serratia* sp. (SB). All three isolates showed significant oil-degrading capacity compared to negative control, when incubated in sterile pond water supplemented with 2% furnace oil, suggesting them as potential bioremediation agents.

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## 1. Introduction

Oil spillage, especially furnace oil, a heavy petroleum product, is a common news around the world nowadays and it has severe effects on the surrounding environment. The consequences

include serious, widespread and long-term destruction to terrestrial life, human health, aquatic ecosystems and natural resources. Oil spillage in developing countries, like Bangladesh, causes damages on the environment severely due to lack of logistics support and trained workforces for prompt measures. Generally, spillage might occur accidentally or operationally during its processing, transportation or storage in underdeveloped settings [1]. Recurrent oil spillage event attracts public concern on the hostile effect on the environment and public health. The toxic effect of petroleum oil on plants, animals, human, and environment is devastating [2].

The Sundarbans, the largest mangrove forest in the world, was declared as a world heritage site by UNESCO in 1997. Beside this, most of the people of the south region of Bangladesh depend on the Sundarbans directly or indirectly for their livelihood. The forest is still standing as a natural defense for Bangladesh against natural calamities like cyclones, tidal effects. Apart from that, different channels and rivers of the Sundarbans are used for transportation routes to Mongla Sea Port. That is why Sundarbans is so important for the economy of southern Bangladesh as well as a pillar against climate change effects in South Asian region. Traffic in channels and rivers of the Sundarbans has increased recently due to some regional transport protocol with south Asian countries and regional developmental activities of southern Bangladesh. As a result, an oil tanker named *Southern Star Seven*, carrying 358,000 L of furnace oil, sunk in the Shela river of Sundarbans following a collision with another cargo vessel on the 09<sup>th</sup> December 2014. The oil spread over 350 km<sup>2</sup> area (Fig. 1b) [2]. Oil slicks spread to a second river and to a network of canals in the Sundarbans and darkened the shoreline. The event was very threatening to trees, planktons and vast population of small fishes, wildlife, and dolphins. This incident was a matter of great concern to national and international communities. However, the National Geographic Society and the United Nations reported that the impact was not as disappointing as initially estimated due to the natural recovery processes [2,3].

Environmentalists are concerned recently, as the number of oil spillage accidents is increasing around the globe. After characterization of oil spill pollutants, several different techniques are suggested for recovery. Among them, physical removal of pollutants, conventional skimming methods, mechanical recovery using oil sorbents, and biological methods i.e. use of hydrocarbonoclastic bacteria, those are able to degrade hydrocarbons, are practiced popularly [4]. Sometimes, combinations of the pollutant removal techniques are also applied. Additionally, abiotic losses due to evaporation of low molecular hydrocarbons, dispersion, and photooxidation (involves only aromatic compounds) play a major role in decontamination of the oil spill environment [5]. The main disadvantage of the application of non-biological methods is that additional chemicals are required for better removal which increase the total cost making it difficult to follow in low-income countries like Bangladesh. That is why environment-friendly bioremediation methods treated as an alternative to chemical or physical methods are promising these days. The components of oil pollutants can be broken down by bacteria and other microorganisms into harmless substances i.e. fatty acids and carbon dioxide. The natural recovery process is initiated by localized microbes and could be speeded up by the addition of higher oil-degrading capacity microbes and nutrients like nitrogen and phosphorous to stimulate the growth

of the microorganisms concerned [6,7]. A number of microbes known as petrophiles, inherently degrade hydrocarbons [8] and can be used externally to boost up the oil removal process. Commonly they are known as “Bioremediation agents” that include microbial cultures, enzyme additives or nutrient additives [9].

The Sundarbans is experiencing oil spillage incidents recently. But there is no report on an overall study about the remedy mechanism of oil pollutants. Some risk assessment based surveys and reports on oil spillage are published to date [2,10]. No research has been reported about the bioremediation microbes (flora) of the Sundarbans oil spillage to date. To combat future oil spillage incidents, a microbial screening based research is needed to get insights into the Sundarbans bioremediation process and local production of bioremediation agents. In the present study, we isolated and characterized three superior hydrocarbonoclastic bacteria from river water, soil sediments, and submerged barks of plant samples collected from the Sundarbans oil spillage area and assessed their oil pollutant bioremediation ability *in vitro*.

## 2. Materials and methods

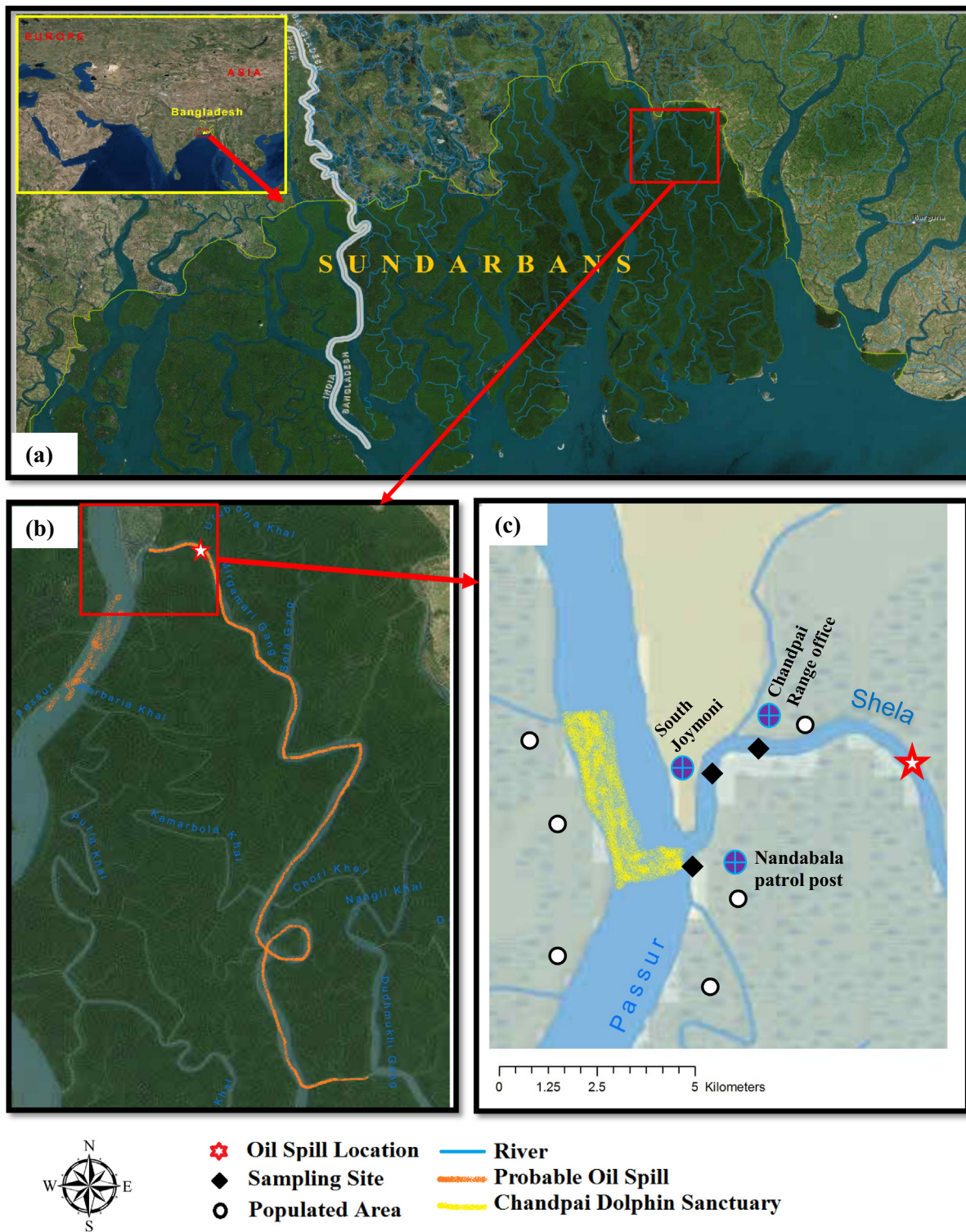
### 2.1. Study and sampling area

Samples were collected from the Sundarbans oil spillage sites, located near the junctions of the Passur and the Shela river on the 28<sup>th</sup> December 2014, just 18 days after the accident (Fig. 1). Submerged bark of plants, soil sediments, and the Shela river water were collected from three different sites situated nearby of the Nandabala patrol post (22°20'53.3"N and 89°38'09.01"E), the South Joymoni (22°21'43.03"N and 89°38'20.16"E), and the Chandpai Range office (22°21'55.24"N and 89°38'39.48"E), respectively (Fig. 1c). Samples were collected in labeled pre-sterilized bottles and shipped to the laboratory in the University of Chittagong. Collected samples were preserved at 4 °C during experiments.

For conducting the bioremediation capability of the isolates and other oil degradation confirmatory tests, raw furnace oil was collected from the Standard Asiatic Oil Company Limited, North Patenga, Chittagong in sterilized airtight bottle and stored at the cool and dark place.

### 2.2. Screening, isolation and identification of hydrocarbonoclastic bacterial isolates

Collected furnace oil contaminated samples namely soil sediments, the Shela river water, and submerged bark of plants were resuspended in 10 mL of 10 mM phosphate buffer (pH 7.0) to prepare a homogenized suspension. Each sample suspension was used separately to inoculate R<sub>2</sub>B broth (Peptone 0.5 g/L, Yeast extract 0.5 g/L, Glucose 0.5 g/L, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.1 g/L, Sodium pyruvate 0.3 g/L, Casein acid hydrolysate 0.5 g/L, Soluble starch 0.5 g/L, and Di-potassium phosphate 0.3 g/L) and incubated 48 h at 37 °C and 150 rpm. After incubation, 0.1 mL of cultured broth from each sample was spread in a mineral salt agar plate (NaNO<sub>3</sub> 2g/L, MgSO<sub>4</sub> 0.5 g/L, KCl 0.5 g/L, Fe<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub>·H<sub>2</sub>O 0.01 g/L, KH<sub>2</sub>PO<sub>4</sub> 0.14 g/L, K<sub>2</sub>HPO<sub>4</sub> 1.20 g/L, Yeast extract 0.02 g/L, Agar 15 g/L). An ethereal solution of furnace oil (10% w/v) was uniformly sprayed over the surface of the agar plate. The ether vaporized immediately



**Fig. 1** Study area map indicating the Sundarbans (a), the oil spillage site with probable oil slick distribution (b), and sampling sites in between the oil tanker spillage site and dolphin sanctuary (c).

and a thin layer of oil retained on the entire surface. Then the plates were incubated for 3 days at 25 °C. Visual reduction in oil content indicated the presence of hydrocarbonoclastic bacteria in collected samples. After preliminary screening of oil-degrading ability of oil spillage samples, a serial dilution was

conducted as described by Azad et al. [11] to isolate bacteria. One gram of each sample was added in 9 mL of sterile water. After a serial dilution ( $10^{-1}$  to  $10^{-6}$ ) of sample suspension with sterile distilled water, 60  $\mu$ L of each diluted suspension was spread separately on LB agar plate (Peptone 10.00 g/L, Yeast

extract 5.00 g/L, NaCl 5.00 g/L, Dextrose Anhydrate 10.00 g/L, and Agar 30.00 g/L) and incubated at 37 °C for overnight. After incubation, depending on the difference in color, morphology, and shape, different colonies were streaked on LB agar plate separately and incubated overnight at 37 °C to isolate pure colonies. Finally, isolated pure colonies were subjected to grow in selective Bushnell-Hass (BH) broth (KH<sub>2</sub>PO<sub>4</sub> 1.0 g/L, K<sub>2</sub>HPO<sub>4</sub> 1.0 g/L, NH<sub>4</sub>NO<sub>3</sub> 1.0 g/L, Cholesterol 0.3 g/L, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.2 g/L, FeCl<sub>3</sub> 0.05 g/L, CaCl<sub>2</sub>·2H<sub>2</sub>O 0.02 g/L, and 2% sterile furnace oil) for estimating furnace oil-degrading ability [12]. Furnace oil was sterilized separately by autoclaving at 121 °C for 15 min in sealed Erlenmeyer flasks and added to the medium separately. Each pure bacterial isolate was inoculated in BH broth supplemented with 2% furnace oil and incubated for 5 days in a rotary shaker (WiseCube®, Korea) at 150 rpm and 30 °C. Degradation of furnace oil is determined in terms of change in biomass (OD at  $\lambda = 600$  nm) and pH. Bacterial isolates showed a rapid change in OD and pH considered as potential furnace oil degraders.

### 2.3. Furnace oil biodegradability experiments

For rating, the relative furnace oil-degrading ability, gravimetric analysis and a visual technique utilizing the redox indicator 2, 6-dichlorophenolindophenol (DCPIP) were carried out. Gravimetric analysis was done as described earlier [13–15]. In the gravimetric analysis, the amount of furnace oil utilized by individual bacterial isolates was analyzed. Firstly, isolates were cultured in a BH broth supplemented with a 2% furnace oil. After 7 days of incubation residual furnace oil in the culture broth was estimated by emulsification with petroleum ether and acetone in a ratio of 1:1 followed by passing through anhydrous sodium sulfate to remove moisture. Finally, percentage of furnace oil utilized was calculated as follows:

% of furnaceoil utilized

$$= \frac{\text{furnace oil utilized (g)}}{\text{furnace oil added to BH broth (g)}} \times 100$$

Furnace oil utilized(g) = furnace oil added to BH broth (g) – furnace oil at the end of fermentation (g)

Along with the gravimetric quantitative analysis, a visual furnace oil biodegradability tests using DCPIP was also carried out. DCPIP test is a quick and reliable method for screening of hydrocarbonoclastic bacteria. When bacteria utilizes hydrocarbon, it generates electron accepted by DCPIP, an electron acceptor dye, changing its color from blue (oxidized) to colorless (reduced) [16]. All bacterial isolates were added (1.5 mL, OD = 0.6 at  $\lambda = 600$  nm) separately to test tubes containing 7.5 mL BH broth. After that, 667  $\mu$ L sterilized furnace oil and 100  $\mu$ g DCPIP were added to each test tube, vortexed shortly and incubated for 24 h at 37 °C and 84 rpm. Isolates showed higher percentage of furnace oil utilization and DCPIP decolorization considered as highly potential furnace oil biodegrading isolates [17].

### 2.4. Phenotypic and biochemical characterization of bacterial isolates

The pure culture of bacterial isolates was identified based on cultural characteristics, Gram staining, motility, and various biochemical tests as described in the Cowan and Steel's

Manual for the Identification of Medical Bacteria [18]. Isolates were biochemically analyzed for the activities of catalase, methyl-red test, citrate utilization, urease test, gelatin hydrolysis, carbohydrate utilization, cetrimide test, and indole production according to the standard methods [18].

### 2.5. 16S rRNA gene sequencing of the bacterial isolates

Genomic DNA was extracted from the pure culture pellet of selected furnace oil-utilizing bacteria (RW<sub>2</sub> and SB isolates) using Spin column based genomic DNA purification kit (Jena Bioscience GmbH, Cat no. PP-215L). A partial fragment of the 16S rRNA gene from the extracted DNA was amplified by polymerase chain reaction (PCR) using universal primers 27F 5' AGA GTT TGA TCMTGG CTC AG 3' and 1492R 5' CGG TTA CCT TGT TAC GAC TT 3'. The amplified PCR product was sequenced bi-directionally by ABI Genetic Analyzer 3500. The sequences were then analyzed in the NCBI BLASTn program ([https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE\\_TYPE=BlastSearch](https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch)) and top matched sequences were retrieved to construct multiple sequence alignment using "Clustal Omega" ([www.ebi.ac.uk/Tools/msa/clustalo/](http://www.ebi.ac.uk/Tools/msa/clustalo/)). Phylogenetic trees from multiple sequence alignment (statistical method: maximum likelihood method; test of phylogeny: no. of bootstrap replications: 500) were constructed using MEGA version 6 [19].

### 2.6. Bioremediation test

After isolation, identification, and confirmation of furnace oil-degrading capacity, selected bacterial isolates were subjected to bioremediation test (triplicate) in laboratory condition. In this regard, five containers (surface sterilized with 70% ethanol) each with 300 ml of autoclaved pond water supplemented with 2% furnace oil (v/v) (autoclaved separately) were used as media. Then 3 mL (OD = 0.5 at  $\lambda = 600$  nm) of fresh liquid culture (overnight culture) of each isolate was added to three containers separately. To compare the oil-degrading capacity of selected isolates, one container left as it was (no inoculation) and another one was inoculated with *Staphylococcus aureus* (hydrocarbon sensitive bacteria collected from laboratory stock) [20]. Then, all containers were incubated at 37 °C for 20 days. After the incubation period, visual observation was made to evaluate the efficiency of selected isolates for hydrocarbon degradation *in vitro*. To measure the relative shrinkage of the oil slick area after inoculation of respective bacterial isolates, the image of each container was taken before inoculation and after 20 days of incubation. The surface oil slick area of all images was measured quantitatively using ImageJ software package (<https://imagej.nih.gov/ij/index.html>) [21]. The percentage of the oil slick free surface (transparent area) and/or fade slicks area (partially transparent area) of each container was measured from the image taken at day 0 and day 20 of incubation for individual 5 experiments presented in Fig. 6a–e using ImageJ. The change in the percentage of the oil slick free area after inoculation was calculated by subtracting the percentage of oil slick free area at day 20 from day 0 and used as a percent of oil slick removal after 20 days. To confirm that the observed change in the percentage of oil slick reduction was statistically significant, Student's t-test was performed.

### 3. Results

#### 3.1. Screening and isolation of hydrocarbonoclastic bacteria

Visual observation of mineral salt agar (with uniform furnace oil sprayed) plates after 3 days of incubation indicated that the typical color of plates changed from dark black to pale (Fig. 2). The decrease of dark color indicated that each of the collected samples contains hydrocarbonoclastic bacteria because the hydrocarbon was the sole carbon source of the screening media. After the preliminary screening of the collected samples, a serial dilution was conducted to isolate distinct bacterial colonies. Total 7 (seven) bacterial isolates were identified, among them three isolates from soil sediment (denotes as SS<sub>1</sub>, SS<sub>2</sub>, and SS<sub>3</sub>), three from river water sample (denotes as RW<sub>2</sub>, RW<sub>3</sub>, and RW<sub>4</sub>), and only one bacterium from submerged bark of plants sample (denotes as SB) (Table 1).

#### 3.2. Comparative analysis for furnace oil-degrading ability of bacterial isolates

To identify the more potential hydrocarbonoclastic bacterial isolates we have conducted Growth curve analysis in a selective BH media containing 2% furnace oil, gravimetric analysis to measure the percentage of furnace oil degradation and visual DCPIP reducing test. The growth curve analysis showed that SB, RW<sub>2</sub>, and SS<sub>3</sub> had superior furnace oil utilizing ability as their sole carbon source over others (Fig. 3). During the incubation period, the culture media pH of SB, RW<sub>2</sub>, and SS<sub>3</sub> were decreased dramatically to ~5 (Fig. 3). The relative furnace oil consumption also measured using gravimetric analysis. Gravimetric analysis showed that isolates SS<sub>1</sub>, SS<sub>2</sub>, SS<sub>3</sub>, RW<sub>2</sub>, RW<sub>3</sub>, RW<sub>4</sub>, and SB degraded 17%, 24%, 56%, 43%, 20%, 30%, and 52% of furnace oil, respectively (Table 2). Among the soil sediments isolate SS<sub>3</sub> showed ~2.5 times more furnace oil-degrading ability than other soil sediments samples whereas RW<sub>2</sub> showed ~2 times more than other river water

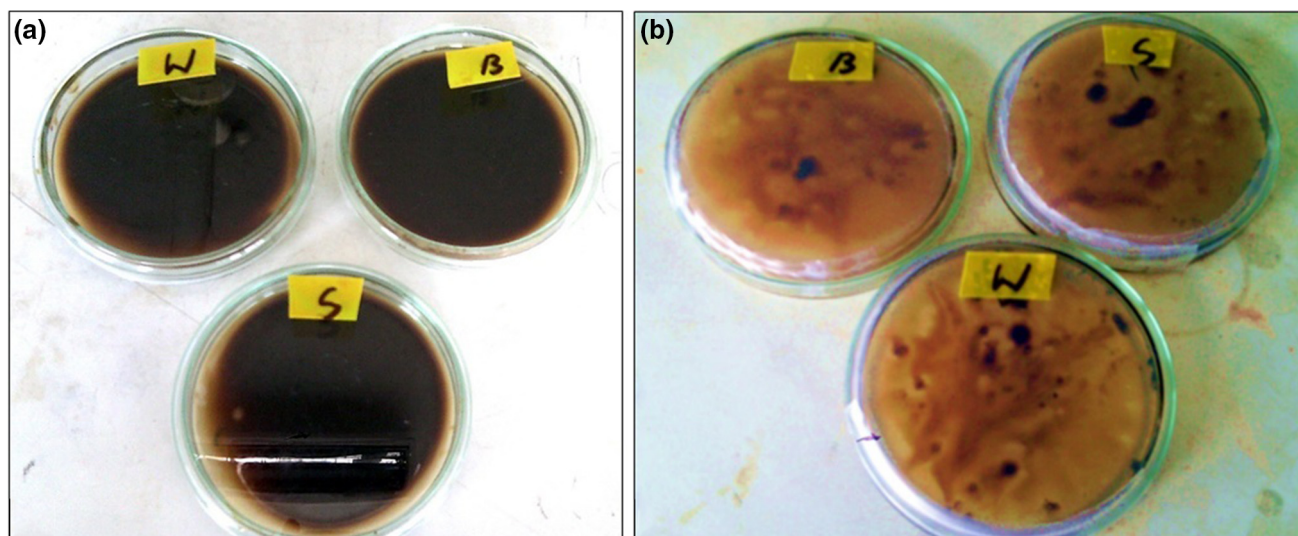
**Table 1** Morphological characteristics of all hydrocarbonoclastic bacterial isolates.

Samples type	Bacterial Isolates	Morphological Characteristics
Soil sediments	SS <sub>1</sub>	White, small
	SS <sub>2</sub>	Yellow, large
	SS <sub>3</sub>	Blue-green, small
River Water	RW <sub>2</sub>	Yellow, large
	RW <sub>3</sub>	Yellow, small
	RW <sub>4</sub>	White, large
Submerged Bark	SB	Brown, large

samples. The only isolates from submerged bark samples might be selected as potential one as it degraded 52%. Furthermore, all the bacterial isolates were subjected to a visual furnace oil oxidization test using DCPIP. This study revealed that isolate SS<sub>3</sub>, RW<sub>2</sub>, and SB completely decolorized typical dark blue color of DCPIP into transparent when compared to other isolates (Fig. 4). After analyzing all the data from comparative growth curve analysis, gravimetric analysis and visual decolorization test, it is clear that SS<sub>3</sub>, SB, and RW<sub>2</sub> might be used as potential biodegrading agents. These three potential isolates have been selected for identification and conducting bioremediation test.

#### 3.3. Identification of highly potential bacterial isolates

After selection of potential hydrocarbonoclastic bacterial isolates, their cultural, morphological, and various biochemical tests were conducted (Table 3). Based on the results of biochemical tests and the cultural and morphological characteristics, isolates SS<sub>3</sub> identified as *Pseudomonas aeruginosa* [22,23] whereas isolates RW<sub>2</sub> and SB showed a close resemblance with *Bacillus* sp. and *Serratia* sp., respectively (Table 3). Furthermore, RW<sub>2</sub> and SB were subjected to 16S rRNA gene



**Fig. 2** Screening of environmental samples collected from the Sundarbans oil spillage sites, Bangladesh: before (a) and after (b) 3 days of incubation in furnace oil sprayed mineral salt agar plates. Samples collected from river water, soil sediments, and submerged barks of plants are labeled as W, S, and B, respectively.

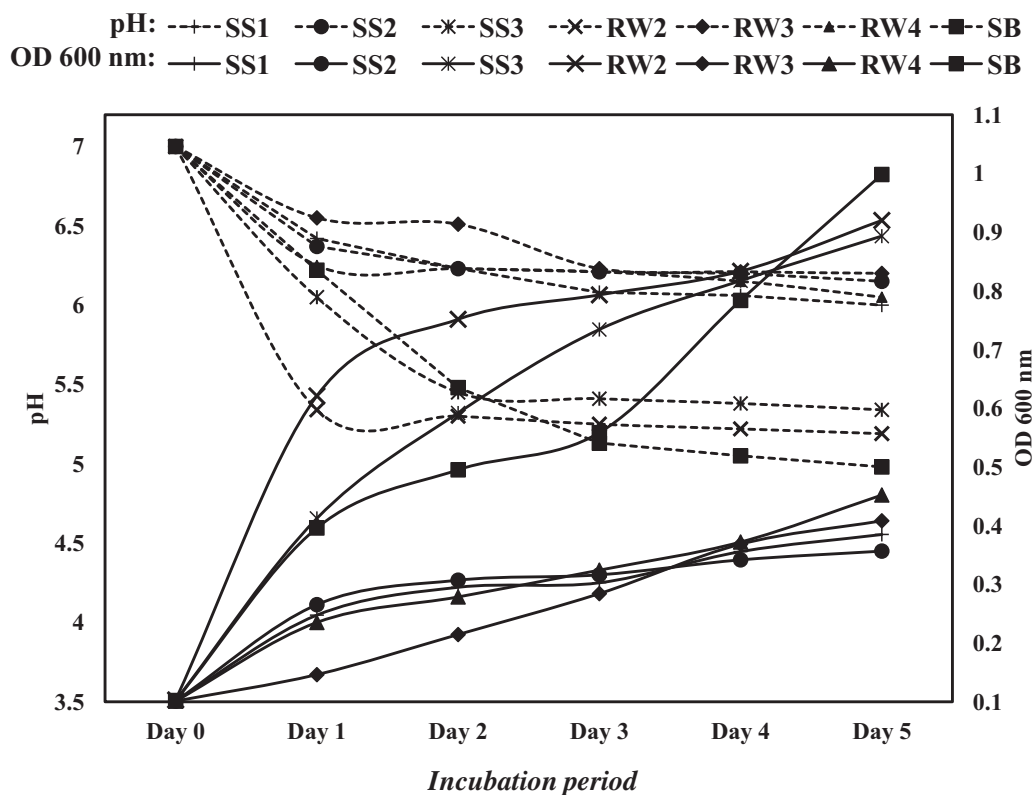


Fig. 3 Growth curve analysis of bacterial isolates in furnace oil containing BH media.

**Table 2** Relative furnace oil degradation capability of bacterial isolates after 7 days of incubation in furnace oil containing BH media.

Bacterial Isolates	Furnace oil added to BH medium (g)	Furnace oil at the end of culture (g)	Furnace oil utilized (g)	% of furnace oil utilized
SS <sub>1</sub>	0.93	0.77	0.16	17
SS <sub>2</sub>	0.93	0.71	0.22	24
SS <sub>3</sub>	0.93	0.41	0.52	56*
RW <sub>2</sub>	0.93	0.53	0.40	43*
RW <sub>3</sub>	0.93	0.74	0.19	20
RW <sub>4</sub>	0.93	0.65	0.28	30
SB	0.93	0.45	0.48	52*

\* Indicates the best result in individual sample type.

sequencing by following standard method. From the sequence similarity analysis using various tools like NCBI BLASTn, CLUSTAL Omega and MEGA6, isolates SB and RW<sub>2</sub> were identified as *Serratia* sp. (Fig. 5a) and *Bacillus* sp. (Fig. 5b), respectively. The use of 16S rRNA in the characterization of microorganisms is more dependable and sensitive than culture-dependent techniques alone [24–27] and the results obtained in this investigation are consistent with other related studies [28–30].

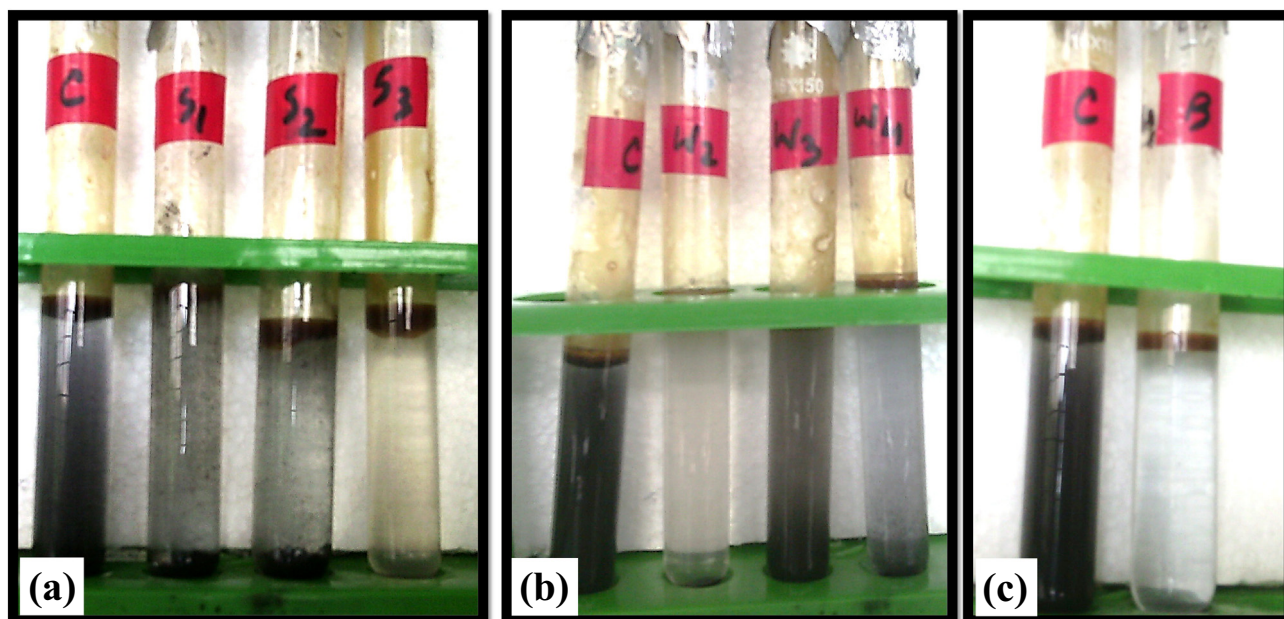
#### 3.4. Bioremediation test

In bioremediation test, 2% furnace oil supplemented sterile pond water was used. A significant ( $P < 0.005$  in all cases) level of oil slicks were removed by isolates *P. aeruginosa*, *Bacillus* sp., and *Serratia* sp. after 20 days of incubation when

compared to negative control (no inoculation) and *S. aureus* (Fig. 6) [20]. In terms of relative removal of oil slicks from the water surface, *P. aeruginosa* removed 42% whereas *Bacillus* sp., *Serratia* sp., *S. aureus*, and no inoculation were 32%, 34%, 3.5%, and 3.1%, respectively (Fig. 7).

#### 4. Discussion

To our best knowledge, this is the first report concerning the characterization and assessment of bacterial bioremediation agents isolated from the sites of oil spillage occurred on the 09<sup>th</sup> December 2014 on the Shela River of the Sundarbans. When publicity about natural remediation was disclosed in media, three different samples i.e. river water, submerged barks of plant and soil sediments were collected on the 28<sup>th</sup> December 2014 just three weeks after spillage accident [3].



**Fig. 4** Relative DCPIP decolorization ability of bacterial isolates from soil sediments (a), river water (b), and submerged barks of plant (c) samples. Test tubes labeled with C denotes negative control, having no inoculation whereas S<sub>1</sub>, S<sub>2</sub>, S<sub>3</sub>, W<sub>2</sub>, W<sub>3</sub>, W<sub>4</sub>, and B indicate isolates SS<sub>1</sub>, SS<sub>2</sub>, SS<sub>3</sub>, RW<sub>2</sub>, RW<sub>3</sub>, RW<sub>3</sub>, and SB, respectively.

**Table 3** Summary of morphological, cultural and biochemical test results where '+' and '-' indicates positive and negative result, respectively.

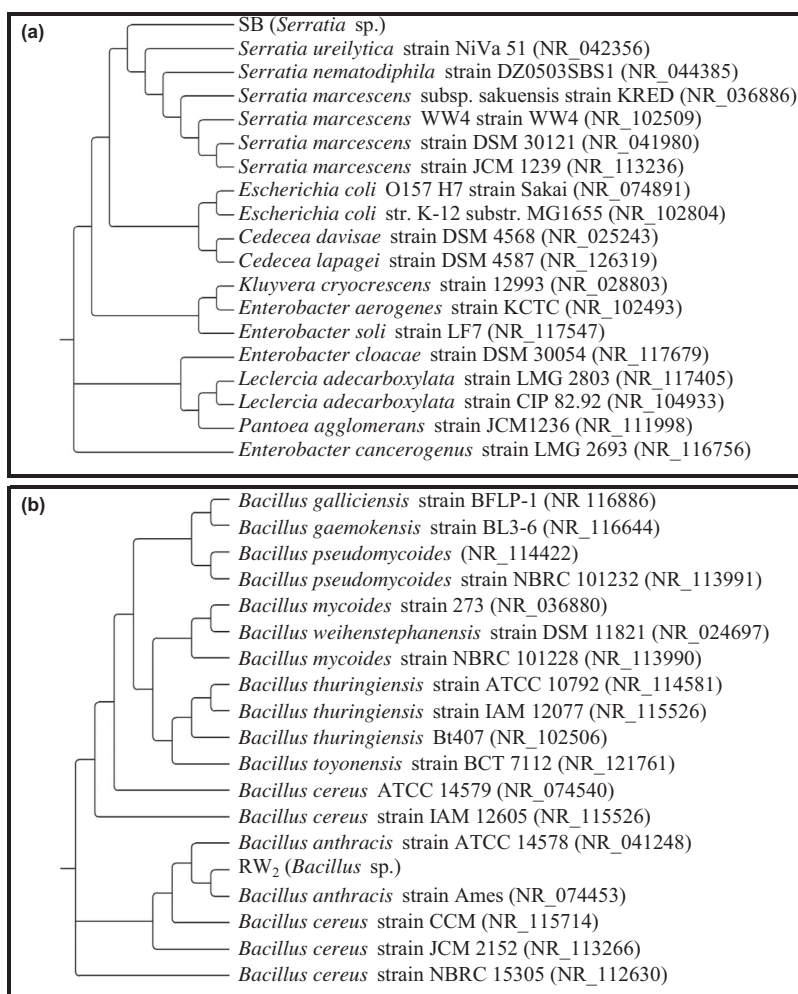
Morphological and biochemical tests	Bacterial isolates		
	SS <sub>3</sub>	RW <sub>2</sub>	SB
Colony color	Blue-green	Yellowish	Brown
Gram staining	Negative	Positive	Negative
Shape	Rod	Rod	Rod
Catalase	+	+	+
Methyl-Red	+	+	-
Citrate Utilization	+	-	+
Indole formation	-	-	-
Gelatin utilization	+	+	+
Urease test	+	-	-
Glucose utilization	+	+	+
Sucrose utilization	-	+	+
Maltose utilization	-	+	+
Cetrimide test	+	-	-
Identified Bacteria	<i>Pseudomonas aeruginosa</i>	<i>Bacillus</i> sp.	<i>Serratia</i> sp.
Reference	[18,22,23]	[18]	[18]

The major focus of this study is to isolate and identify the major bioremediating bacterial agents that help the natural recovery of the Sundarbans and assess their comparative furnace oil remediation capability to select few isolates that might be used as bioremediation agents in future spillage accidents. Moreover, this work might be a foundation work for screening and production of bacterial bioremediation agents for commercial use.

Preliminary screening of the collected samples for oil-degrading ability showed that all three types of samples

degraded furnace oil. This indicates that the oil was spread not only in the water surface but also on the soil and the submerged plant surface where bacteria plays a major role in natural bioremediation of the Sundarbans. Serial dilutions of all three samples yield 7 distinct isolates (three from both soil sediments and river water whereas only one from submerged bark). After conducting various analyses, i.e. growth curve analysis on a media where furnace oil was the sole carbon source, the percentage of furnace oil utilization by gravimetric analysis and visual reduction of DCPIP, proved that all the 7 isolates can degrade furnace oil to some extent. Based on the above-mentioned tests we have chosen three better oil-degrading isolates namely SS<sub>3</sub>, RW<sub>2</sub>, and SB for identifications using biochemical and 16S rRNA gene sequencing. From, morphological, biochemical, and 16S rRNA gene sequencing results we have confirmed that isolates SS<sub>3</sub>, RW<sub>2</sub>, and SB were *P. aeruginosa*, *Bacillus* sp., and *Serratia* sp., respectively.

The growth curve analysis in BH selective media (furnace oil was only carbon source in this media) determines the top three isolates *P. aeruginosa*, *Bacillus* sp., and *Serratia* sp. reached the OD 600 nm of 0.8932, 0.8965, and 0.9982, respectively on day 5 of incubation (Fig. 3). Bacterial isolates degrading crude oil significantly reached to OD 600 nm ~0.80 at the day 4 of incubation in the same media [31]. As culture media (furnace oil) degraded the pH of the culture media also falls as different acids may be produced as secondary metabolites [31]. Gravimetric analysis followed by DCPIP tests results of all bacterial isolates showed the overall relative oil-degrading capacity. The isolate *P. aeruginosa* degraded ~56% of crude furnace oil after day 7 of incubation and completely decolorize DCPIP solution (Fig. 4 and Table 2). According to previous reports, *P. aeruginosa* had degraded ~90% engine oil in four weeks of incubation [8]. The result observed in this study indicated strongly that this *P. aeruginosa* might act as a potential bioremediation agent. The isolate *Serratia* sp. and *Bacillus* sp.



**Fig. 5** Phylogenetic relationship of isolate SB (a) and RW<sub>2</sub> (b) with other *Serratia* and *Bacillus* species. The NCBI GenBank accession number for each of the sequence used in the phylogenetic analysis is bracketed out. 16s rDNA sequences of isolate SB and RW<sub>2</sub> were deposited to NCBI GenBank with the accession number KX101073 and KX101074, respectively.

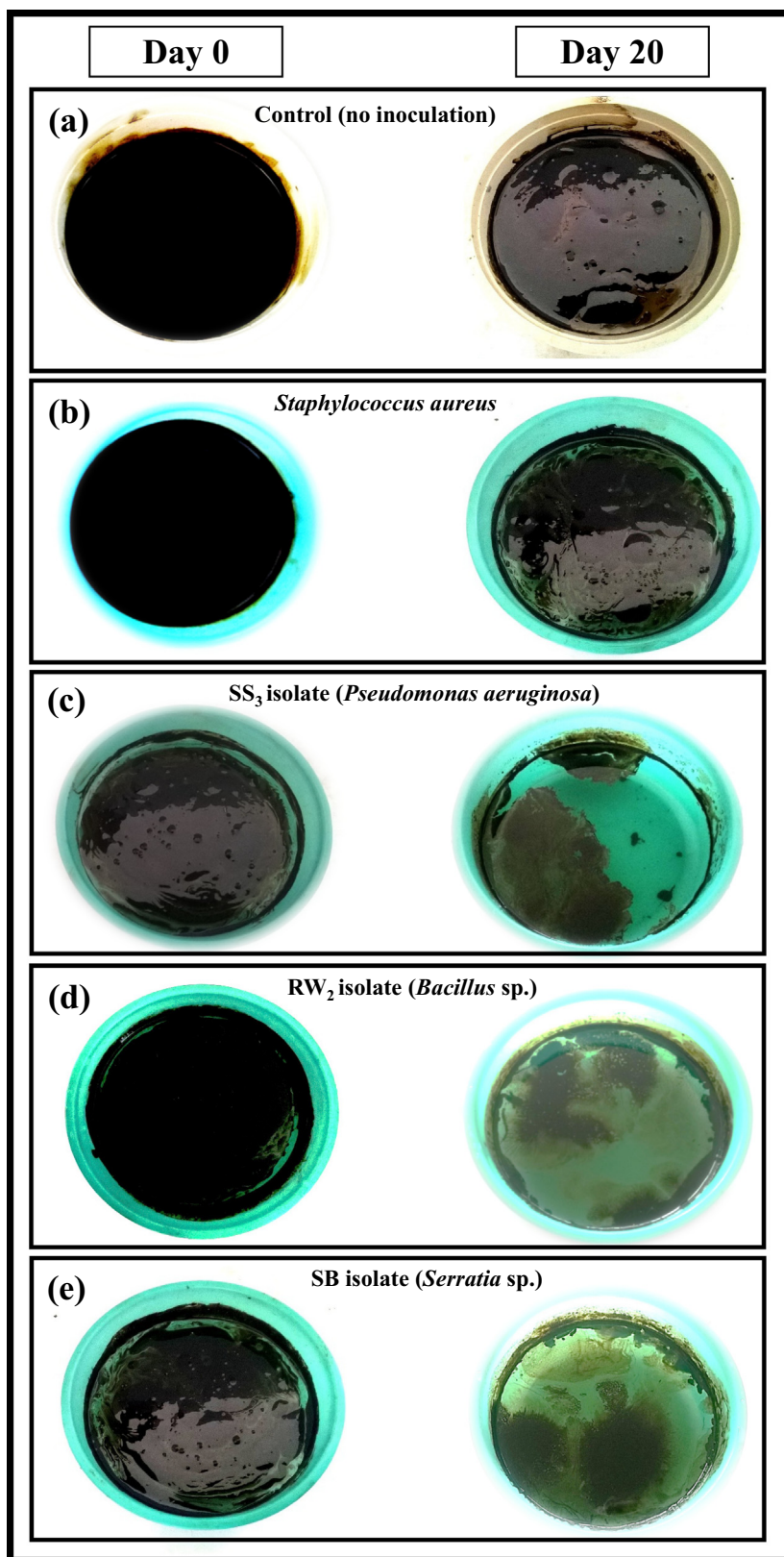
degraded 52% and 43% of furnace oil, respectively and completely decolorize DCPIP (Fig. 4 and Table 2). From the data of gravimetric analysis and DCPIP test depicted that *Serratia* sp. and *Bacillus* could also become good bioremediation agents. The previous report showed that *Serratia marcescens* significantly degraded 50–60% of hydrocarbons present in gasoline, kerosene, diesel and lubricating oil [32]. *Serratia* sp. also have some biosurfactant producing ability and used for petroleum degradation [33]. Different species of *Bacillus* are well known for their hydrocarbon degrading capacity. *Bacillus anthracis* was found to be degrading 67, 57, 72 and 42% of diesel, kerosene, crude oil and used engine oil, respectively after 28 days in the absence of nitrogen and phosphorus supplements [34]. Some *Bacillus* species are better petroleum degraders among them *B. flexus*, *B. cereus*, and *B.adius* were notable [35].

According to the assessment of the Joint United Nations/ Government of Bangladesh Sundarbans Oil Spill Response Mission and the National Geographic Society report, only ~20% of oil slicked out in the Shela River was recollected by local residents and the Bangladesh Navy by January 2015 [2,3]. The rest of the furnace oils have undergone natural reme-

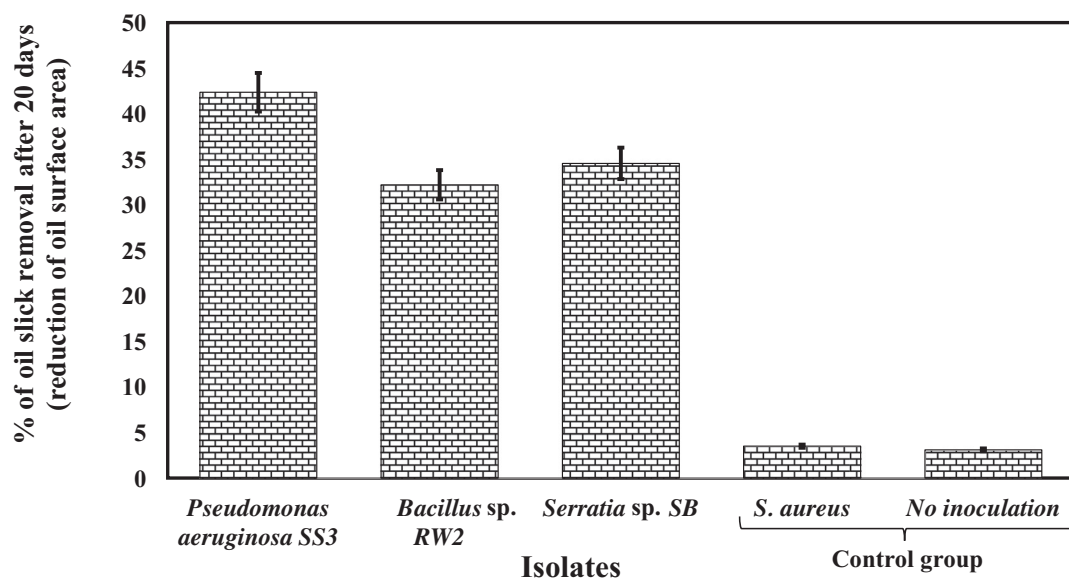
diation process. Here we studied the bioremediation capacity in terms of % removal of surface oil slicks by three selected isolates. In this experiment, autoclaved pond water supplemented with 2% furnace oil was used to make the media. Results of bioremediation tests showed that *P. aeruginosa* could degrade 42% of the floating oil slicks completely and turned the surface to transparent when compared to *Serratia* sp. and *Bacillus* sp. isolates. *P. aeruginosa* might produce some bio surfactants that make oil slicks more accessible to degrade furnace oil efficiently [33].

The study reported herein elucidated the major bacterial player behind the bioremediation process of Sundarbans after oil spillage occurred on the 09<sup>th</sup> December 2014. From the data it is clear that the three isolates namely *P. aeruginosa* SS<sub>3</sub>, *Bacillus* sp. RW<sub>2</sub>, and *Serratia* sp. SB might be used as potential bioremediation agents. Further study of the effects of different supplements to the isolates and their mixture as cocktail definitely added some merits to the current study. Although, a fundamental study of this type focusing on oil spillage accidents of the Sundarbans might be helpful to develop local production of bioremediation agents.





**Fig. 6** Observation of surface oil removal after 20 days of incubation of sterile pond water supplemented with 2% furnace oil inoculated without any inoculum (a), *Staphylococcus aureus* (b), *Pseudomonas aeruginosa* SS<sub>3</sub> (c), *Bacillus* sp. RW<sub>2</sub> (d) and *Serratia* sp. SB (e).



**Fig. 7** Relative removal of surface oil slicks after bioremediation of sterile pond water supplemented with 2% furnace oil. Triplicate measurements were conducted and compared with the control group each case. Error bar indicates  $\pm$ SD.

## 5. Conclusions

In this study, three different samples were collected from oil spillage sites and assessed for their oil-degrading potentiality. From three samples, 7 distinct bacterial colonies have been isolated. The isolated bacterial colonies were subjected to rigorous growth curve analysis, furnace oil utilizing analysis and visual DCPIP reduction tests to select best three bioremediation candidates that might be used further for bioremediation of oil pollutants. Based on the various biochemical tests and 16S rRNA gene sequencing data, we identified them as *P. aeruginosa*, *Bacillus* sp., and *Serratia* sp. These three isolates were further assessed in the laboratory by incubating them in sterile pond water supplemented with furnace oil. All the results presented in this study support the notion that *P. aeruginosa*, *Bacillus* sp., and *Serratia* sp. had significant bioremediation potential and might be used as starting bacterial strains to formulate effective bioremediation agents to combat future oil spillage accidents in Bangladesh as well as other parts of the world.

## Authors' contribution

LWM and MMH conceived and designed the project. TS carried out the laboratory experiments except 16s-rDNA sequencing and prepared the manuscript draft. LWM, MRI, MMH, and SAM monitored laboratory experiments. LWM and MMH interpreted all the results. AGMR and MIHK carried out the 16s-rDNA sequencing. LWM, MRI, and MMH prepared and reviewed the final manuscript. All authors read and approved the final manuscript.

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The nucleotide sequences reported in this paper has been deposited to NCBI GenBank will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number KX101073 and KX101074 denote *Serratia* sp. SB1 and *Bacillus* sp. RW2, respectively.

## References

- [1] M. Shukor, F. Dahalan, A. Jusoh, R. Muse, N. Shamaan, M. Syed, *J. Environ. Biol.* 30 (1) (2009) 145–150.
- [2] The Joint UNEP/OCHA Environment Unit, Sundarbans oil spill assessment. <<http://whc.unesco.org/document/140155>>, 2015 (accessed on 26 April, 2016).
- [3] C. Alexander, After oil spill, unique mangrove forest faces more threats, National Geographic Society, <<http://news.national-geographic.com/2015/05/150507-sundarbans-india-bangladesh-oil-spill-royal-bengal-tiger-irrawaddy-dolphin-bay-of-bengal/>>, 2015, (accessed on 6 June, 2016).
- [4] E. Riser-Roberts, *Bioremediation of Petroleum Contaminated Sites*, CK Smoley, 1992.
- [5] A. Mills, C. Breuil, R. Colwell, *Can. J. Microbiol.* 24 (5) (1978) 552–557.
- [6] J. Mueller, C. Cerniglia, P. Pritchard, in: D. Crawford (Ed.), *Bioremediation of Environments Contaminated by Polycyclic Aromatic Hydrocarbons*, Cambridge University Press, Idaho, 1996, p. 125.
- [7] S. Cappello, G. Caruso, D. Zampino, L. Monticelli, G. Maimone, R. Denaro, B. Tripodo, M. Troussellier, M. Yakimov, L. Giuliano, *J. Appl. Microbiol.* 102 (1) (2007) 184–194.
- [8] T. Mandri, J. Lin, *Afr. J. Biotechnol.* 6 (2007) 1.
- [9] X. Zhu, A.D. Venosa, M.T. Suidan, in: National Risk Management Research Laboratory (Ed.), *Literature Review on the Use of Commercial Bioremediation Agents for Cleanup of Oil-Contaminated Estuarine Environments*, U.S. Environmental Protection Agency, Cincinnati, OH 45268, 2004.
- [10] S. Raha, *Asian J. Multidiscip. Stud.* 3 (2015) 5.

- [11] A.K. Azad, A. Nahar, M.M. Hasan, K. Islam, M.F. Azim, M.S. Hossain, M.R. Rahman, R.K. Ojha, G.M.S. Mahmud, R. Kayes, *Asian J. Microbiol. Biotechnol. Environ. Sci.* 15 (2) (2013) 365–374.
- [12] K. Hanson, J.D. Desai, A.J. Desai, *Biotechnol. Tech.* 7 (10) (1993) 745–748.
- [13] S. Panda, R. Kar, C. Panda, *Int. J. Environ. Sci.* 3 (5) (2013) 2013.
- [14] R. Latha, R. Kalaivani, *Adv. Appl. Sci. Res.* 3 (5) (2012) 2789–2795.
- [15] M.K. Subathra, G. Immanuel, A.H. Suresh, *Bioinformation* 9 (3) (2013) 150.
- [16] A.P. Mariano, D.M. Bonotto, D.d.F.d. Angelis, M.P.S. Piróllo, J. Contiero *Braz. J. Microbiol.* 39 (1) (2008) 133–142.
- [17] S. Varjani, V. Upasani, *Int. J. Innov. Res. Sci. Eng. Technol.* 2 (10) (2013) 5377–5383.
- [18] G.I. Barrow, R.K.A. Feltham, *Cowan and Steel's Manual for the Identification of Medical Bacteria*, Third ed., Cambridge University Press, 2003.
- [19] K. Tamura, G. Stecher, D. Peterson, A. Filipinski, S. Kumar, *Mol. Biol. Evol.* (2013).
- [20] T. Kayode-Isola, K. Eniola, A. Olayemi, O. Igunnugbemi, *American-Eurasian J. Agron.* 1 (1) (2008) 06–09.
- [21] E.C. Jensen, *The Anatomical Record* 296 (3) (2013) 378–381.
- [22] H. Lilly, E. Lowbury, *J. Med. Microbiol.* 5 (1) (1972) 151–153.
- [23] D.L. Paterson, *Clin. Infect. Dis.* 43 (Supplement 2) (2006) S43–S48.
- [24] E.M.S. Brito, R. Guyoneaud, M. Goñi-Urriza, A. Ranchou-Peyruse, A. Verbaere, M.A. Crapez, J.C.A. Wasserman, R. Duran, *Res. Microbiol.* 157 (8) (2006) 752–762.
- [25] R.J. Brooijmans, M.I. Pastink, R.J. Siezen, *Microb. Biotechnol.* 2 (6) (2009) 587–594.
- [26] K. Jyothi, K. Babu, N. Clara, A. Kashyap, *Helix* 2 (2012) 105–111.
- [27] H. Al-Awadhi, N. Dashti, M. Khanafer, D. Al-Mailem, N. Ali, S. Radwan, *SpringerPlus* 2 (1) (2013) 369.
- [28] J.E. Kostka, O. Prakash, W.A. Overholt, S.J. Green, G. Freyer, A. Canion, J. Delgado, N. Norton, T.C. Hazen, M. Huettel, *Appl. Environ. Microbiol.* 77 (22) (2011) 7962–7974.
- [29] A. Alonso, F. Rojo, J.L. Martínez, *Environ. Microbiol.* 1 (5) (1999) 421–430.
- [30] D. Borah, R. Yadav, *J. Environ. Sci. Technol.* 7 (3) (2014) 176–184.
- [31] M. Ahmad, W. Sajjad, Z.U. Rehman, M. Hayat, I. Khan, *Int. J. Curr. Microbiol. App. Sci.* 4 (2) (2015) 338–346.
- [32] P. Wongsu, M. Tanaka, A. Ueno, M. Hasanuzzaman, I. Yumoto, H. Okuyama, *Curr. Microbiol.* 49 (6) (2004) 415–422.
- [33] T. Wu, W. Xie, L. Yi, X. Li, B. Yang, J. Wang, *Plant Soil Environ.* 58 (9) (2012) 412–416.
- [34] D. Borah, R. Yadav, *J. Environ. Sci. Technol.* 7 (3) (2014) 176.
- [35] M. Meenakshisundaram, C. Bharathiraja, *Indian J. Appl. Res.* 4 (7) (2014) 39–42.