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Exopolysaccharide (EPS) Producing Bacteria of Sundarbans Mangrove Forest Soil

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Abstract

The present study was undertaken to investigate the exopolysaccharide (EPS) producing bacteria from Sundarbans Mangrove Forest (SMF) soil of Bangladesh. The pH and aerobic heterotrophic bacterial counts of the soil samples ranged from 5.83 to 7.77 and 0.87×10^7 to 7.2×10^7 cfu/g, respectively. Potential 18 EPS producing bacterial isolates were selected for detailed study among which 15 were provisionally identified as members of the genus *Bacillus*, 2 were recognized as *Dinococcus* sp. and another one as *Micrococcus* sp. The genus *Bacillus* includes 7 distinct species *viz*. *B. stearothermophilus*, *B. subtilis*, *B. brevis*, *B. marinus*, *B. schlegelli*, *B. pumilus* and *B. globisporous*. The highest EPS production was found in the LB medium. The pHs of 6.5-7.5, 0% salinity, and 37°C temperature were found to be optimum for better growth of EPS producing bacteria. The three EPS producing bacterial isolates *i.e. B. pumilus, B. globisporus* and *B. strearothermophilus* were further confirmed through 16S rDNA sequence analysis. The culture and sensitivity (C/S) test results revealed streptomycin (S 10) as the most effective antibiotic to control the tested bacterial isolates.

Introduction

The Sundarbans Mangrove Forest (SMF) is situated in south-west of Bangladesh lying in between the Baleswar river in East and the river Harinbanga in West. It is the Bay of Bengal adjoining largest mangrove forest in our planet. SMF covers an area of 10,000 km^2 with a unique ecological niche for diverse flora, fauna and microflora (Chaudhuri et al. 1994; Ahmad et al. 2009). The rich microbial diversity is found in mangroves where dying mangrove vegetation is continuously transformed into nutrients *viz*. phosphorous, nitrogen and more nutrients by the activities of various microbial communities. Fan et al.

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(2006) predicted that the total quantity of bacteria of mangroves is progressively correlated to the content of organic matter. The halophilic environment of mangroves leads the bacteria to produce different vital substances such as pigments, exopolysaccharides (EPSs), or other secondary metabolites to cope with harsh environmental conditions. Hence, the study on EPS producing bacteria may give us a clear idea to know the defense mechanism of SMF bacteria against the hostile condition.

Microbial EPS has been shown to play a key role in micro and macro environments, nutrition and cellular associations. EPSs are mainly carbohydrate polymers with high molecular weight that make up an important element of the extracellular polymers which surrounds the cell of microbes live in the marine environment. Bacterial polysaccharides play a vital role in strengthening the bioremediation process due to having the properties of ion uptake and the capability to bind cations. EPS producing bacteria can also be applied as a bioinoculant to increase the water retention capacity and aggregation of rhizosphere soil (Sutherland 1985). In addition, EPS induces nodulation in plants. EPS also plays a probable role in upkeeping the integrity of biofilm formation and its structure as well (Di Martino 2018). Microbial EPS may play an indirect role in genetic materials exchange through enhancing the cell attachment on solid surface which eventually promote aggregate formation (Sutherland 1985). Recently, EPSs are increasingly used in detergents, food, petroleum, textile, beverages, paint, paper, biotechnology, agricultural and pharmaceutical industries (Quesada et al. 1993). Moreover, EPS synthesized by bacteria plays a major role in manufacturing of milk-based desserts and fermented dairy products (Duboc and Mollet 2001). Considering the increasing demand for microbial polysaccharides, the present study aimed to isolate and identify EPS producing bacteria from SMF soil. Subsequently, special attention has been paid to finding out optimum temperature, pH, and salinity both for bacterial growth and EPS production. To the best of our knowledge, this may be the first comprehensive report on promising EPS producing bacteria from SMF soil in Bangladesh. The findings of this experiment may provide the new insight into the biotechnological application of EPS producing bacteria.

Materials and Methods

To obtain a fair idea about the EPS producing bacteria the Patcosta of SMF was selected as a sampling site for this study. The Patcosta site is situated at 22º1.5' N and 89º26'E of Khulna region in SMF. The sampling site includes five quadrates. At each quadrate, the sample soil was collected in depth wise which includes the upper layer (U, 0-10 mm), middle layer (M, 11-20 mm) and lower layer (L, 21-30 mm). Samples were collected aseptically in sterile ziplock bags and labeled properly. Thereafter, the soil samples were brought to the laboratory as early as possible for analyses. The pH of the samples was measured with a digital pH meter (Jenway 3310 pH meter, U.K) by preparing paste of soil at 1:2 ratio of soil and water. The samples were kept in a refrigerator at 4ºC and preserved them before and after analyses.

The serial dilution plate technique was followed using nutrient agar (NA) medium for enumeration and isolation of aerobic heterotrophic bacteria and fungi associated with the collected soil samples of SMF (Widawati et al. 2005; Gupta and Mohapatra 2002). The pH of the culture media was adjusted according to the recorded pH of soil samples. After 24 h of incubation at 37°C, distinct bacterial colonies were developed on serial dilution agar plates. The colonies developed on dilution plates were counted using a digital colony counter (OSK 10086, DC-3, Japan). Followed by some colonies with distinct characters were selected as EPS producing bacterial isolates and screened based on visual estimation for detailed study. The morphology of bacterial colonies was observed including colony form, elevation, margin, surface, color and optical characteristics as well (Eklund and Lankford 1967). The screened bacterial colonies were purified through the repeated streak plate technique. Gram-reaction and spore staining of the isolates were observed under the microscope (Nikon, FX 35 WA, Japan). A series of biochemical tests *viz*. catalase, methyl Red (MR), Voges-Proskauer (VP), NO_3 reduction, amylase production, hydrolysis of casein, Simmons citrate agar test, egg albuminase, lipase production, propionate test, lecithinase test etc. were performed for provisional identification of the selected EPS producing bacterial isolates. The Bergey's Manual of Systematic Bacteriology was consulted for the identification of Gram-positive aerobic heterotrophic bacterial isolates (Brenner et al. 2005, Sneath et al. 1986).

The heat-thaw method was employed for DNA extraction of the EPS producing bacterial isolates (Dashti et al. 2009). The broth cultures (1 ml) were transferred to Eppendorf tube and centrifuged at 13,000 rpm for 1 min. Followed by the pellets were collected and mixed comprehensively by adding 100 µl DNase free water. In a water bath the tubes were boiled for 10 min at 100°C and immediately transferred in ice for subsequent 10 min. Thereafter, the tubes were centrifuged for 5 min at 10000 rpm. The bacterial DNA containing supernatant was collected and preserved at -20°C. In this study, the 16S rRNA gene segment was amplified using universal primer pairs (27F, 5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492R, 5′-GGTTACCTTGTTACGACTT-3′). The PCR amplification reactions were conducted in a 30 µl mixture containing 2.0 µl DNA template, 1.2 µl of each forward and reverse primer, 22.5 µl of PCR Super Mix and 3.1 µl DNase-free water for 35 cycles using a thermal cycler. Amplification states comprised of 94°C for 1 min, 60°C for 30 sec, 72°C for 30 sec and final extension was performed at 72°C for 5 min. After amplification, the PCR products were separated with 1.0% agarose gel in 1.0×TAE buffer by electrophoresis (Cleaver Scientific, UK) and DNA bands were visualized with ethidium bromide under UV transilluminator (Micro-Doc CSL, UK). The sequences generated by the automated sequencing of PCR amplified DNA were analyzed through the BLAST program (http://blast. ncbi.nlm.nih.gov/) to find out the correct match of the bacterial isolates. Sequence alignment and phylogeny reconstruction were performed on MEGA6 using CLUSTALW and Neighbor-Joining packages, respectively. The consensus tree generated was tested by bootstrapping (1000 times).

Results and Discussion

The microbial load of SMF soil plays a vital role in nutrient recycling of the mangrove ecosystem and contributes to its high productivity. In the present study, the Patcosta sampling site of SMF revealed diverse pH values ranging from 5.83 to 7.77 where the pH of most of the samples was slightly acidic to neutral. In addition, a wide range of bacterial counts was observed where the recorded aerobic heterotrophic bacterial counts of collected 15 samples varied from 0.87×10^7 to 7.2×10^7 cfu/g soil (Table 1). In the case of all samples, the overall high bacterial load was accounted for the middle layer of each quadrate which might be due to the deposition of organic matter in the soil. The average aerobic heterotrophic bacterial count of each quadrate ranged between 0.33×10^7 and 5.20×10^7 cfu/g. The highest average count of 5.20×10^7 cfu/g was recorded in quadrate-16 while the lowest number (0.33×10^7 cfu/g) was found in quadrate-19. On an average, the bacterial count in SMF soil was 2.43×10^7 cfu/g. The bacterial count of this study had shown resemblance with the results of our previous studies (Saha et al. 2017, Anzum et al. 2022). A similar result was found in earlier reports demonstrated in the mangrove of Marambaia (2.0×10^7 cfu/g) in Brazil (Maciel-Souza et al. 2006) and the mangrove forest (1.38×10^7 to 4.13×10^7 cfu/g) of Odisha, India (Ranjan Mishra et al. 2012). Furthermore, a few numbers of fungi populations were recorded in this study (Table 1) showing agreement with the findings of fungal frequency from the mangrove in southern China (Wang et al. 2010). The low abundance of fungi in SMF soil might be attributed to the tough competition of fungi with bacteria and neutral soil pH as well. During this study, a total of 55 bacterial isolates were isolated from different soil samples among which 18 isolates were finally selected and purified by repeated streak plate for detailed study.

Microbial EPS is important for bacterial life that provides an ideal environment for chemical reactions, nutrient entrapment and protection against environmental stresses such as salinity and drought (Jayathilake et al. 2017). However, the microbial biodiversity of the SMF ecosystem is relatively unexplored. During this investigation, various types of EPS producing bacteria were isolated and identified. Table 2 shows the degrees of EPS producing bacterial growth on ten different specialized media. The LB medium was found to be the most suitable medium for better growth of EPS producing bacterial isolates.

Some morphological characteristics of the selected EPS producing bacteria have been shown in Fig. 1. The colony form results of eighteen bacterial isolates revealed 44.44% irregular, 38.89% circular and 16.67% rhizoidal colony. However, a varying degree of diversity was noticed in the case of colony margin of these isolates representing 55.56% entire, 27.78% undulate, and 16.67% rhizoidal. Accordingly, all the selected isolates were grouped under four different colony colors *viz.* off-white (61.11%), orange (16.67%), brown (16.67%), and yellow (5.56%) demonstrating the diversity in colony colors.

Table 1. pH and microbial load of collected soil samples of SMF.

Sampling site	Quadrate No.	Sample No.	pH	Bacterial Count (cfu/g)	Average bacterial count (cfu/g) in each quadrate	Average bacterial count (cfu/g)	Fungal count* (cfu/g)
Patcosta	16	U	6.91	1.60×10^7	5.20×10^7	2.43×10^7	31
		M	7.21	6.70×10^7			19
		L	7.31	7.20×10^7			13
	17	U	7.31	2.40×10^7	3.20×10^7		1
		M	7.31	3.65×10^7			5
		L	7.77	0.92×10^7			Nil
	18	U	6.54	3.08×10^7	2.00×10^7		12
		M	6.81	3.95×10^7			13
		L	7.14	0.89×10^7			2
	19	U	5.84	1.15×10^7	0.33×10^7		31
		M	5.83	0.92×10^7			14
		L	6.56	0.88×10^7			14
	20	U	6.87	0.87×10^7	1.42×10^7		1
		M	5.98	1.70×10^7			6
		L	6.71	1.70×10^7			18

*During isolation of bacteria, some fungal colonies were noticed on NA media and these colonies were recorded for only quantitative studies.

Table 2. Visual estimation of potential EPS producing bacterial growth on different media.

Isolates No.	NA	SA	PSA	GYA	LB	HIM	PGS	PGS(F)	PGS(S)	PGS(L)
16U/1/1	3	5	5	1	5	3	2	4	3	4
17U/1´/1	2	1	5	2	3	3	1	1	1	1
17U/4´/1	3	5	2	2	4	2	1	3	5	3
17M/4/1	2	3	3	1	4	3	1	0	1	1
17M/1´/1	4	1	4	1	5	5	1	2	2	2
17M/5´/1	2	5	5	2	4	3	2	1	2	1
17L/4/1	2	2	3	2	3	1	2	4	2	3
18U/1´/1	2	2	1	1	3	1	1	1	1	3
18M/2/1	2	5	2	2	3	5	1	2	5	4
18M/3/1	2	3	3	2	4	2	1	2	1	1
18L/4/1	1	2	2	3	3	2	1	3	2	3
18L/1´/1	2	2	4	3	5	1	1	1	2	3
18L/1´/2	3	5	5	2	5	5	1	1	1	3
19U/1/4	3	5	5	2	5	5	2	3	4	3
19M/5´/1	1	1	1	1	3	1	1	0	1	1
20L/2/1	1	1	1	3	3	1	1	2	5	1
20L/4/1	2	1	1	2	2	5	2	2	1	1
20L/4/2	2	2	4	1	2	1	2	3	1	1

"1" to "5"=Degree of EPS producing bacterial growth, '0'=No growth; NA= Nutrient agar, SA=Sucrose agar, PSA=Peptone sucrose agar, GYA=Glucose yeast ammonium agar, LB=Luria Bertani, HIM=Heart infusion medium, PGS=Peptone glucose salt agar, PGS (F)=Peptone glucose salt agar supplemented with fructose, PGS (S)=Peptone glucose salt agar supplemented with sucrose, PGS (L)=Peptone glucose salt agar supplemented with lactose.

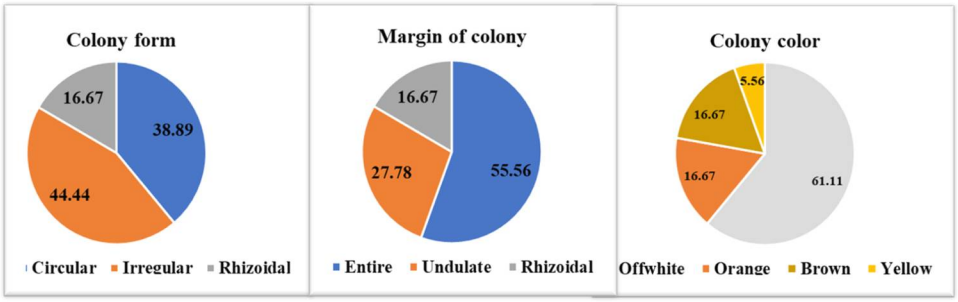

Fig. 1. Percentile presentation of colony morphology of eighteen selected EPS producing bacterial isolates

The microscopic observation and Gram reaction results revealed that the EPS producing isolates were Gram-positive and motile where most of them were rod-shaped and spore former exhibiting in agreement with the previous study conducted by Devendran et al. (1987) in the sediment of mangrove from Pichavaram, India. A range of biochemical tests was carried out for provisional identification (Table 3). Based on morphological characterization, biochemical tests and microscopic observation, the isolates were identified provisionally by consulting Bergey's Manual of Systematic Bacteriology (Sneath et al. 1986). Among 18 EPS producing isolates, 15 were under the genus *Bacillus* while 2 belonged to *Micrococcus* and another was recognized as the genus *Dinococcus*. There were 7 distinct species under the genus *Bacillus viz*. *B. brevis*, *B. marinus*, *B. subtilis*, *B. stearothermophilus*, *B. schlegelli*, *B. pumilus* and *B. globisporous*. In this study, the obtained results unveiled *Bacillus* as the dominant genus in SMF soil which represented 83.33% of all identified bacteria. In a previous study (Kathiresan et al. 2006), the genus *Bacillus* was reported as one of the vital components of microbial community in the mangrove soil justifying the obtained results of this study. The dominance of *Bacillus* in SMF soil might be attributed to the spore forming nature of bacteria which helped them to survive in soil habitats even under distressed conditions or nutrient deprivation. The presence of *Micrococcus* in the mangrove soil samples was also reported by Prescott et al. (1986) and Kumar et al. (2007).

The bacterial isolates were tested with a wide range of temperatures (4, 10, 30, 37, 45, 50, 55°C), pH (4.5, 5.5, 6.5, 7.5, 8.5), and salt concentrations (0, 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20%) to observe the optimum temperature, pH, and salt concentrations for highest bacterial growth and EPS production (data not shown). According to the obtained data, temperature of 37°C, 0% salinity and pH 6.5-7.5 were found to be optimum both for bacterial growth and EPS production.

The provisionally identified three EPS producing isolates were further confirmed with molecular analysis based on 16S rDNA gene sequences (Table 4). Based on BLAST search analyses, the isolates were identified as *Bacillus* sp., *B. pumilus* and *B. strearothermophilus*.

Table 3. Biochemical characteristics of the provisionally identified EPS producing bacterial isolates from SMF soil.

Isolates Name	Biochemical tests													Provisional Identification
	Catalase	VP	MR	Indole	Phenyl alanine	Citrate	NO_3 Reduction	Amylase	Casein	Egg albuminase	Propionate	Lipase	Egg yolk Lecithinase	
16U/1/1	+	+	-	-	-	-	+	+	+	+	+	+	+	*B. stearothermophilus*
17U/1´/1	+	+	-	-	-	-	+	+	+	+	+	+	+	*B. stearothermophilus*
17U/4´/1	+	+	-	-	-	+	+	-	+	+	+	+	+	*B. subtilis*
17M/4/1	+	-	-	-	-	-	+	+	+	-	-	+	-	*B. brevis*,
17M/1´/1	+	+	-	-	-	-	+	+	+	+	+	+	+	*Dinococcus* sp.
17M/5´/1	+	-	-	-	-	-	+	+	+	+	+	+	+	*B. marinus*
17L/4/1	+	+	-	-	-	-	+	-	+	+	+	+	+	*B. subtilis*
18U/1´/1	+	+	-	-	-	+	+	+	+	+	-	+	+	*B. schlegelli*
18M/2/1	+	+	-	-	-	-	+	+	+	+	-	+	-	*B. schlegelli*
18M/3/1	+	-	-	-	-	-	+	+	+	+	+	+	-	*B. pumilus*
18L/4/1	+	-	-	-	-	-	+	-	+	+	+	+	-	*B. globisporous*
18L/1´/1	+	+	-	-	-	-	+	-	+	+	+	+	-	*B. brevis*
18L/1´/2	+	-	-	-	-	-	+	+	+	+	-	-	-	*Dinococcus* sp.
19U/1/4	+	+	-	-	-	+	+	-	+	+	+	+	-	*B. stearothermophilus*
19M/5´/1	+	-	-	-	-	-	+	-	+	-	+	+	+	*B. pumilus*
20L/2/1	+	+	-	-	-	-	+	-	+	-	-	-	-	*B. pumilus*
20L/4/1	+	-	-	-	-	-	+	+	+	+	+	+	+	*B. brevis*
20L/4/2	+	+	-	-	-	+	-	-	+	+	+	+	+	*Micrococcus* sp.

"+" =Positive, **"-"** =Negative.

Table 4. Molecular identification and basic bioinformatics of some EPS producing bacteria.

Isolates name	Molecular Information			
	Scientific name	Query coverage (%)	E-value	Identity match
18M/3/1	*B. pumilus*	83	0.0	99.50%
18L/4/1	*Bacillus sp.*	72	0.0	99.00%
19U/1/4	*B. strearothermophilus*	87	0.0	98.00%

A phylogenetic tree was constructed by using MEGA4 (Tamura et al. 2007) software with the Neighbor-Joining (NJ) algorithm (Fig. 2). The tree was tested based on 1000 bootstrap replications values. The associated taxa clustered together is shown next to the branches.

At genus level, the molecular based identified three isolates were identical with the provisionally identified bacteria where two isolates had shown similarity even at species level (Table 3 and 4). Therefore, the conventional identification of bacteria based on their morphology, physiological characterization and biochemical profile had shown the validity to some extent and reliability in comparison to the molecular based identification.

Fig. 2. The Phylogenetic tree of the isolated EPS producing bacteria of SMF soil

The emergence and spread of antibiotic resistant pathogenic bacteria pose a severe public health challenge worldwide in the twenty-first century (FAO 2016). Hence, a culture and sensitivity (C/S) test was carried out in this study to unveil the antibiogram profile of the identified bacteria. Falkiner (1990) demonstrated that antibiotic *viz.* gentamicin resists the bacterial cell wall synthesis which eventually inhibits bacterial growth. Therefore, it can be inferred that the antibiotic discs used in the present study might control the bacterial growth by preventing the development of their cell wall. The diameter of the inhibition zone (mm) for different antibiotics at their respective concentration were measured and evaluated to conclude them as effective or non-effective against test organisms.

As shown in Table 5 and Fig. 3, all of the bacterial isolates had shown susceptibility against streptomycin (S-10) which agreed to the earlier reports (Reed et al. 1995 and Ali et al. 2018). Gentamycin (CA 10) and vancomycin (VA 30) revealed antibacterial activities against all bacteria except *B. schlegelli*. Among four antibiotics penicillin (P10) was found to be less effective against EPS producing bacteria showing a resemblance with our earlier report (Ali et al. 2011). Conversely, *B. schlegelli* demonstrated resistance against the three antibiotics *viz.* gentamycin (CA 10), vancomycin (VA-30), and penicillin (P 10).

All the antibiotics used in this study were found to be effective to inhibit the growth of most of the tested bacterial isolates hinting the control potentiality of tested organisms. The results suggested that streptomycin (S 10) could be used to control the growth of *Bacillus* which are capable to produce EPS. The uses of particular drugs could play a key role in controlling pathogenic bacteria during EPS production which ultimately could lead to ascertaining the health safety of EPS produced by the selected organisms.

Table 5. Culture and Sensitivity test of the selected EPS producing bacteria.

Isolates No.	Identified bacteria	Antibiotics and inhibition zone (mm)			
		P 10	CA 10	VA 30	S 10
16U/1/1	*B. stearothermophilus*	28.0	14.5	6.5	14.0
17U/1´/1	*B. stearothermophilus*	28.0	14.5	6.5	29.0
17U/4´/1	*B. subtilis*	R	18.0	11.0	18.4
17M/4/1	*B. brevis,*	41.5	28.5	17.7	19.0
17M/1´/1	*Dinococcus* sp.	18.5	18.0	11.0	18.5
17M/5´/1	*B. marinus*	35.5	25.5	17.5	18.5
17L/4/1	*B. subtilis*	R	23.5	10.5	11.0
18U/1´/1	*B. schlegelli*	R	26.5	10.0	13.0
18M/2/1	*B. schlegelli*	R	R	R	23.0
18M/3/1	*B. pumilus*	11.5	12.6	25.8	19.0
18L/4/1	*B. globisporous*	11.0	26.5	32.4	18.5
18L/1´/1	*B. brevis*	28.0	14.5	6.5	14.0
18L/1´/2	*Dinococcus* sp.	28.0	14.5	6.5	14.0
19U/1/4	*B. stearothermophilus*	R	18.0	11.0	13.5
19M/5´/1	*B. pumilus*	41.5	28.5	17.7	24.0
20L/2/1	*B. pumilus*	18.5	18.0	11.0	43.0
20L/4/1	*B. brevis*	35.5	25.5	17.5	22.0
20L/4/2	*Micrococcus* sp.	R	23.5	10.5	12.0

Disc size= 6mm; R= Resistant, P= Penicillin, CA= Gentamycin, VA= Vancomycin, S= Streptomycin.

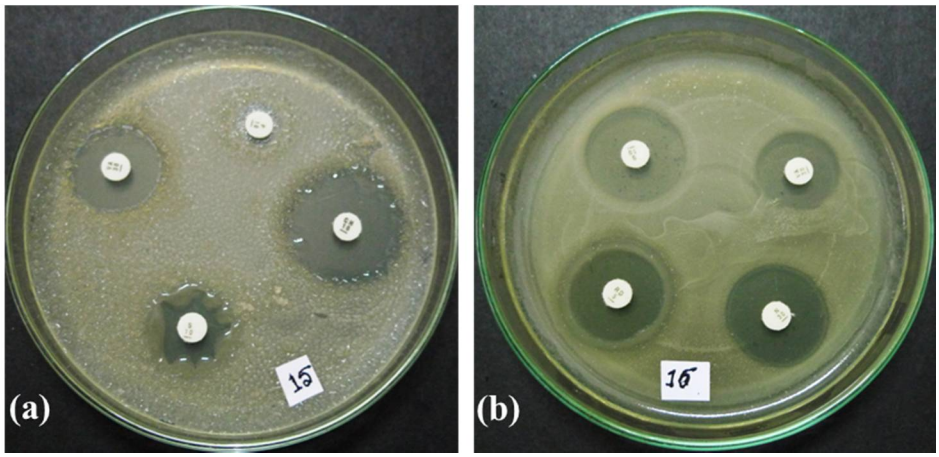

Fig. 3. Culture and sensitivity test of selected bacterial isolates using different antibiotic discs.

The finding of high aerobic heterotrophic bacterial load in SMF soil indicates the rich microbial community in SMF soil even under saline conditions. EPSs are necessary for the survival of microbes live in the extreme marine environment which resulted the ubiquity of microbial EPS for marine environment. EPS could assist the microbial communities to endure extreme environmental conditions. Hence, the presence of bacteria in SMF soil ratifies the presence of EPS producing bacteria which could help us to know the defense mechanism of EPS producing bacteria to survive under stress conditions. EPS producing bacteria have great biotechnological potential in metal bioremediation, food processing, pharmaceutical industries as emulsifier, solidifying agent and solubilizer. The isolation and identification of EPS producing bacteria in this study may provide extensive opportunities for new fields of biotechnology. The optimization results of EPS producing bacterial growth might induce EPS production which could be applied for industrial purposes. The susceptibility of all tested bacterial isolates against four antibiotics discloses the control potentiality of pathogenic bacteria during EPS production. Streptomycin (S 10) was found to be the most effective antibiotic to control the growth of all bacteria. Based on the findings of this study, it can be concluded that more advanced study is needed to explore the defense mechanism and development of biotechnological application of EPS producing bacteria in industrial processes.

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