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EXOPOLYSACCHARIDE PRODUCING BACTERIA OF SUNDARBAN MANGROVE FOREST SOIL AND THEIR ANTIBIOTIC SENSITIVITY PROFILE

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Key words: EPS producing bacteria, Microbial diversity, SMF soil bacteria, Antibiotic sensitivity

Abstract

This study focused on the investigation of Exopolysaccharide (EPS) producing bacteria from Sundarbans mangrove forest (SMF) soil, Bangladesh. The heterotrophic bacterial loads in the soil samples varied from 0.44×10^7 to 4.2×10^7 cfu/g indicating high bacterial load even under hostile environment. Fifteen EPS producing bacterial isolates were identified provisionally where thirteen isolates belonged to the genus *Bacillus* including *B. badius* (n=1, 6.67%), *B. subtilis* (n=3, 20.0%), *B. pumilus* (n=3, 20.0%), *B. brevis* (n=2, 13.33%), *B. stearothermophilus* (n=2, 13.33%), *B. sphaericus* (n=1, 6.67%) and *B. alcalophilus* (n=1, 6.67%). The remaining two isolates were recognized as the genus *Micrococcus* sp. (n=2, 13.33%). The genus *Bacillus* was predominant representing 86.67% abundance frequency. The LB medium was proven to be the most suitable medium for the growth of EPS producing bacterial isolates. 16S rDNA sequence analysis was conducted for three EPS producing bacterial isolates and they were identified as *Bacillus subtilis*, *B. strearothermophilus* and *Micrococcus* sp. The antibiogram profile of this study revealed streptomycin as the most effective antibiotic to control the growth of bacteria. The presence of antibiotic resistance bacteria in SMF soil is alarming for human health associated with this marine ecosystem. The multidrug resistance bacteria may come to the soil of SMF through the untreated discharged wastewaters and agricultural runoff from adjacent areas.

Introduction

Sundarban is the largest Mangrove Forest (MF) in the world which is one of the prominent marine ecosystems in Bangladesh. It is geographically situated in between the latitude of 21°56′59″N and the longitude of 89°10′59.988″E in the southern portion of Bangladesh lying in the gigantic delta on the Bay of Bengal. Sundarbans mangrove forest (SMF) is a unique ecological niche to various flora, microflora and fauna covering an area

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of 10,000 km^2[1,2]. In SMF soil, the microbial diversity performs significant role in the biotransformation of organic matter and minerals[3]. Hence it can be suggested that microbial diversity is an essential component to maintain the balance of marine ecosystem. The diverse and rich microbial diversity of mangrove forest simultaneously transform the nutrients of deceased vegetation of mangrove into the sources of phosphorus, nitrogen, carbon and other nutrients. Fan *et al*.[4] speculated that the total numbers of prevailed bacteria in mangroves are positively correlated with the organic matter content. To withstand the hostile environmental condition of marine ecosystem, different types of major substances such as exopolysaccharides (EPS), pigments or other secondary metabolites are secreted by the bacteria live in marine environments. Therefore, the investigation on EPS producing bacteria may provide us a guide to understand their interaction with marine ecosystem and activities against hostile environmental condition.

Polysaccharides or glycans are naturally abundant chemical compound exhibiting different physical and chemical properties, and biological functions. The natural sources of polysaccharides are mainly animals, plants, algae, fungi, yeast and bacteria[5]. Generally, there is a layer of polysaccharide attached outside of bacterial cell wall which is recognized as glycocalyx. When the polymers are tightly attached on the surface of bacterial cell to form a capsule by covalent bonds, they are termed as capsular polysaccharides. Conversely, the loosely attached polymers that form a slime layer on the surface of bacterial cell is known as exopolysaccharide (EPS)[6]. EPSs are extracellular high molecular weight macromolecules excreted as loosely attached slime layer surrounding the cells of most of the microorganisms in marine environment. The microbial extracellular polysaccharides are metabolic by-products of microorganisms e.g. bacteria, yeast, fungi algae, etc. and those are either soluble or insoluble in nature[7,8]. As suggested, EPS has numerous functions such as it enhances the bacterial interactions with environment, helps bacteria to survive under environmental stresses (changes in temperature, pH, osmotic pressure etc.) and provides protection of bacteria from hostile conditions[6,9]. In addition, the nodulation in plants is induced due to the presence of EPS. EPS also play probable role in maintenance of integrity and structure of biofilm formation[10]. Microbial EPS helps in aggregation by enhancing the entrapment of cells to solid surfaces which indirectly assists to exchange the genetic materials[11]. Now a days, the bacteria mediated EPS play a vital role in the manufacturing of milk-based desserts and fermented dairy products[12]. Nonetheless, the real functions of microbial EPSs are still unknown in mangrove forest ecosystem. Therefore, this study focuses on the investigation of microbial EPS from SMF soil ecosystem.

Considering the above facts, this study attempts to conduct a comprehensive and in-depth examination of microbial population in SMF soil along with the identification of EPS producing bacteria from marine ecosystem. Finally, the culture and sensitivity (C/S) test of EPS producing bacteria has been critically examined to reveal their antibiotic sensitivity and speculate the possible sources of antibiotic resistant bacteria in this

natural habitat. The findings of this study may open a new window for understanding the microbial diversity in SMF soil with special reference to EPS producing bacteria.

Materials and Methods

Two distinct sampling sites of SMF *viz.* Katka and Arpanggacia were selected in this study to investigate the microbial community of SMF with special reference to EPS producing bacteria. The soil samples were collected from five sampling location covering the two sampling sites. In case of each sampling location, the soil samples were collected from three different depths of soil denoting as upper layer (U, 0-10 mm), middle layer (M, 11-20 mm) and lower layer (L, 21-30 mm). Samples were collected aseptically in properly labeled sterile ziplock bags and brought immediately to the laboratory for both physical and microbial analyses. The collected samples were stored in a refrigerator at 4ºC before and after the analyses. The soil suspension was prepared by mixing soil and water at the ratio of 1:2. The pHs of soil suspension were measured using a digital pH meter (Jenway 3310 pH meter, U.K).

The enumeration and isolation of mangrove microbiota were examined by conducting the serial dilution plate technique supplemented with nutrient agar (NA) medium[13]. The pH of the culture media for each set of dilution plate was kept very similar with the recorded soil samples pH. The inoculated dilution plates were incubated at 37°C for 24 h to develop discrete bacterial colonies on those plates. The colonies developed on dilution plates were enumerated by using a digital colony counter (OSK 10086, DC-3, Japan). Some colonies developed with mucoid characters on agar plate were assumed as EPS producing bacterial colonies and isolated them for detailed study. The isolated bacterial colonies were purified by repeated streak plate technique. The morphology of bacterial colonies was studied under the heading of colony form, elevation, margin, surface, color and optical characteristics[14]. The differential staining e.g. Gram-reaction and endospore staining of the selected isolates were done and followed by observed under microscope (Nikon, FX 35 WA, Japan). Some important physiological and biochemical tests *viz*. catalase, oxidase, casein hydrolysis, production of lipase & amylase, methyl Red (MR), Voges-Proskaure (VP), NO_3 reduction, egg albuminase, lecithinase tests etc. were conducted for conventional identification of the selected EPS producing bacterial isolates. The Gram-positive aerobic heterotrophic bacterial isolates were identified through consulting the Bergey's Manual of Systematic Bacteriology[15,16].

The DNA of three selected EPS producing bacterial isolates were extracted following the heat-thaw method[17]. The Eppendorfs containing broth cultures (1 ml) of bacteria were centrifuged at 13,000 rpm for 1 min. After centrifuge, the pellets were collected and then 100 µl RNase free water was added to mix thoroughly. The tubes were boiled at 100°C in a water bath for 10 min and instantly placed in ice for another 10 min; then, the tubes were centrifuged at 10000 rpm for 5 min. The supernatant was collected and

preserved at -20°C that contains bacterial DNA. In this study, 16S rDNA gene was amplified using a universal oligonucleotide primer pair 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) in a thermal cycler. For PCR amplification, the reactions were done in a 30 µl mixture compiled of 22.5 µl PCR Super Mix, 1.2 µl of each forward and reverse primer, DNA template 2 µl and 3.1 µl RNase free water 35 cycles using a thermal cycler. Amplification states were 94°C for 1 min, 60°C for 30 sec, 72°C for 30 sec and final extension was carried out at 72°C for 5 min. After amplification, PCR products were examined with 1.0% agarose gel in 0.5X TAE buffer by electrophoresis (Compact XS/S, Biometra) and DNA bands were visualized with ethidium bromide under UV transilluminator (Biometra). The sequences generated by the automated sequencing of PCR amplified DNA was analyzed through 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 MEGA4 using CLUSTALW and Neibour-Joining packages, respectively. The consensus tree generated was tested by bootstrapping (1000 times).

The Culture and sensitivity (C/S) test was carried out following the Kirby-Bauer method[18]. This test was conducted by inoculating the selected EPS producing bacterial isolates on Mueller-Hinton agar medium. The antibiogram test of EPS producing bacterial isolates was performed using four different antibiotic discs *viz.* Penicillin (P, 10 µg), Gentamycin (CA, 10 µg), Vancomycin (VA, 30 µg) and Streptomycin (S, 10 µg). The antibiotic discs were aseptically placed on inoculated bacterial plates and incubated at 37 ºC for 24 h. The diameters of the developed inhibition zones around each inoculated disc were measured in mm and recorded accordingly for further analysis.

Results and Discussion

The microbial load of SMF soil plays a vital role in nutrient recycling of mangrove ecosystem and contributes to its high productivity. In present study, the pH of soil samples collected from SMF varied from 6.65 to 7.56 with an average pH of 7.10 demonstrating the neutral pH which could be conducive for the growth of neutrophile microorganism in mangrove forest soil. The saline condition of mangrove forest may hinder the growth of microbial community in the soil of SMF. The mechanism of biogeochemical cycling and pollutant removal from marine environment by soil bacteria prevailed in the mangrove[19] can be realized through exploring the microbial diversity of SMF soil.

The observed bacterial load of 15 soil samples ranged from 0.44×10^7 to 4.12×10^7 cfu/g soil (Table 1) revealing the high bacterial load even under saline habitat. The top layer soil of Katka sampling site had shown more bacterial count while for Arpanggacia sampling site, the higher bacterial count was found in low soil layer which might be attributed to the high nutrient leaching in Arpanggacia site as compared to Katka site. The highest aerobic heterotrophic bacterial count (4.12×10^7 cfu/g) was observed in the

area of Katka while the lowest number (0.44×10^7 cfu/g) was found in Arpanggacia. On an average, the Katka site demonstrated higher bacterial count (2.14×10^7 cfu/g) as compared to Arpanggacia sampling site (0.67×10^7 cfu/g). The average bacterial count in SMF soil was 1.41×10^7 cfu/g showing agreement with the bacterial count of previous reports conducted in mangrove forest of Marambaia (2.0×10^7 cfu/g), Brazil[20] and Odisha (1.38×10^7 to 4.13×10^7 cfu/g), India[21]. Unlike total bacterial counts, a few numbers of fungi (up to 64 cfu/g) were recorded in this study (Table 1) revealing the similar trend for fungal count with the earlier report carried out for mangrove in southern China[22]. The low abundance of fungi in this study might be accredited to the strong competition of fungi with bacteria for nutrients. Moreover, the neutral soil pH of SMF are limiting factors for fungal growth. As discussed in previous report, the microbial diversity in SMF play a pivotal role in biotransformation of the organic matter and mineral[3]. Hence, the high abundance of bacterial population along with prevalence of few numbers fungal population in this study could open a new window for understanding the dynamic characters of marine ecosystem of SMF. Initially 33 bacterial colonies were isolated from collected soil samples among which 15 isolates were screened and purified by repeated streak plate for detailed study.

Table 1. The microbial counts and pH of collected soil samples from SMF.

Sampling sites	Sampling location	Layer wise samples	pH	Bacterial count (cfu/g)	Av. bacterial count (cfu/g) in each sample	Av. bacterial count (cfu/g) in each site	Fungal count* (cfu/g)
Katka	14	U	6.68	2.30×10^7	1.30×10^7	2.14×10^7	60
		M	6.65	0.78×10^7			06
		L	7.21	0.93×10^7			17
	15	U	6.72	3.45×10^7	2.99×10^7		62
		M	7.05	4.12×10^7			01
		L	6.94	1.42×10^7			08
Arpang gacia	21	U	6.75	0.44×10^7	0.66×10^7	0.67×10^7	14
		M	7.30	0.77×10^7			64
		L	7.20	0.79×10^7			06
	22	U	7.30	0.59×10^7	0.68×10^7		23
		M	7.20	0.80×10^7			22
		L	7.16	$0.89\ 10^7$			21
	23	U	7.20	0.58×10^7	0.67×10^7		Nil
		M	7.52	0.59×10^7			02
		L	7.56	0.85×10^7			03
Average			**7.10**			**1.41×10^7**	**20.6**

EPSs are fundamental structural constituent of bacterial cell wall which play an important role in protecting bacteria against distress environmental conditions including

osmotic pressure, extreme salinity, temperature and dehydration by forming a layer surrounding the bacterial cells[23]. Microbial EPS also delivers favorable environment for nutrient entrapment, chemical reactions, and safeguard against environmental stresses such as drought, salinity etc.[24]. Microbial diversity in SMF plays crucial role in biotransformation of organic matter and minerals which ultimately help to maintain the ecosystem balance[3]. Hence, this study focused on the unearthing the microbial diversity of SMF which eventually could open the door to know about abundance and adaptation mechanism of EPS producing bacteria in marine environment. As mentioned in Table 1, numerous types of EPS producing bacteria were prevailed in the soil of SMF. Immediately after isolation, the selected EPS producing bacteria were grown on ten different specialized media to explore the most effective media for high pigment production along with vigorous bacterial growth (Fig. 1) where LB medium was observed as the most suitable media.

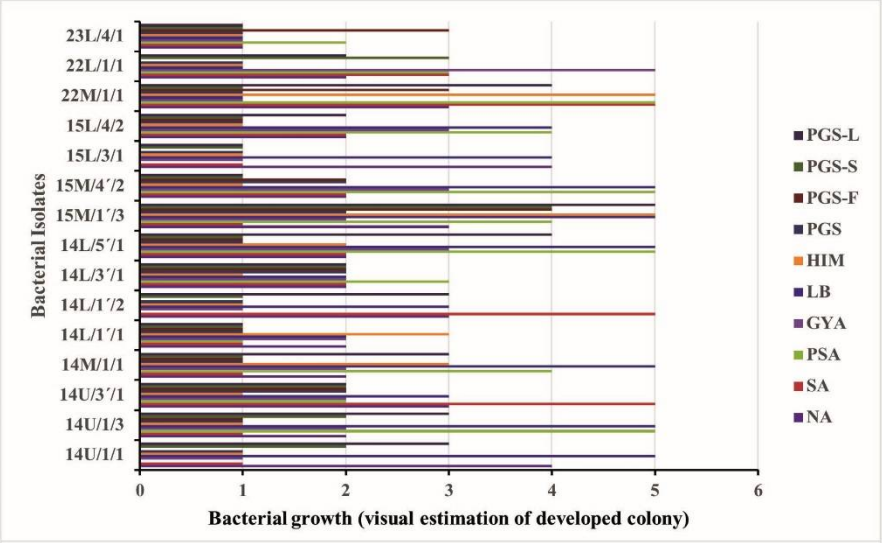

Fig. 1 Visual estimation (0-5) of potential EPS producing bacterial growth (colony development) on different media. ["1" to "5"=Degree of EPS producing bacterial growth, 'NA= Nutrient agar, SA=Sucrose agar, PSA=Peptone sucrose agar, GYA=Glucose yeast ammonium agar, LB=Luria bertani, HIM=Heart infusion 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.]

All the isolates of this study were found to be motile, Gram positive and rod shaped, some were endospore former exhibiting resemblance with the study of Devendran *et al*.[25] conducted in the mangrove sediment of Pichavaram, India. The EPS producing bacterial isolates were tested with a range of temperatures (4, 10, 30, 37, 45, 50 and 55°C), pHs (4.5, 5.5, 6.5, 7.5 and 8.5) and salt concentrations (0, 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20%) where the temperature of 30-37°C, pH 6.5-7.5 (data not shown here) were found to

be favorable for the EPS producing bacterial growth while the isolates withstand at variable concentration of salt (0-7%) revealing their high adaptability under different saline conditions.

Table 2. Physiological and biochemical characteristics of the selected Gram positive EPS producing bacterial isolates and their identification.

Isolates Name	Biochemical tests														Provisional Identification
	Catalase	Oxidase	VP	MR	Indole	Phenyl alanine	Citrate	NO_3 reduction	Amylase	Casein	Egg albuminase	Propionate	Lipase	Lecithinase	
14U/1/1	+	-	-	-	-	-	-	-	-	+	+	-	+	+	*Bacillus badius*
14U/1/3	+	-	-	-	-	-	-	+	+	+	+	-	+	+	*B. subtilis*
14U/3´/1	+	-	+	-	-	-	-		+	+	+	-	+	+	*B. subtilis*
14M/1/1	+	-	+	-	-	-	-	-	+	+	+	-	+	+	*B. pumilus*
14L/1´/1	+	-	+	-	-	-	-		-	+	+	-	+	+	*B. pumilus*
14L/1´/2	+	-	+	-	-	-	-	-	-	+	+	-	-	-	*B. brevis*
14L/3´/1	+	-	+	-	-	-	+	+	-	+	+	-	+	+	*B. stearothermophilus*
14L/5´/1	+	-	-	-	-	-	-	-	+	+	+	-	+	+	*B. sphaericus*
15M/1´/3	+	-	+	-	-	-	-		+	+	-	-	+	-	*B. brevis*
15M/4´/2	+	-	-	-	-	-	-	+	+	+	-	-	+	-	*Micrococcus* sp.
15L/3/1	+	-	-	-	-	-	-	+	+	+	+	-	+	+	*B. alcalophilus*
15L/4/2	+	-	-	-	-	-	-	+	+	+	+	+	+	+	*B. stearothermophilus*
22M/1/1	+	-	-	-	-	-	-	+	-	+	+	+	+	+	*B. pumilus*
22L/1/1	+	-	+	-	-	-	-	-	-	+	+	-	+	-	*B. subtilis*
23L/4/1	+	-	+	-	-	-	+	-	-	+	+	+	+	-	*Micrococcus* sp.

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

The EPS producing bacterial isolates were identified (Table 2) by consulting Bergey's Manual of systematic Bacteriology[15] based on the morphological and microscopic observation, a series of physiological and biochemical tests results. Out of fifteen isolates, thirteen isolates were identified as the genus *Bacillus* which includes seven distinct species such as *B. badius* (n=1, 6.67%), *B. subtilis* (n=3, 20.0%), *B. pumilus* (n=3, 20.0%), *B. brevis* (n=2, 13.33%), *B. stearothermophilus* (n=2, 13.33%), *B. sphaericus* (n=1, 6.67%) and *B. alcalophilus* (n=1, 6.67%). Conversely, the remaining two isolates were recognized as *Micrococcus* sp. (n=2, 13.33%). *Bacillus* was found to be the most dominant (Fig. 2) genus in this study with an abundance frequency of 86.67% revealing the similarity with the findings of our previous study[26]. The high abundance of *Bacillus* in the present study might be attributed to the endospore forming properties of this bacteria which helped

them to endure in distress conditions e.g. saline soil habitats. Moreover, the presence of *Micrococcus* in mangrove soil is agreed with the previous studies[26, 27] justifying the finding of our study. The identified *Micrococcus* sp. and *Bacillus* sp. of this study were also reported earlier[28] as the indigenous bacteria which play a crucial role in leaf litter decomposition of mangrove forest soils and thus maintain the ecological balance by recycling nutrients.

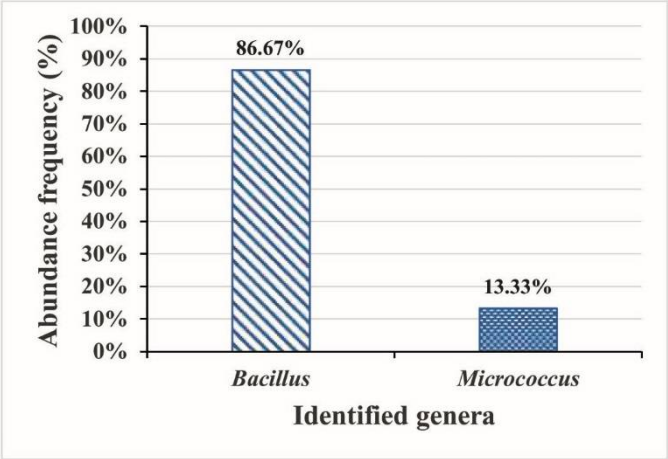

Fig. 2. Abundance frequency of the identified bacteria obtained from soil of SMF.

On the basis of 16S rDNA gene sequences, three EPS producing bacterial isolates were identified at species level (Table 3). Considering the BLAST search analyses the isolates were confirmed as *Bacillus subtilis, Micrococcus* sp. and *B. strearothermophilus.* The molecular based identified three isolates were found to be identical with the provisional identification at genus level. Hence, the provisional identification of bacteria still has the validity to some extent in contrast to molecular identification.

Table 3. Molecular identification of potential EPS producing bacteria through 16S ribotyping.

Isolates name	Molecular information			
	Scientific name	Query coverage (%)	E-value	Identity match
14U/1/3	*B. subtilis*	87	0.0	99.65%
15M/4´/2	*Micrococcus* sp.	77	0.0	99.50%
15L/4/2	*B. stearothermophilus*	87	0.0	98.00%

The phylogenetic tree (Fig. 3) was constructed by using MEGA4 software with the Neighbor Joining (NJ) algorithm[29]. The tree was tested based on 1000 bootstrap replications values. The associated taxa clustered together is shown next to the branches (Fig. 3).

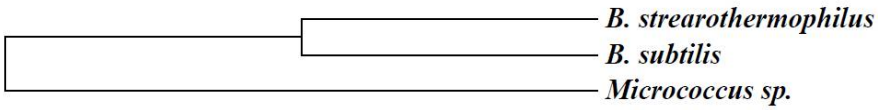

Fig. 3. The Phylogenetic tree of the identified EPS producing bacteria of SMF soil.

According to FAO[30], the appearance and spread of antibiotic resistant infective bacteria cause a serous public health challenge worldwide. Therefore, we have conducted a culture and sensitivity (C/S) test in this study to evaluate the antibiotic susceptibility and resistance pattern of the tested EPS producing bacteria (Table 4).

The diameter of the developed inhibition site (mm) was measured, and based on its diameter the tested antibiotics were inferred as effective or non-effective against the EPS producing organisms. Four commonly used antibiotics discs *viz*. penicillium (P 10 µg), gentamycin (CA 10 µg), vancomycin (VA 30 µg) and streptomycin (S 10 µg) were used in this study to assess the antibiogram profile of pigment producing bacteria isolated from SMF soil. As observed in Table 4 and Figs. 4-5, almost all of the tested bacteria exhibited sensitivity against streptomycin (S-10) which was also reported in previous studies[26, 31].

Table 4. Culture and Sensitivity test of the selected EPS producing bacteria.

Isolates No.	Antibiotics and inhibition zone (mm)			
	P 10	CA 10	VA 30	S 10
14U/1/1	R	25.5	17.0	31.0
14U/1/3	20.0	26.5	16.5	30.5
14U/3´/1	20.5	7.5	12.5	8.5
14M/1/1	22.5	R	24.0	16.5
14L/1´/1	10.5	26.5	R	30.0
14L/1´/2	23.0	R	23.0	36.5
14L/3´/1	36.5	26.0	18.5	16.0
14L/5´/1	R	18.5	12.0	10.5
15M/1´/3	R	27.5	16.0	20.0
15M/4´/2	R	25.5	19.5	R
15L/3/1	R	R	R	19.0
15L/4/2	R	R	R	14.0
22M/1/1	R	26.5	10.0	34.0
22L/1/1	R	R	R	12.0
23l/4/1	11.5	12.6	25.8	13.0

[Disc size=6mm; R=Resistant; P=Penicillin; CA=Gentamycin; VA=Vancomycin and S=Streptomycin]

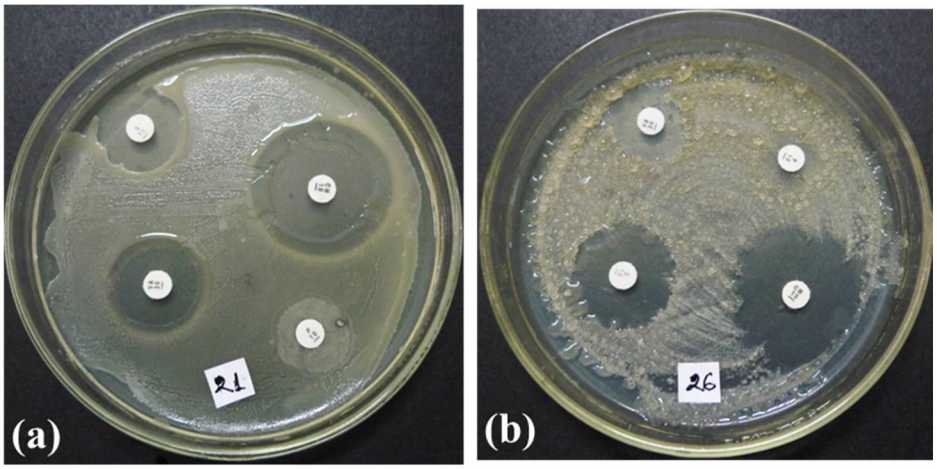

Fig. 4. Antibiotic sensitivity test of EPS producing bacterial isolates against four different antibiotics.

The high sensitivity of tested organisms against streptomycin implying the potentiality to control pathogenic organism which eventually could provide safety during the handling of these organisms for pigment production. The gentamycin (CA 10 µg) and vancomycin (VA 30 µg) antibiotics had shown sensitivity against 66.67% and 73.33% EPS producing bacterial isolates, respectively. The penicillin (P 10) was found to be less effective antibiotic to control the organisms in this study since only 46.67% organism revealed sensitivity against this antibiotic which was in accordance with our earlier report[32].

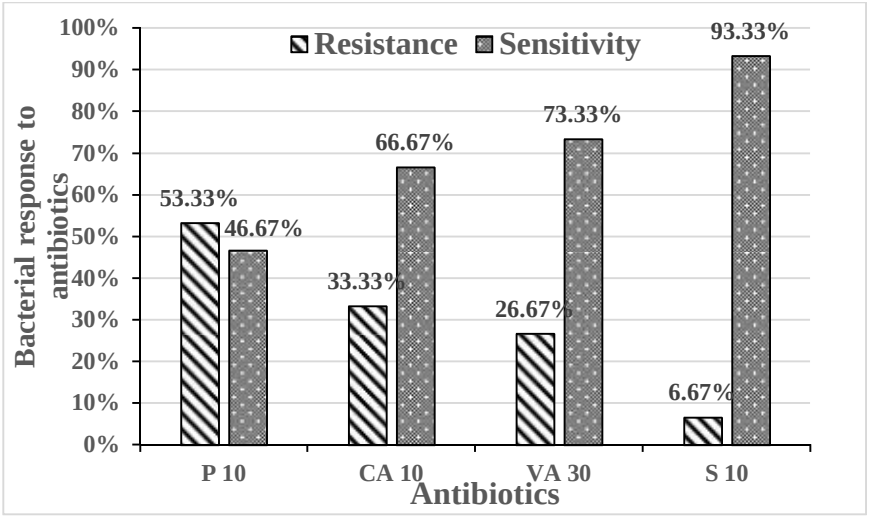

Fig. 5. The antibiotic sensitivity and resistance profile of EPS producing bacterial isolates against four commercially available antibiotics.

Overall, most of the tested antibiotics had shown the ability to resist the growth of EPS producing bacterial isolates paving the way to control the tested organisms. However, a good number of organisms of this study were multidrug resistant. In C/S test (Fig. 5), 53.33% bacteria revealed resistant against penicillin where 33.33% and 26.67% bacteria were found to be resistant against gentamycin and vancomycin antibiotics which is alarming for human health during the handling of these organisms. The presence of antibiotic resistant bacteria in the microbial community of SMF soil is threatening to human and animal population associated with SMF ecosystem. It may be assumed that a group of multidrug resistant bacteria has got exposed to Sundarbans mangrove forest soil through dumping of untreated wastewaters from different industries and agricultural run-off into the rivers and streams as well. The triggering of antibiotic resistance properties of the bacteria in SMF might be directly or indirectly linked to the discharge of untreated industrial wastes and chemical fertilizers run-off into streams and adjacent rivers that merge with the Sundarbans estuary.

The prevalence of high aerobic heterotrophic bacterial load in SMF soil even under saline condition inferring the rich microbial diversity in SMF soil with high adaptability. It is well known that EPS is essential for microbial survival and it assists the microbes to endure in extreme marine environment. Therefore, the presence of high microbial diversity in distress condition of mangrove forest ecosystem implying the prevalence of EPS producing bacteria in SMF soil microbial community. The identification of EPS producing bacteria from SMF soil may provide an opportunity to develop a new field of biotechnology. The antibiotic resistance properties of EPS producing bacteria of SMF soil is alarming for human health. The multidrug resistant bacteria may come in SMF soil through the dumping of industrial wastes and agricultural run off into the adjacent rivers which should be controlled by implementing stringent controlling measures. The defense mechanism of EPS producing bacteria in extreme environmental conditions can be explored in subsequent study which could lead to develop a new biotechnological application in industrial processes.

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Declaration

This is to certifying that the work has been carried out by the authors themselves and the contents of the paper were neither published nor submitted for publication to any other journal except to the Editorial Committee of Dhak University Journal of Biological Sciences.

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