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Study of the antimicrobial activity of the solvent extracts of mangrove plants associated *Bacillus* species

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Abstract

Mangrove associated bacterial species are the major inhabitants that represent a rich reservoir for production of bioactive compounds. The isolates procured from the mangrove environment were enriched and screened for potent compounds using agar strip method, agar streak method and Wilkin's overlay method. The isolates showing inhibition against the test pathogens were subjected to identification using partial 16s rRNA gene sequencing, which were found to be *Bacillus species.* Isolates were bulk seeded into the nutrient medium, grown, followed by removal of bacterial cells using membrane filtration technique. The filtrate was mixed with equal amounts of solvent and kept overnight under shaken conditions. The two layers were separated using a separating funnel. The solvent layer was concentrated and its efficacy was checked against various pathogens like *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Proteus mirabilia, Klebsiella pneumoniae* and *Salmonella typhi* using agar cup method. The solvent concentrates showed inhibition against the pathogens which confirmed the antimicrobial activity of *Bacillus species*.

Key words : *Bacillus* spp., antimicrobial activity, bioactive compounds, mangrove.

Background:

The demand for new antibiotic discovery is urgent due to the rise in virulent disease frequency and the quick development of multidrug resistance[13]. Global public health is at risk due to the rise in microbial resistance to antibiotics, which reduces treatment efficacy and raises morbidity, death, and medical expenses[6]. Due to the severe lack of new

antibacterial medication options, medical professionals are obliged to deal with the scenario known as "Bad Microbes, No Drugs," which has made the search for novel antibiotics a crucial and urgent problem[13].

Mangroves are found along tropical and subtropical coastlines in the intertidal zone. They have a harsh, distinct climate with a very productive ecosystem[13]. One of the most expressive parts of this ecosystem is the greenery, and the majority of the surroundings is reflected in the leaves. Mangrove forests serve as a link between freshwater, marine, and terrestrial forests due to their geographical location. Through photosynthesis, plants significantly affect the atmosphere, and the organic matter they contribute to the planet Earth through their leaves also affects a larger portion of the energy. In a healthy environment, various microorganisms from various microbial classes, including bacteria, archaea, and fungi, coexist and interact with plants to form what is known as the plant microbiome. The aerial portion of a plant, which is twice as large as the terrestrial surface, is its primary component: the leaves. The aerial or upper portion of a plant is known as the phyllosphere ecosystem, and the foliar or upper surface is known as the phylloplane. The creatures that live in these environments are known as epiphytes[9]. While some chemicals, like steroids, terpenoids, alkaloids, and phenols, are products of secondary metabolism and are thought to have pharmacological, ecological, and toxicological significance, others, like carbohydrates, proteins, and amino acids, are products of primary metabolism and are essential for the maintenance of life processes[2].

It has been demonstrated that a number of mangrove plant species can produce bioactive substances that may regulate specific bacteria growth. According to preliminary research, mangrove plant extracts have demonstrated effectiveness against multidrug-resistant strains of *Proteus* sp. and *Staphylococcus* sp. as well as pathogenic bacterial strains such as *Escherichia coli, Pseudomonas* sp., and *Staphylococcus* sp. Mangrove species have been utilized extensively in traditional remedies, and their extracts have been shown to prevent diseases that affect people, animals, and plants[12].

Sampling :

A sample of thirty healthy individual mangrove plants leaves were collected. Ten leaves were taken from each plant, placed in sterile plastic bags, and kept in the refrigerator at 10°C for fewer than twenty-four hours[8].

Isolation of Phylloplanes /Epiphytic microbes :

The samples were exposed to the following procedure in order to separate the epiphytic microorganisms: ten 1 cm discs were cut from each plant leaf using a sterile cork borer. The discs were put into 10 millilitre phosphate buffered saline (PBS) tubes, pH 7. The PBS is a 0.1 M phosphate buffer that contains 0.1% Bacto Peptone. Next, the suspension was vortexed for two minutes. From the obtained suspension, serial dilutions were performed in PBS up to a factor of 10^{-4}. Trypticase soy agar plates (TSA: tryptone 17 g, soytone 3 g, dextrose 2.5 g, NaCl 5.0 g, K_2HPO_4 2.5 g, agar 15 g, distilled water 1000 ml, pH

7.3 ± 0.2) were plated with aliquots of 100 µl suspension. At 25°C, plates were incubated. After 24 to 48 hours, colonies were seen[10].

Screening :

Giant colony inhibition spectrum (Agar strip method/Agar streak method): The cultures whose activity has to be assessed are streaked in a small section across the centre of the plates or injected onto the central regions of the designated media plates. After that, the plates are incubated till growth is attained. Then, test microorganism strains are streaked from the plate edges up to the central cultures, avoiding contact with the central growth, in order to assess the strains susceptibility to the bioactive compounds generated. The plates are then further incubated to aid in the growth of the test organisms, and the distance in millimetres across which each test organism's growth has been suppressed by bioactive substances generated nearby the core culture is then noted[1]. In agar streak method, the isolate to be checked for bioactive compounds were streaked at the central part of the plate and allowed to grow, further the test cultures were streaked from the edges of the plate to the centre followed by incubation at 37°C for 24 hours. In agar strip method the test strains are streaked continuously from one end of the plate to other and a strip is cut aseptically from a pre-grown swabbed culture (isolate) plate and is placed perpendicular to the streaks of the test cultures. Further on incubation inhibition is checked.

Crowded plate approach (Wilkins Overlay method): This is the most straight forward screening method for finding microorganisms that produce bioactive compounds. The source of the microorganisms is diluted to a cell concentration where 300–400 individual colonies per plate are packed onto the agar surface of the dilutions formed from these dilutions. A region of agar surrounding a colony that is devoid of other colonies development indicates that the colony is manufacturing bioactive compounds. The colony's metabolism and quick uptake of essential nutrients in the immediate proximity of the colony cause the medium's pH to shift. In order to create well-isolated colonies, dilutions of microbial sources are applied to the surface of agar plates, and test organisms are utilized as a signal for the existence of particular bioactivity. To produce bioactive compounds, the plates are cultured until the colonies reach a specific diameter. After applying a test organism suspension to the agar plate's surface in some way, the plates are incubated to promote the test organism's growth. Zones where an organism's growth is inhibited surrounding bioactive compounds, leading to colonies, are indicative of bioactivity[1]. For this research this test is carried out to check production of bioactive compounds. The suspension of the isolates was spot inoculated on the nutrient agar medium and allowed to grow well up to a certain diameter, later Wilkins overlay medium containing the test organism was overlayed onto the grown isolate spots and further incubated at 37°C for 24 hours. Then the plates were further observed for inhibition around the spotted isolates.

Identification of Bacteria :

Partial 16S rRNA gene sequencing: The isolated colony's 16S ribosomal RNA (rRNA) gene was amplified using PCR and

then sequenced[7].

Fermentation and filtration of secondary metabolites :

The isolates that showed bioactivity were inoculated (5–10% inoculum of 0.5 McFarland) in 100 millilitres of pH 7.0 nutrient medium and cultured for four days at 28°C with constant shaking[4]. The broth was then centrifuged for ten minutes at 10,000 rpm in order to extract the bacterial cells. To get the cell-free supernatant, the extracts were filtered using membrane filter paper (45µ pore size) using a vacuum filtration pump equipped with a filtering unit[5].

Extraction of secondary metabolites using solvent extraction :

Ethyl acetate solvent was used to extract bioactive compounds from the centrifuged culture broth (supernatant). Equal amounts (1:1) of the supernatant and ethyl acetate were placed in a separating funnel and was agitated for approximately half an hour. After the solvent layers were divided, ethyl acetate was once more used to extract the supernatant. At 40°C, the solvent layers were combined, evaporated, and then concentrated. After that, the crude solvent extract was tested for antibacterial activity[11].

Check for antibacterial activity of the extract :

Using the agar well diffusion method, the solvent extract's antibacterial properties were assessed against a variety of bacteria, including *Salmonella typhi MTCC 98, Proteus mirabilis MTCC 1429, Escherichia coli MCC 2412, Pseudomonas aeruginosa MCC 2080,* and *Klebsiella pneumoniae MCC 2451*. In wells measuring 6 mm in diameter on nutrient agar plates, test microorganisms (24 hours old) were inoculated followed by 50 µl of the solvent extracts, and the plates were then incubated at the appropriate growth temperature of 37°C. Zones of inhibition were seen and evaluated after a 24-hour period[3].

Mangrove plant leaves samples were collected from four different sampling areas namely–Dahisar– Kandarpada, Dahisar – RTO, Versova (below metro station) and Gorai creek. In total 44 samples were collected out of which 97 isolates were obtained in total. Isolated colonies were obtained on trypticase soy agar after 48 hours after carrying out serial dilutions for the samples and spread plate technique. Fig. 1.

Fig. 1. *Excoecaria agallocha Linn. Acanthus ilicifolius Linn. Rhizophora mucronate Poir*

After screening of all the 97 isolates it was found out that only 3 isolates namely S4WR, S6A and isolate no. 8 showed inhibition against the test pathogens (*Escherichia coli MCC2412* and *Staphylococcus aureus NCIM 5022*). S4WR was isolated from the samples obtained from Dahisar (kandarpada), S6A from Versova (below Versova metro station) and isolate no 8 from Gorai creek. These three cultures have shown inhibition against the test pathogens during the screening process *i.e.* Agar strip method, Agar streak method and Wilkins Overlay method. Fig. 2, 3 and 4.

Fig. 2. Agar Strip Method (Inhibition effect of isolates S4WR and S6A on Test pathogens *Escherichia coli* and *Staphylococcus aureus*).

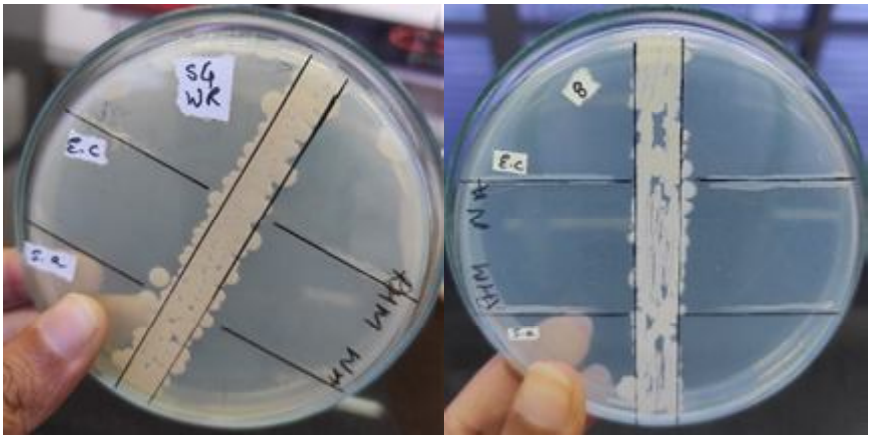

Fig. 3. Agar Streak Method (Inhibition effect of isolates S4WR and isolate no 8 on Test pathogens *Escherichia coli* and *Staphylococcus aureus*).

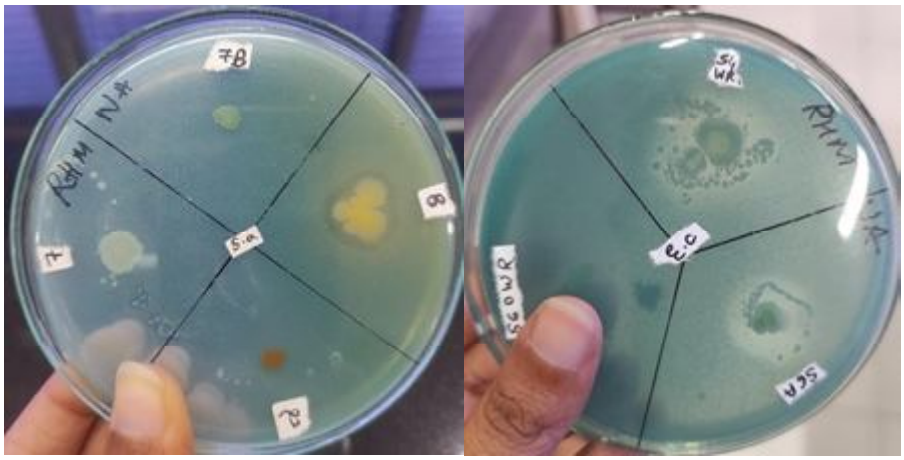

Fig. 4. Wilkins Overlay Method (Inhibition effect of isolate no 8 on test pathogen *Staphylococcus aureus*, Inhibition effect of isolates S4WR and S6A on test pathogen *Escherichia coli*).

Table 1. Results for Partial 16S rRNA gene sequencing.

Name of the Isolate	Result
S4WR	*Bacillus subtilis*
S6A	*Bacillus subtilis*
Isolate No. 8	*Bacillus licheniformis*

Phylogenic Tree

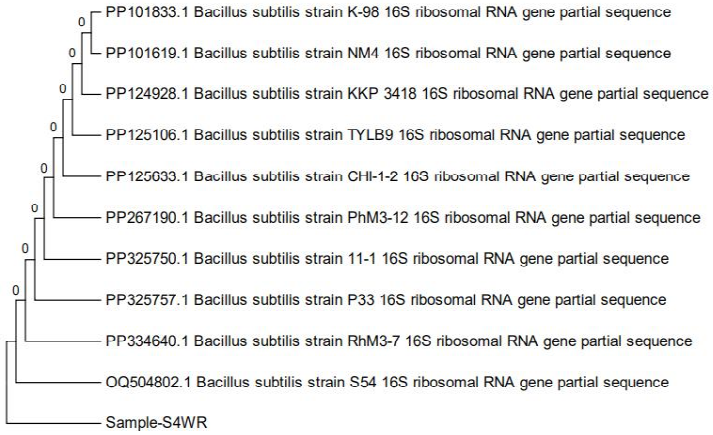

Figure. Evolutionary relationships of taxa

The evolutionary history was inferred using the Neighbor-Joining method [1]. The optimal tree with the sum of branch Length is greater than 0 shown. The confidence probability (multiplied by 100) that the interior branch length is greater than 0, as estimated using the bootstrap test (500 replicates is shown next to the branches [2, 3]. The evolutionary distances were computed using the Maximum Composite Likelihood method [4] and are in the units of the number of base substitutions per site. The analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 620 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

Phylogenic Tree

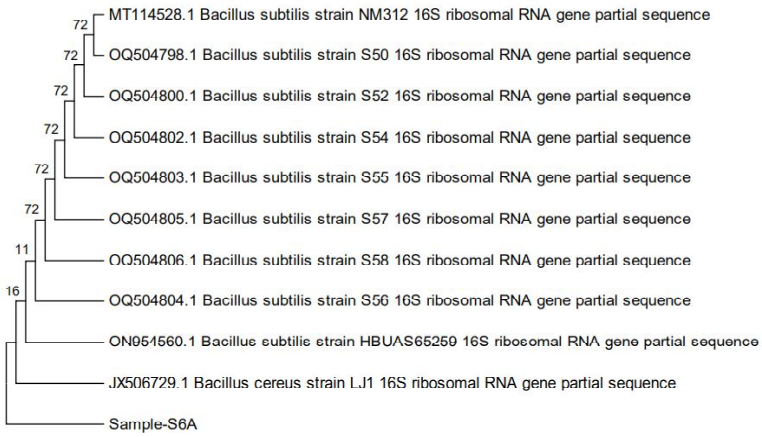

Figure. Evolutionary relationships of taxa

The evolutionary history was inferred using the Neighbor-Joining method [1]. The optimal tree with the sum of branch Length = 4.33948777 is shown. The confidence probability (multiplied by 100) that the interior branch length is greater than 0, as estimated using the bootstrap test (500 replicates is shown next to the branches [2, 3]. The evolutionary distances were computed using the Maximum Composite Likelihood method [4] and are in the units of the number of base substitutions per site. The analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 725 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

Phylogenic Tree

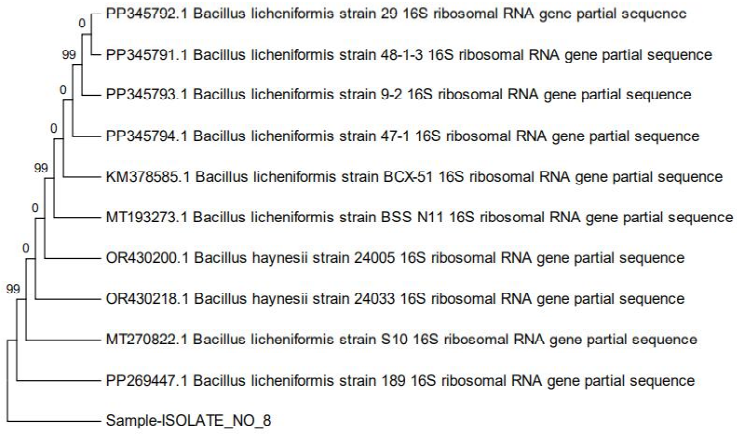

Figure. Evolutionary relationships of taxa

The evolutionary history was inferred using the Neighbor-Joining method [1]. The optimal tree with the sum of branch Length = 1.05018344 is shown. The confidence probability (multiplied by 100) that the interior branch length is greater than 0, as estimated using the bootstrap test (500 replicates is shown next to the branches [2, 3]. The evolutionary distances were computed using the Maximum Composite Likelihood method [4] and are in the units of the number of base substitutions per site. The analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 791 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

Table-2. Antibacterial activity of solvent extracts against test pathogens.

Test Organism	Zone of Inhibition (in mm)			
	Solvent extract of S4WR	Solvent extract of S6A	Solvent extract of Isolate No 8	Ethyl acetate (Control)
Escherichia coli MCC 2412	18 mm	12 mm	19 mm	10 mm
Staphylococcus aureus NCIM 5022	26 mm	19 mm	22 mm	-
Pseudomonas aeruginosa MCC 2080	13 mm	15 mm	14 mm	-
Klebsiella pneumoniae MCC 2451	12 mm	11 mm	13 mm	11 mm
Proteus Mirabilis MTCC 1429	15 mm	12 mm	16 mm	-
Salmonella typhi MTCC 98	17 mm	14 mm	15 mm	11 mm

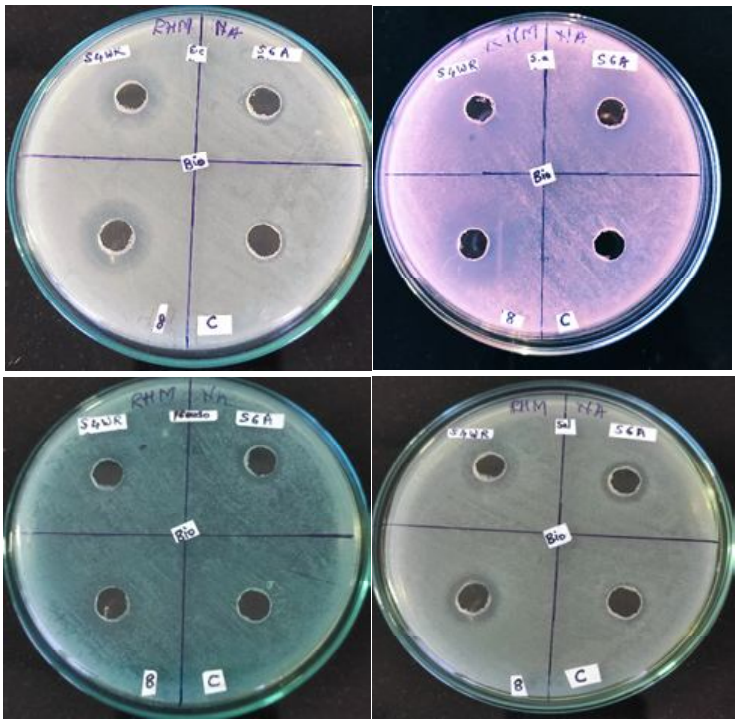

Fig. 5. Results for solvent extracts of the isolates S4WR, S6A and isolate no 8 against the test organisms *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa* and *Salmonella typhi.*

On the basis of the results obtained we conclude that the bacterial strains (*Bacillus spp*) isolated from the mangrove area are producing novel bioactive compounds that are having antimicrobial activity. The inhibition observed against the various pathogenic reference strains can conclude that these bioactive compounds can be separated, identified and further used for synthesis of various potent drugs.

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