ISOLATION AND CHARACTERIZATION OF BACTERIA ISOLATED FROM MANGROVES SOIL

Jaya Singh, Sapna Yadav and Annika Durve-Gupta
Department of biotechnology, B.K. Birla College of Arts, Science and Commerce, Kalyan DistThane. MS

ABSTRACT

Bacterial diversity from Mangroves ecosystems has been studied for their unique biochemical processes and their use in various applications. In the present study, mangroves soil sample from two different places of Retibandar area, Dombivli (west), were collected for the isolation of bacteria. Twenty-five bacterial isolates were isolated and were studied for their gram nature and colony characters. These bacterial isolates were screened for the production of extracellular enzymes like Protease, Amylase, β-galactosidase, Cellulase, Lipase etc. They were also checked for their ability to degrade dyes and for production of different PGPR traits like phosphate solubilization, Production of auxin (IAA), Ammonia production, HCN production, Siderophore production, Nitrogen fixation and Sulphate oxidation. They were also tested for their Antibacterial ability and Salt tolerance. They were also tested for their resistance/sensitivity against different antibiotics. The study has overall concluded that bacteria isolated from mangroves soil could serve as potential bioresources for many biotechnological applications. Bacterial diversity from mangroves soil ecosystems has been studied for their unique biochemical processes and their use in various applications. The study has overall concluded that bacterial isolated from mangroves soil could serve as potential bioresources for many biotechnological applications.

KEYWORDS: Mangroves bacteria, enzymes, dye degradation, PGPR traits, salt tolerance, antibacterial activity, antibiotics sensitivity.

INTRODUCTION

Mangroves are a highly productive ecosystem and are of ecological and economic importance. Mangroves are located on the coastal area andare considered to have highly developed root systems. Mangroves provide a unique ecological niche to the various microbes present in that area. Because of richness in carbon and other nutrients mangrove ecosystem harbours diverse microbial communities which have adapted in the extreme conditions there.Microorganisms play important roles in the mangroves ecosystem and make essential contributions to its productivity.

Bacteria create mutualistic relationship with the mangrove trees. The bacteria provide services such as N-fixation while the mangroves trees provide root exudates stimulating microbial growth activity. There is a lot of competition among the microorganism because of the limited amount of the nutrients present. Mangroves provides a unique ecological niche to different micro-organisms, which plays various different roles in nutrient recycling as well as environmental activities. Distribution of micro-organisms mainly depends on the changes in the temperature, salinity and other different parameters. Due to high concentration of salinity, halophilic bacteria are predominant in the ecosystem. It plays an important role in serving food to the marine organisms and maintains the nature of the environment (Masilamani and Kathiresan, 2010).

Some soil bacteria, isolated from the root region are known to enhance the growth of the plants. Soil bacteria present in root regions are known to enhance plant growth. This beneficial effect is mediated through either direct or indirect mechanisms. The direct effects are commonly attributed to the supply of biologically fixed nitrogen and the production of plant hormones, such as auxins. These bacteria are termed as plant growth promoting rhizobacteria (PGPR) which include nitrogen fixing bacteria, phosphate solubilizing bacteria, etc. The PGPR also improves the growth of the plants by indirect mechanisms such as suppression of bacteria, fungal and pathogens, and also production of siderophores, HCN, ammonia, antibiotics, etc. (Masilamani and Kathiresan, 2010).Different enzymes like proteases, amylases are also obtained from the mangrove's bacteria.

In the present study, the isolation of bacteria was carried from the mangroves soil. The potential of microorganisms presents in mangroves soil which can be utilized for various enzyme processes and biodegradation processes. The ability to produce PGR's and siderophores was also checked and to relate them with the nutrient's levels in the soil.

MATERIALS AND METHODS

Test chemicals:

All the chemicals and media were prepared in distilled water. The media components were procured from Himedia, India.

Sample collection

Mangroves soil and water sample were collected from Retibandar, Dist Thane. Soil was collected from 4cm deep area using a sterile spatula. The soil samples were collected in sterile plastic bags and stored at 4ºC till further processing.

Isolation of bacteria

Suspensions of the soil sample were prepared using Sterile normal saline (0.75% NaCl). Bacteria were isolated on Sterile Nutrient agar plates. The plates were incubated at 37ºC for 24 hours. After incubation, well isolated bacterial colonies were selected and were subcultured for purification on Sterile Nutrient agar plates. The bacterial isolates were studied for their Gram nature and Colony characters.

Screening for production of extracellular enzymes

All bacterial isolates were screened for the production of extracellular enzymes namely amylase, lipase, cellulase, beta-galactosidase, protease using simple plate assay.

For Amylase activity, SterileNutrient agar medium supplemented with 0.2% starch as substrate was used. A24 hours old culture was spot inoculated on the plates and incubated for 24 hours at 37ºC. After incubation the plates were flooded with Grams iodine, to check the formation of clear zones around the colony for the production of amylase(Jain*et al*., 2017).

For Protease activity, Sterile Nutrient gelatin medium was used. A 24 hours old cultures were spot inoculated on the nutrient gelatin medium and incubated for 24 hours at 37ºC. After incubation, the plates were observed for the clear zones around the colony for the production of protease activity(Durve *et al*., 2015).

For Lipase activity, Sterile Nutrient agar containing ethidium bromide and oil was used. A 24 hours old cultures were spot inoculated on the agar plate and incubated for 24 hours at room temperature. After incubation the plates were observed under UV transilluminator for a clear zone around the colony for the production of lipase activity(Jain*et al., 2017*).

For Cellulose activity, Sterile Carboxymethylcellulose (CMC) agar was used. A 24 hours old cultures were spot inoculated on the plates and the plates were kept for incubation for 24 hours at room temperature. After incubation, the plates were flooded with congo red and 10% NaCl for 10 mins. The clear zone was observed around the colony for the production of cellulose (Jain *et al.*,2017).

For Beta-galactosidase activity, Sterile Luria Bertani agar containing X-gal and IPTG was used. The cultures were spot inoculated on the agar plates and incubated for 24 hours at 37ºC. After incubation, blue-green colonies were observed for the production of beta-galactosidase (Durve *et al.*, 2015).

Dye degrading ability

The isolates were checked for their ability to degrade dyes using Sterile Bushnell Hass broth. The biological dyes like Crystal violet and Phenol red was used. The 24 hours old cultures were inoculated in the Sterile Bushnell Hass broth and incubated for 24 hours at 37ºC. After incubation, 0.5 ml of 100 ppm of respective dyes were added in the broth and were incubated for 48 hours at 37ºC. After incubation, the tubes were centrifuged at 5000 rpm for 10 mins and supernatant was used for the colorimetric analysis. Percentage decolourization (%) was calculated using the formula(Jain *et al.*,2017).

Decolourization (%) = Initial absorbance – Final absorbance x 100 / Initial absorbance

Characterisation of the isolated bacteria for PGPR traits

Screening for phosphate solubilizing bacteria

For the screening of Phosphate solubilizing bacteria, Sterile Pikovaskaya's phosphate solubilizing agar was used. The 24 hours old culture was spot inoculated on the agar plate and incubated for three days at 28±2ºC. After incubation, the clear zones around the colony were observed. Phosphate solubilization index (SI) was calculated by measuring the diameter of the zones using the formula(Jain *et al.*,2017).

SI(solubility index)= colony diameter(mm)+solubilization zone(mm) / colony diameter (mm)

Production of auxin(IAA)

For the production of auxin(IAA), a medium containing 1% peptone water, 1% tryptophan was used. The 24 old cultures were inoculated in the medium and incubated for 24 hrs at 28±2ºC. After incubation, the medium were centrifuged at 5000 rpm for 10 mins. For IAA estimation, the supernatant was mixed with two drops of orthophosphoric acid and 4ml of Salkowski's reagent (50 ml of 35% perchloric acid, 1 ml of 0.5 N $FeCL_3$ solution). The development of pink colour indicated the production of IAA. Optical density was measured at 540nm. Concentrationof IAA was estimated by using the standard IAA(Jain *et al., 2017*).

HCN production

For HCN production, Sterile Nutrient agar amended glycine was used. The 24 hours old cultures was spot inoculated on the modified agar plates. A Whatman filter paper no.1 was soaked in 2% sodium carbonate in 0.5% of picric acid solution and placed on the top of the plate. Plates were sealed with parafilm and incubated at 28±2ºC for 4 days. Development of orangeto red colour indicated the production of HCN(Jain *et al.*, 2017).

Ammonia production

For Ammonia production, the peptone water medium was used. The 24 hours old culture was inoculated in the medium and were incubated at 37ºC for 48-72 hours. For Ammonia estimation, 0.5 ml Nessler's reagent was added on the respective colonies. The development of yellow colour indicated the production of ammonia(Jain *et al.*, 2017).

Siderophores detection

For Siderophore production, Sterile Chrome Azurol S (CAS) agar, where 60.5 mg Chrome azurol S (CAS) dissolved in 50 ml distilled water and mixed with 10ml $FeCL_3$ (1 mmol/lit) solution. While constantly stirring, this solution was slowly added to 72.9 mg HDTMA dissolved in 40 ml distilled water. The resultant dark blue mixture was diluted 20-fold and autoclaved at 121ºC for 15 min. Agar (3%, w/v) was used as gelling agent. Petri dishes (10 cm in diameter) were prepared with CAS- blue agar (dye solution 10ml) as bottom agar plate. After solidifying, the CAS- blue agar was overlayed with an appropriate MM9 agar (7ml) containing (g/L) Na2HPO4 (6.8); KH2PO4 (0.3); NaCl (0.5); NH4CL (1.0); Glucose (4% w/v); Agar (3%, w/v). The 24 hours old culture were spot inoculated on the agar plates and incubated at 37ºC for 24-48 hours. Development of pink halo surrounding the colonies indicated the production of siderophore(Jain *et al.*, 2017).

Nitrogen fixation

To determine the ability of isolates to fix atmospheric nitrogen, Sterile Norris Glucose N-free medium (1g K2HPO4, 0.2g MgSO4.7H2O, 1g CaCO3, 0.2g NaCl, 5mg FeSO4.7H2O, 10g glucose, 5mg NaMoO4 per litre, and 1.5% agar at pH 7.0) containing Bromothymol blue (BTB) as an indicator were used. The 24 hours old cultures was spot inoculated on the agar plates and incubated for 24 hours at 37ºC. After incubation, the blue coloured zones producing isolates were considered to be nitrogen fixers. The colouring zone was calculated by deducting the colony diameter from the colouring zone diameter(Jain *et al.*, 2017).

Resistance/sensitivity pattern to antibiotics

All the bacterial isolates were tested for the resistance and sensitivity against four different antibiotics *i.e.*Erythromycin, Carbenicillin, Aztreonam, Tetracycline (Himedia, India). The 24 hours old culture was swabbed on the nutrient agar plates and the respective antibiotic discs (6mm diameter)were placed on the agar plates and was incubated for 24 hours at 37ºC. After incubation, the clear zones around the colony was observed. The diameter (mm) was measured and the results were compared with Kirby Bauer chart(Amir *et al.*, 1993; Bhat *et al.*, 2013).

Salt tolerance

Salinity tolerance was tested using different concentrations of salinity (30-110%) prepared in Sterile Nutrient agar plates. The 24 hours old cultures were spot inoculated on the agar plates and incubated at room temperatures for 48 hours. After incubation, growth was observed on the plates(Amir *et al.*, 1993).

Antibacterial activity

For Antibacterial activity, the Wilkin's agar overlay method was used. The 24 hours old cultures were spot inoculated on the agar plates and incubated at 24 hours at room temperature. After incubation the plates were overlayed with Wilkin's agar containing test organisms *E. coli* and *S. aureus*respectively in different plates. The plates were incubated for at 37ºC for 24 hours(Durve *et al.*, 2015).

RESULTS AND DISCUSSIONS

Twenty-five morphological differentbacterial isolates were isolated on the Nutrient agar plates after incubation at 37ºC for 24 to 48 hours.

(M1,M2,M3,M4,M5,M6,M7,M10,M11,M12,M13,M14,M15,M16,M17,M18,M19,M20,M21,M22,M23,M24,M25)

Colony characteristics were studied and Gram staining was performed for all the isolates and it was observed that fourteen isolates were Gram positive (M1, M2, M5, M7, M9, M11, M12, M13, M14, M16, M17, M18, M19, M21) and eleven isolates were Gram negative (M3, M4, M6, M8, M10, M15, M20, M22, M23, M24, M25).

Screening for production of extracellular enzymes

For Amylase activity, seven bacterial isolates (M4, M9, M10, M11, M12, M14, M16) showed a clear zone around the colony on nutrient agar supplemented with 0.2% starch as substrate after treating with grams iodine. Thus, indicating the production of amylase activity (Fig 1) (Table 1).

Fig 1: Bacterial isolates producing amylase enzyme

For Protease activity, sixteen bacterial isolates (M2, M3, M4, M5, M6, M7, M9, M12, M13, M14, M15, M17, M18, M19, M20, M25) showed a clear zone around the colony on nutrient gelatin medium. Thus, indicating the production of protease activity (Fig 2) (Table 1).

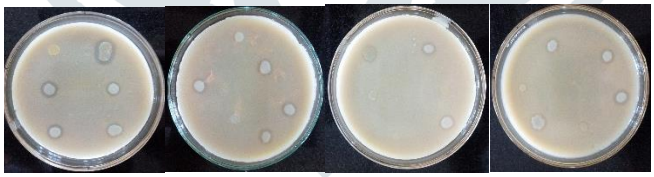

Fig 2: Bacterial isolates producing protease enzyme

For Lipase activity, ten bacterialisolates (M2, M3, M5, M7, M8, M10, M12, M19, M20, M21) showed a clear zone around the colony on nutrient agar containing ethidium bromide and oil. Thus, indicating the production of lipase activity (Fig 3) (Table 1).

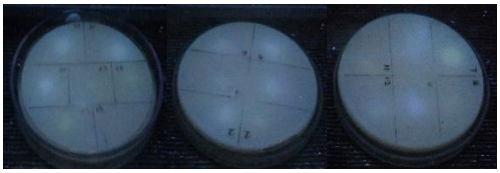

Fig 3: Bacterial isolates producing lipase enzyme

For Cellulase activity, ten bacterial isolates (M7, M12, M14, M15, M16, M17, M19, M20, M24, M25) showed a clear zone around the colony on carboxymethylcellulose (CMC) agar plates. Thus, indicating the production of cellulase activity (Fig 4) (Table 1).

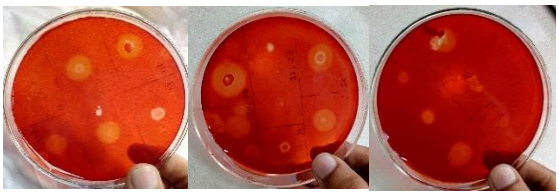

Fig 4: Bacterial isolates producing cellulase enzyme

For Beta-galactosidase activity, six bacterial isolates (M4, M9, M10, M13, M17, M23) showed blue-green colonies on the Luria Bertani agar containing X-gal and IPTG. The presence of blue green indicates the production of beta-galactosidase activity (Fig 5) (Table 1).

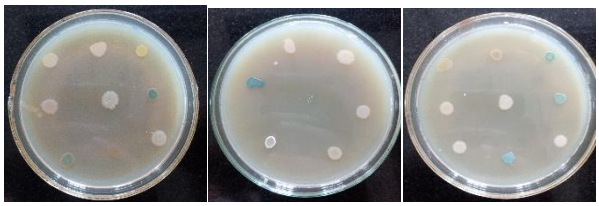

Fig 5: Bacterial isolates producing beta-galactosidase enzyme

Table 1: Enzyme activity

Enzymes	M 1	M 2	M 3	M 4	M 5	M 6	M 7	M 8	M 9	M 10	M 11	M 12	M 13	M 14	M 15	M 16	M 17	M 18	M 19	M 20	M 21	M 22	M 23	M 24	M 25
Amylase	-	-	-	+	-	-	-	-	+	+	+	+	-	+	-	+	-	-	-	-	-	-	-	-	-
Protease	-	+	+	+	+	+	+	-	+	-	-	+	+	+	+	-	+	+	+	+	-	-	-	-	+
Lipase	-	+	+	-	+	-	+	+	-	+	-	+	-	-	-	-	-	-	+	+	+	-	-	-	-
Cellulase	-	-	-	-	-	-	+	-	-	-	-	+	-	+	+	+	+	-	+	+	-	-	-	+	+
β-galactosidase	-	-	-	+	-	-	-	-	+	+	-	-	+	-	-	-	+	-	-	-	-	-	+	-	-

Keywords: '+'-Positive, '-'- Negative

Dye degrading ability

All bacterial isolates were able to degrade the biological dyes within 3 days indicating that the isolates have utilized the hydrocarbon from the biological dyes as a carbon which is necessary for the growth of the bacteria. For crystal violet, 60% was the maximum decolourization and 5.14% was the minimum decolourization observed. For phenol red, 80% was the maximum decolourization and 10% was the minimum decolourization observed (Fig 6).

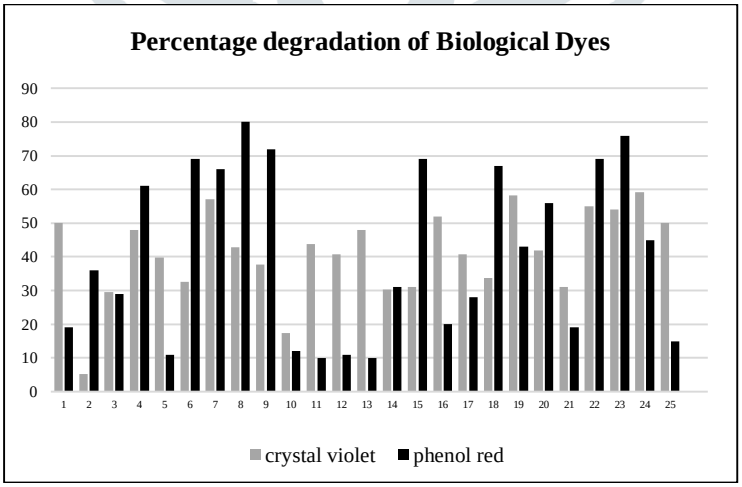

Fig 6: Percentage dye degradation

Characterisation of the isolated bacteria for PGPR traits

Screening for phosphate solubilising bacteria

The microbial isolates were screened for their phosphate solubilizing ability using Pikovaskaya's phosphate solubilizing agar plate. None of the bacterial isolates showed the clear zone around the colony indicating that phosphate was not solubilized by any of the isolates.

Production of auxin (IAA)

The bacterial isolates were tested for their ability to produce IAA by inoculating them in 1% peptone water, 1% tryptophan. Eight bacterial isolates (M4, M6, M8, M9, M10, M14, M18, M20) showed the presence of IAA in different concentrations(Fig 7).

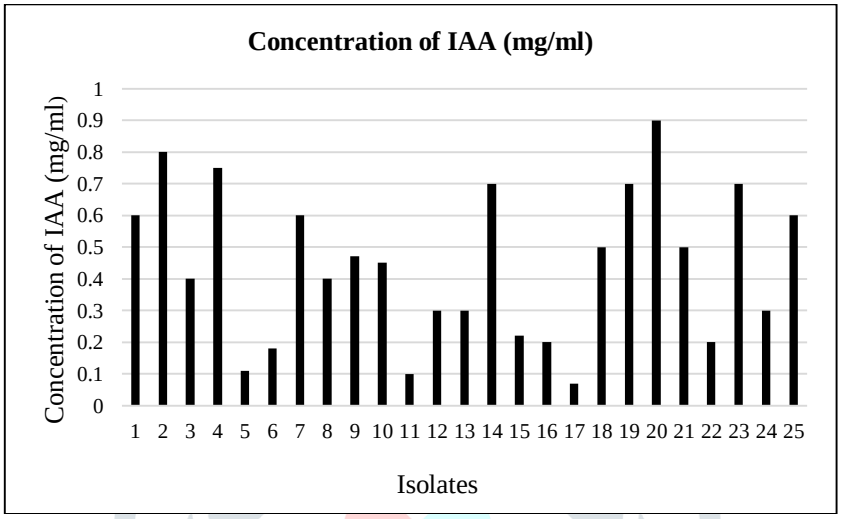

Fig 7: Production of IAA by bacterial isolates

HCN production

The bacterial isolates were tested for their ability to produce HCN using nutrient agar amended with glycine. None of the bacterial isolates showed the growth indicating that HCN was not produced.

Ammonia production

The bacterial isolates were tested for their ability to produce ammonia. Only one bacterial isolate showed the production of ammonia *i.e.*M8 bacterial isolate (Fig 8).

Fig 8: Bacterial isolates producing ammonia

Siderophores detection

All the bacterial isolates were screened for Siderophore production on Chrome Azurol S (CAS) agar plates. Only M13 bacterial isolate showed the development of pink colour indicating utilization of Fe and thus the production of siderophore (Fig 9).

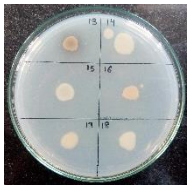

Fig 9: Bacterial isolates producing siderophore

Nitrogen fixation

All the bacterial isolates were tested for Nitrogen fixation by spot inoculating on Norris glucose N-free medium supplemented with Bromothymol blue (BTB) as an indicator. Yellow coloured zone indicated that organisms maybe methanogenic in nature, having the ability to fix nitrogen by dropping the pH around 5.Eight bacterial isolates (M1, M4, M13, M21, M22, M23, M24, M25) showed the development of yellow colour indicating that these isolates are nitrogen fixers in nature (Fig 10).

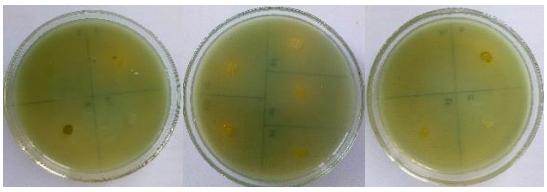

Fig 10: Nitrogen fixing bacterial isolates

Resistance/sensitivity of antibiotics

All the bacterial isolates were tested for theirantibiotic sensitivity against different antibiotics using nutrient agar plates. All the isolates were found to be resistance against Tetracycline and Erythromycin and only one isolate showed resistance against Carbenicillin*i.e.*M25 isolate and none of the isolates showed resistance against Aztreonam (Fig 11) (Table 2)

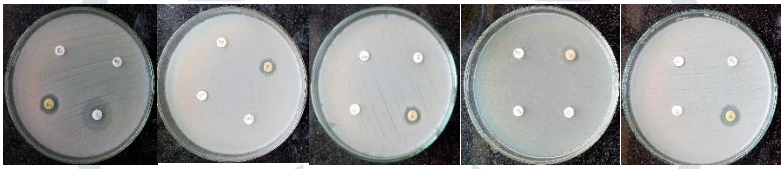

Fig 11: Bacterial isolates showing resistance to antibiotics

Table 2: Antibiotic Susceptibility test

Antibiotics	M 1	M 2	M 3	M 4	M 5	M 6	M 7	M 8	M 9	M 10	M 11	M 12	M 13	M 14	M 15	M 16	M 17	M 18	M 19	M 20	M 21	M 22	M 23	M 24	M 25
Tetracycline (T30)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Erythromycin (E10)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Carbenicillin CB100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R
Aztreonam (AT30)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Antibiotic efficacy: R=Resistant, I=Intermediate, S=Susceptible, NS= Nonsusceptible (Based on Kirby Bauer Chart)
Keywords: '-' - No zone of inhibition

Salt tolerance

Salinity tolerance of the bacterial isolates were tested using Nutrient agar plates with different concentrations of salt. All bacterial isolates showed tolerance up to 50% concentration (Fig 12).

Fig 12: Bacterial isolates showing salt tolerance

Antibacterial activity

All the bacterial isolates were tested for their ability to show Antibacterial activity against two organisms *i.e. E.coli*and *S.aureus*. Only M13 bacterial isolate showed Antibacterial activity against *E. coli*. None of the bacterial isolates showed Antibacterial activity against *S.aureus* (Fig 13).

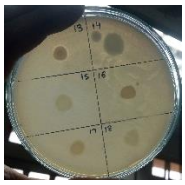

Fig 13: Bacterial isolates showing antibacterial activity against *E. coli*

CONCLUSION

Mangrove regions are unique regions with water region being alkaline in nature and sediment or soil region having a neutral to slightly acidic pH.Mangroves provide a unique ecological niche for diverse bacterial communities. Heterotrophic bacteria are very important in mangrove habitats as the bacteria decompose the mangrove litter, recycle the nutrients and produce the detritus food for many fishes (Agate et al., 1988; Tam and Diep, 2017). Abundance and activities of the bacteria are controlled by various physicochemical parameters in the mangrove environment (Palaniappan, and Krishnamurthy, 1985; Kathiresan et al., 1995; Tam and Diep, 2017). Among heterotrophic bacteria, N2-fixing bacteria are efficient in using a variety of mangrove substrates (Pelegri and Twilley, 1988; Tam and Diep, 2017).

A total of twenty-five bacterial strains were isolated from mangroves soil. Ten isolates were Gram positive and fifteen were Gram negative. These isolates were screened for the production of different extracellular enzymes. The isolates were able to produce enzymes like protease, amylase, beta-galactosidase, lipase, cellulase. The bacterial isolates were also screened for different PGPR traits which promotes plant growth and health benefits as well as provide a good source of bioactive compounds.These isolates were able to produce IAA, ammonia and siderophores. Maximum production of IAA was estimated in M19 isolate and minimum IAA production was estimated in M17 isolate. Siderophore production was estimated by only one isolate. Ammonia production was also estimated by only one isolate. Eight bacterial isolates were found to be nitrogen fixers.All the isolates showed high resistance towards tetracycline and erythromycin. Mangroves bacterial flora are generally tolerant to high salinity. All these bacterial isolates showed tolerance to high concentrations of salinity, which tolerated up to 50%. Bacterial isolates were also tested for their ability to degrade biological dyes (*i.e.* crystal violet and phenol red). 60% was the maximum decolourization of the crystal violet dye and 80% was maximum decolourization of the phenol red dye observed. The isolates were also tested for their antibacterial activity against *E. coli* and *S. aureus*. Only one isolate showed antibacterial activity against *E. coli*.Preliminary screening of these isolates has given a brief idea of their abilities. The bacterial strains have shown novel genetic markers which could be exploited through genetic engineering to produce salt tolerant plants. Which will also help in lessening the hazards of salinity and increase in the availability of land. Further purification, characterization and structural analysis is needed for studying the various applications of the microbes.

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