Monitoring of fecal coliform bacteria and plant growth promoting rhizobacteria from Chilika Lagoon, Odisha

Thesis

Submitted in partial fulfilment of the requirements for the award of the degree of

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Under the supervision
Of

Dr. GURDEEP RASTOGI
Senior Scientist,
Wetland Research and Training Centre,
Chilika Development Authority, Balugaon, Odisha, India

Dr. SUDHIR KUMAR
(Administrative Guide)

Submitted By

CHINMAYEE PRIYADARSINI (Roll no: 197808)

to

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY
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HIMACHAL PRADESH, INDIA
MAY 2021

CERTIFICATE

This is to certify that the thesis entitled **"Monitoring of fecal coliform bacteria and plant growth promoting rhizobacteria from Chilika Lagoon, Odisha"** submitted to Jaypee University of Information Technology, Waknaghat, Solan, Himachal Pradesh, India in the partial fulfilment of the requirements for the award of the degree of Master of Science in Biotechnology is a faithful record of bonafide research work carried out by Ms. Chinmayee Priyadarsini, a student of M.Sc. semester IV from 1st February 2021 to 1st May 2021 under my guidance and supervision in Wetland Research and Training Centre, Chilika Development Authority, Balugaon, Odisha, India. It is further certified that no part of this thesis has been submitted for any other degree or diploma or published in any form and submitted for final evaluation by examiner.

Date: 17/05/2021

Administrative Guide

Dr. Sudhir Kumar
HOD, Department of Biotechnology
Jaypee University Of Information Technology
Waknaghat, Solan – 173234
Himachal Pradesh, India

Supervisor

Gurdeep Rastogi

Dr. Gurdeep Rastogi
Senior Scientist,
Wetland Research and Training Centre,
Chilika Development Authority,
Balugaon, Odisha, India

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DECLARATION

I hereby declare that the thesis entitled **"Monitoring of fecal coliform bacteria and plant growth promoting rhizobacteria from Chilika Lagoon, Odisha"** submitted to the Jaypee University of Information Technology, Waknaghat, Solan, Himachal Pradesh, India for the partial fulfilment of the requirement for the degree of Master of Science (Biotechnology) is an authentic record of the work carried out by me under the guidance of Dr. Gurdeep Rastogi, Senior Scientist, Wetland Research and Training Centre, Chilika Development Authority, Balugaon, Odisha, India. I further declare that any part of the result has not been presented for the award of any degree, diploma, or any equivalent qualification.

Date: 17/05/2021

Place: Bhubaneswar, Odisha

Name: Chinmayee Priyadarsini

Roll No: 197808

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Ms. Chinmayee Priyadarsini

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CHAPTER-1

MONITORING OF COLIFORM BACTERIA

1.1. ABSTRACT

The focus of this study was to determine the level of fecal pollution in Chilika waters especially using fecal indicator bacteria as an indicator. A total of 99 surface water samples were collected from 33 sampling points across four different sectors having different salinity gradients inside the Chilika Lagoon. Sampling was conducted on monthly basis over a period 3 month study period i.e., from January − March 2021. The mos probable number (MPN) method is used to determine the number of fecal coliform bacteria. In the present study, fecal coliforms were measured using multiple tube fermentation technique with presumptive medium being lactose broth and confirmatory medium being eosin-methylene-blue agar and results were expressed as MPN/100 ml. The results indicated that the mean concentrations of fecal coliform bacteria in water samples collected during January, February, and March were 1.0, 0.1, 0.7 MPN/100 ml, respectively. MPN index of all of the stations showed below 100 (threshold value according to the guidelines of Central Pollution Control Board (CPCB), New Delhi for class II surface water used for Bathing, Contact Water Sports and Commercial Fishing) in presumptive test during the study period. Positive presumptive result was found at station no. 4, 9, 19, 24, and 33 during January, station no. 33 during February and station no. 5, 6, 12, 16, 20, 25 and 33 during March 2021. The mean MPN index of 100 ml of samples in stations of Nalabana Island were 1.3, 0.5, and 0.5 during January, February, and March, respectively. None of the samples of the 33 stations of the Chilika Lagoon during the 3 month showed fecal contamination (negative confirmatory result). Thus, the MPN index of the Lagoon was within the threshold prescribed by CPCB. The study has direct relevance with respect to monitoring and management of lagoon. This study is necessary for ongoing monitoring of coliforms and human pathogens in Chilika Lake to evaluate water quality. A robust Fecal Indicator Bacteria Index would be developed to assess the risks associated with potential human pathogens.

1.2. INTRODUCTION

Water is one of the most important components of life. Not only humans, but every life form on Earth requires water for its survival including growth, reproduction, repair and maintenance [1]. Water is generally used for drinking purpose and to perform house chores. Humans are continuously exploiting this resource resulting in its contamination leading to water-related illness [2].

Water contains pathogenic microorganisms which get transmitted to humans and it grows inside the gastrointestinal tract. Later the pathogens are released from human body via feces [3]. The human feces are carried by sewage and dumped into rivers and lakes. This results in microbial contamination of these water bodies. Thus, it is necessary to keep a regular check on water bodies for microbial contamination. Coliform bacteria are the indicators of fecal contamination. Therefore, the presence of coliform needs to be checked.

1.2.1. Coliform Bacteria

Coliform bacteria are mostly used as an indicator of water and food sanitary quality. The bacteria are rod shaped, gram negative and non-spore forming. They can either be motile or non-motile. Coliforms are commonly found in aquatic environment, soil and vegetation, but majorly in the feces of warm-blooded animals. Normally, coliforms don't cause serious illness, but its presence can be used to indicate the presence of pathogenic organism of fecal origin such as protozoans, viruses, parasites.

Coliforms have the ability to ferment lactose to lactic acid and carbon dioxide at 35^{o} C [4]. *E. coli*, a coliform ferments lactose at 44^{o} C.

The 5 genera of coliform are: *Citrobacter, Enterobacter, Hafnia, Klebsiella, Escherichia.*

Coliform bacteria are of 3 groups:

1. Total coliform
2. Fecal coliform
3. *Escherichia coli*

Total coliform is commonly found in the environment and is harmless. Its presence in water might have been influenced by surface water. Fecal coliform is a sub-group of total coliforms and is commonly found in the gut and feces of warm-blooded animals. *Escherichia coli* is a major group of fecal coliforms and is mostly harmless. It is found in the gut of warm-blooded animals. Its presence in water indicates recent fecal contamination. O157:H7is a pathogenic strain of *E. coli*.

This strain produces a toxin, Shiga toxin which results in the development of hemolytic uremic syndrome (HUS). HUS is a life-threatening medicalsyndrome in which the small blood vessels of kidney are damaged and inflamed resulting in kidney failure [5]. Figure 1 shows the relationship between various groups of coliform bacteria.

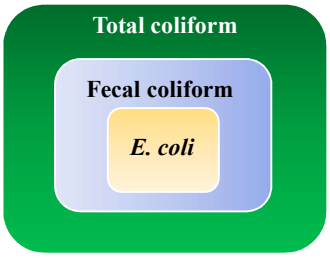

Fig. 1: Shows relationship between various groups of coliform bacteria.

1.2.2. CPCB Guidelines:

Certain guidelines have been set by Central Pollution Control Board (CPCB) to keep a check on the sanitary quality of Chilika lagoon water since its being exploited by humans resulting in its contamination. A standard MPN index has been set and remarks have been provided on the basis of the standard. Regular tests are done to keep a check on the water quality.

Table 1: CPCB standards of fecal coliforms for coastal waters primary water quality criteria for Class SW-II waters PCB (For Bathing, Contact Water Sports and Commercial Fishing)

Parameter	Standards	Rationale/Remarks
Fecal coliform	100/100 ml (MPN)	1. The MPN value of each sample not exceeding 100/100 ml 2. The average MPN value not exceeding 200/100 ml in 20 percent of samples in the year 3. The average MPN value not exceeding 200/100 ml in 3 consecutive samples in monsoon months.

1.2.3. Coastal Lagoons or estuaries

These are the ecosystems of ecological and economic importance. They are the source of many valuable ecosystem goods and services [6]. However, these ecosystems are frequently exposed to various environmental pressures. Since, human populations are concentrated within coastal zones, the pressure is mostly associated with human activities.. Urbanization, intensive agriculture, sewage disposal and recreation can lead to an increased stream of microbial pollutants. Because

of the increased tourism in coastal areas and the needs of local communities for recreation sites, there is a request to have sanctioned beaches not only along the sea, but also in interior coastal waters like lagoons or bays [7, 8].

1.3. OBJECTIVE

The primary objective is to monitor the fecal contamination of water samples from the 33 GPS fixed stations of the Chilika Lagoon and 11 GPS fixed stations of Nalabana Island during January-March 2021.

1.4. REVIEW OF LITERATURE:

The presence of a spatial inclination in fecal pollution levels among checking stations may mirror the impacts of actual cycles or "compelling capacities" that establish slopes in the actual climate [9]. The interaction between physical forces, biological and biochemical processes affects fecal pollution and this may vary considerably depending on the water body [10,11]. Comparable amounts of tubidity and organic matter are known to promote the survival of faecal bacteria[11, 12, 13].

There have been several studies of fecal coliform contamination from coastal environments, such as coastal waters in Virginia [9], Del Rey Lagoon, City of Los Angeles, California, USA [14], Akyatan Lagoon [15], Curonian Lagoon, Baltic Sea [16]. Huang, 2011 investigated the variability and distribution of fecal coliform contamination levels that are expressed at different temporal and spatial scales. In addition, they also accounted various effects such as at tidal, regional, land state and climatic. These results not only provide inmorfations related to management decisions, but also direct subsequent quantification of fecal bacterial loadings. The lagoon was the sink or source of FIB in the adjacent estuary, and these are potentially pathogenic bacteria present in the lagoon waters, and their diversity varies depending on the tide [14]. Furthermore, Yetis et al., 2015 have investigated the relationships between the total coliform and fecal coliform and physical parameters. They found that no significant relation was observed between fecal indicator bacteria and environmental parameters. Turbidity, oxygen, pH, suspended particulate matter of natural beginning and chlorophyll are the main estimated water quality boundaries in calm estuarine tidal pond like Curonian Lagoon [16]. The key sources of the pollution in this lagoon are the drainage outlets, water birds, and sediments especially of virulent and multi-resistant *E. coli* strains, but their role still needs to be monitored.

1.5. MATERIALS AND METHODS

1.5.1. Study site and Period

1.5.1.1. Chilika Lagoon

It is the largest brackish water lagoon in the world and the largest coastal lagoon in India. It spreads over the districts of Puri, Khurdha and Ganjam of Odisha state on the east coast on India. Based on the salinity regimes, Chilika has been divided into 4 sectors: northern sector which predominantly contains fresh water, central and southern containing brackish water, and outer channel which connects to the marine. On its shore 132 villages are located in which 1,50,000 fisher-folks survive. During the peak migratory season over 160 bird species arrive on this lagoon. It has numerous islands, 6 of which are: Parikud, Phulbari, Berhampur, Nuapara, Nalabana, and Tamapara.

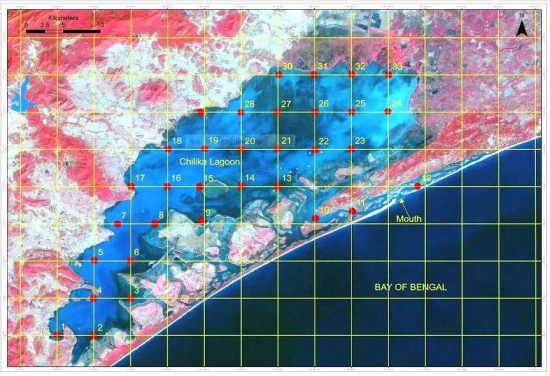

Fig. 2: 33 GPS fixed Stations of Chilika Lagoon

The study was conducted on Chilika Lake water source to assess the extent of fecal coliform contamination from January to March 2021 in Wetland Research and Training Center, Chilika, Odisha. A total of 44 samples were collected from 33 GPS fixed Chilika stations (Fig. 2) and 11 GPS fixed Nalabana stations (Fig. 3).

1.5.1.2. Nalabana Island

It is a shallow boggy environment that is situated in the focal area of the Chilika Lagoon. The island gets lowered during rainstorm and steadily reveals in post-storm period. The island has been pronounced as a bird safe-haven in 1973 and the safe-haven is named as Chilika Wildlife Sanctuary.

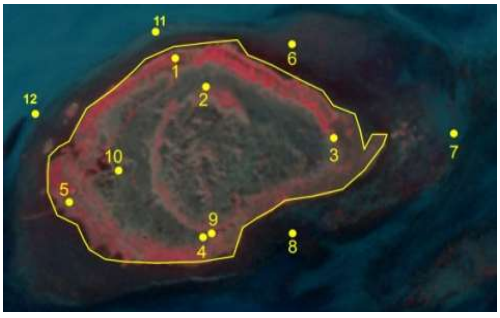

Fig. 3: 11 GPS fixed Stations of Nalabana Island

1.5.2. Sample Collection

The samples were collected in sterile bottles. The bottles were sterilized by washing with detergent and drying in hot air oven. The water was first taken in buckets and then the bottles were filled carefully avoiding any water bubble formation. This is because if water bubbles are produced then the microbial population of the sample might be affected leading to error during the experiment. After sample collection, the bottles were immediately placed inside ice box. When reached laboratory, the bottles were transferred to 4 ° C refrigerators.

Fig. 4: Collection of water samples from Chilika Lagoon for fecal coliform analysis

1.5.3. Procedure for Fecal Coliform Analysis

1.5.3.1. Presumptive Test

The test is name so because the reaction observed might be due to organisms other than coliform bacteria and this presumption needs to be confirmed. For the presumptive test, Lactose broth (Refer Appendix) of 1X and 2X concentration were used. Sterilized test tubes were taken and 5ml of 2X and 1X media were poured into it. Durham tubes were carefully filled with media avoiding any air bubbles inside it and placed inside the test tubes and autoclaved. The media as cooled to room temperature and water samples were added to it. 10ml sample was inoculated to 2X lactose broth and 1ml and 0.1ml to 1X lactose broth. The test was performed in triplets. The inoculated media was placed inside a shaking incubator at 44 ° C and 110 rpm for 48 hours.

Lactose broth contains a pH indicator Bromocresol purple which remains purple colored at 6.8 pH and when the pH reduces to 5.2, it changes color from purple to yellow. Fecal coliform bacteria

have the ability to ferment lactose at 44 ° C. Thus, when the inoculated lactose is incubated at 44° C, the coliform ferments lactose to lactic acid resulting in pH reduction and color change. During lactose fermentation, carbon dioxide is also produced which gets trapped inside the Durham tube. If the broth color changes from Purple to yellow and gas is produced inside Durham tube then the test is positive (Fig. 5a). But if not, then the test is negative (Fig. 5b).

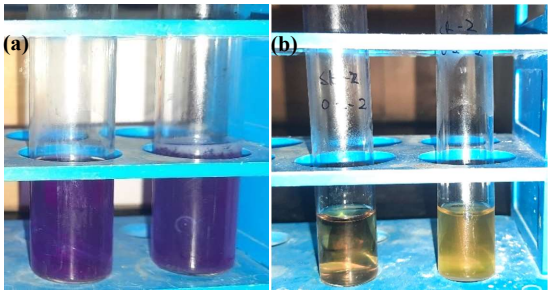

Fig. 5: Presumptive test result of fecal coliform (a) negative and (b) positive

Based on the number of presumptive tubes produced from the presumptive test, MPN (Most Probable Number) Index is calculated. MPN is used to calculate the approximate number of viable cells in a given volume of sample.

1.5.3.2. Confirmatory Test

Confirmatory test is performed by streaking a loopful of culture on Eosin Methylene Blue (EMB) agar plate from positive presumptive test tubes. For confirmatory test, EMB agar plates were used because it is a selective and differential medium used to isolate fecal coliform. The methylene blue present in the agar inhibits the growth of Gram-negative organism and promotes the growth of Gram-positive organism. The plate is then incubated at 44° C for 24 hours. If green shiny colonies are produced, the test is positive which means *E.coli* presence is confirmed (Fig. 6a). If colonies other than green (like purple, deep blue, pink etc.) are produced then the test for confirmatory result is negative (Fig. 6b).

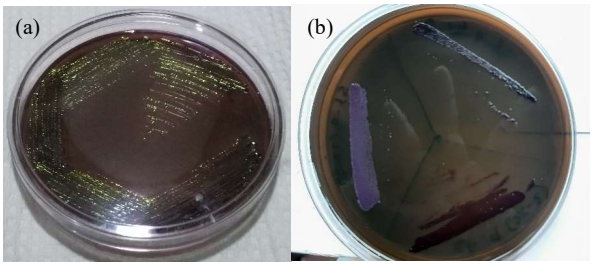

Fig. 6: Confirmatory test result of fecal coliform (a) positive and (b) negative.

Table 2: Different patterns showing positive and negative **results of** MPN index per 100 ml of sample (when three 10 ml, three 1 ml, and three 0.1 ml test portions are used) [17].

Pattern	3 of 10 ml each	3 of 1 ml each	3 of 0.1 ml each	MPN index per 100 ml
1	0	0	1	3
2	0	1	0	3
3	1	0	0	4
4	1	0	1	7
5	1	1	0	7
6	1	1	1	1
7	1	2	0	1
8	2	0	0	9
9	2	0	1	14
10	2	1	0	15
11	2	1	1	20
12	2	2	0	21
13	2	2	1	28
14	3	0	0	23
15	3	0	1	39
16	3	0	2	64
17	3	1	0	43
18	3	1	1	75
19	3	1	2	120
20	3	2	0	93
21	3	2	1	150
22	3	2	2	210
23	3	3	0	240
24	3	3	1	460
25	3	3	2	1100
26	3	3	3	2400

1.6. RESULTS

MPN index of all of the stations showed below 100 (threshold value according to the guidelines of Central Pollution Control Board (CPCB), New Delhi for class II surface water used for Bathing, Contact Water Sports and Commercial Fishing) in presumptive test during the study period. The average fecal coliform bacteria concentrations in water samples collected during January, February, and March were 1.0, 0.1, 0.7 MPN/100 ml, respectively. Positive presumptive result was found at station no. 4, 9, 19, 24, and 33 during January, station no. 33 during February and station no. 5, 6, 12, 16, 20, 25 and 33 during March 2021. Within Nalabana, NB1, NB9, NB11, and NB con during January, NB9 and NB con during February, and NB5 and NB9 during March 2021 showed positive presumptive result. None of the positive presumptive cultures showed positive confirmatory results.

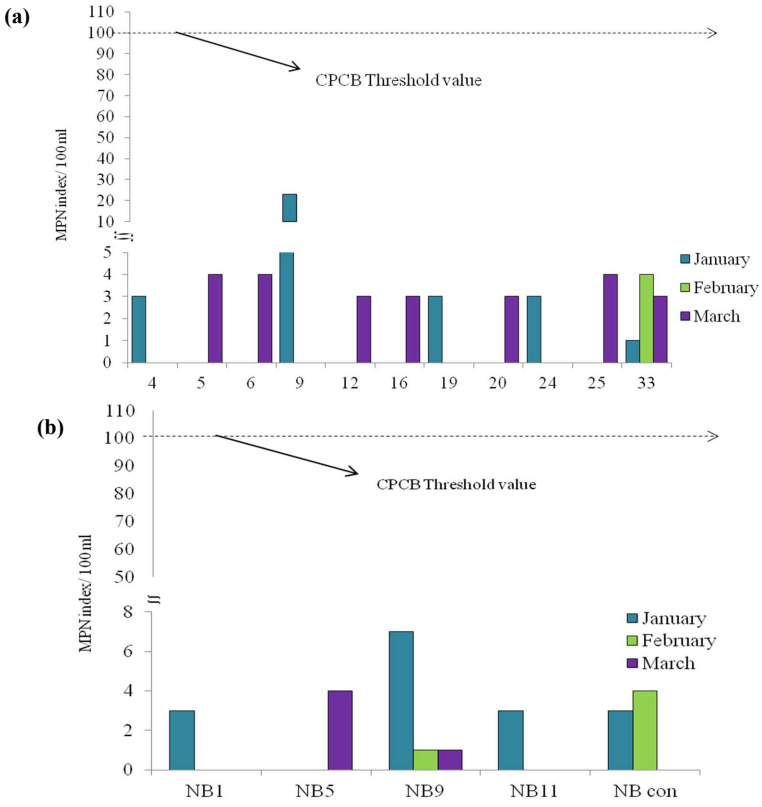

Fig. 7: MPN Index of fecal coliforms in Chilika Lagoon (a) and Nalabana Island (b) during January, February, and March 2021.

1.7. CONCLUSION

The above result revealed that the fecal coliform counts in terms of MPN index per 100 ml in Chilika water samples was within the threshold value for all stations. Station no. 4 (Badakuda-Samalnasi), 9 (Veteswara), 19 (Kalijugeswar-Tuagambhari), 24 (Haridaspur-Barakudi), and 33 (Teenimuhani Nali) during January, station no. 33 during February and station no. 5 (Gopakuda), 6 (Budhibaranasi), 12 (Arakhkuda), 16 (WRTC-Nalabana), 20 (Kalijugeswar-Tuagambhari), 25 (Teenimuhani) and 33 (Timimuhani Nali) during March 2021 showed positive presumptive result. Within Nalabana Island, NB1, NB9, NB11, and NB con during January, NB9 and NB con during February, and NB5 and NB9 during March 2021 showed positive presumptive result. None of the samples of the 33 (Timimuhani Nali) stations of the Chilika Lagoon during the 3 month showed fecal contamination (negative confirmatory result). Since the following stations are either situated

near villages or rivers where human population is found. Thus, water is used by humans for performing their chores and by animals also resulting in its contamination. The primary reason for fecal contamination is, animals as well as humans excrete in the banks of Chilika Lagoon and Nalabana Island. Even though fecal contamination is found in these stations. Still there is no threat as the MPN Index does not exceed the limit which has been set by CPCB.

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CHAPTER-2

ASSESSMENT OF PLANT GROWTH PROMOTING RHIZOBACTERIA FROM *PHRAGMITES KARKA*

2.1. ABSTRACT

The present study is focused on isolation and screening of PGPR stains from rhizospheric sediment samples of P.karka collected from Chilika Lagoon. In this study, a total 35 rhizobacterial isolates showed several multiple PGP features. All of the isolates showed catalase activity and ammonia production except PK63. Out of 35 isolates, only 6 isolates (PK4, PK7, PK21, PK30, PK54, PK78) were positive for cellulase activity and 4 isolates (PK11, PK24, PK29, and PK72) were positive for lipolytic activity. The PK37and PK79 isolates demonstrated excellent amylase activity. 18 isolates were recorded positive for proteolytic activity. 15 isolates such as PK3, PK8, PK10, PK22, PK23, PK24, PK28, PK33, PK41, PK47, PK52, PK57, PK63, PK72, and PK74 were recorded positive for HCN production, postulating that the production of HCN by rhizobacteria plays a significant role in biological control of pathogens. PK29, PK11, and PK23 were the most efficient P-solubilizers observed from the study. Alot of the isolates produces siderophore except 5 isolates (PK5, PK11, PK23, PK63, and PK64). All the isolates exhibited N_2-fixing characteristic except few isolates i.e., PK8, PK52, PK54, PK57, PK63, PK76, and PK78. However, only 6 isolates (PK5, PK11, PK23, PK24, PK28, and PK33) were able to produce IAA. It can be concluded that isolates obtained from the rhizosphere region of *P. karka* showed several distinct PGPR characteristics and can be used for the formulation of bio-fertilizers.

2.2. INTRODUCTION

Phragmites, the common reed is widely distributed throughout the world. These are highly invasive plant species forming dense strands in the shoreline of freshwater and brackish water wetlands. Chilika Lagoon, a wetland in Odisha has a wide variety of flora and fauna. *Phragmites karka*, locally known as "Nala dala" has invaded in the north and north-western regions of the lagoon. This acts as an ecological filter to sequester nutrients and pollutants (pesticides, heavy metals, and pharmaceuticals) before entering the lake. *P. karka*can survive at a wide range of pH, salinity, nutrient and hydrological regime.

P. karka are commonly used in phytoremediation of polluted water bodies. This ability depends on the microbial community present in the rhizosphere of the reed. The effect of rhizosphere and sediment salinity helps in shaping the bacterial community. Most commonly found bacteria are *Bacillus, Methylotenera, Steriodobacter, Thiobacillus, Escherichia/ Shigella.*

PGPR rhizobacteria colonize plant roots and are potentially useful for enhancing plant growth and the crop yield. PGPR influence the growth, yield, and nutrient uptake by an array of mechanisms like soil stabilisation, prevention spreading of metalcontaminated soil, and reduction of metal mobility by rhizospheric adsorption and precipitation.

Biochemical assays can be used to determine the metabolism of the bacterial community and differentiating them based on these activities. Bacterial species are able to perform these activities due to the presence of certain enzymes. These enzymes would help determine differences at structural as well as metabolic level. It would also help in study of intracellular as well as extracellular enzymes present in a bacterial strain. Thus, biochemical assays can be performed on the bacterial community of *P. karka* to understand the its metabolism and abilities.

2.3. OBJECTIVE

The primary objective of this study is to perform antibiotic susceptibility test and to characterize microbial strains based on various biochemical assays.

2.4. REVIEW OF LITERATURE

PGPR influences plant development twoly: indirectly and directly. The direct influence by PGPR includes either giving a compound to the plant which a bacterium has integrated, for instance phytohormones, or speeding up the take-up of important supplements from the general climate. The indirect influence by PGPR happens when PGPR lessens or hinders the undesireable impacts

of at least one phytopathogenic creatures. This happens either by delivering hostile substances or by actuating protection from microorganisms. A specific PGPR may influence plant development and improvement by utilizing any, at least one, of these instruments. PGPR, as bio-control specialists, can act through different components, paying little heed to their job in direct development advancement, for example, by known creation of auxin phytohormone, decline of plant ethylene levels or nitrogen fixing related with roots [1].

Several studies have been carried out from *P. karka* rhizospheric sediment and water samples that are potentially toxic degraders, help in biofilm formation, siderophore production, and metabolism of phosphorus, maintain nitrogen cycles in rhizospheric region, showed ACC deaminase activity, protein, carbohydrate, and multiple aromatic compounds degradation [2, 3, 4]. Few starins are also found to be producing plant growth hormones such as IAA [4, 5].

2.5. MATERIALS AND METHODS

2.5.1. Sampling site and period

Sampling was done in 2014-2015 at Chilika Lagoon, Odisha. Bacterial strains were isolated from the rhizospheric sediments of *Phragmites karka* (*P. karka*). Previously, Behera et al. (2020) [6] performed molecular identification of rhizobacterial strains of *P. karka* through 16S rRNA gene sequencing which are displayed in Table 3.

Table 3: Molecular identification of *P. karka* rhizobacterial isolates using NCBI BLAST percent identity scores of 16S rRNA gene sequences.

Bacterial Strain	Nomenclature	Bacterial Strain	Nomenclature
PK2	*Enterobacter ludwigii*	PK37	*Bacillus altitudinis*
PK3	*Bacillus velezensis*	PK41	*Bacillus velezensis*
PK4	*Bacillus siamensis*	PK46	*Leclerciaadecarboxylata*
PK5	*Enterobacter ludwigii*	PK47	*Bacillus vietnamensis*
PK7	*Bacillus altitudinis*	PK52	*Bacillus altitudinis*
PK8	*Bacillus drentensis*	PK54	*Exiguobacterium indicum*
PK9	*Bacillus velezensis*	PK57	*Enterobacter cloacae subsp. dissolvens*
PK10	*Exiguobacteriumacetylicum*	PK63	*Bacillus altitudinis*
PK11	*Bacillus altitudinis*	PK64	*Bacillus altitudinis*
PK12	*Bacillus siamensis*	PK67	-
PK21	*Pseudomonas stutzeri*	PK68	*Enterobacter hormaechei subsp. oharae*
PK22	*Bacillus subtilis subsp. inaquosorum*	PK72	*Bacillus cereus*
PK23	*Enterobacter ludwigii*	PK74	*Bacillus velezensis*
PK24	*Pseudomonas stutzeri*	PK76	*Bacillus altitudinis*
PK28	*Exiguobacterium indicum*	PK78	*Bacillus velezensis*

PK29	*Enterobacter xiangfangensis*	PK79	*Staphylococcus hominis subsp. novobiosepticus*
PK30	*Bacillus altitudinis*	PK92	*Bacillus subtilis subsp. stercoris*
PK33	*Bacillus altitudinis*		

2.5.2. Antibiotic Susceptibility Test (AST)

Antibiotic susceptibility test or AST was performed by Kirby-Bauer disc diffusion method on LB plates. Bacterial samples were streaked on the sterile Luria-Bertani (LB) agar plates. The plate was divided into 4 sections and each section consists of an inserted antibiotic disc. The plates were placed in incubator at 37°C for 24 hours. Sensitivity of the antibiotic was determined by the inhibition zone produced. The diameter of the inhibition zone was measured in millimeters.

For this test, 24 commercially available antibacterial discs were used including Amikacin (AK, 10 µg),Amoxycillin (AMX, 30 µg), Bacitracin (B, 10 µg), Carbenicillin (CB, 100 µg), Cefadroxil (CFR, 30 µg), Ciprofloxacin (CIP, 30 µg), Cefalexin (CN, 30 µg), Cefaloridine (CR, 30 µg), Chlortetracycline (CT, 30 µg), Co-trimoxazole (COT, 25 µg), Cloxacillin (COX, 30 µg), Erythromycin (E, 10 µg), Furazolidone (FR, 50 µg), Kanamycin (K, 30 µg), Nalidixic Acid (NA, 30 µg), Nitrofurantoin (NIT, 300 µg), Novobiocin (NV, 30 µg), Norfloxacin (NX, 10 µg), Oxytetracycline (O, 30 µg), Ofloxacin (OF, 5 µg), Sulphamethiozole (SM, 300 µg), Sulphadiazine (SZ, 300 µg), Tobramycin (TOB, 10 µg), Trimethoprim (TR, 5 µg).

2.5.3. PLANT GROWTH PROMOTING ACTIVITIES

2.5.3.1. Catalase Test

Bacterial samples were grown on sterile Luria-Bertani agar plates and incubated at 30°C for 24 hours. A drop of 3% (v/v) Hydrogen peroxide (H_2O_2) was added on the microbial colony and observed. Production of air bubble indicates positive catalase activity and no bubble formation indicates catalase negative activity [7].

2.5.3.2. Cellulase Test

Bacterial colonies were grown on sterile M9 media (Appendix) supplemented with 1% Carboxymethyl cellulase platesand incubated at 28° C for 3-5days. The inoculated agar plates were flood with 1% Congo red dye and incubated for 15minutes. The dyed plated were washed with 1N NaCl and observed. If halozone is produced then, the test for cellulase enzyme is positive, if not the result is negative.

2.5.3.3. Lipolytic activity

Bacterial colonies were grown on sterile Nutrient agar supplemented with 1% Tributyrin plates and inoculated at 28℃ for 24 hours. Presence of halozone indicates positive activity and absence of halozone indicates negative activity.

2.5.3.4. Amylolytic activity

Bacterial colonies were grown on sterile Nutrient agar supplemented with 0.2% soluble starch plates and pH as maintained at 6. The inoculated plates were incubated at 28℃ for 24 hours. After 24 hours, the plates were flood with Iodine solution and incubated for a minute [8]. Presence of halozone indicates amylase positive result and absence indicates negative result.

2.5.3.5. Proteolytic activity

1L media containing Casein 2.5g, Yeast extract 1.25g, Glucose 0.5g, Skimmed milk 35g, and Agar 18g was prepared and poured onto sterile petri plates. Bacterial colonies were streaked on the plates and incubated at 28℃ for 3-5days [8]. Presence of halozone indicates protease positive result and absence indicates negative result.

2.5.3.6. HCN Production

Bacterial colonies were grown on sterile Nutrient agar supplemented with 4.4g glycine (per liter) plates. Whatman filter paper No 1 was soaked in a solution of 2% Sodium carbonate and 0.5% Picric acid and placed on the top of the plate. The plates were sealed with parafilm and incubated at 30℃ for 4 days. The change in color was observed. If the color changes from yellow to orange or brownish-red, HCN was produced and if color doesn't change, no HCN was produced [9].

2.5.3.7. Phosphate solubilization

Bacterial sample was gown in LB medium and incubated till 1.5 OD was recorded at 600nm. A loopful of the bacterial culture was inoculated in sterile National Botanical research Institute's Phosphate Growth Medium (NBRIP) and incubated at 30℃. After 24 hours, an aliquot of 5ml from the medium was taken and centrifuged at 7000 rpm for 20minutes. 1ml of supernatant was transferred into a tube and 2.5 ml Barton's reagent or Vanadate-Molybdate reagent (Appendix) was added to it and incubated for 10minutes. The OD was recorded at 430nm. For creating a Standard Phosphate Solubilization Curve, 5ml of solution was taken with different phosphate concentration. 1ml of Vanadate-molybdate reagent was added and incubated for 10 minutes. Standard curve was plotted at 430nm.

Table 4: Standardize table for Phosphate Solubilization

Final concentration of K	Volume of K (in ml)	Volume of distilled water (in ml)	Total volume (in ml)
0	0	5	5
1	0.1	4.9	5
2	0.2	4.8	5
3	0.3	4.7	5
4	0.4	4.6	5
5	0.5	4.5	5
6	0.6	4.4	5
7	0.7	4.3	5
8	0.8	4.2	5
9	0.9	4.1	5
10	1	4	5

2.5.3.8. Siderophore Production

Bacterial samples were grown in LB media and incubated at 30°C for 24 hours. After 24 hours, the inoculated media was centrifuged at 10,000rpm for 15 minutes [9]. The supernatant was transferred to another tube and CAS dye (Appendix) was added to it and incubated. If the color remains blue, the result is negative. If the dye chelates the supernatant and turn to yellow color, the result is positive.

2.5.3.9. Nitrogen Free

Bacterial samples were first grown in LB media. Then a loopful of the bacterial culture was inoculated in sterile Nitrogen fixation media (NFM) (Appendix) and incubated at 30°C for 5 days. After 5 days, change in color is observed. If the color changes from blue to yellow, the test for nitrogen free is positive and if not, the test is negative.

2.5.3.10. Indole Acetic Acid (IAA) Production

Bacterial sample was inoculated in autoclaved LB media supplemented with 5mM Tryptophan. The media was incubated at 30°C for 3-4 days. The media was then centrifuged at 10,000rpm for 15 minutes and the supernatant was transferred to another tube [9]. To the supernatant Salkowski reagent (Appendix) is added and incubated for 25 minutes in dark. If the color changes to pink IAA is produced. But if the color doesn't change, IAA isn't produced.

2.5.3.11. Ammonia Production

Bacterial cultures were inoculated in autoclaved Peptone water (Appendix) and incubated at 30°C for 2-3 days. The inoculated media was then centrifuged at 10,000rpm for 15 minutes and the supernatant was transferred to another tube. To the supernatant 1ml Nessler's reagent was added

and change in color was observed [10]. If the color changes from yellow to orange, ammonia is produced.

2.6. RESULT AND DISCUSSION

2.6.1. Antibiotic Susceptibility Test

In AST, the placed antibacterial discs get diffused in the medium. If the bacterial strain is doesn't grow in the diffused region, it means that the antibiotic has inhibited the bacterial growth and thus, the strain is susceptible to that particular antibiotic. If the bacterial strain grows in the diffused region, then the bacterium is resistant to the antibiotic (Fig. 8).

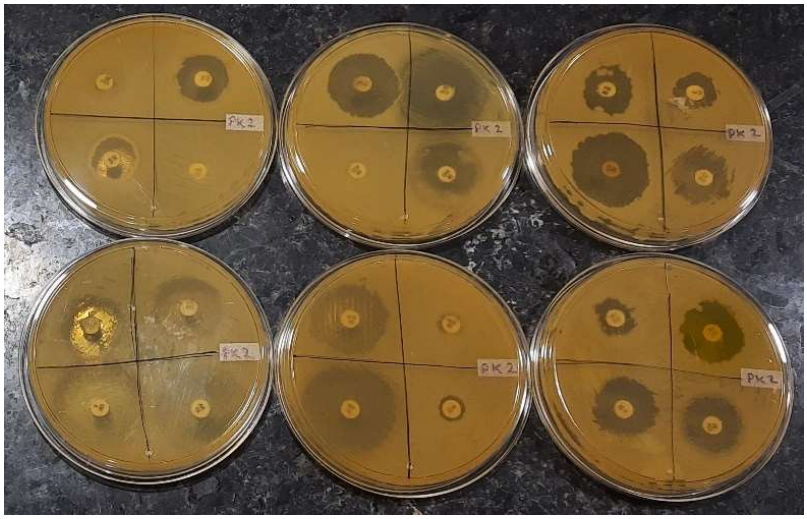

Fig. 8: AST results of PK 2 bacterial strain.

After 24 hours of incubation, the AST plates were observed and the diameter of the inhibition zone is noted in millimeter (Table 5).

Table 5: Diameter of zone of inhibition in mm

	AK10	AMX30	B10	CB100	CFR30	CIP30	CN30	CR30	CT30	COT25	COX30	E10	FR50	K30	NA30	NIT300	NV30	NX10	O30	OF5	SM300	SZ300	TOB10	TR5
PK2	20	0	8	12	20	32	18	20	30	0	0	18	16	22	14	20	18	38	24	20	20	20	20	0
PK3	22	0	10	22	30	40	34	30	22	30	22	20	0	26	24	20	26	30	26	30	18	40	22	38
PK4	24	30	16	24	0	36	22	24	12	40	24	26	12	24	22	20	0	34	30	30	26	46	30	32
PK5	16	0	0	16	0	40	0	0	0	20	0	0	16	14	20	14	0	30	14	28	14	20	20	24
PK7	22	26	20	30	0	32	24	26	26	36	8	24	16	20	20	22	0	36	22	36	34	34	30	36
PK9	26	20	12	26	30	42	32	30	24	32	22	28	12	28	22	20	28	24	8	28	24	30	26	38
PK10	24	34	12	34	20	42	26	26	34	42	26	28	20	22	22	20	24	40	28	36	0	46	30	46
PK11	18	0	0	18	0	0	0	0	10	24	0	0	12	0	16	0	0	34	14	26	20	26	16	10
PK16	22	0	0	16	0	42	0	0	20	28	0	0	16	20	18	16	0	32	14	28	10	22	10	20
PK21	26	32	12	36	30	36	22	24	28	30	20	28	12	22	20	20	14	24	12	20	20	32	24	20
PK22	26	40	12	40	32	34	22	24	30	30	24	30	24	24	26	20	26	36	16	28	24	38	28	30
PK23	18	0	0	16	38	32	0	0	34	20	0	0	24	10	20	12	26	16	0	32	6	24	0	26
PK24	24	0	14	0	28	36	22	20	32	0	0	22	22	26	0	18	20	28	28	30	0	0	26	0
PK26	14	10	10	8	26	30	26	30	28	20	22	28	14	20	22	18	20	22	12	24	22	34	20	28
PK28	20	40	22	34	26	30	26	22	26	18	28	28	16	16	32	14	16	22	24	24	20	26	20	22
PK29	20	32	10	24	22	30	20	18	24	0	16	20	10	14	22	20	14	20	20	22	0	10	22	0
PK30	24	30	10	30	30	42	22	20	24	38	12	22	0	20	18	14	20	30	38	36	32	44	24	40
PK46	20	30	10	38	24	38	34	0	20	32	0	16	18	20	10	10	20	32	36	38	40	38	24	0
PK47	28	38	16	36	32	40	26	22	28	32	28	28	20	38	20	28	24	40	34	34	38	42	24	30
PK54	22	26	10	26	30	40	28	22	30	36	18	24	22	20	20	20	20	34	0	36	30	40	28	38
PK62	22	0	12	34	36	32	22	26	32	32	20	28	22	26	28	24	26	38	10	32	40	40	26	34
PK63	14	14	12	0	0	20	0	0	24	20	0	8	0	0	0	20	18	20	30	18	0	0	0	20
PK64	20	30	22	40	34	30	34	30	26	30	28	30	0	18	0	20	32	20	22	22	20	26	24	0
PK67	20	34	10	38	24	32	28	28	26	38	24	18	0	20	26	18	24	20	16	26	26	30	24	28
PK72	20	0	10	0	24	20	20	22	18	0	0	22	12	20	16	18	18	26	8	36	0	16	20	0
PK78	26	30	14	36	36	38	26	28	36	38	20	28	22	24	18	24	24	36	36	34	38	42	30	42

Based on the recorded diameter, the bacterial strain is Resistant (R) or Susceptible (S) to the particular antibiotic or not has been recorded (Table 6).

Table 6: Bacterial strain is Resistant (R) or Susceptible (S).

	AK10	AMX30	B10	CB100	CFR30	CIP30	CN30	CR30	CT30	COT25	COX30	E10	FR50	K30	NA30	NIT300	NV30	NX10	O30	OF5	SM300	SZ300	TOB10	TR5
PK2	S	R	S	S	S	S	S	S	S	R	R	S	S	S	S	S	S	S	S	S	S	S	S	R
PK3	S	R	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S
PK4	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S
PK5	S	R	R	S	R	S	R	R	R	S	R	R	S	S	S	S	R	S	S	S	S	S	S	S
PK7	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S
PK9	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
PK10	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
PK11	S	R	R	S	R	R	R	R	S	S	R	R	S	R	S	R	R	S	S	S	S	S	S	S
PK16	S	R	R	S	R	S	R	R	S	S	R	R	S	S	S	S	R	S	S	S	S	S	S	S
PK21	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
PK22	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
PK23	S	R	R	S	S	S	R	R	S	S	R	R	S	S	S	S	S	S	R	S	S	S	R	S
PK24	S	R	S	R	S	S	S	S	S	R	R	S	S	S	R	S	S	S	S	S	R	R	S	R
PK26	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
PK28	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
PK29	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	R	S	S	R
PK30	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S
PK46	S	S	S	S	S	S	S	R	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	R
PK47	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
PK54	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S
PK62	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
PK63	S	S	S	R	R	S	R	R	S	S	R	S	R	R	R	S	S	S	S	S	R	R	R	S
PK64	S	S	S	S	S	S	S	S	S	S	S	S	R	S	R	S	S	S	S	S	S	S	S	R
PK67	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S
PK72	S	R	S	R	S	S	S	S	S	R	R	S	S	S	S	S	S	S	S	S	R	S	S	R
PK78	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

2.6.2. PLANT GROWTH PROMOTING ACTIVITIES

2.6.2.1. Catalase Test

Catalase is an enzyme which is found in microorganisms. This enzyme has the ability to decompose Hydrogen peroxide (H_2O_2) to Oxygen (O_2) and water (H_2O) resulting in the formation of bubble (Fig. 9).

Fig. 9: Bubble formation due to presence of Catalase enzyme.

Table 7: Shows the catalase activity result for the various microbial strains ("+" represents positive result and "-" represents negative result)

Bacterial Strain	Catalase Activity	Bacterial Strain	Catalase Activity
PK2	+	PK37	+
PK3	+	PK41	+
PK4	+	PK46	+
PK5	+	PK47	+
PK7	+	PK52	+
PK8	+	PK54	+
PK9	+	PK57	+
PK10	+	PK63	-
PK11	+	PK64	+
PK12	+	PK67	+
PK21	+	PK68	+
PK22	+	PK72	+
PK23	+	PK74	+
PK24	+	PK76	+
PK28	+	PK78	+
PK29	+	PK79	+
PK30	+	PK92	+
PK33	+		

2.6.2.2. Cellulase Test

Congo-red is a dye which binds to cellulase. In the presence of cellulase enzyme, cellulose is degraded into simple sugars. Therefore, when the dye is washed using 1N NaCl, the dye is washed off where cellulase has been degraded forming the halo zone (Fig. 10).

Halozone was measured and the result has been recorded in Table 5.

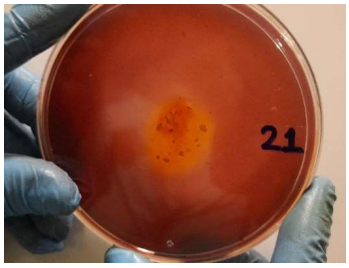

Fig. 10: Halo zone produced due to presence of Cellulase enzyme in PK22.

Table 8: showing halo zone measurements produced by bacterial strains showing Cellulase

Bacterial Strain	Radius of bacterial culture and halozone (in mm) (R)	Radius of culture (in mm) (r)	Halozone measurement (in mm) (R-r)
PK4	9	7	2
PK7	6	5	1

PK21	13	12	1
PK30	10	9	1
PK54	10	6	4
PK78	10	8	2

2.6.2.3. Lipolytic activity

The presence of lipase enzyme results in the degradation of Tributyrin forming the halo zone (Fig. 11 (A) (B)). The halo zone is measured and recorded in Table 9.

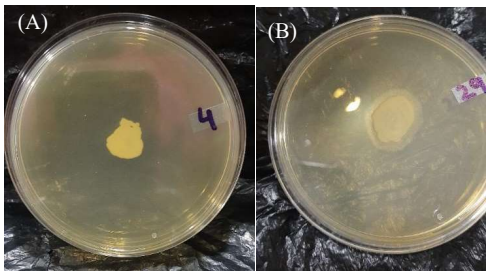

Fig. 11: (A) Lipase negative result, (B) Lipase positive result

Table 9: showing halo zone measurements produced by bacterial strains showing Lipase activity

Bacterial Strain	Radius of bacterial culture and halozone (in mm) (R)	Radius of culture (in mm) (r)	Halozone measurement (in mm) (R-r)
PK 11	10	7	3
PK24	13	10	3
PK29	12	10	2
PK72	13	10	3

2.6.2.4. Amylolytic activity

Amylase is an enzyme which catalyzes the hydrolysis of starch into simple sugars. When iodine combines with starch, blue-black color is produced. But when amylase is present, this color is not produced resulting in the formation of halo zone (Fig. 12). The halo zone is measured and recorded in Table 10.

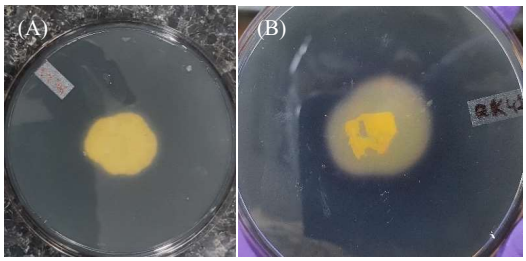

Fig. 12: (A) Amylase negative result
(B) Amylase positive result

Table 10: showing halo zone measurements produced by bacterial strains showing Amylase activity

Bacterial Strain	Radius of bacterial culture and halozone (in mm) (R)	Radius of culture (in mm) (r)	Halozone measurement (in mm) (R-r)
PK3	23	17	6
PK8	17	14	3
PK9	28	22	6
PK12	16	9	7
PK21	15	10	5
PK22	28	24	4
PK28	7	3	4
PK33	20	15	5
PK37	20	8	16
PK41	17	8	9
PK52	16	13	3
PK54	10	7	3
PK64	13	7	6
PK67	18	15	3
PK68	13	8	5
PK72	18	10	8
PK74	23	17	6
PK76	20	13	7
PK78	22	18	4
PK79	30	17	13
PK92	20	13	7

2.6.2.5. Proteolytic activity

The protease enzyme degrades casein present in the media resulting in the formation of halo zone (Fig. 13). The halo zone is measured and recorded in Table 11.

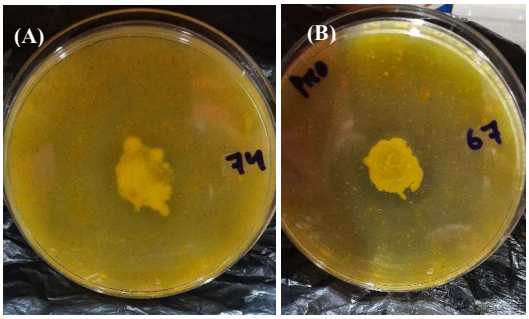

Fig. 13:

(A) Protease negative result

(B) Protease positive result

Table 11: showing halo zone measurements produced by bacterial strains showing Proteolytic activity

Bacterial Strain	Radius of bacterial culture and halozone (in mm) (R)	Radius of culture (in mm) (r)	Halozone measurement (in mm) (R-r)
PK2	13	7	6
PK3	15	13	2
PK8	15	12	3
PK9	15	10	5

PK12	15	10	5
PK21	13	10	3
PK22	15	12	3
PK24	10	9	1
PK33	13	9	4
PK41	13	9	4
PK52	10	8	2
PK54	16	15	1
PK67	14	10	4
PK68	13	10	3
PK72	10	8	2
PK76	15	11	4
PK79	14	9	5
PK92	15	12	3

2.6.2.6. HCN Production

Picric acid reacts with HCN and produces red iso-purpurate. An alkali is required to carry out this reaction since free HCN doesn't react with picric acid. Thus, sodium carbonate is used as an alkali. The production of iso-purpurate gives the brownish-red color (Fig. 14). HCN producing bacterial strains have been listed in Table 12.

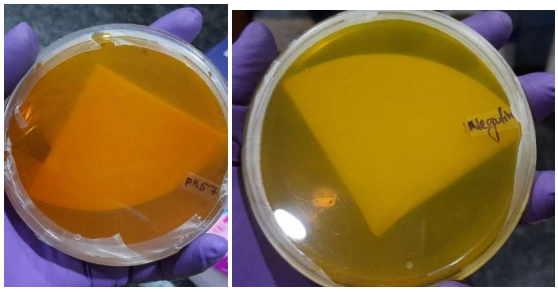

Fig. 14: HCN produced by PK 57 strain resulting in the formation of red colour. Negative plate for comparison.

Table 12: Shows the HCN production result for the various microbial strains ("+" represents positive result and "-" represents negative result)

Bacterial Strain	HCN Production	Bacterial Strain	HCN Production
PK2	-	PK37	-
PK3	+	PK41	+
PK4	-	PK46	-
PK5	-	PK47	+
PK7	-	PK52	+
PK8	+	PK54	-
PK9	-	PK57	+
PK10	+	PK63	+
PK11	-	PK64	-
PK12	-	PK67	-
PK21	-	PK68	-
PK22	+	PK72	+
PK23	+	PK74	+
PK24	+	PK76	-
PK28	+	PK78	-

PK29	-	PK79	-
PK30	-	PK92	-
PK33	+		

2.6.2.7. Phosphate solubilization

The standard graph for Phosphate solubilization is shown in Fig. 15 and the OD at 430 nm has been recorded and plotted (Fig. 16).

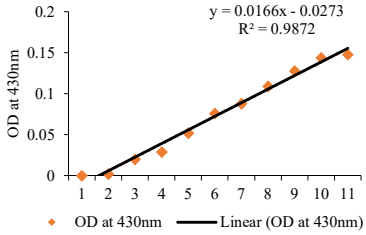

Fig. 15: Standard Phosphate curve

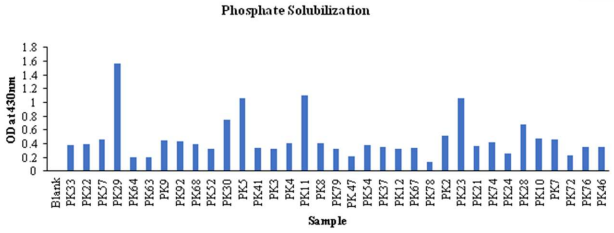

Fig. 16: Phosphate solubilization test, OD at 430nm.

2.6.2.8. Siderophore Production

Siderophores are ligands used to chelate ferric iron. When CAS forms complex with ferric iron, blue color is produced. But when an iron chelator such as siderophore is present it removes iron from the CAS-ferric iron complex resulting in color change from blue to yellow (Fig. 17). Siderophore producing bacterial strains have been listed in Table 13.

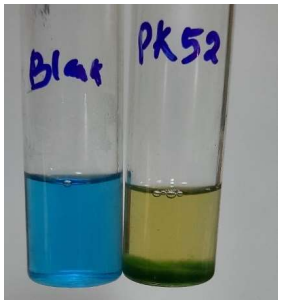

Fig. 17: showing Blank and Positive result for Siderophore activity.

Table 13: Shows the Siderophore activity result for the various microbial strains ("+" represents positive result and "-" represents negative result)

Bacterial Strain	Siderophore Production	Bacterial Strain	Siderophore Production
PK2	+	PK37	+
PK3	+	PK41	+
PK4	+	PK46	+
PK5	-	PK47	+
PK7	+	PK52	+
PK8	+	PK54	+

PK9	+	PK57	+
PK10	+	PK63	-
PK11	-	PK64	-
PK12	+	PK67	+
PK21	+	PK68	+
PK22	+	PK72	+
PK23	-	PK74	+
PK24	+	PK76	+
PK28	+	PK78	+
PK29	+	PK79	+
PK30	+	PK92	+
PK33	+		

2.6.2.9.Nitrogen Free

NFM medium contains a pH indicator, Bromothymol blue. When nitrogen is produced, the pH decreases which results in the color change from blue to yellow (Fig. 18). Nitrogen producing bacterial strains have been listed in Table 14.

Fig. 18: showing Blank and Positive result for Nitrogen free activity.

Table 14: Shows the Nitrogen production result for the various microbial strains ("+" represents positive result and "-" represents negative result)

Bacterial Strain	Nitrogen Production	Bacterial Strain	Nitrogen Production
PK2	+	PK37	+
PK3	+	PK41	+
PK4	+	PK46	+
PK5	+	PK47	+
PK7	+	PK52	-
PK8	-	PK54	-
PK9	+	PK57	-
PK10	+	PK63	-
PK11	+	PK64	+
PK12	+	PK67	+
PK21	+	PK68	+
PK22	+	PK72	+
PK23	+	PK74	+
PK24	+	PK76	-
PK28	+	PK78	-
PK29	+	PK79	+
PK30	+	PK92	+
PK33	+		

2.6.2.10. Indole Acetic Acid (IAA) Production

Salkowski reagent is used to detect IAA production. This reagent consists of perchloric acid and ferric chloride, which yields pink color when reacts with IAA (Fig. 19). Pink color is produced due to IAA complex formation along with reduction of $FeCl^{+3}$. Nitrogen producing bacterial strains have been listed in Table 15.

Fig. 19: showing Blank and Positive result for IAA Production

Table 15: Shows the IAA production result for the various microbial strains ("+" represents positive result and "-" represents negative result)

Bacterial Strain	IAA Production	Bacterial Strain	IAA Production
PK2	-	PK37	-
PK3	-	PK41	-
PK4	-	PK46	-
PK5	+	PK47	-
PK7	-	PK52	-
PK8	-	PK54	-
PK9	-	PK57	-
PK10	-	PK63	-
PK11	+	PK64	-
PK12	-	PK67	-
PK21	-	PK68	-
PK22	-	PK72	-
PK23	+	PK74	-
PK24	+	PK76	-
PK28	+	PK78	-
PK29	-	PK79	-
PK30	-	PK92	-
PK33	+		

2.6.2.11. Ammonia Production

Nessler's reagent when reacts with Ammonia, produces a complex known as Amido-iodo-mercury which results in the orange color (Fig. 20). Ammonia producing bacterial strains have been listed in Table 16.

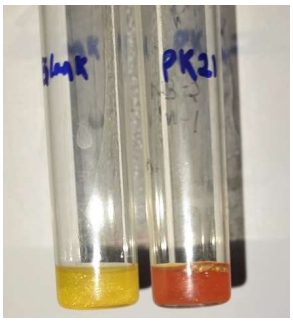

Fig. 20: showing Blank and Positive result for Ammonia Production

Table 16: Shows the Ammonia production result for the various microbial strains ("+" represents positive result and "-" represents negative result)

Bacterial Strain	Ammonia Production	Bacterial Strain	Ammonia Production
PK2	+	PK37	+
PK3	+	PK41	+
PK4	+	PK46	+
PK5	+	PK47	+
PK7	+	PK52	+
PK8	+	PK54	+
PK9	+	PK57	+
PK10	+	PK63	-
PK11	+	PK64	+
PK12	+	PK67	+
PK21	+	PK68	+
PK22	+	PK72	+
PK23	+	PK74	+
PK24	+	PK76	+
PK28	+	PK78	+
PK29	+	PK79	+
PK30	+	PK92	+
PK33	+		

2.7. CONCLUSION

In the AST, 7 isolates (PK9, PK21, PK22, PK26, PK28, PK47, PK78) were susceptible to all the antibiotics and only 2 (PK11 and PK63) isolates were resistant to 11 antibiotics. In this study, a total 35 rhizobacterial isolates showed several multiple PGP features. All of the isolates showed catalase activity and ammonia production except PK63. Out of 35 isolates, only 6 isolates (PK4, PK7, PK21, PK30, PK54, PK78) were positive for cellulase activity and 4 isolates (PK11, PK24, PK29, and PK72) were positive for lipolytic activity. The PK37and PK79 isolates demonstrated excellent amylase activity. 18 isolates were recorded positive for proteolytic activity. 15 isolates such as PK3, PK8, PK10, PK22, PK23, PK24, PK28, PK33, PK41, PK47, PK52, PK57, PK63, PK72, and PK74 were recorded positive for HCN production, postulating that the production of HCN by rhizobacteria plays a significant role in biological control of pathogens. PK29, PK11, and

PK23 were the most efficient P-solubilizers observed from the study. Alot of the isolates produces siderophore except 5 isolates (PK5, PK11, PK23, PK63, and PK64). All the isolates exhibited N_2-fixing characteristic except few isolates i.e., PK8, PK52, PK54, PK57, PK63, PK76, and PK78. However, only 6 isolates (PK5, PK11, PK23, PK24, PK28, and PK33) were able to produce IAA.

2.8. REFERENCES

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APPENDIX

Lactose Broth (2X)

Peptone- 5g/L

Beef extract- 3g/L

Lactose- 5g/L

Bromocresol purple- 25mg/L

Add the components in distilled water and makeup the volume to 1L. To mix the components vortex at 760rpm.

M9 Media

Potassium dihydrogen phosphate- 2g/L

Magnesium sulphate- 0.3g/L

Potassium nitrate – 0.3g/L

Diazanium sulfate- 1.4g/L

Calcium chloride- 0.3g/L

Iron (II) sulfate monohydrate- 5mg/L

Zinc sulfate heptahydrate- 1.4mg/L

Iron (III) chloride- 2mg/L

Manganese chloride- 1.5mg/L

Agar- 18g/L

pH= 7

National Botanical research Institute's Phosphate Growth Medium (NBRIP)

Glucose- 10g/L

Calcium phosphate- 5g/L

Magnesium chloride hexahydrate- 5g/L

Magnesium sulfate heptahydrate- 0.25g/L

Potassium chloride- 2g/L

Diazanium sulfate- 0.1g/L

Nitrogen fixation media (NFM)

Sucrose- 5g/L

Potassium phosphate dibasic- 0.8g/L

Mannitol- 3g/L

Potassium dihydrogen orthophosphate- 0.2g/L

Magnesium sulfate- 0.2g/L

Sodium chloride- 20g/L

Calcium chloride- 0.02g/L

EDTA Ferric monosodium- 28mg/L

0.5% Bromothymol blue- 2ml/L

Adjust pH to 7 by adding 0.2N KOH.

Peptone water

Peptone- 10g/L

Sodium chloride- 5g/L

pH= 7

Barton's reagent or Vanadate-Molybdate reagent

Solution A: Dissolve 25g Ammonium molybdate in 300ml distilled water

Solution B: Dissolve 1.25g Ammonium metavandate by heating to boiling in 300ml distilled water.

Reagent: Add 330ml conc HCl. Cool Solution B to room temperature and pour Solution A into Solution B. Dilute to 1L.

Standard phosphate solution: Dissolve 0.2195g Anhydrous Potassium dihydrogen phosphate in 1000ml distilled water.

CAS dye

0.5M $FeCl_3$- 40µL/20mL

1M HCl- 0.2ml/20mL

CTAB- 36.45mg/20mL

CAS dye- 121mg/20mL

Salvonski reagent

0.5M Ferric chloride- 2ml

70% Perchloric acid- 50ml

Makeup the volume to 100ml and store in dark.