International Journal of Pharmacy and Biological Sciences-IJPBS™ (2021) 11 (3): 131-138
Online ISSN: 2230-7605, Print ISSN: 2321-3272
Research Article | Biological Sciences | OA Journal | MCI Approved | Index Copernicus

Isolation And Characterization of Endophytic Microorganisms from Plants and Their Application as Biofertilizer

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Received: 14 Mar 2021 / Accepted: 12 Apr 2021 / Published online: 1 Jul 2021
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Abstract
Endophytes, by residing within the specific chemical environment of host plants, form a unique group of microorganisms. Microbially unexplored plants can have diverse groups and potential microbial association. The present investigation was aimed to isolate and characterize endophytic microorganisms from *Avicennia, Acacia* and coconut with ability to solubilize phosphate, fix nitrogen and produce various enzymes. Total 18 bacterial and 12 fungal isolates were isolated from the surface sterilized stem, root, and leaf of plants. Ten bacterial isolates and nine fungal isolates have capacity to solubilize phosphate. Studies on nitrogen fixation suggested that sixteen isolates have the ability to fix nitrogen. All the endophytic bacterial and fungal isolates were screened for enzyme production. The result of the study revealed that maximum bacterial isolates have positive L-asparaginase activity, followed by Amylase and Lipase activity. Similarly maximum fungal isolates have positive Lipase activity followed by Amylase activity. Bacterial isolates and bacterial plus fungal isolated gave efficient results in seed assay and pot assay respectively. Thus, results indicate that endophytes isolated from different plants could be used as a biofertilizer.

Keywords
Biofertilizer, Endophytes, Nitrogen fixation and Phosphate solubilization.

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INTRODUCTION:
Endophytes are organisms living as symptomless colonies, maybe during a part of their life cycle, inside the host plants [1]. The term 'endophyte' was coined by de Bary (1866) to distinguish the epiphytic organisms living on the surface of plants. [2]. The diversity between the endophytes will impact greatly on plant communities through the increase of fitness. This increase of fitness in plants is achieved through the conferring of abiotic and biotic stress tolerance, increasing biomass, decreasing water consumption and/or decreasing fitness by altering the allocation of resources from soil to the plants [3]. There is evidence that the presence of endophytes may not only influence plant growth, developments, fitness, and diversity but also population dynamics, plant community diversity and ecosystem functioning [4,5]. Endophytes have evolved mechanisms to live within the plant by defending themselves against all physical and chemical weapons of the plants, e.g., in plants like Camptotheca acuminata produces anticancer compound Camptothecin which binds to topoisomerase I to stop cell divisions [2]. Endophytes have evolved with the plants themselves, and during this long period, they have developed all strategies

to live, survive, evolve and refine the relationship with the plant. [6,7,8,9]. During the same evolutionary period, endophytes have adapted themselves to the plant microenvironment by genetic variation including uptake of some plant's DNA [10]. Due to this adaptation and genetic material uptake, endophytes started producing plant metabolites or their precursors [11,12]. Now, endophytes are known to occur in all habitats and in different plants such as mosses, ferns, lichens, shrubs, grasses, and deciduous and coniferous trees [13]. So, endophytes are an important part of our ecosystem.

Endophytes are transferred from generation to generation through seeds (vertical transmission) or may be transferred to allied species through plant part decay/soil (horizontal transmission) [14,15]. Endophytes may produce an overabundance of substances of potential use to agriculture, industry, and modern medicine such as novel antibiotics, antimycotics, immunosuppressant and anticancer compounds [16]. Endophytes share everything with an invading pathogen in the host plant Increasing evidence suggest that endophytes interact with the pathogen in different ways in different hosts, and resultantly, altered physiology may suppress the growth of the pathogen, alter nutrient balance in favor of endophyte or stimulate the plant's defense mechanism [14,17]. Several important medicines are obtained from plants such as vincristine, vinblastine, camptothecin, quinine and Taxol [18]. Endophytes are gaining importance because of their role in plant growth stimulation, protection against biotic and abiotic stresses and pest via modulation of growth hormone signaling, higher seed yield and plant hormones [19].

Hyde and Sorting proposed five statements 'why the endophytes are so important'.

1. Studies provide high taxon diversity; can be completed in the relative comfort of a laboratory with minimal fieldwork and use a well- established traditional methodology that any motivated student can follow.
2. Most sporulating isolates are relatively easily identified (at least to genus) as they belong to less than 50 characteristic genera.
3. Various methodologies can be applied to mycelia sterile to promote sporulation; alternatively molecular methods can be utilized to identify these relatively fast growing morphotypes.
4. Sophisticated statistics can be applied to the isolates which "appear" to have been derived from single random units and will satisfy the demands of any unforgiving non-fungal economist.
5. The relatively fast growing and "highly" diverse endophytes provide ideal tools for screening and novel compound discovery, and they can easily be lodged in culture collections.

MATERIALS AND METHODS:

Collection of samples:

Samples of various plants such as *Avicennia, Acacia* and coconut were collected from different places. *Avicennia* samples were collected one from Udvada beach and second from a place near Vekariya hanuman temple, Hingraj Valsad. Acacia samples were collected from a place near M2 mall, Tithal road Valsad and near GEB office, Tithal crossroad Valsad. Coconut sample was collected from one of the vendors from Vapi.

Isolation of microorganisms:

Isolation of organisms was done by direct inoculation method. Firstly, the sample plants were thoroughly washed under running tap water to remove all soil and other dirt. Then the small thin section of roots, stem and leaves were cut. For the surface sterilization process, it was dipped in a solution of 70% alcohol for 1 minute, then it was dipped in a solution of sodium hypochlorite for 3 minutes and at last it was washed with sterile distilled water to remove the above solution. It was dried in sterile laminar air flow and then directly inoculated on the medium. Plates were incubated at room temperature for 6-7 days. After incubation plates were observed for growth. For characterization, colony characteristic was written from colonies observed on plate, gram staining was performed for bacterial isolates obtained from nutrient agar plate, and fungal mounting was done for fungal isolates obtained from potato dextrose agar plate.

Analysis of characterization of isolates:

Production potential of various enzymes such as amylase, lipase and L-asparaginase was tested. Amylase, lipase and L-asparaginase enzyme production was checked on starch agar plate, tributyrin agar plate and medium containing L-asparagine agar plate respectively. Plates were incubated at room temperature for 3 days and 7 days respectively for bacterial and fungal isolates. Zone of hydrolysis was observed (by the addition of iodine in case of starch agar plate), color change was observed (in case of L-asparaginase enzyme), transparency of medium was observed (in case of lipase enzyme), after the incubation and results were recorded. Various biochemical tests such as citrate utilization test was done by using Simmons's citrate agar slant that contain citrate as substrate was observed for

color change from green to blue after 2-3 days of incubation. Second test done was a gelatin hydrolysis test by using gelatin nutrient broth that contains gelatin as substrate and tube for liquid in the tube after incubating the tube at low temperature for a specific time period. Third test performed was catalase test done on slide using H_2O_2 observed for bubbles formation produced by isolates. Fourth test was an oxidase test done using an oxidase disc that contains p- phenylenediamine as reagent and was observed for purple color formation on slide after spreading some growth of isolate. Fifth test is Hydrogen sulphide production test, was done using 2% peptone broth. Isolates were inoculated in the broth and lead acetate paper strip was placed in the neck of the tube such that 1/4 to 1/2 of strip projects below the cotton plug. Tubes were observed for Blackening of paper strips after 2-3 days of incubation.

Screening of phosphate solubilizing isolates:

Screening of phosphate solubilizing isolates was done on Pikovskaya's medium containing calcium phosphate. Isolates were grown on the plate by line inoculation and a zone of hydrolysis was observed produced due to presence of phosphatase enzymes, after 3 days and 7 days respectively for bacterial and fungal isolates.

Screening of nitrogen fixing isolates:

Screening of nitrogen fixing isolates was done on Jensen medium, the medium devoid of nitrogen source. Isolates were grown on medium by line inoculation and only those isolates were able to grow, who were able to fix nitrogen. Colonies were observed on plates after 2-3 days of incubation.

Screening for IAA (Indole acetic acid) producing isolates:

For screening of IAA producing isolates, an indole test was performed using 1 % peptone as a medium. The medium was inoculated with inoculants that were incubated at room temperature for 24 to 48 hours, after incubation Kovac's reagent is added and observed for pinkish red color ring formation that indicated positive test. Indole test positive isolates were further selected for the IAA production test. Isolates were inoculated in tryptone yeast extract broth and were incubated at room temperature for 5 to 7 days, then the broth was centrifuged, and supernatant was collected. Add 1ml of Salkowski reagent was added in supernatant and the mixture was again incubated for 1 hour in dark condition, after incubation red color appearance in the medium indicated IAA production.

Preparation of inoculum for antimicrobial activity and seed/pot assay:

For the inoculum preparation, in case of bacterial isolates nutrient broth and for fungal isolates Sabouraud's broth were used. After inoculation, broths were incubated for 5 to 7 days at room temperature on a rotatory shaker. After incubation the broths were used as inoculum.

Antimicrobial activity:

To carry out the antimicrobial activity supernatant required is prepared from above step. Broth was centrifuged to separate out supernatants and it is used to carry out antimicrobial activity. Agar well diffusion assay is used for antimicrobial activity experiment, in which nutrient agar plates were streaked with test microorganism like *Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa, Staphylococcus aureus.* With the help of sterile cup borer wells are created on the agar plate, supernatant was added in wells. Plates were incubated at 37^0 C for 24 hours. After incubation plate was observed for the zone of inhibition.

Seed germination assay:

For seed germination assay the seeds of Vigna radiata (moong) were first surface sterilized with the help of 0.1% $HgCl_2$ for 3 minutes followed by washing with water to remove mercury traces present on the surface. Then seeds were added in inoculum for 30 minutes and transferred on moist sterilized filter paper on a petri plate and left as it is at room temperature, undisturbed for 7 days. Seeds incubated with water only acted as negative control. The distilled water was sprayed regularly for 7 days on control and experiment seeds and then the length of root and shoot were measured.

Pot assay:

For pot assay, the soil used was repeatedly sterilized. Then soil was transferred to the pot. Seeds and inoculum were added. Plants were watered daily for 7 to 14 days, after that growth of roots and shoots of plants were measured.

RESULTS AND DISCUSSION:

Sample collection:

Different plant samples such as *Avicennia, Acacia* and Coconut were collected from different places such as Valsad, Vapi and Udvada. Total, 2 *Acacia,* 2 *Avicennia* samples and 1 Coconut sample were collected.

Isolation of microorganisms:

Plant's part was cut into thin sections and placed on the medium for the growth of Endophytes. From different samples collected from different places, 18 bacterial isolates and 12 fungal isolates were isolated. Out of 18 bacterial isolates 17 were gram negative and 1 was gram positive.

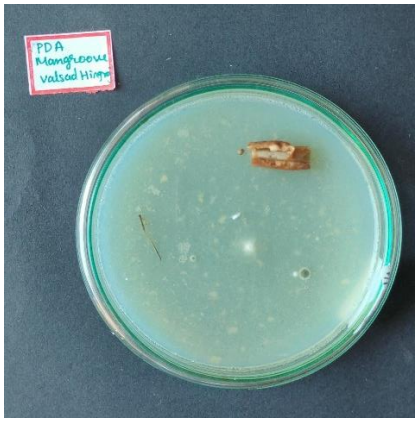


Figure 1: Plates with growth of Bacterial and Fungal isolates

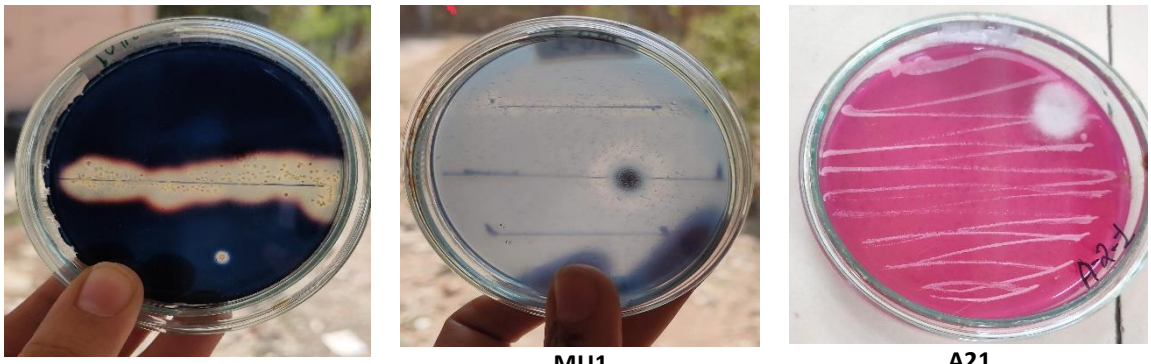
Characterization of isolates:

Out of 18 bacterial isolates, 3, 4, 13 and 14 isolates gave positive Citrate utilization test, Catalase test, Oxidase test and Gelatin hydrolysis test, H_2S production test respectively. Isolates producing Amylase enzymes have capacity to utilize Starch, giving a zone of hydrolysis after the addition of Iodine. Isolates having the capacity to utilize Lipid by Lipase enzyme give a zone of hydrolysis on plates. Isolates having capacity to degrade L-asparagine into aspartate and ammonia turns medium pink due to Phenol red as indicator dye. We have analyzed enzyme production capacity of both Bacterial isolates and Fungal isolates. Out of 18 bacterial isolates, 8, 4 and 9 isolates gave positive results for Amylase, Lipase and L-asparaginase enzymes respectively. Out of 12 fungal isolates, 1and 5 isolates gave positive results for Amylase and Lipase enzyme production capacity respectively. No proper citrate utilization, catalase, oxidase, gelatin hydrolysis, H_2S production test and L-asparaginase activity was shown by any of the fungal isolates.

Table 1: Characterization of isolates

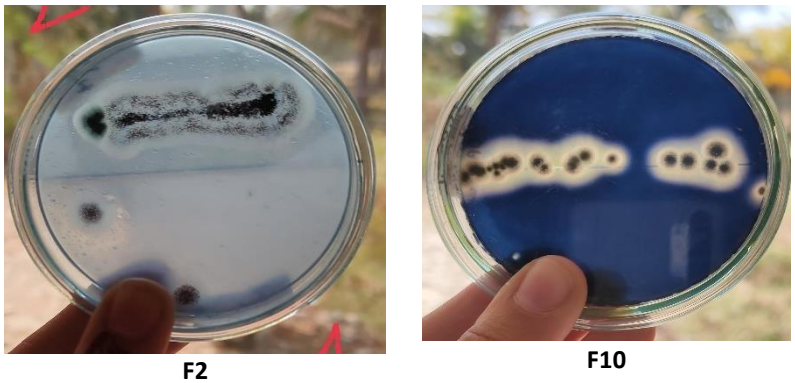
Isolates	Citrate utilization test	Catalase test	Oxidase test	Gelatin hydrolysis test	H_2S production test	Amylase enzyme	Lipase enzyme	L-asparaginase enzyme
MV1	_	_	+	+	_	_	_	_
MV2	_	+	+	+	_	+	+	_
MV3	_	+	+	+	_	_	+	+
MV4	_	_	+	+	_	_	_	_
MV5	_	_	+	_	_	_	_	_
MU1	_	_	+	+	_	+	+	+
MU2	_	_	+	+	_	+	_	+
MU3	_	_	+	+	_	_	_	+
CF1	_	_	+	+	_	+	_	_
A11	+	_	+	+	_	+	+	+
A12	+	_	+	+	_	_	_	+
A13	+	+	_	+	_	_	_	+
A21	_	+	_	_	_	_	_	+
A22	_	_	_	+	_	+	_	_
A23	_	_	_	_	_	_	_	_
CW1	_	_	+	+	_	+	_	+
CW2	_	_	_	_	_	_	_	_
CW3	_	_	+	+	_	+	_	_
F1	_	_	_	_	_	_	_	_
F2	_	_	_	_	_	_	+	_
F3	_	_	_	_	_	_	+	_
F4	_	_	_	_	_	_	_	_
F5	_	_	_	_	_	_	_	_
F6	_	_	_	_	_	_	_	_

F7	–	–	–	–	–	–	+	–
F8	–	–	–	–	–	–	–	–
F9	–	–	–	–	–	–	–	–
F10	–	–	–	–	–	+	+	–
F11	–	–	–	–	–	–	–	–
F12	–	–	–	–	–	–	+	–



MV2 MU1 A21

Figure 2: Plates showing Amylase, Lipase and L-asparaginase enzyme production by Bacterial isolates



F2 F10

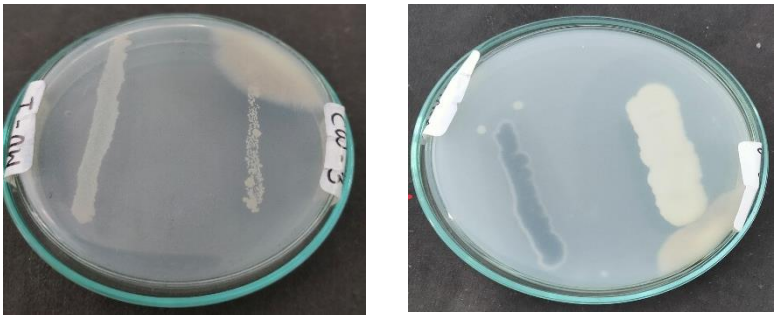
Figure 3: Plates showing Amylase and Lipase enzyme production by Fungal isolates

Phosphate solubilization:

Phosphate solubilizing activity was conducted to observe the potential of isolates to utilize phosphate. Isolates were grown on Pikovskaya's medium containing $Ca_3(PO_4)$. The ability of endophytes to solubilize phosphate was identified by the formation of clear zones around the colonies. We have analyzed Phosphate solubilizing activity on both Bacterial and Fungal isolates. We have obtained the effective result from both isolates. Out of 18 bacterial isolates, 10 isolates were able to solubilize phosphate. Out of 12 fungal isolates 9 were able to solubilize phosphate.

Table 2: Phosphate solubilization by isolates

Bacterial Isolates	Result	Bacterial Isolates	Result	Bacterial Isolates	Result	Fungal Isolates	Result	Fungal Isolates	Result
MV1	+	MU1	+	A11	_	F1	+	F7	_
MV2	+	MU2	+	A12	+	F2	+	F8	_
MV3	_	MU3	+	A13	_	F3	+	F9	+
MV4	_	CW1	+	A21	_	F4	+	F10	+
MV5	_	CW2	_	A22	+	F5	+	F11	_
CF1	+	CW3	+	A23	_	F6	+	F12	+



MU1 & CW3 **F3 & F4**

Figure 4: Plates showing clear zone of Phosphate solubilization by Bacterial and Fungal isolates

Nitrogen fixation capabilities of isolates:

Nitrogen is an essential element for plant growth, but plants cannot utilize atmospheric nitrogen directly. Bacteria fix atmospheric nitrogen as they possess nitrogenase enzymes and help plants in their growth and development. We screened isolates on nitrogen deficient medium (Jensen medium). Out of 18 bacterial isolates 16 isolates were able to fix nitrogen.

Table3: Nitrogen fixation by isolates

Isolates	Result	Isolates	Result
MV1	+	A11	+
MV2	+	A12	+
MV3	+	A13	+
MV4	+	A21	+
MV5	_	A22	+
MU1	+	A23	+
MU2	_	CW1	+
MU3	_	CW2	_
CF1	+	CW3	+

CF1

Figure 5: Plate showing growth of Nitrogen fixing isolates

IAA (Indole Acetic Acid) production given by bacterial isolates:

Plants have phytohormones for growth and regulation. IAA is a type of phytohormone of auxin class which helps in apical dominance. Some Endophytes have the capacity to produce these 2° metabolites that act as phytohormones. Out of 18 isolates only 2 isolates were able to produce Indole and further when they were screened for IAA production no color change was observed that indicated no IAA production.

Antimicrobial Assay:

One isolate out of 18 for antimicrobial activity to test against test pathogens pathogens *Escherichia coli*, *Proteus vulgaris*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* using Agar well diffusion

method. But isolate did not give any zone of inhibition against any test pathogens.

Seed germination assay:

Isolates having capacity to utilize phosphate and fix nitrogen were used as inoculum to coat the seeds surface. The effect of isolates on seed germination is determined by observing the length of shoot and root from germinating seeds. Out of both types of isolates, bacterial isolates gave promising results. Fungal isolates didn't have much effect on shoot and root of the plant as compared to control.

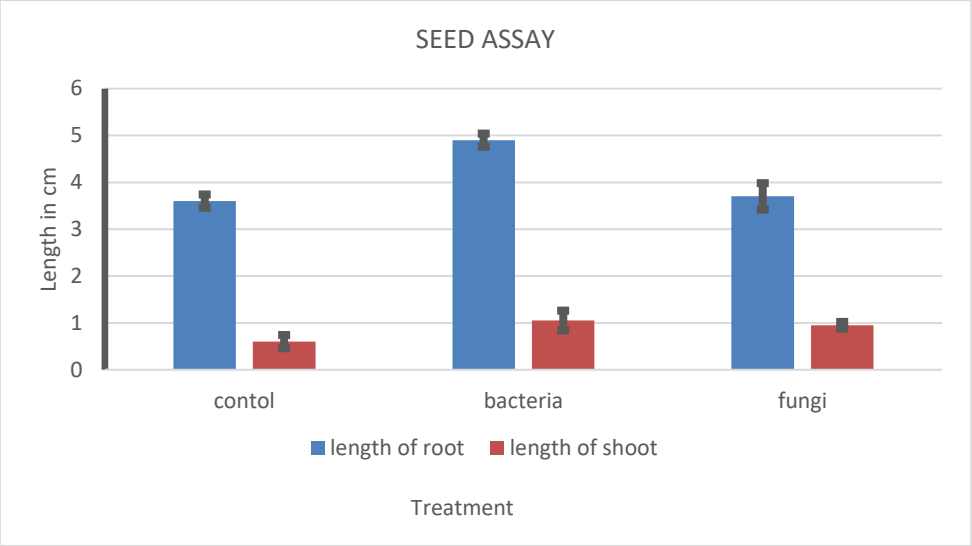


Figure 6: Effect of morphological parameter of *Vigna radiata* seeds inoculated with microbial biofertilizer

Pot assay:

Phosphate and nitrogen are important elements for plant growth and development. Hence isolates having capacity to utilize and fix phosphate and nitrogen respectively were used as biofertilizers. We tested Endophytic Bacteria and Fungi both for Biofertilizer activity. Four sets such as Control [C], Bacteria [B], Fungi [F] and last was Bacteria and Fungi mix [BF]. Out of this, Bacterial isolates [B] and Bacterial and Fungal isolates mix [BF] gave promising results.

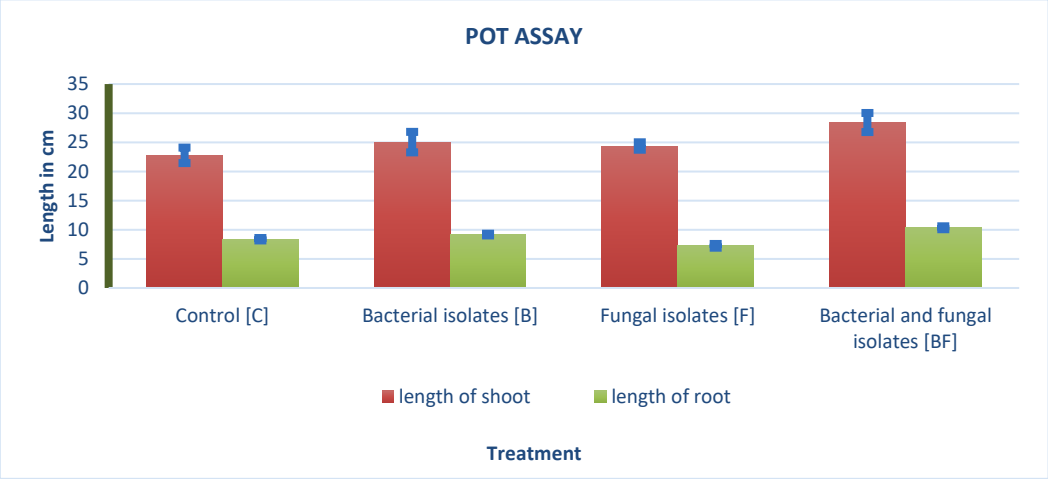


Figure 7: Effect of morphological parameter such as length of shoot and root were recorded from seedling raised with biofertilizer (inoculum)

CONCLUSION:

The result from the study demonstrated the diverse community of endophytic microorganisms associated with *Avicennia*, *Acacia* and coconut. Among the endophytic microorganisms isolated, 18 were Bacterial isolates and 12 were Fungal isolates. Characterization of these isolates were done by certain Biochemical tests such as Citrate utilization test, Oxidase test, Catalase test, Gelatin hydrolysis test. Isolates were screened for Amylase, Lipase and

L-asparaginase enzyme production and many isolates gave positive results. These enzymes have many medical and industrial applications. Isolates were also tested for IAA (Indole Acetic Acid) production and antimicrobial activity, but no efficient results were found. The isolates were also found to have the capacity of Phosphate solubilization and Nitrogen fixation which have high impact on plant growth and hence isolates can be used as Biofertilizer. To test the efficiency of isolates as Biofertilizer, Seed assay and Pot assay were carried out and promising results were given by Bacterial isolates.

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