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Isolation and molecular characterization of plant growth promoting fungi from rhizosphere soil

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Abstract
Plant growth-promoting fungi (PGPF) have attracted ample interest as bio-fertilisers due to their multiple advantageous outcomes on plant quantity and quality. In this study, the potential of rhizosphere fungi is revealed. Samples were collected from the rhizosphere of rubber plant at Kothamangalam. Microorganisms were isolated by serial dilution method and further inoculating in SDA media for fungal growth. Pure colonies were isolated by repeated subcultures. Enzyme production assay was checked with the isolated fungi and it showed the production of amylase, cellulase, protease and lipase enzyme. Molecular identification was performed by analysing sequences of the nuclear ribosomal RNA genes from internal transcribed spacer (ITS) region. This studied the phylogenetic relationship of the isolated samples by comparing the ITS sequences with those stored in the GenBank database. Among the isolated fungi, *Penicillium* species showed potential enzyme production and lipase enzyme production as greater for this fungus. So this *Penicillium* species can be used for the large scale production of lipase enzyme and also it can be utilized as a biofertilizer for agricultural uses.

Keywords: PGPF, lipase, rhizosphere, 18S rRNA, ITS, *Penicillium* species

1. Introduction
Plants, animals and microorganisms are different kinds of sources from where we obtain enzymes. Due to the limitation of enzymes that can be obtained from animals and plants, microorganisms become an interesting source for enzymes. They are capable to produce different variety of enzymes. Nowadays, microbial enzyme production based on biotechnology is making great progress [1]. Enzymes produced from micro-organisms are already being used in various industrial applications. For the commercial production of various enzymes selected micro-organisms such as bacteria, fungi or yeasts are globally used [2]. These enzymes are used for several purposes like diagnosis, biochemical investigation, treatments and as catalytic agents in food etc.
Microbial enzymes are currently gaining much attraction due to the rapid development of enzyme technology. Microbial enzyme production mainly focuses on hydrolytic enzymes like proteases, amylases, lipases, pectinases etc that decompose polymers such as proteins, starches, lipids or pectin. When microorganisms are grown in suitable nutrient medium, they secrete these enzymes into the corresponding medium. It is easy to harvest these enzymes from the medium and its yield can be increased by optimising the growth conditions. So microbial enzymes are gaining attention due to their high yield, economic feasibility, ease of product recovery, optimization and modification. Once optimized the product and growth conditions, micro-organisms provide a regular supply of enzymes. Its growth and production are not influenced by the seasons and growth of microorganisms are rapid, less costly and have greater catalytic activities.
The term "rhizosphere" was initially used to describe the portions between root and plant, [3, 4]. Hiltner noted the rhizosphere soil as the soil covering the plant root and it is inhabited by a variety of population of microorganisms. These micro-organisms utilize the chemicals released from the roots of plants and vice versa. There is competition between the rhizosphere microorganisms for water, nutrients and space and form a good relation with plants[5]. These microorganisms are key components for the growth and ecological fitness of their plant host. Since these micro-organisms are producing a variety of enzymes and other factors that help in the host plant growth, it can be utilized for an environmentally friendly and sustainable agriculture practices. The organisms that help in the plant growth are known as plant growth promoting micro-organisms. Plant growth promoting rhizobial micro-organisms enhance crop growth, productivity and can help a secure, clean environment. The rhizosphere soil contains many different types of PGPR species, which have beneficial effects on crop growth and productivity [6]. Plant growth-promoting fungi (PGPF) are a heterogeneous group of non-pathogenic fungi that reside freely in the root surface or the interior of the root itself or of the rhizosphere. It also improve seed germination, seedling vigour, plant growth, production and flowering of a wide variety of host plants [7].
Objectives of this study include isolation of fungal colonies that produce potential enzymes, molecular characterization of selected isolates and determination of plant growth promoting factors.

2. Materials and Methods
2.1. Sample collection
The soil samples were collected from Kothamangalam, Ernakulam district, Kerala [10°4'33.38"(N) and 76°40'46.96"(E)]. The collected soil sample were placed in Zip-lock plastic bags and taken to the laboratory immediately.

2.2. Isolation of fungi
The fungi were isolated by serial dilution plate method.

1gm of soil sample was suspended in 100ml of distilled water to make microbial suspension of 10^{-2}dilution and was serially diluted upto 10^{-6} dilutions. 0.1ml of samples was plated in Sabourauds Dextrose Agar plates. The plates were kept for incubation at room temperature for 3 days for the growth of fungus.

2.3. Screening tests for hydrolytic enzyme production

2.3.1. Catalase enzyme

A loop full of test culture was transferred to sterile glass slide and a drop of 3% Hydrogen peroxide was added to the culture at once and observed for effervescence. Evolution of effervescence indicated catalase production [8].

2.3.2. Protease enzyme

The protease production was performed on sterile skim milk agar plates. Isolates were spot inoculated and followed by incubation at 30 C and zone of clearance around the colony indicating the enzymatic degradation of protease [9].

Table 1: Requirements for Skim milk agar

Pancreatic digest of casein	**5.0g**
Yeast extract	2.5g
Glucose	1.0g
Agar	15.0g
Distilled water	1000ml
Skim milk 7% was added as inducer.	

2.3.3. Amylase enzyme

The microbial isolates were spot inoculated on starch agar medium plates and incubated at 30 C for 3 days. At the end of inoculation period, the plates were flooded with iodine solution, kept for a minute and then poured off. Iodine reacts with starch to form a blue colour compound. This blue colour fades rapidly. Hence the colour less zone surrounding colonies indicates the production of amylase [8].

Table 2: Requirements for Starch agar

Beef extract	**3.0g**
Peptone	5.0g
Soluble starch	2.0g
Agar	15.0g
Distilled water	1000ml

2.3.4. Cellulase enzyme

Inoculated CMC agar plates were incubated at 30 C for 5 days. End of incubation, agar medium was flooded with an aqueous solution of congo red 1% (w/v). Formation of clear zone indicates the cellulose degradation [8].

Table 3: Requirements for CMC agar

Beef extract	**3.0g**
KH2PO4	1.0g
MgSO4.7H20	0.5g
NaCl	0.5g
FeSO4.7H20	0.01g
MnSO4.H2O	0.01g
NH4NO3	0.3g
CMC	10.0g
Agar	15.0g
Distilled water	1000ml

2.3.5. Lipase enzyme

The culture were streaked on tributyrene agar and incubated at 30° C for 72 hrs. Development of clear zones around the microbial colonies indicated lipase activity [8].

Table 4: Requirements for Tributyrene agar

Tributyrene	1.0g
Peptone	0.5g
Beef extract	0.3g
Agar	2g

2.4. Fungal identification test

LPCB staining

The screened fungus was identified by LPCB Staining. LPCB wet mount preparation is the most widely used method of staining and observing fungi. The preparation has three components: phenol, which will kill any live organisms; lactic acid which preserves fungal structures, and cotton blue which stains the chitin in the fungal cell walls. The samples were immersed in a drop of alcohol and it was viewed under the microscope after the addition of two drops of the lactophenol cotton blue stain.

2.5. Molecular identification

18SrRNA was amplified using specific primers. The PCR products were sequenced using the forward and reverse primers, ITS1 and ITS4.

Forward Primer: 5'-TCCGTAGGTGAACCTGCGG-3'

Reverse Primer: 5' TCCTCCGCTTATTGATATGC-3'

The obtained sequence was analysed using Basic Local Alignment Search Tool (BLAST), which is the most frequently used tool for calculating the sequence similarity. ITS data analysis was also done.

3. Results and Discussion

PGPF colonize plant roots and exert beneficial effects on plant growth and development by a wide variety of mechanisms. To be effective PGPF, fungi must be able to colonize roots because fungi need to establish itself in the rhizosphere at population densities sufficient to produce the beneficial effects. The exact mechanism by which PGPF stimulate plant growth is not clearly established, although several hypothesis such as production of phytohormones, suppression of deleterious organisms, activation of phosphate solubilization and promotion of the mineral nutrient uptake are usually believed to be involved[10, 11, 12, 13].

This study mainly focuses on the screening for a potential PGPF strain on the basis of plant growth promoting activity viz., catalase, amylase, lipase, protease and cellulase enzyme production.

The fungal colonies obtained during serial dilution from the Rhizosphere soil of rubber plants was sub cultured onto a new medium to obtain pure individual colonies. A total of twenty fungal isolates, showing maximum frequency were successfully sub cultured. The selected isolates were used for characterisation and screened for the production of different kinds of enzymes. Enzyme assays for catalase, protease, Amylase cellulase and lipase were done and the results are shown in Table.1. Out of 20 isolates 16 showed catalase enzyme production. 15 fungal colonies produced protease enzyme with a little zone ranging from 1mm to 3mm diameter. The isolate F2, F15 and F18 showed highest zone (3mm). The enzyme proteases helps to fight against phyto pathogens and protect them from diseases [14]. Enzyme amylase was produced by 17 isolates and the

largest zone of hydrolysis was shown by the isolate F20 (10mm) followed by F5 (8mm). Cellulase enzyme production was shown by only 6 isolates. Highest zone of cellulose hydrolysis was shown by F9 and F18 (10mm).

Lipase was produced by 10 isolates with highest zone produced by F5 (27mm). Lipase production by *Penicillium restrictum* was studied by Gombert tal., 1999 [15]. Lipase production by *Penicillium verrucosum* was also studied [16]. Lipases are biotechnologically important enzymes that has applications in food, detergent and pharmaceutical industries. They catalyze the breakdown of fats and oils as well as various synthetic reactions such as esterification, transesterification and interesterification in organic solvents. Microbial lipases stand out as the major sources of the enzyme because of their diversity in catalytic activity, high yield and low cost of production, as well as relative ease in genetic manipulation. This paper describes the lipase production by the selected fungi.

In the present study, we investigated the effectiveness of PGPF isolates whether they could increase the seed germination rate as well as growth of seedlings. For this we use fungal strains like F5, F9 and F18, which showed the production of all the enzymes under study. It significantly increased plant height, root length and dry matter production of shoot and root. Seed germination was also increased when seeds were pre-treated with PGPF isolates. F5 isolate showed better performances in aspects of seed germination and growth of seedlings. In a study, mung bean plants inoculated with PGPR shows increase in shoot length, number of pods and seed weight etc [17]. It is also reported that *V. radiata* inoculation with rhizospheric organisms enhanced the its plant growth in pot trials [18, 19].

The isolate then subjected to the field application by using pot assay. In this analysis the F5 fungal inoculum treated seeds were shown high rate of plant growth than control seeds. From this analysis it is relived that F5 have potent PGPF activity and can be used for the cost-effective production of bio-fertilizer. Further investigations, including efficiency test under greenhouse and field conditions are needed to clarify the role of PGPF as biofertilizers that exerts beneficial effects on plant growth and development. Such type of study is necessary as it advocates that use of PGPR as inoculants or bio-fertilizers is an efficient approach to replace chemical fertilizers.

From these fungal isolates, F5 which produce all the enzymes and showed highest lipase activity was used for the molecular characterization by genomic DNA isolation and PCR amplification. Genomic DNA was isolated from the fungal strain F5 and was amplified in PCR using ITS primers. Bands were visualized under UV trans illuminator and were send for sequencing. The DNA sequence obtained were trimmed at BIOEDIT and then submitted to BLAST to check sequence similarity and sequence authenticity. Sequence analysis were done by comparing the sequence in the database.

Many approaches to molecular classification for fungi have been tested to date [20]. Of these, the most successful was PCR amplification of genomic DNA followed by the sequencing of resulting amplicones. Ideally, target regions for sequence-based approaches should evolve slowly and display sufficient fungal conservation to enable cross-species amplification, while being sufficiently variable to Allow for robust discrimination between closely related species. In studies with limited numbers of different isolates, the sequencing of segments of the nuclear ribosomal RNA gene cassette, and precisely the internal transcribed spacer (ITS) regions, has proved sufficient to distinguish between several species of fungi [21]. and useful for phylogenetic analysis.

The species identification and homology between the sequences was identified using BLAST method. DNA sequences obtained were manually aligned using Clustal W software. The analysis of DNA sequence was conducted by Neighbor-joining to assess topology with MEGA 6.0. All the regions containing gaps and missing data were eliminated. The analysis of per site from averaging over all sequence pairs between groups and the number of base substitutions per site between the sequences were conducted using the Kimura 2-parameter model. The phylogenetic tree was developed by Neighborjoining (NJ) method which was tested with Kimura 2-parameter for evolutionary distances in MEGA6.0[22].

The phylogenetic analysis showed that there are two Clades, Clade A & Clade B. In Clade B, F5 ITS1 clustered with *Pencillum vanoranjei, Pencillium johnkrugii, Pencillium sclerotiorum, Pencillium mallochii & Pencillium glabrum* with 61%, 60%, 86%, 72%, 26% & 36% boot strap value respectively. The sequence analysis using BLAST and BOLD of F5 ITS shows 99% identity to *Pencillium johnkrugii, Pencillium sclerotiorum and Pencillium mallochii*.

Eco-friendly and sustainable agricultural practices include the use of genetically modified plants and growing plants with the help of plant growth promoting microorganisms. Here, the enzyme production by the plant growth-promoting bacteria is discussed. In the nearby future, plant growth-promoting organisms will begin to replace the use of chemicals and pesticides in agriculture and environmental clean-up processes.

4. Tables and Figures

Table 5: Screening for enzyme production

Fungal Isolates	Catalase	Protease	Amylase	Cellulase	Lipase
F1	_	2mm	-	_	_
F2	+	3mm	4mm	_	9mm
F3	+	1mm	5mm	_	1mm
F4	+	2mm	-	_	_
F5	+	2mm	8mm	4mm	27mm
F6	+	-	-	_	_
F7	+	-	-	2mm	_
F8	+	2mm	-	_	_
F9	+	2mm	7mm	10mm	23mm
F10	+	2mm	-	6mm	_
F11	+	2mm	-	_	5mm
F12	_	2mm	-	_	_
F13	+	-	-	_	17mm
F14	+	2mm	5mm	_	15mm
F15	_	3mm	2mm	_	_
F16	_	-	-	_	_
F17	+	2mm	-	_	_
F18	+	3mm	3mm	10mm	18mm
F19	+	-	-	8mm	25mm
F20	+	-	10mm	_	2mm
Total positive isolates	16	15	17	6	10

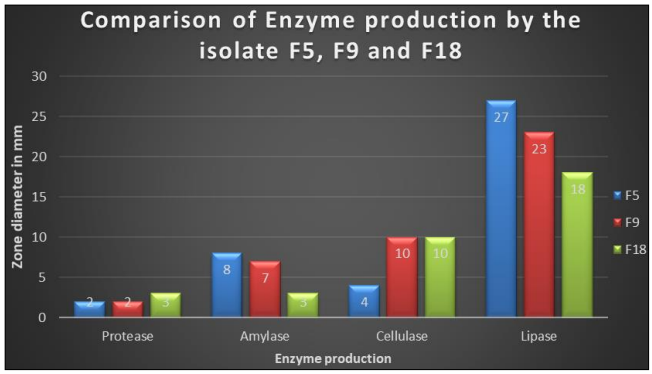

Fig 1: Comparison of enzyme production by the fungal isolates

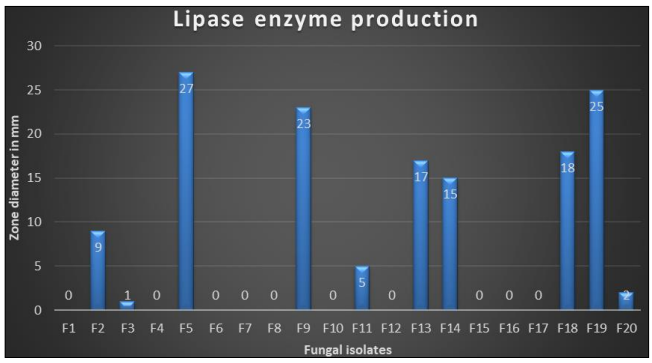

Fig 2: Lipase enzyme production by the fungal isolates

Table 6: Seed germination assay

Period of study	Shoot length(cm)				Root length(cm)			
	Control	F5	F9	F19	Control	F5	F9	F19
1st Day	-	-	-	-	1.2	1.8	2	1.8
2nd Day	-	-	-	-	1.5	2.2	2.4	2
3rd Day	0.5	1	0.8	1.2	2	2.9	3	2.7
4th Day	0.8	1.7	1.2	1.7	2.5	3.9	3.2	3.3
5th Day	1.4	2.3	1.6	2	3	4.8	3.8	3.7

Table 6: Pot experiment assay

Period of study	Shoot length (cm)			
	control	F5	F9	F19
10th day	2.5	8.5	9	7
15th day	4	22	15	12

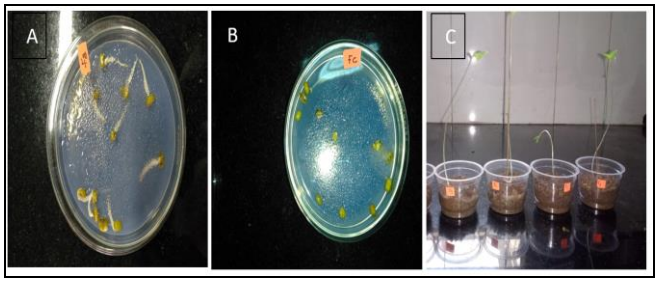

Fig 3: Seed germination assay and Pot experiment; (A) treated seedling, (B) untreated seedling, (C) Pot experiment.

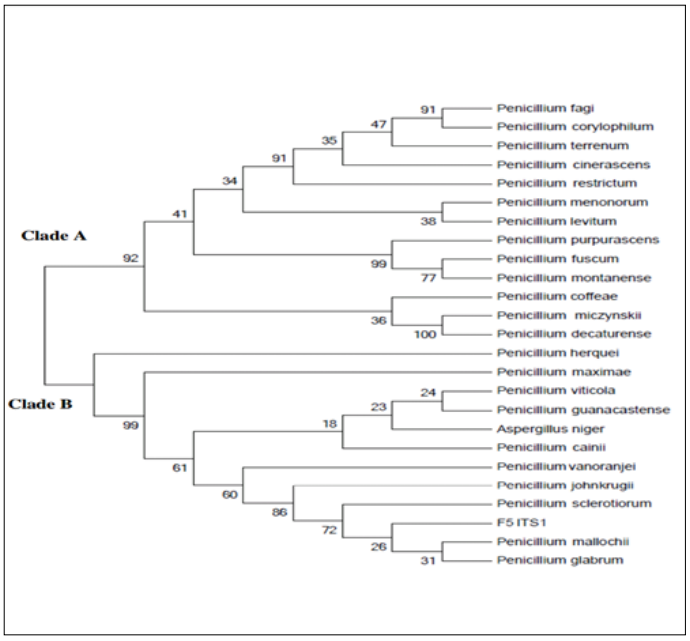

Fig 4: Phylogenetic tree of F5

5. Conclusion

It can be concluded from the above discussion that Plant growth promoting fungi can be used for crop growth and improvement. In the same context, present study was focused for the isolation and characterization of PGPF and its field applications. Enzyme production assays such as catalase, amylase, protease, cellulase and lipase production was considered for the present study. Isolate with good enzyme production was characterized and it was identified as *Penicillium* species. This was capable of lipase production in large quantity. It will help in the evelopment of lipase technologies for the synthesis of novel compounds and will also result in their expansion into new areas, and a major impact on a range of industries. The results are promising for design of potentially active plant growth promoting and lipase producing PGPF strain based formulation which would be beneficial for crop improvement.

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