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Isolation, Morphological characterization and Molecular identification of Endophytic fungi isolated from Mangroves.

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Abstract

Mangroves are salt-tolerant plants of tropical and subtropical intertidal regions of the world. Pichavaram has a well-developed mangrove forest with *Rhizophora* sp, *Avicennia marina, Exocaria agallocha, Bruguiera cylindrica, Lumnitzera racemosa, Ceriops decandra* and *Aegiceras corniculatum* as the dominant flora. Microbes that inhabit plant tissues for their life without causing any harm to their host plant are endophytes. Endophytes lives symbiotically within the plants. The aim of this study was to confirm the ability of endophytic fungi in production of extracellular enzymes with economic importance. In the present study, 32 strains of endophytic fungi were isolated from mangrove plants viz. *Rhizophora annamalaiana, Rhizophora appiculata, Suaedo monoica, Ceriops decandra* and *Rhizophora mucarneta*. Enzymes from microbial sources are more stable, economical and are of great significance in food, textile and paper industries. The isolated fungi were investigated for production of the above mentioned industrially important enzymes. The fungal strains produced various extracellular enzyme of industrial importance viz. Amylase, Gelatinase, Cellulase, Protease, Catalase, Pectinase, Chitinase and Laccase. Based on the enzyme activity growth medium was standardized. Effect of pH, temperature on the enhanced enzyme production was also tested.

Keywords

Amylase, cellulase, casein degrading, protease.

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INTRODUCTION

Microorganisms dwelling inside plants are known as endophytes [**1].** Endophytes, mostly fungi and bacteria inhabit plant hosts during either a part or whole of the life cycle. Endophytes form inconspicuous infections in healthy plants and appear symptomless, and become parasitic when the hosts are stressed **[2].** Endophytic fungi are an ecological, polyphyletic group of highly diverse fungi, mostly belonging to ascomycetes and anamorphic fungi **[3].** Endophytic fungi are known to be a rich source of bioactive compounds like therapeutics and enzymes. Fungi are one of the important microbial components of the plants. Fungi make a very important part of the ecosystem along with other microbes **[4, 5].** Fungi growing on mangroves from

different parts of the world have been reported **[6]**. Enzymes of microbial origin have high biotechnology importance in the processing of foods, manufacturing of detergents, textiles, pharmaceutical products, medical therapy and in molecular biology **[7]**. Endophytic fungi occupy a relatively unexplored area in microorganism isolation and thus represent a new source for obtaining enzymes with different potentialities **[8].** Endophytes are rich sources of bioactive metabolites, which have important potentials in medicine, agriculture, foods and manufacturing detergents. Therefore, it is necessary to find microorganisms that produce enzymes for specific substrate, with different requirements in temperature range, pH and presence of different ions, for different production processes **[9].** The aim of this study was to isolate endophytic fungi from *Rhizophora appiculata, Rhizophora annamalayana, Suaedo monoica, Rhizophora mucronata, Ceriops decandra* and the produce of enzymes such as amylase, gelatinase, cellulase, protease, catalase, pectinase, chitinase and laccase.

MATERIALS AND METHODS

Chemicals

All chemicals used for the study were of AR grade, it was procured from Merck Ltd.,
Hi Media Laboratories Pvt Ltd.

Collection of plant samples

Samples were collected from mangrove forest situated in Pichavaram, TamilNadu, India. Healthy leaves of *Rhizophora annamalaiana, Rhizophora appiculata, Suaedo monoica, Ceriops decandra* and *Rhizophora mucarneta* were collected in sterile bags and transported to the laboratory aseptically.

Surface- sterilization and isolation of endophytic fungi

Isolation of endophytic fungi from *R.appiculata, R.annamalayana, S.monoica, R.mucronata and C.decandra* were carried out using the protocol of Sunitha et al., 2013 **[10]**. Briefly, these samples were cut into small pieces of 1 cm^2 in size and washed under running tap water for 10 min. It was sterilized in series with 70% ethanol for 1 min, 1.0% sodium hypochlorite for 10 min and further cleaned by passing through two sets of sterile distilled water. The sterilized bits were carefully laid on the Potato Dextrose Agar (PDA) medium plates and incubated for 5 days at 25± 2˚C, for further studies the fungal cultures were sub cultured, and pure culture was maintained in PDA slants.

Morphological analysis

The fungal isolates were identified by studying the morphology of the fungal culture on the upper and lower surface of the culture plate.

Microscopical analysis

Isolated endophytic fungi were stained using lactophenol cotton blue staining procedure and visualized under microscope at 40X magnification. The fungi were identified on the basis of mycelia and spore characteristics.

Molecular taxonomy of the potent isolates

PCR amplifications of the 28s rRNA gene were performed for the isolates and was compared with other fungal sequences by using NCBI.

Primer details

LR7 5' TAC TAC CAC CAA GAT CT 3' 17
LROR 5' ACC CGC TGA ACT TAA GC 3' 17 was used as primer for the given study

Sequencing protocol

Single-pass sequencing was performed on each template using 28s rRNA universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

Bioinformatics protocol:

The sequence was blast using NCBI blast similarity search tool. The phylogeny analysis of query sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment. The program MUSCLE 3.7 was used for multiple alignments of sequences **[11].** The resulting aligned sequences were cured using the program Gblocks 0. 91b.This Gblocks eliminates poorly aligned positions and divergent regions (removes alignment noise) **[12]**. Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as Substitution model. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. The program Tree Dyn 198.3 was used for tree rendering **[13].**

SEM ANALYSIS

Fungi were also subjected for scanning electron microscope (SEM) Ho et al., 1999 **[14].** The specimens first fixed with 2.5% glutaraldehyde in phosphate buffer (PBS, Ph 6.8) was carried out , followed a graded series of ethanol (50,70,80,90 and 95%,100) for about 10 mts at each step of ethanol for every 10 mts and followed by 100% ethanol for 20 mts and then freeze dried and coated with gold – palladium and were examined with a scanning electron microscope at different magnification under a SEM (Jeol, JSM-6390LV,Japan) at an accelerating voltage of 5Kv.

ENZYME PRODUCTION

QUALITATIVE METHOD

Qualitative screening was done using agar plate method. Procedure followed for the qualitative estimation Amylase, Gelatinase, Cellulase, Protease, Catalase, Pectinase, Chitinase, and Laccase are given below**.**

Amylase activity

Amylase activity was carried out using the protocol of Maria et al., 2005 [**15].** Amylase activity was assessed by growing the fungi on 2% Agar with 1% soluble starch. After incubation, the plates were flooded with 1% iodine in 2% potassium iodide. The clear zone against the purple blue background of the plate formed surrounding the colony was considered positive for amylase activity.

Gelatinase activity

Gelatinase activity was carried out using the protocol of Maria et al., 2005 **[15]**. Gelatinase activity was performed by growing the fungi on 1% Nutrient Gelatin. After incubation, the plates were flooded with 1% iodine in 2% potassium iodide. The clear zone formed surrounding the colony was considered positive for gelatinase activity.

Cellulase activity

Cellulase activity was carried out using the protocol of Maria et al., 2005 **[15]**. For cellulase activity, the fungi were grown on PDA medium supplemented with 0.5% with Sodium carboxymethyl cellulose (CMC). After incubation, the plates were flooded with 0.2% aqueous Congo red and destined with 1M NaCl for 15 minutes. The clear zone formed surrounding the colony was considered positive for cellulose activity.

Protease activity

Protease activity was carried out using the protocol of Sahoo et al., 2014 **[16].** Protease activity was performed by growing the fungi on PDA medium amended with 0.4% gelatin. After five days of incubation, plates were flooded with saturated aqueous ammonium sulphate. The undigested gelatin will precipitate with ammonium sulphate and digested area around the fungal colony would appear as clear zone.

Catalase activity

Catalase activity was carried out using the protocol of Andro et al., 1984 **[17].** Catalase activity, the fungi were cultured on PDA medium. After incubation, transfer a small amount of fungal colony to a surface of clean, dry glass slide using a loop or sterile wooden stick. Place a drop of 3% H_2O_2 to the slide and mix. A positive result is the rapid evolution of oxygen (within 5-10 sec) as evidenced by bubbling. A negative result is no bubbles or only a few scattered bubbles.

Pectinase activity

Pectinase activity was carried out using the protocol of Rahul Yadav et al., 2015 **[18].** The fungi were cultured on PDA medium. After the incubation, the plates were flooded with 1% aqueous solution of hexadecyl trimethyl ammonium and a clear zone formed surrounding the fungal colony was considered positive for pectinase activity.

Keratinase activity

Keratinase activity was carried out using the protocol of Viera, J.D.G, 1999 **[19].** Keratinase activity, PDB medium with feathers were autoclaved, the test tubes were inoculated and incubated at 26˚C. After incubation, the fungus was growing on the medium and when fungi broken the feathers that is positive for keratinase activity.

Chitinase activity

Chitinase activity was carried out using the protocol of Rahul Yadav et al., 2015 **[18].** Chitinase activity, the fungi were cultured on PDA medium with 1g chitin the plates were inoculated and incubated at 26˚C up to 72 h. The clear zone formed surrounding the colony was considered positive for chitinase activity.

Laccase activity

Laccase activity was carried out using the protocol of Rahul Yadav et al., 2015 [18]. Laccase activity, the fungi were cultured on PDA medium amended with 0.005% 1-napthol (pH, 6). After incubation, the fungus was growing on the colorless medium turns blue due to oxidation of 1-napthol by laccase.

QUANTITATIVE METHOD

Amylase assay activity

Amylase activity was assayed by dinitro salicylic acid (DNS) method. In a test tube, the reaction mixture (containing 1 ml of soluble starch solution was mixed with 1 ml of 50mM potassium phosphate buffer, pH 6.9) was mixed with 0.1 ml of the crude enzyme sources and incubated for 15 mts at room temperature. After the incubation, 2 ml of the DNS

reagent was added and the reaction terminated by immersing the tube in a boiling water for10 mts. The same technique was simultaneously applied to the rest of the crude enzyme sources. The absorbance was read at 440 nm with starch as standard. The reducing sugars given off were estimated by the DNS method. One unit of enzyme activity has been defined as the amount of enzyme that hydrolyses 1 mg of starch /min under assay conditions. [20].

Cellulase assay activity

The cellulase was assayed using Dinitro salicylic acid DNS reagent by estimating the reducing sugars released from Carboxy methyl cellulose (CMC). 0.1ml of crude enzyme was mixed with 0.9 ml of Sodium acetate buffer (50mM, pH5.0).1ml of 0.1% CMC was also added and incubated at 50˚ C for 10min. After incubation, the reaction was heated by adding 1 ml of DNS reagent. This was followed by the incubation of the tube at 100˚C for 10 min in a water bath was read the absorbance at 660 nm with glucose as standard. The cellulose activity was determined by using calibration curve for glucose. One unit of enzyme activity was defined as the amount of enzyme that released 1 mμ mole of glucose/minutes. [**20].**

Protease assay

Protease activity was measured by the methods of Lowry et al.,1951[**21]** used to different reagent A-NaOH 100ml water dissolved and Na_2CO_3 dissolved NaOH, B-$CuSO_4$ in dissolved 100ml 1% sodium or potassium tartrate mixed A and B solution this complex reagent. 0.1ml of sample added 0.1ml of 2N NaOH incubate water bath at 100˚ C in 10 mts. After 10 mts added 1 ml of complex reagent incubated in10mts at room temperature.0.1 ml of foline reagent were added to each test tubes and incubated 30 mts at room temperature. The absorbance at 660 nm. The standard curve was generated using solution 25 mg of Bovine serum Albumin.

PH

The effect of pH for amylase, cellulase and protease production was determined by culturing the fungi in the production media with different pH. The experiment was carried out individually at various pH 1,3,5,7,9,11 and 13. **[22].**

TEMPERATURE

Temperature is an important role for the production of amylase, cellulase and protease. The effect of temperature on amylase, cellulase, protease production was studied by the incubating the culture media at various temperature -20˚, 20˚, 40˚, 60˚, 80˚, 100˚ C. **[22].**

PROTEASE

ACID, ALKALINE AND NEUTRAL PROTEASE

Assay of Acid protease

The acid protease activity in crude enzyme extract was assayed according to the modified method of Srividya shivakumar 2012 **[23]**. Using BSA as substrate, the reaction mixture containing 0.5ml of enzyme solution and 0.5ml of 1 % (w/v) BSA in 0.2M Phosphate buffer was incubated at 30°C for 10mts. By adding 1ml of 10% Trichloroacetic acid containing 0.22M acetic acid and 0.33M sodium acetate and the enzyme reaction was stopped. The reaction mixture was allowed to stand for 30 mts at 30°C and then was filtered. Take 2 ml of the filtrate and add 5ml of 0.55M sodium carbonate, followed by the addition of 1ml (3 times diluted) phenol reagent. The blue colour was measured at 660nm by using a spectrophotometer. One unit of activity was defined as the amount of enzyme producing a change of absorbency equivalent to 1μg of tyrosine per min, under the above conditions.

Assay of Neutral protease

The Neutral protease activity in crude enzyme extract was assayed according to the modified method of Srividya shivakumar 2012 **[23]**, using Casein as substrate, the reaction mixture containing 0.2ml of enzyme solution and 0.5ml of 1% (w/v) Casein in 50mM Phosphate buffer was incubated at 40°C for 20mts. Except where specified, enzyme reaction was carried out at pH7.0. The enzyme reaction was stopped by adding 1ml of 10% Trichloroacetic acid. The reaction mixture was allowed to stand for 15 mts at Room temperature. After centrifugation (10,000 rpm 5 mts) and 2.5ml of 0.4M sodium carbonate was added, followed by the addition of 1ml of 3 times diluted phenol reagent and incubated at room temperature in dark 30 mts. The blue colour was measured at 660nm by using a spectrophotometer. One unit of activity was defined as the amount of enzyme producing a change of absorbency equivalent to 1μg of tyrosine per min, under the above conditions.

Assay of Alkaline protease

The alkaline protease activity in crude enzyme extract was assayed according to the modified method of Srividya shivakumar 2012 **[23]**. Using casein as substrate, the reaction mixture containing 1ml of enzyme solution and 1ml of 0.5% (w/v) casein in 0.2M Tris-HCl buffer was incubated at 75°C for 1hours. Except where specified, enzyme reaction was carried out at pH7.4. The enzyme reaction was stopped by adding 2ml of 10% Trichloroacetic acid. Reaction mixture was allowed to vortex mixer after centrifuged at 3000rpm for 10 mts, Supernatant was

measured at 440nm by using a spectrophotometer. One unit of activity was defined as the amount of enzyme producing a change of absorbency equivalent to 1µg of tyrosine per min, under the above conditions.

RESULTS AND DISCUSSION

Sample Collection, Isolation of endophytic fungi

Endophytic fungus in mangrove plant leaves of *R.appiculata, R.annamalayana, S.monoica, R.mucronata and C.decandra* was isolated to evaluate their capacity to produce extracellular enzyme. A total of 32 endophytic fungi were isolated from leaves of *R.appiculata, R.annamalayana, S.monoica, R.mucronata and C.decandra*. Each isolate was sub cultured in PDA Medium and stored at 4˚C for further studies. The shape, structure and type of colony were analyzed on different types of medium and further the lacto phenol staining was performed.

MOPRHOLOGY OF COLLECTED PLANTS

1. *Rhizophora appiculata:* Simple, opposite, narrowly elliptical, leathery midrib on lower part is pink, leaf tip acute, cuneate at base.

2. *Rhizophora annamalayana*: Simple, opposite, broadly obviate, darkish green, leathery.

3. *Suaeda monoica:* Alternately crowded, linear-oblong or spathulate, flat or sub terete, obtuse or rounded at tip, narrowed at base, black on drying.

4. *Rhizophora mucronata*: Simple, opposite, broadly elliptical, tooth- like leaf tip, cuneate at base, leathery, leaf lower surface yellowish green, black dots scattered.

5. *Ceriops decandra*: Simple, opposite, obovate, leaf tip rounded, uneate at base

Identification

Morphological and microscopic analysis **(**Table1)

Molecular identification of the potent most productive isolate

The most productive fungal isolates namely, BWC F-9, F-13, F-25, and F31 was identified using molecular technique. (Table2 and fig 1). The isolates were deposited in NCBI. The accession numbers are BWCF9- EJNCKXNN014, BWCF13- EJNMH4UW015, BWCF25- A4CSDPF801R and BWC F31-EJP8607301R.

SEM STUDY

SEM analysis were identifying fungi spore morphology under the Electron microscope HV: 20KV MAG: 5.00 KX 5µm VEGA3 TESCAN,

Penicillium brevicompactum

Penicillium brevicompactum is a mould species. Conidiophores stipes smooth walled, 500-800µm long. Conidia smooth walled or slightly Verruculose, ellipsoidal.

Warcupiella spinulosa

Hyphal ascomata without stromata. Stromata with lacking, cleistothecia hyphal, interwoven white hyphae, ascospores hyaline. The conidiophore is septate apically, broad.

Scedosporium apiospermum

The conidiophores are hyaline and long. Conidiogenous cells are annellides. Conidia unicellular and obovate, truncate, single or in balls.

Agaricus guizhouensis

It is a new species characterized by a white pileus with small ellipsoid spores.

QUALITATIVE ANALYSIS

Qualitative screening was done using agar plate method. Procedure followed for the qualitative estimate Amylase, Gelatinase, Cellulase, Protease, Catalase, Pectinase, Chitinase, Keratinase and Laccase is given below. In particular amylase is an important enzyme that is used in numerous applications in a variety of industries and there is growing interest in amylases with a wider spectrum of biological science. Out of 32 endophytic fungi 60% fungi isolates were able to degrade starch by amylase production, which is shown by significant area of clear zone around fungal mycelia growth. Microbial pectinases are important in the decomposition of dead plant material, degradation of host tissue by phytopathogens generally begins with the production of pectinolytic enzymes. In pectinase production, 50% fungi isolates were able to produce pectinase, which is shown by significant area of clear zone around fungal mycelia growth. All the endophytes tested showed cellulose activity similar to that of leaf inhabiting salt marsh fungi **[24]**. Gessner, 1979 **[25]** reported protease activity in 13 out of 14 marine fungi. Protease activity was seen in some endophytes of current study. Seventy percent of the isolates showed positive for Protease activity which is shown by significant area of clear zone around fungal mycelia growth. None of the fungi showed laccase although laccase activity is seen in many marine fungi **[26, 27].** The endophytic nature of these fungi might be the reason for the lack of laccase activity, since an active enzyme might damage the host plant. None of the chitinase activity and Keratinase activity showed in our study out of 32endophytic fungi. while Gelatinase and cellulase activity were found in 95% endophytes, which is shown by significant area of clear zone around fungal mycelia growth the cellulose production in mangrove plants **[28]**. Marine microorganisms are attracting increasing attention as a resource for new enzymes because the enzymes derived from marine microbes are relatively more stable and active than the others.

QUANTITATIVE ASSAY OF ENZYME ACTIVITY

Amylase activity

Penicillium brevicompactum, *Warcupiella spinulosa*, *Scedosporium apiospermum* and *Agaricus guizhouensis* the four fungal isolates were selected by starch hydrolysis can be produced detectable quantities of amylase activity. Among these isolates *Warcupiella spinulosa* showed maximum amylase production in liquid media. The use of starch nutrient agar and iodine for detecting amylase (hydrolytic enzyme) producing microorganisms have been reported by Iverson and Millis (1974); Forgarty and Kelly (1979) **[29, 30]** that starch hydrolysis can be detected. The work done by Kathiresan *et al.,* 2006 **[31]** and among fungal community *Aspergillus niger* were known as an efficient degrader of starch by producing amylolytic enzymes which proved that *Penicillium fellutanum* and *Chrysosporium tropicum* respectively can produce high yield of Amylase using wastes agro–industrial by products **[32, 33]**.

Cellulase activity *Penicillium brevicompactum, Warcupiella spinulosa, Scedosporium apiospermum* and *Agaricus guizhouens* are the four fungal isolates selected on basis of their cellulase producing capacity. Among these isolates *Scedosporium apiospermum* showed maximum cellulase production in liquid media. Many microorganisms have been reported with cellulosic activities including many bacterial and fungal strains both aerobic and anaerobic.

Protease activity *Penicillium brevicompactum, Warcupiella spinulosa, Scedosporium apiospermum and Agaricus guizhouensis* the four fungal isolates were selected by protein can be produced detectable quantities of protease activity. Among these isolates *Warcupiella spinulosa* showed maximum protease production in liquid media. Fungal proteases are widely used in industry; however, they demand heavy investments in filtration methodology in order to clear the microorganisms from enzymatic preparations. Proteases are a highly complex group of enzymes that differ in their substrate specificity, catalytic mechanism and active site **[34]**. Acid proteases offer a variety of applications in the food and beverage industry and medicine. The worldwide requirement for these enzymes with specific application is increasing rapidly. Although several reports of acid protease from fungal sources have appeared in the recent past, the enzymes have low reaction rates and are not well characterized. With the advent of modern biotechnological tools, it is possible to produce these enzymes in bulk quantities for use as crude preparations in food industries. High enzyme production in protease into the comparation of Amylase and Cellulose activity. Further analysis is done on protease activity (**acid, neutral and alkaline protease**). In compared with Acid, Neutral and Alkaline protease, **acid protease** is highly produced. (Figure 2and 3)

Effect of pH of the culture media on Amylase, Cellulase and Protease activity

pH is very important factors that will help in determining the growth and morphological structure of the microorganism as they are sensitive to the concentration of hydrogen ions present in the medium. This pH is known to affect the secretion of Amylase, Cellulase and Protease. The pH was selected for optimization was 1, 3, 5, 7, 9, 11 and 13. The pH 5 showed the optimum growth and production. This effect is shown in (**fig 4, 5, 6).** These results confirmed the optimum protease activity of endophytic fungus in these media is at pH5. As reported Kamath p et al., 2010 **[35]** the organism produced maximum protease at pH 4. According to Mohanasrinivasan V. et al., 2012 **[36]** it was found that maximum protease enzyme production was at pH 9 after 96 hours of incubation and 0.05% inducer concentration. pH had a major effect on the morphology of the organism. *E. coli, Pseudomonas fluorescens, Bacillus subtillis and Serratia marscens* all the four bacteria showed the maximum amylase enzyme activity was measured in medium of pH 7.0 Shyam Sunder Alariya et al., 2013 **[22].** Maria et al., 2005 **[14]** reported that maximum enzyme activity of amylase production was found in pH 5. However, the broad range of its protease activity might be useful for different industrial and medicinal applications **[37].** The results of the current study showed that the endophytic fungi of *Penicillium brevicompactum, Warcupiella spinulosa, Scedosporium apiospermum* and *Agaricus guizhouens* produced Amylase, Cellulase and Proteases in a wide range of pH (1-13). However, the enzyme activity was observed in medium of pH 5 in Amylase, and cellulase and pH 13 protease activity in case of the four isolated organisms. Agrawal et al., 2016 **[38]** reported that the pH 7 showed the optimum growth and production of protease. (fig 4, 5, 6)

TEMPERATURE

Amylase production was maximum at Room temperature, Cellulase was effective at 100˚C and Protease was effective in almost all temperature. Even at 100˚C proteases were active**.** Kamath et al., (2010) **[35]** reported that the maximum proteolytic activity was found at 28˚ C at 120 hrs. Shyam Sunder Alariya et al., 2013 **[22]** reported that *E.coli, Pseudomonas fluorescens, Bacillus subtillis and Serratia marscens* all the four bacteria yielded

maximum amylase production at 35-40° C. Agrawal et al., (2016) **[38]** reported that the optimum incubation temperature for protease production at 27° C (fig 7, 8, 9).

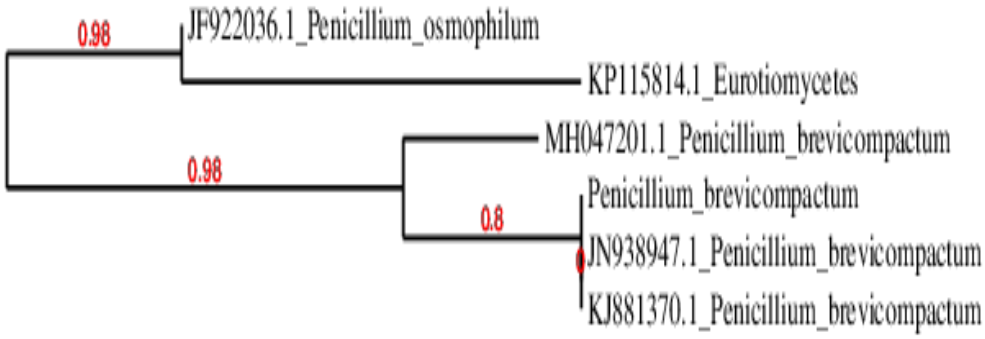
Table 1: Morphological Characteristics of endophytic fungi

S.N	Identified strains	Colony morphology on PDA Medium
1.	*Penicillium brevicompactum*	Dull grey with white margin and black dots /black and brown with white margin.
2.	*Warcupiella spinulosa*	Brown powdery.
3.	*Scedosporium apiospermum*	Center white puffy and golden yellow with grey margin and Golden Yellow water Dots /Brown with Black
4.	*Agaricus guizhouensis*	Many Cluster of Black colony

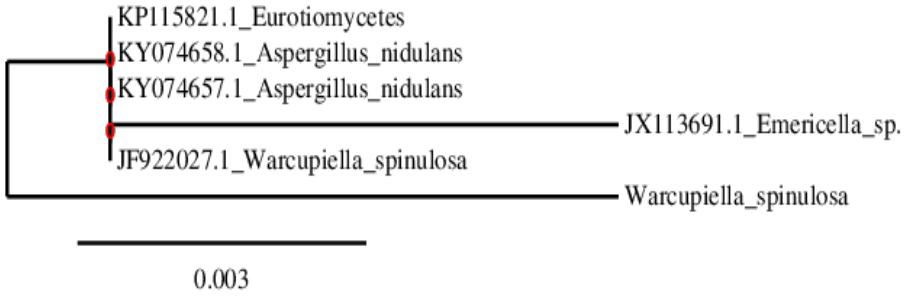
Table 2: Identification of fungal isolates

S.N	Given names	Identified strains
1.	BWC F9	*Penicillium brevicompactum*
2.	BWC F13	*Warcupiella spinulosa*
3.	BWC F25	*Scedosporium apiospermum*
4.	BWC F31	*Agaricus guizhouensis*

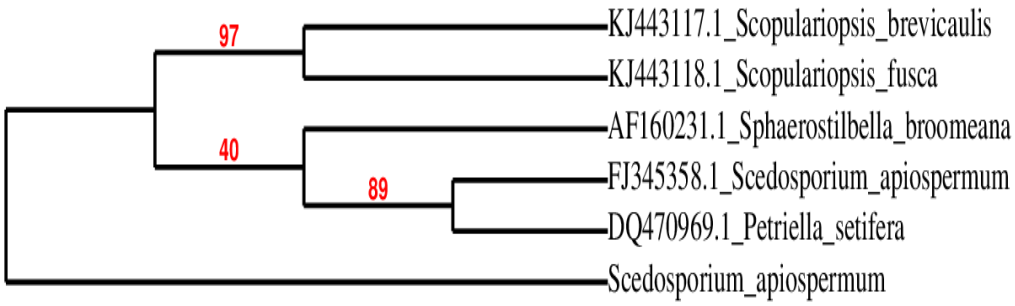
phylotree structure of *Penicillium brevicompactum*



phylotree structure of *Warcupiella spinulosa*



phylotree structure of *Scedosporium apiospermum*



Phylotree structure of *Agaricus guizhouensis*

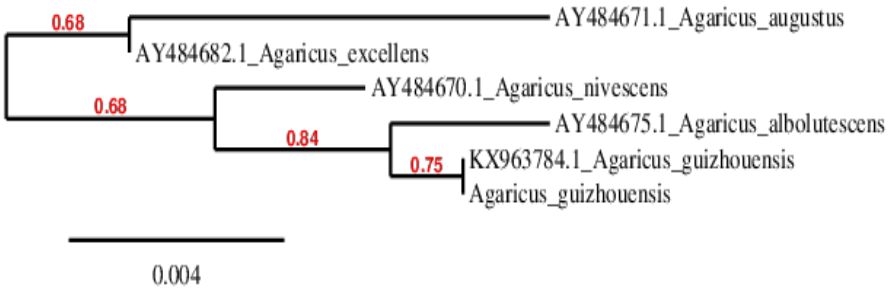


Fig 1: Phylotree structure of fungal strains. (a) *Penicillium brevicompactum,* (b) *Warcupiella spinulosa*, (c) *Scedosporium apiospermum* (d) *Agaricus guizhouensis*.

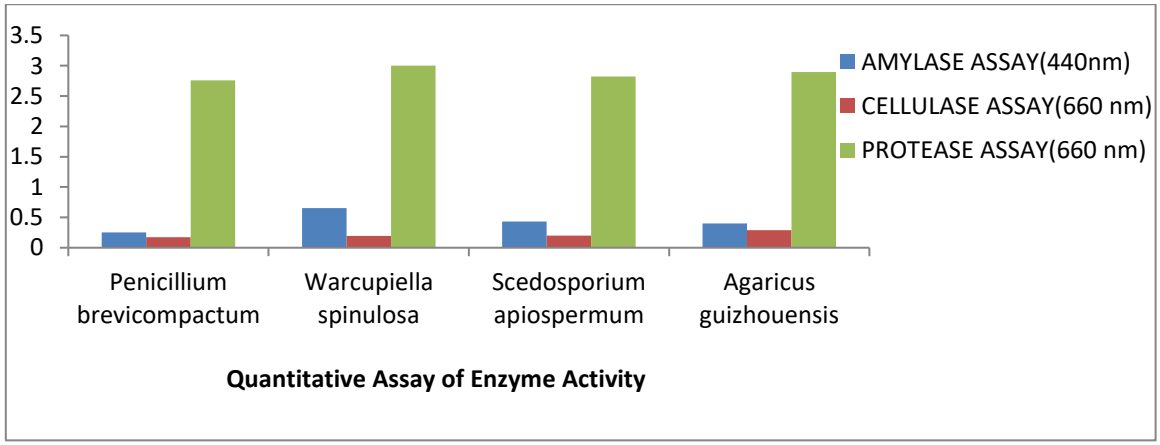


Fig 2: Quantitative Assay of Enzyme Activity

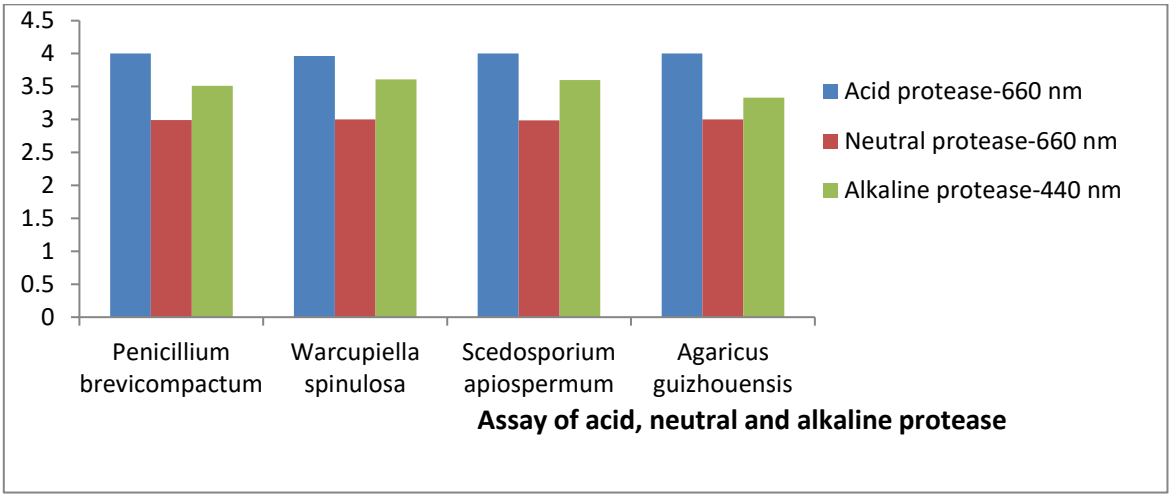


Fig3: Assay of acid, neutral and alkaline protease

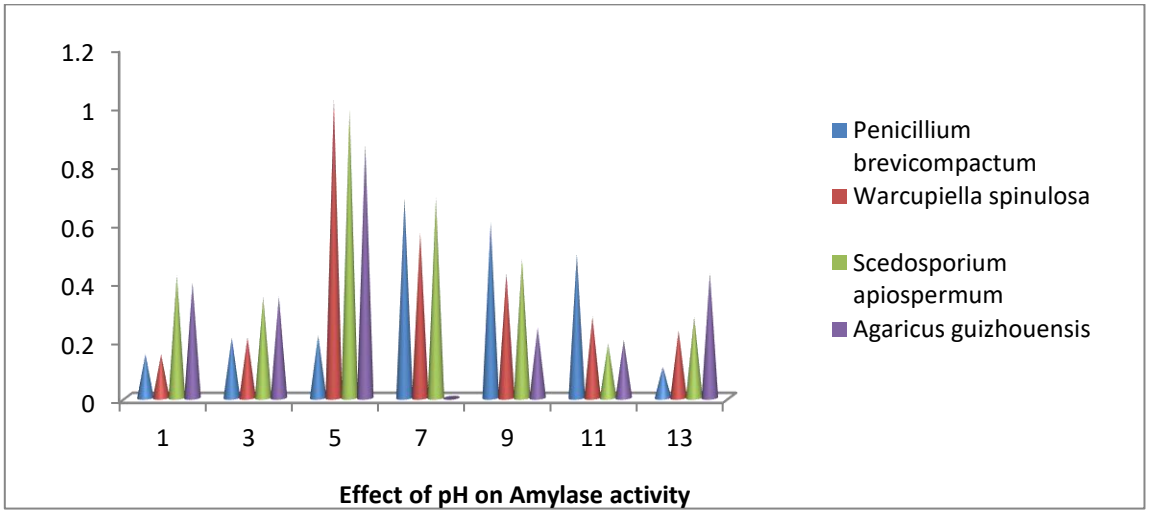


Fig 4: Effect of pH on Amylase activity

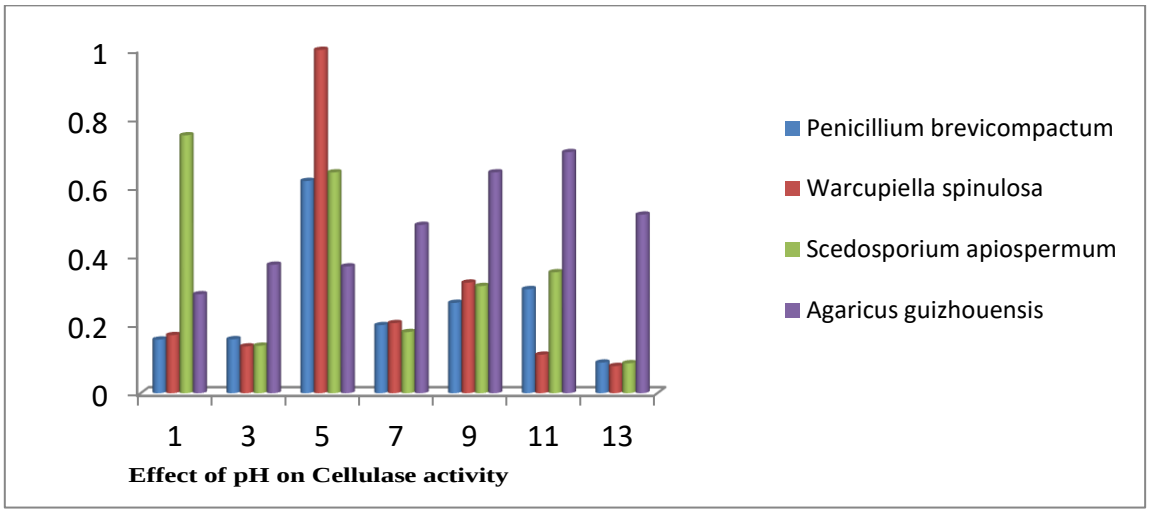


Fig 5: Effect of pH on Cellulase activity

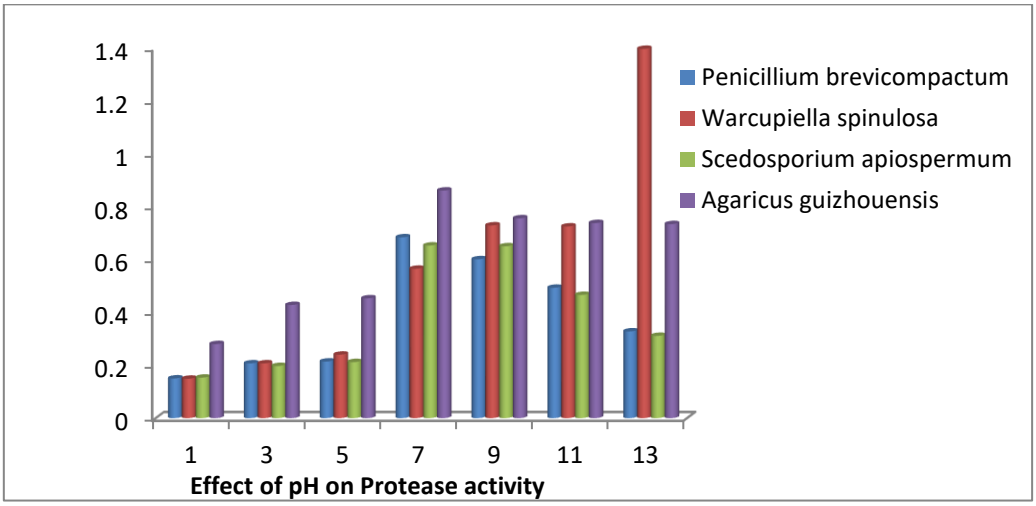


Fig 6: Effect of pH on Protease activity

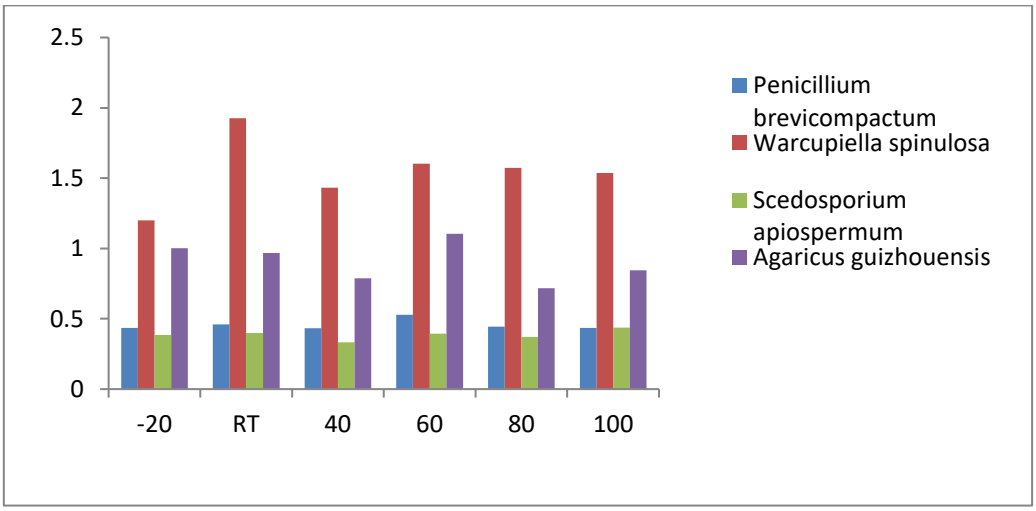


Fig 7: Effect of Temperature on Amylase activity

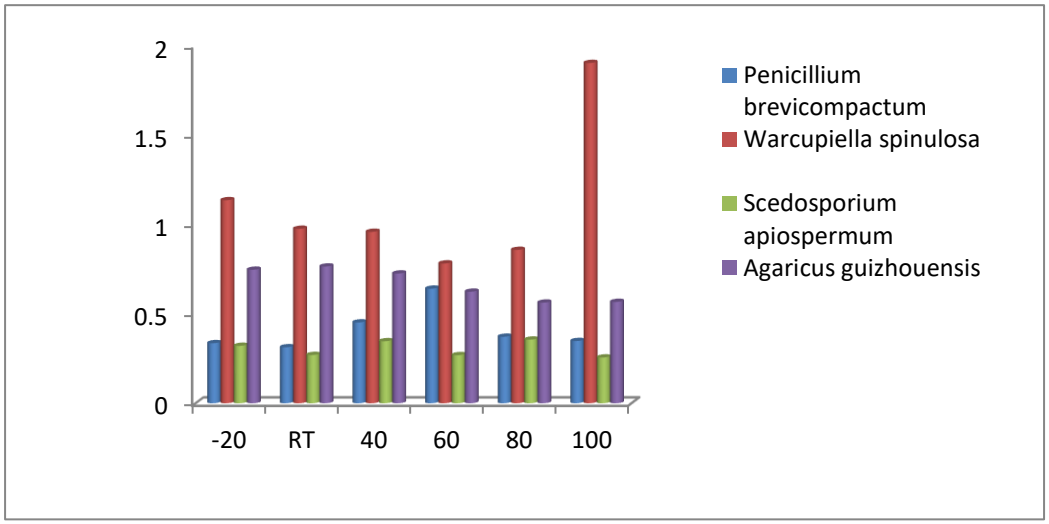


Fig 8: Effect of Temperature on Cellulase activity

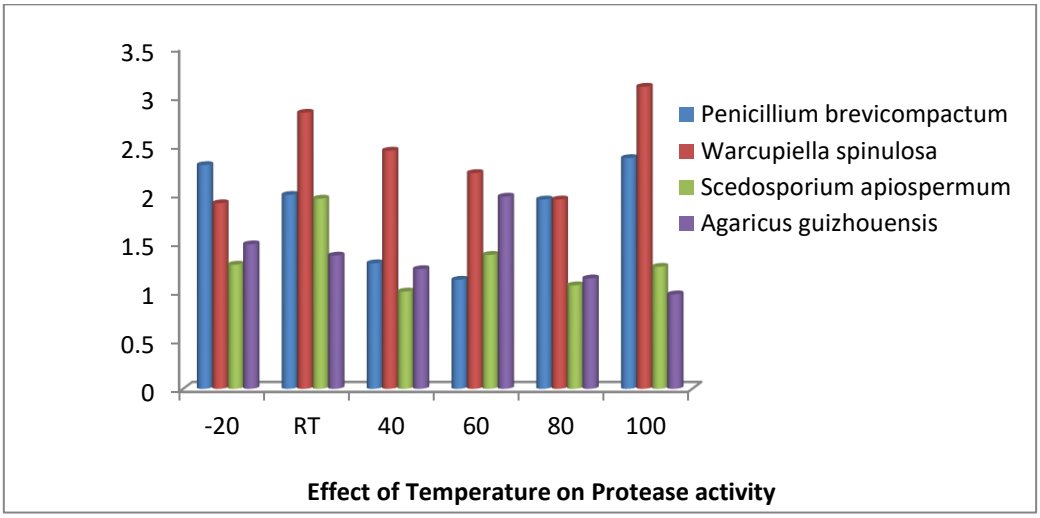


Fig 9: Effect of Temperature on Protease activity

CONCLUSION

Out of the 32 isolates, four strains were found to possess more activity after screening and were observed under microscope by using molecular identification method *Penicillium brevicompactum*, *Warcupiella spinulosa*, *Scedosporium apiospermum* and *Agaricus guizhouensis*. 32 fungal strains of endophytic fungi were isolated from mangrove plants and 4 best strains were shortlisted for further analysis. Nine types of industrially important enzymes were screened, top three enzymes produced by fungal strains were amylase, cellulose and protease. Optimization of growth media and culture conditions studies were carried out of the five carbon sources dextrose was instrumental in enhancing the enzyme production in all the three enzymes. Amylase and Cellulase was effective at pH 5, protease pH 13. Temperature's effect varied with the enzyme types, Amylase Room temperature, Cellulase was effect at 100˚C and Protease was effective in almost all temperature. Even at 100˚C proteases were active.

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