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# Evaluation of Antifungal and Enzymatic Potential of Endophytic Fungi Isolated from *Cupressus torulosa* D. Don

**Mohd Mahfooz[1], Sushmita Dwedi[1], Arun Bhatt[1], Sharad Raghuvanshi[1], Manoj Bhatt[2] and Pavan Kumar Agrawal[1*]**

[1]Department of Biotechnology, G.B. Pant Engineering College, Ghurdauri, Pauri, Garhwal, Uttarakhand, India
[2]Guru Gobind Singh Indraprastha University, Delhi, India
**Corresponding author*

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**A B S T R A C T**

Today, endophytic fungi have become new sources of industrially useful enzymes such as cellulases, chitinases, lipases, amylases and proteases. When endophytes reside on the plant surface, they produce enzymes to hydrolyze plant cell walls. Therefore, these enzymes can also suppress plant pathogen activities directly and are capable of degrading the cell walls of fungi. In dual plate assay fungal endophytes inhibited *Fusarium solani*; few isolates were inhibitory towards *Ganoderma lividense* and *Colletotrichum demati*. Microbial enzymes are also biotechnologically important products employed in biotechnology, agriculture, and the pharmaceutical, photographic and food industries.

## Introduction

The term endophyte is applied to fungi or bacteria which live within plant tissues, for all or part of their life cycle and causes no apparent infections (Wilson, 2000). Like other organisms invading plant tissues, endophytic fungi produce extracellular hydrolases as a resistance mechanism against pathogenic invasion and to obtain nutrition from host. Such enzymes include pectinases, cellulases, lipases, laccase from the endophytic fungus *Monotospora* sp., xylanase, -1, 4-glucanlyase, phosphotases and proteinase (Sunitha *et al.,* 2013). Hydrolytic enzymes (amylase, proteases, cellulases, chitansae and laccase) with various industrial applications are also of major interest.

Biological control through microorganisms which inhibit or antagonize plant pathogens and pests reduces or eliminates the use of chemical products. Fungal endophytes are effective antagonists (Azevedo *et al.,* 2000) and constitute a taxonomically and metabolically diverse group of organisms that colonize internal plant tissues without causing apparent harm to the host plant. Indeed, endophyte-mediated biological control has been investigated both *in vivo* and *in vitro*

through screening experiments to verify the activity of endophytes against phytopathogens and pests (Flores *et al.,* 2013).

Endophytic and phytopathogenic fungi compete and interact within the same ecological niche through the action of hydrolytic enzymes such as proteases and chitinases, which degrade the hyphal cell walls of pathogenic microorganisms (Almeida *et al.,* 2007). This enzymatic activity is closely associated with the fungus-host specificity. To facilitate the entry of endophytes into host tissues through natural or artificial openings, hydrolytic enzymes including pectinases, cellulases and lipases are secreted (Polizeli *et al.,* 1991).

It has been observed that some strains of bacteria and fungi shows beneficial effects on plants and this type of evaluation is based on better emergence of seedling's fast growth. The mechanism of plant growth promotion include, i] solubilization of insoluble phosphates (ii) ability to produce phytoharmone auxin iii) production of HCN iv) siderophore production. The Plant growth promoting activity of microorganism showed beneficial association with the plants by increasing uptake of N, P and Fe (Kloepper *et al.,* 2009). There are many reports on the use of micro-organisms as biocontrol agents as an alternate to agricultural chemical fungicides (Kaur *et al.,* 2013).

In present investigation, considering the shortage of information concerning the antifungal and enzymatic activities of the endophytes from this plant, the aim of the present study was to evaluate the antagonism interactions between endophytic fungi and phytopathogenic fungi in dual culture experiments and to detect the extracellular enzyme activity of these fungal endophytes isolated from *Cupressus torulosa* D. Don using a plate assay and different growth substrates.

## Materials and Methods

### Isolation of endophytic fungi

The sampling procedure was designed with the intention of isolating as many endophytic fungal species as possible from the different tissues samples. Tissues of the leaves of *Cupressus torulosa* D. Don were cut into 5 mm long segments. Surface sterilization followed the method of (Arnold *et al.,* 2007) with minor modification.

Segments were surface sterilized by consecutive immersion for 1 min in 75 % Ethanol, treated for 1 min in 0.1 % mercuric chloride, followed by several washing in sterile distilled water (Sharma *et al.,* 2016). The time of the dilution and immersion in ethanol and Mercuric chloride varies with tissues and host (At least three washing require).

Under sterile conditions, tissue segments were allowed to surface-dry before plating. Five segments were then evenly placed in each 90 mm Petri dish containing Potato dextrose agar and water agar Medium. The dishes were sealed with parafilm and incubated at 27ºC ± 2ºC for 2-4 weeks in incubator.

Fungal growth was observed from the tissues segments inoculated on the different media plates as colored cottony outgrowth. Hyphal tips, from germinating fungi, were isolated, sub cultured onto PDA and brought into pure culture by incubating at 28ºC for 5-7 days.

The purified fungal isolates were maintained at 4ºC. The cultures were also submitted to the Gujarat State Biotechnology Mission microbial repository for preservation.

## Preservation of endophytic fungi cultures

### PDA slants

The PDA slants were inoculated with hyphal tips taken from the PDA plates. The slants were incubated at 28ºC for 5-7 days to observe the growth of pure culture of fungal endophyte. This was followed by refrigeration of the slants at 4ºC.

### 20% glycerol

Mycelial agar plugs were placed in sterile cryovials containing PDB with 20% glycerol under sterile laminar conditions and stored at -80°C.

## Identification of endophytic fungi

Fungal growth and sporulation was facilitated by placing the isolates onto PDA culture medium. The plates were continuously monitored for spore formation. Isolates were identified on the basis of cultural characteristics, colour and morphology of fruiting bodies and spores. Fungal isolates were stained with lactophenole cotton blue and examined under light microscope (Olympus, USA).

## Metabolic activity of fungal endophytes

### Screening for secretion of amylase

To test for the production of amylase, endophytes were inoculated on glucose yeast extract peptone (GYP) agar medium (glucose 1g, yeast extract 0.1g, peptone 0.5g, agar 15 g, and distilled water 1000 mL, pH 6) containing 1% soluble starch. After incubating for 5 days, 1% iodine in 2% potassium iodide was flooded in the fungal colony plates. A clear zone appearance surrounding the colony was considered positive for amylase enzyme (Bhardwaj *et al.*, 2015).

### Screening for secretion of protease

Protease assay was performed by growing the fungi on GYP media amended with 1% skim milk and pH was adjusted to 6.5, after 5 days incubation the clear zone appeared around the fungal colony indicated the presence of protease enzyme.

### Screening for secretion of lipase

For lipase activity measurement, the fungus were grown on Peptone Agar Medium (Peptone 10 g, NaCl 5 g, Agar 16 g, Distilled water: 1000ml and pH 6) supplemented with 1% w/v tween 20 (separately sterilized). A clear zone around the colony indicated the presence of lipase enzyme.

### Screening for secretion of cellulase

For Cellulase test, GYP Agar media was used as the medium to grow the fungus. To GYP agar media, 1% CMC (carboxy methyl cellulose) was added which act as a substrate. After 4 days of incubation at 27°C, the plates were flooded with 0.2% of aqueous Congo red solution for 20 minutes and distained with 1 M NaCl for 15 min. Appearance of yellow areas around the fungal colony indicates the presence of cellulase activity.

### Screening for secretion of chitinase

The final chitinase detection medium consisted of a basal medium comprising (all amounts are per litre) 4.5 g of colloidal chitin, 0.3 g of $MgSO_4.7H_2O$, 3 g of $(NH_4)$ $SO_4$, 2 g of $KH_2PO_4$, 1 g of citric acid monohydrate, 15 g of agar, 0.15g of bromocresol purple and 200 µl of tween-80, pH was adjusted to 4.7 and then autoclaved at 121°C for 15 min. After cooling the media was poured in to petri plates and allowed to solidify. The fresh culture plugs of the isolates to be tested for chitinase activity was inoculated into the

medium and incubated at 27°C for 2-3 d and observed for colored zone formation. Chitinase activity was identified due to the formation purple colored zone.

## Plant growth promoting attribute of fungal endophytes

### Phosphate solubilising activity

The fungal cultures were inoculated on centre of Pikovskaya's medium under aseptic. Inoculated plates were incubated for 7-10 days at 27°C. The clear zone around the colony showed positive phosphate solubilisation ability (Agrawal and Agrawal, 2013).

### Production of ammonia

Fungal isolates were tested for the production of ammonia peptone water is used. In each tube of 10ml peptone water freshly grown cultures were inoculated and incubated for 48–72 h at 27°C.

Nessler's reagent 0.5 ml of was added in each tube. Development of brown to yellow colour indicate positive test for ammonia production.

### Production of HCN

For HCN production, fungal isolates were screen for the production of hydrogen cyanide by adapting the method of Lorck (1948).

Briefly nutrient broth was amended with 4.4g glycine/l and fungal streaked on modified agar plate. Then a Whatman filter paper No.1 soaked in 0.5% picric acid and 2% $Na_2CO_3$ was placed in the lid of the Petri dish, which was then sealed with paraffin film. After four days of incubation at 27°C, yellow to brown disc coloration of the paper indicated HCN production (Agrawal *et al.*, 2015).

### Antagonistic activity of endophytic fungi

Test pathogens, *Aspergillus niger Fusarium solanii* and, *Fusarium oxysporum* were obtained from department of Biotechnology, GBPEC, Pauri, Garhwal, Uttrakhand. Dual culture technique was adopted for antagonistic test against these pathogens on PDA plates. Six-day-old mycelia disks of (5mm diameter) of test pathogens were placed on one corner of Petri plates containing PDA medium. Fungal endophytes were inoculated on the other corner of PDA plates. Plates were incubated at 28˚C for 6 days and antagonistic index was accessed according to the following formula:

Antagonistic Index: $\frac{RM-rm}{RM} \times 100$

RM: radius of the pathogen in the control plate

rm: radius of pathogen in the dual culture plate

## Result and Discussion

A systematic study about the endophytic fungal biodiversity in a forest plant, *Cupressus torulosa* D. Don. located in Govind Ballabh Pant Engineering College Campus, Pauri Garhwal, Uttarakhand were carried out to evaluate their extracellular enzyme production and it role in biocontrol activity. A total of eight endophytic fungi were isolated from leaves of *C. torulosa* D.Don by using different culture media. These endophytic fungi were characterized morphotypically using lactophenole cotton blue using scotch tape techniques (Table 1). All fungal endophytes were preserved in PDA slants at 4ºC as well -80º C in glycerol stock for further use.

The Majority of the recovered endophtytes belong to the Ascomycota. Fungal endophytes are especially common among the Ascomycota, representing at least five classes,

dozens of families, and large numbers of previously unknown species (Sharma *et al.*, 2016). Only one species from the collected isolates in this study belong to the *Dothideomycetes* (Petrini, 1986).

## Functional attributes of fungal endophytes

The relationship between the endophyte and its host plant may range from latent phytopathogenesis to mutualistic symbiosis (Panutahi *et al.*, 2012). Like other organisms invading plant tissues, endophytic fungi produce extracellular hydrolases (Table 2) as a resistance mechanism against pathogenic invasion and to obtain nutrition from host. Such enzymes include pectinases, cellulases, lipases, laccase from the endophytic fungus *Monotospora sp.*, xylanase, -1, 4-glucanlyase, phosphotases and proteinase (Sunitha *et al.*, 2013). Hydrolytic enzymes (amylase, cellulase and laccase) with various industrial applications are also of major interest. Hydrolases also reported from *Pinus rouxburghii* by Bhardwaj *et al.*, (2015)

## Amylase activity

All fungal endophytes were able to produce extracellular amylase (Fig. 1). The amylolytic potential of these endophytes may help them to degrade starch which is available when the plant senesces. The isolate KCTS34 was show highest amylase activity with the diameter of 76 mm The Amylase activity was found to be higher with *Tuber aestivum* than with *Tuber maculatum* (Nadim *et al.*, 2015). *Rhizoctonia sp*. showed highest production of amylase enzyme i.e. 0.26U/ml (Patil *et al.*, 2015). Endophytic fungi such as *Penicillium frequentans* isolated from *Pinus rouxburghii* were screened for amylolytic activity. Influence of various physical and chemical factors such as pH, temperature, carbon and nitrogen sources on amylase production by *P. frequentans* in liquid media was studied (Bhardwaz *et al.*, 2015). The maximal amylase productivity achieved at 30˚C of incubation was 0.28µ/ml and maximum fungal biomass was 5.618 g/25 ml and at pH 7.0 of the cultural media showed maximum amylase activity of 0.461µ /ml and the maximum fungal biomass production was 5.511 g/25 ml.

## Protease activity

Out of eight a four x endophytes exhibited protease activity (Fig. 2). The protease activity was observed in *Biosporus sp.*, *Aspergillus sp*., *Cladosporium sp.*, *Curvularia sp.*, *Rhizoctonia sp.*, *Chaetomium sp.*, *Cladosporium sp.* indicated by formation of clear zone around the colony because of degradation of gelatin, while other two isolates *Colletotrichum sp.*, *Fusarium sp.* from the medicinal plants *Citrus limon* and *Gossypium hirsutum* respectively indicated negative results. Similar result was reported by Pavithra *et al.*, 2012, where out fungal isolates from *Ocimum sanctum* were found positive for protease.

## Lipase activity

These all eight isolates were screened for lipase activity seven isolates such as WCTS21, WCTS33, PCTS13, PCTS21, PCTS25, KCTS15 and KCTS34 produce lipase activity while one isolate WCTS31 was not produce lipase activity (Fig. 3). The isolate PCTS21 was show highest lipase activity with the diameter of 64 mm. *Cladosporium sp.* of *Phyllanthus emblica* medicinal plant was the maximum producer of lipase activity (Patil *et al.*, 2015). Amirita *et al.*, (2012) reported lipolytic activity of *Curvularia brachyspora*, *C. vermiformis*, *Drechslera hawaiiensis*, *Colletotrichum falcatum* and *Phyllosticta sp*. isolated from medicinal plants. Venkates agowda *et al.*, (2012) reported lipolytic endophytic fungal isolates from the different oil-bearing seeds.

## Cellulase activity

All fungal endophytes were able to produce extracellular cellulase (Fig. 4). The isolate KCTS34 was show highest cellulase activity with the diameter of 50 mm. Cellulolytic activity was prominent in *Talaromyces emersonii* (Ci1), followed by *Discosia sp*. (Ci 5), of *Calophyllum inophyllum* and *Drechsclera sp*. (Cr2) from *Catharanthus roseus*. The production of cellulase was not significant from isolates of other two plants. Only 32% of the endophytes tested were able to produce cellulose (Sunitha *et al.,* 2013).

Similar result was reported by (Maria *et al.*, 2005) from mangrove angiosperm isolates. However, 66% cellulolytic activity was reported by (Choi *et al.,* 2005) from isolates of *Brucea javanica*. Bezerra *et al.,* (2003) reported 53.84% cellulolytic activity of endophytes from *Opuntiaficus-indica* Mill., *Cladosporium cladosporioides* with maximum activity.

## Chitinase activity

All fungal isolates exhibited the chitinase activity. The isolate PCTS13 showed the highest chitinase activity with the diameter of 53 mm while the isolate WCTS31 showed the lowest chitinase activity with the diameter of 20mm. the chitinase activity of 0.00093 U/mL in Streptomyces sp., which corresponds to a specific activity of 0.050 U/mg protein, was four times lower than that in *S. viridificans* (0.0038 U/mL) (Gupta *et al.*, 1995).

As with *Trichoderma* sp, *S. elegans* is capable of degrading *Rhizoctonia solani* cell walls (Morissette *et al.,* 2003) and releases the CWDEs, β-1,3-glucanases and chitinases, into culture medium amended with *R. solani* cell wall fragments or with chitin as a carbon source (Tweddell *et al.*, 1994).

## Plant growth promoting activity of endophytic fungi

Many endophytes are reported to be capable of nitrogen (N) fixation, solubilization of phosphate, enhance uptake of phosphorus (P), production of siderophores, ACC deaminase, and plant hormones such as auxin, abscisins, ethylene, gibberellins, and indole acetic acid (IAA), which are important for plant growth and development regulation (Selim *et al.,* 2012).

## Phosphate solubilizing activity

Eight isolates were screened for Phosphate solubilizing activity, only six isolates such as WCTS21, WCTS33, PCTS13, KCTS15, PCTS25 AND KCTS34 showed the Phosphate solubilizing activity while two isolates such as WCTS31 and PCTS21 were not produce Phosphate solubilizing activity (Fig. 5 and Table 3).

Nahas (1996) reported that among the microbial group, fungi are more efficient in solubilizing phosphate than bacteria. The highest range was observed in *T. peudokoningii* (37.45±2.78 to 64.32±2.87) µg/ml followed by *C. globosum* (33.62±5.92 to 69.32±3.21) µg/ml, *F. semitectum* (32.64±1.89 to 57.63±2.11) µg/ml, *A. versicolor* (31.63±2.02 to 63.72±2.36) µg/ml (Chadha *et al.,* 2015).

## Production of ammonia

All fungal isolates exhibited the ammonia production (Fig. 6, Table 3). Most of the ammonia produced by fungi will be transformed to ammonium in solution. However, at higher pH a larger proportion of the ammonium becomes toxic unionized ammonia; the approximate ratios of $NH_3^+$ % to $NH_3$ will be 1800:1 at pH 6, and 9:1 at pH 8±3. Ammonia is very toxic because it is lipid

soluble and raises intracellular pH, thus inhibiting protein synthesis and enzyme activity (Doyle and Butler, 1990).

## Production of HCN

All eight fungal endophytes isolates screened for the production of HCN and any isolates was not produce HCN. HCN is the common secondary metabolite produced by rhizosphere *Pseudomonas* (Schippers, 1988). Meena *et al.,* (2001) compared the HCN production of several strains of *P. fluorescens* and their efficacy in controlling root rot of groundnut caused by *M. phaseolina.*

## Antagonistic activity of endophytic fungi isolates

The isolates were tested for antagonistic activity by the dual culture technique. A total of eight endophytic fungal isolates out of seventeen were showed antagonism activity against *A. niger, F. oxyspourm* and *F. solani.* Radial growth of the pathogen was considerably hindered by all the test antagonists under the conditions of this study. WCTS21 was the most antagonistic and inhibited the radial growth of the pathogen most while KCTS34 was the least antagonistic. The fungal isolate PCTS25 was more active against *A. niger* with antagonistic index 75.55 while WCTS31 more active against *F. oxysporum* with antagonistic index 42.18 (Figs. 8, 9 and 10; Table 4) and isolate WCTS21 is more active against *F. solani* with antagonistic index 51.66. *Aspergillus flavus, A. fumigatus, A. niger, A. sydowi, A. sulphureus, Penicillium sp.* and three biocontrol agents namely *Trichoderma harzianum, T. Koeningii* and *T. viride* were tested against one plant pathogen namely *Pythium debaryanum*. The percentage inhibition of growth of pathogen against *Trichoderma harzianum, T. koeningii* and *T. viride* were 66.6, 62.5, 60.4 percentage with respectively (Gomathi *et al.*, 2011).

**Table.1** Morphotypic characterization of endophytic fungi

| Code of isolate | Source of Endophytic Fungi | Colony charecterstics on PDA media | Probable endophytic fungus | Class |
|---|---|---|---|---|
| PCTS13 | Leaves | Appears olivaceous brown in colour | *Penicillium oxalicum* | Ascomycetous |
| PCTS21 | Leaves | Appears grayish green in colour | *Alternaria alternata* | Dothideomycetes |
| KCTS34 | Leaves | Appears cottony white in colour | *Daldinia sp.* | Zygomycetes |
| WCTS31 | Leaves | Whitish appearance, rapid growth horizontally | *Fusarium circinatum* | Sordariomycetes |
| WCTS33 | Leaves | Whitish appearance, cotton growth | *Pestalotiopsis versicolor* | Sordariomycetes |
| WCTS21 | Leaves | Brown | *Penicillium megasporum* | Sordariomycetes |

**Table.2** Extracellular enzymatic activity of endophytic fungi

| S.NO. | Isolates | Diameter of clear zone (in mm) | | | | |
|---|---|---|---|---|---|---|
| | | Amylase activity | Protease activity | Lipase activity | Cellulase activity | Chitinase activity |
| 1 | WCTS21 | 30 | 50 | 48 | 48 | 36 |
| 2 | WCTS31 | 65 | 40 | - | 42 | 20 |
| 3 | WCTS33 | 20 | 63 | 60 | 20 | 30 |
| 4 | PCTS13 | 40 | - | 48 | 34 | 53 |
| 5 | PCTS21 | 60 | - | 64 | 50 | 25 |
| 6 | PCTS25 | 54 | - | 52 | 36 | 34 |
| 7 | KCTS15 | 68 | 46 | 30 | 30 | 30 |
| 8 | KCTS34 | 76 | - | 54 | 50 | 26 |

**Table.3** Plant growth promoting activity of endophytic fungi

| Strain/test | Phosphate solubilizing activity | Production of ammonia | Production of HCN |
|---|---|---|---|
| WCTS21 | + | + | - |
| WCTS31 | + | + | - |
| WCTS33 | + | + | - |
| PCTS13 | + | + | - |
| PCTS21 | + | + | - |
| PCTS25 | + | + | - |
| KCTS15 | + | + | - |
| KCTS34 | + | + | - |

**Table.4** Antagonistic activity evaluation of endophytic fungi against fungal pathogens

| S. No. | Isolates | Antagonistic index (%) | | |
|---|---|---|---|---|
| | | *Aspergillus niger* | *Fusarium oxyspourm* | *Fusarium solani* |
| 1. | WCTS21 | 44.44 | 34.37 | 51.66 |
| 2. | WCTS31 | 55.55 | 42.18 | 28.33 |
| 3. | WCTS33 | 55.55 | 21.87 | 15.00 |
| 4. | PCTS13 | 60.00 | 37.50 | 43.33 |
| 5 | PCTS21 | 48.88 | 37.50 | 15.00 |
| 6. | PCTS25 | 75.55 | 34.37 | 23.33 |
| 7. | KCTS15 | 44.44 | 37.50 | 46.66 |
| 8. | KCTS34 | 44.44 | 34.37 | 28.33 |

**Fig.1** Amylase activity of endophytic fungi

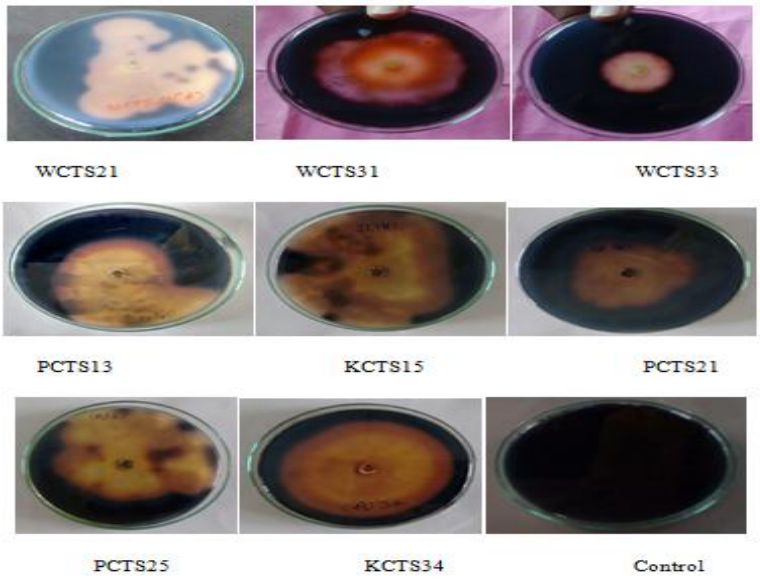



**Fig.2** Protease activity of endophytic fungi

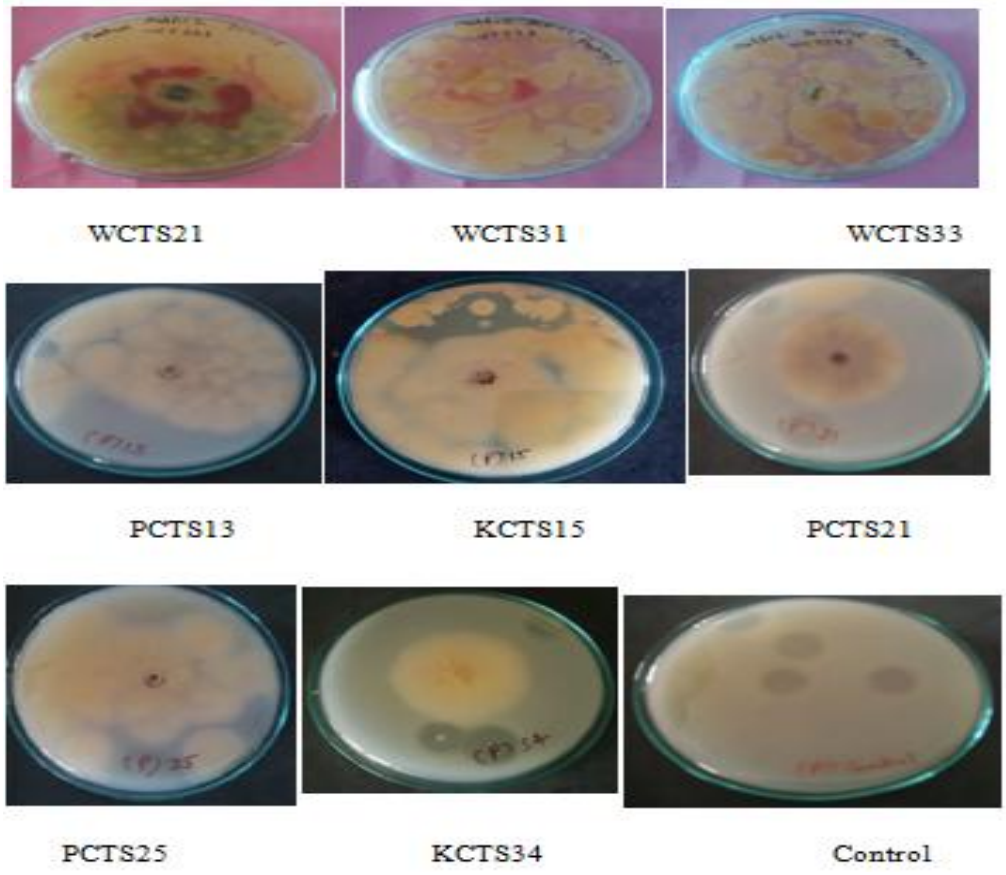


**Fig.3** Lipase activity of Endophytic fungi

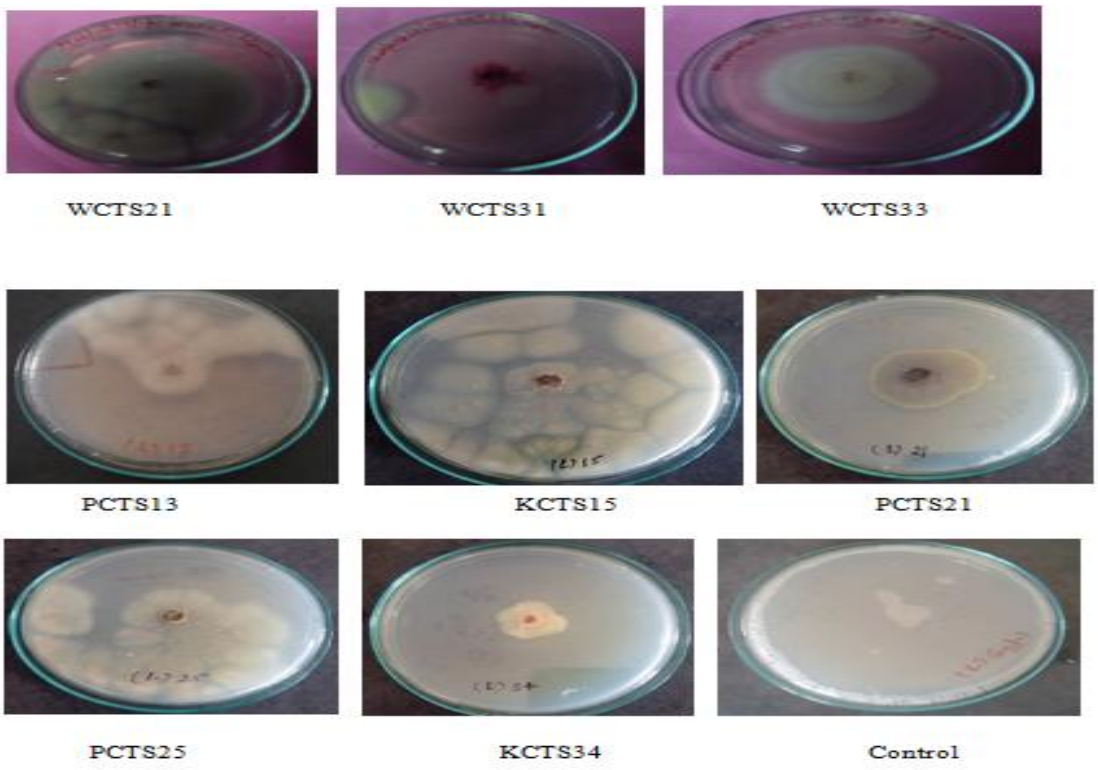



**Fig.4** Cellulase activity of endophytic fungi

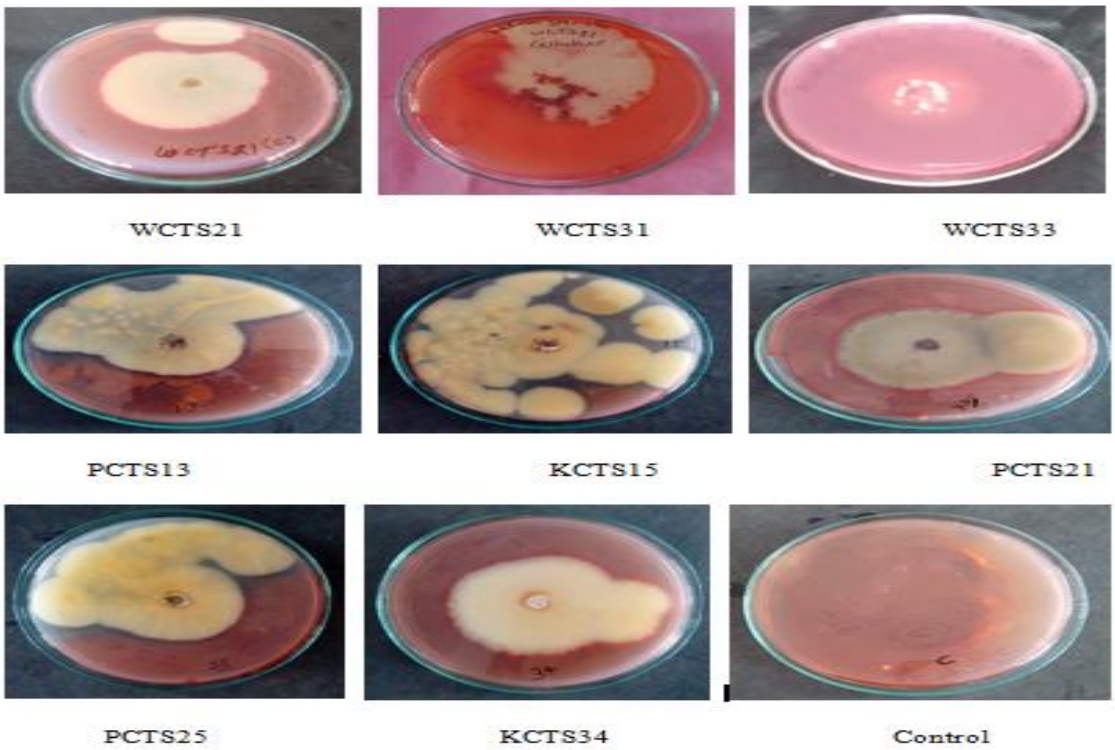


**Fig.5** Chitinase activity of endophytic fungi

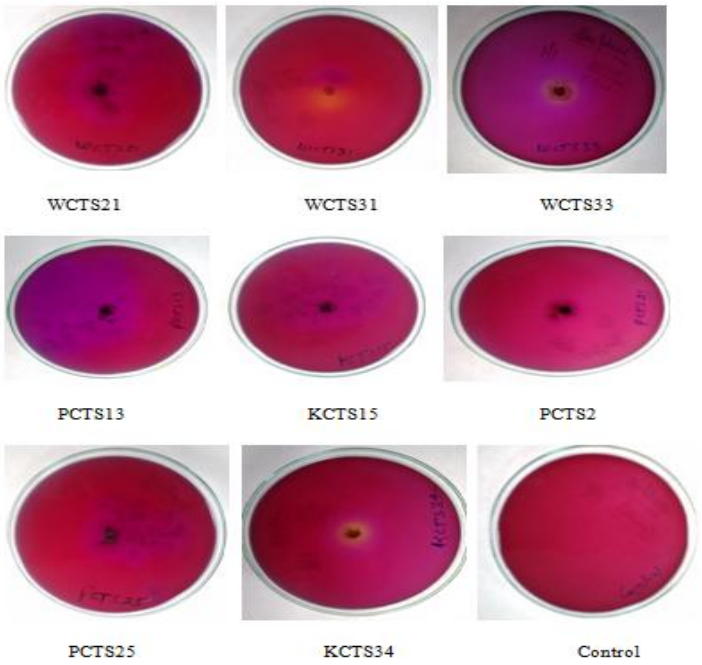



**Fig.6** Phosphate solubilizing activity of endophytic fungi

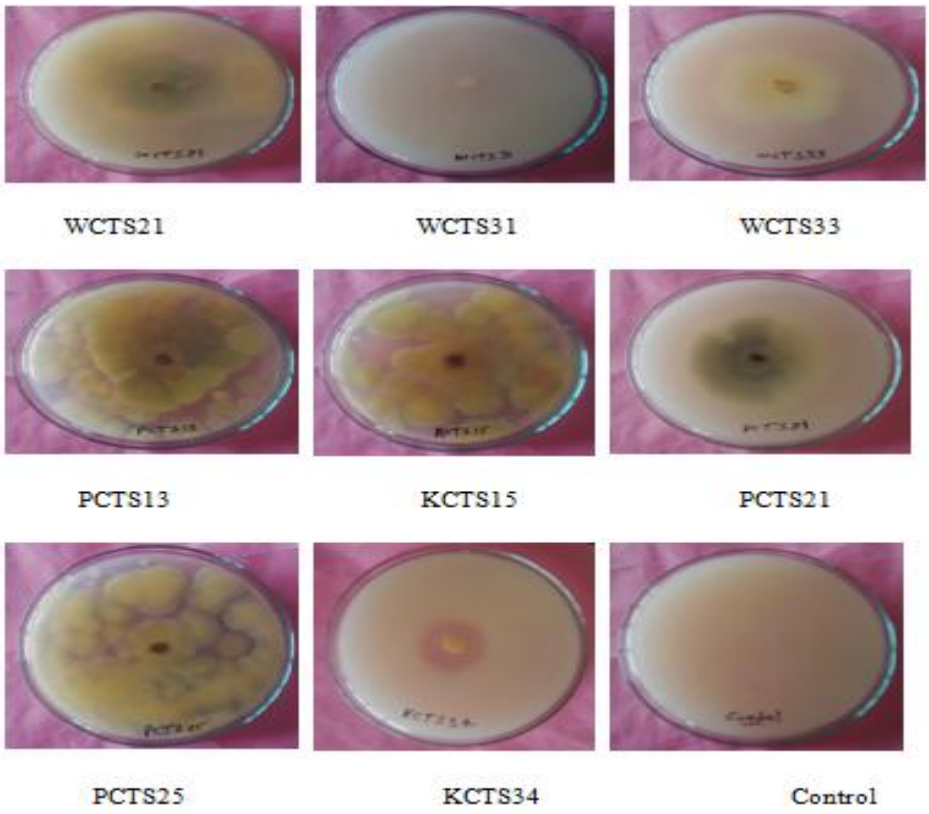


**Fig.7** Ammonia production by endophytic fungi

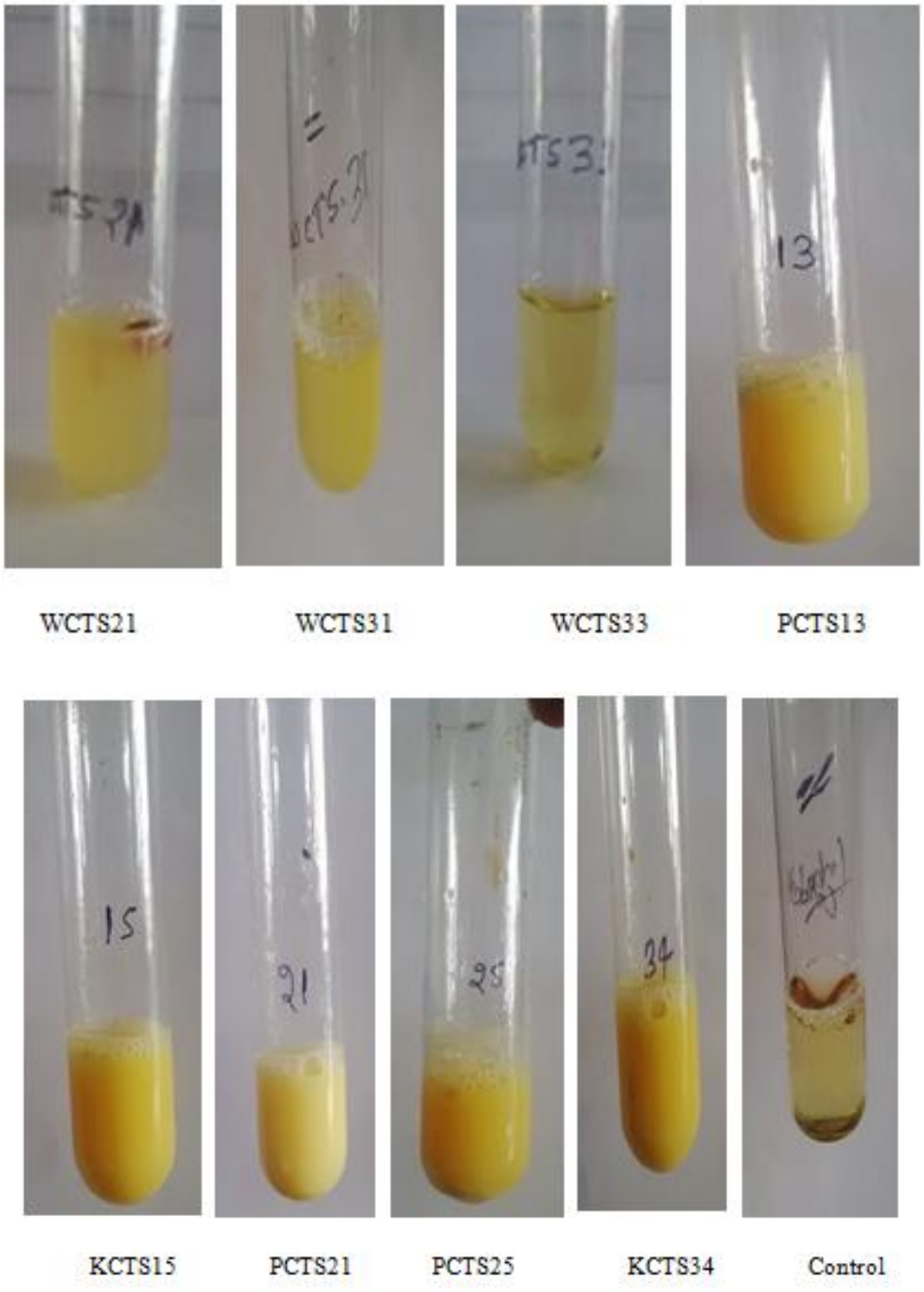


**Fig.8** Production of HCN by endophytic fungi

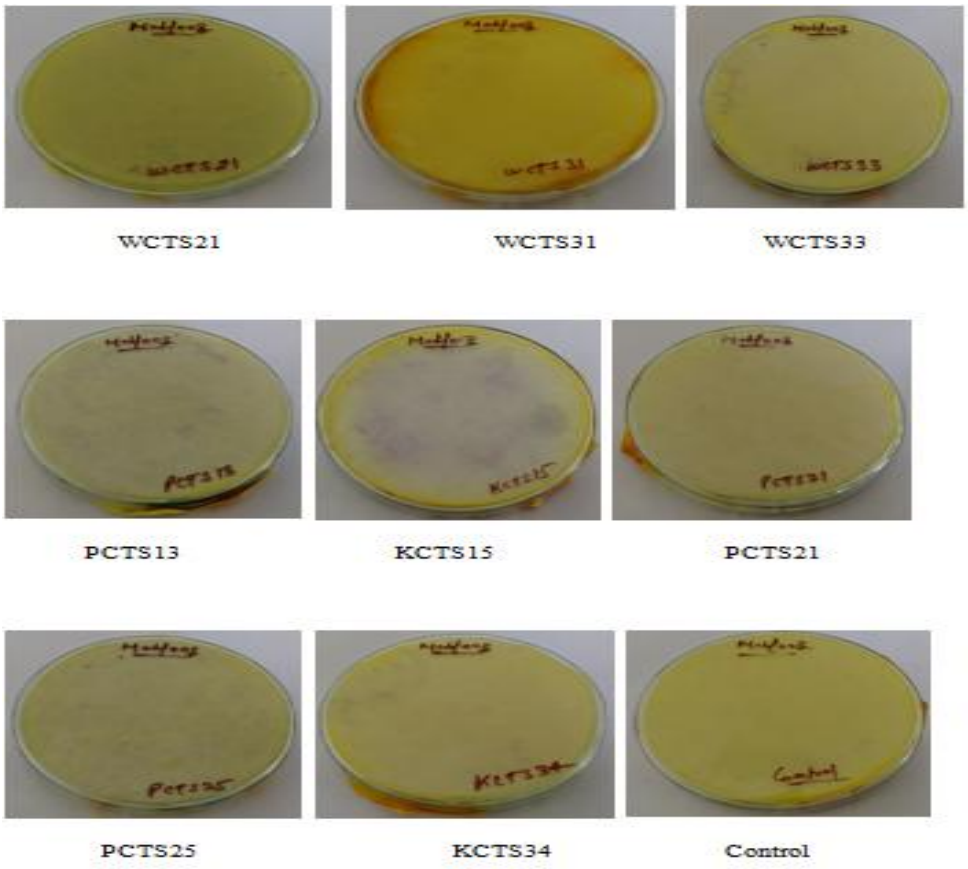



**Fig.9** Antagonistic activity of endophytic fungi against fungal pathogen *A. niger*

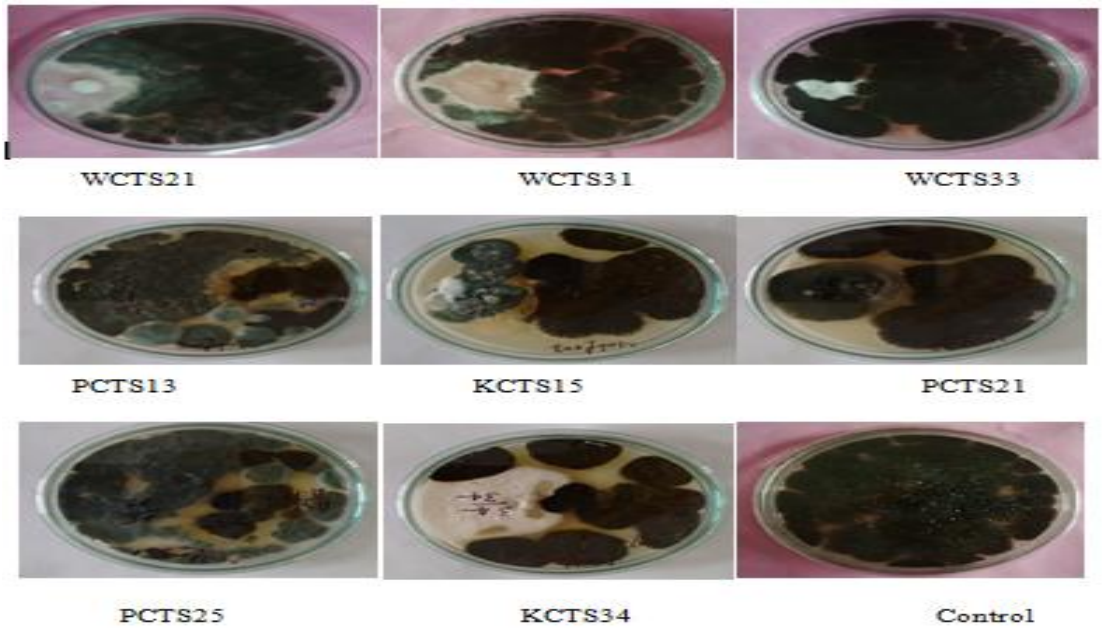


**Fig.10** Antagonistic activity of endophytic fungi against fungal pathogen *F. oxysporum*

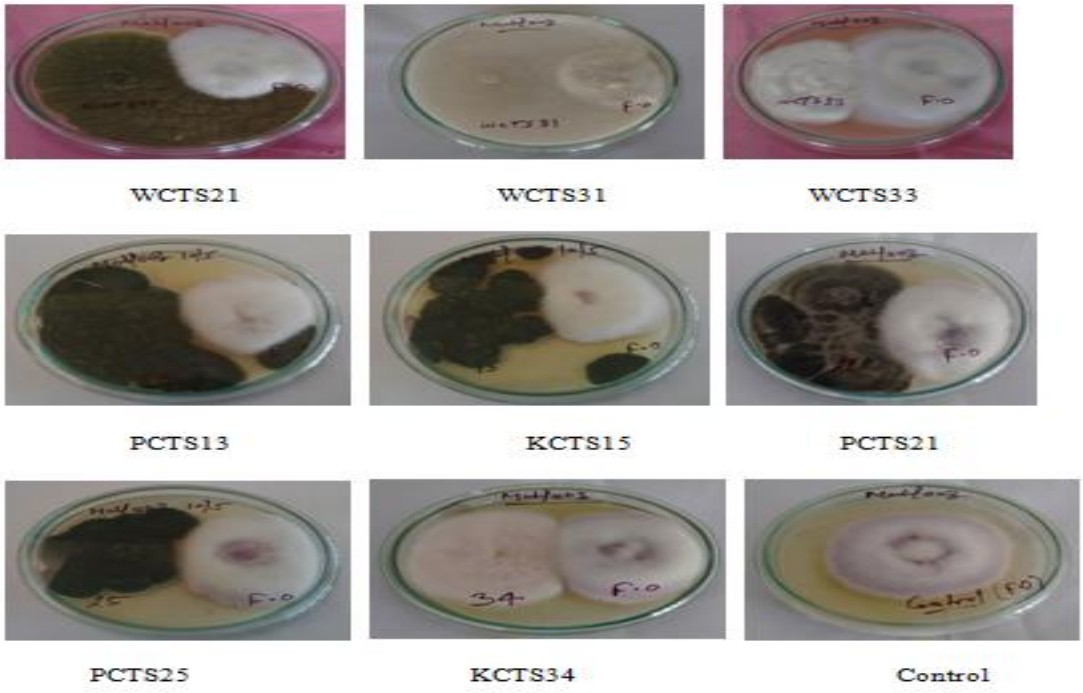
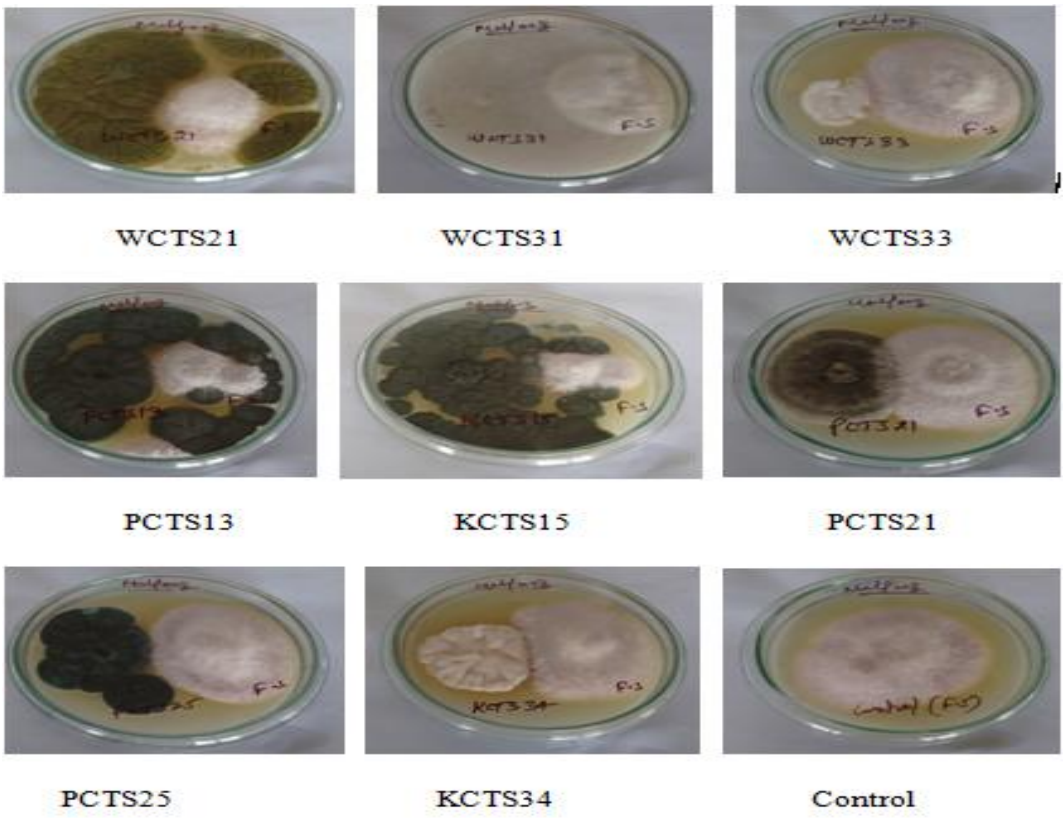



**Fig.11** Antagonistic activity of endophytic fungi against fungal pathogen *F. solani*



These endophytic fungal isolates are not pathogens for their antagonism against phytopathogens. These fungi could be adapted to this host and be antagonists of their pathogens. Depending on their antagonistic capacity, they would be able to displace, reduce, suppress or induce resistance against them (Bisht *et al.,* 2016). Those active endophytic fungi inside the plants may play an important role in protecting the plant host against pathogenic microorganisms and have an intimate correlation with the development and physiological activity of wheat (Tian *et al.,* 2004). Antagonism might be due to the production of biologically active compounds in media).

In conclusion, endophytes are being considered for use in biological control, and the extracellular enzymes they secrete might facilitate their initial colonization of internal plant tissues and direct interactions with microbial pathogens. Endophytic fungi produce enzymes which hydrolyses several plant-derived macromolecules and several secondary metabolites. Fungal enzymes are used in textiles, leather industry, food, beverages and confectionaries to simplify the processing of raw materials.

In the present study eight fungal endophytes were screened for qualitatively for the presence of extracellular enzymes such as amylase, protease, lipase and cellulase which has grown in specific medium. The order of enzymes activity found in this study for the isolated microorganism is amylase>cellulose> lipase>protease. Based on the result presented, it can be clearly seen that endophyte isolates from *Cupressus torulosa* D.Don may be beneficial to the host.

In this study fungal strains also showed several enviable features of plant growth promoting traits and multiple action mechanism which suggests their potential for plant growth promotion. The Results indicated that selected fungi may be helping the plants in protecting from pathogenic fungi. Endophytic fungi PCTS25 identified as *Penicillium oxalicum* found active in the study can be explored for its potential as a biocontrol agent against *A. niger*, while WCTS33 identified as *Pestalotiopsis versicolor* and isolate PCTS21 identified as *Alternaria alternata* showed least antagonistic activity against pathogen *Fusarium solani.*

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