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Isolation and Biological evaluation of Endophytic Fungus from *Ziziphus nummularia*

Sabba Fareed, Urooj Nawaz Jadoon, Ikram Ullah, Muhammad Ayub Jadoon, Mujaddad Ur Rehman, Zainib Bibi, Muhammad Waqas and Sobi Nisa

Abstract
The aim of the current research work was to analyzed Endophytic fungi and there enzymatic activity. The research work was carried out in the Microbiology Lab, University of Haripur, during May 2015 to September 2015.The plant was collected and fungi were isolated by inoculating the leaf sections on different Medias. Six fungal species were isolated and identified on Sabouraud Dextrose agar medium. The results indicate highest colonization frequency at Sabouraud Dextrose Agar media that is 83%. Frequency of colonization was 76%, 26% and 23% on Malt Extract Agar, Potato Dextrose Agar and Czapak Dox Agar respectively. Identification was done by comparing with available literature and by microscopy. The study on this plant revealed that plant contains endophytic fungi that have ability to produce amylase, protease and lipase enzymes, although better activity for production of amylase enzyme was observed.

Keywords: Endophytic fungi, *Ziziphus nummularia*, enzymatic activity

1. Introduction
Enzymes are large biological molecules responsible for all those important chemical inter conversions that are required to sustain life [1].
The enzymes speed up the reaction. In the absence of catalyst most of the cellular reaction would not occur even over time period of year. In the absence of cellular reaction life in its present form would not be possible [2].
Enzymes are produced from verity of microorganism e.g, bacteria, fungi and yeast. Amylase can be obtained from different species of microorganisms, but for commercial use, α-amylase derived from *Bacillus licheniformis*, *Bacillus stearothermophilus*, and *Bacillus amyloliquefaciens* has number of application in different industries such as in food, fermentation, textiles and paper industries [3-4]. For the extraction of enzymes other methods are used which includes chromatography, filtration and electrophoresis. The disease diabetes mellitus caused by the deficiency of insulin enzyme [5].

1.1 Endophytic Fungi
Endophytic fungi are the fungi that is present intracellular in parts of plant for at least a portion of their lives without causing appearance of symptoms of disease [6]. Endophytic fungi belong to the phylum *Basidiomycota* while some species belong to the phylum *Ascomycota* and *Zygomycota* [7]. Approximately 1.3 million species of endophytic fungi are to be known by Dreyfuss & Chapela [8]. Endophytic fungi are the source of enzyme production and bioactive compound which act as a reservoir of other compounds e.g, antibiotic, antiparasitic, anticancer and antioxidant compounds, use in the pharmaceutical industries [9].
Z. nummularia is a shrub plant up to 2 meters high and a multipurpose species valued for edible fruits, leaves as forage, branches for fencing and as folk medicine. This plant grows in warm and dry climate and on sandy and silicic soil. A much branched shrub, flexuous, tomentose, young branches puberulous, grey, spines in unequal pairs, smaller recurved, bark light colour. Leaves 1-8 cm long, 8-10 mm broad, orbicular or ovate-orbicular to elliptic, dark green and densely pubescent above, densely woolly beneath, entire or serrate, apex obtuse apiculate or mucronate, base round to sub cordate, lateral nerves prominent, pedicel 2-4 mm long. Flowers in axillary, sessile pubescent cymes; 3-4 mm in diameter. Calyx pubescent outside, cleft about halfway down; lobes 1 mm, ovate lanceolate.

Petals cuneate longer than stamens c. 1.25 mm long, rounded or truncate at apex. Stamen included c. 1 mm long. Disc.10-lobed, pitted opposite each lobe. Styles 2, united to above the middle. Drupe globose, reddish brown-black when ripe, 2 celled, 2 seeded, 5-10 mm long [10].

The leaves of Ziziphus *nummularia* are collected, dried and stored. The leaves are antipyretic and reduce obesity. The fruit is cooling, tonic, digestible, laxative aphrodisiac and removes biliousness, thirst, vomiting and burning sensations [11].

The shrub show wind erosion, it also provides barrier, support and boundary. The plant is host of larva of butterfly in Africa, Iran, Lebnon. The species can withstand harsh conditions such as salinity, drought and temperature [12].

2. Material and Methods

This research work was conducted at the Microbiology research laboratory Department of microbiology, University of Haripur, Pakistan. During May 2015 to September 2015.

2.1 Collection of plant samples

For the purpose of research Plant *Ziziphus nummularia* was collected from Hattar, Haripur.

2.2 Media preparation

Different media were used e.g. Potato Dextrose Agar (PDA), Saboraud Dextrose Agar, Malt extract agar and Czapak dox agar etc. Before autoclave the media, 0.5g drug (0.15g amoxicillin) was added, to avoid contamination and stop the growth of bacteria.

2.3 Sterilization of Plant sample

The collected plant material is washed under running tap water for 10 minutes for removal of dust particles and debris and dried it for few minutes. Then section of plant e.g stem or leave removed from plant and dipped in autoclave petri plates containing 0.1% Mercuric chloride for one minute. The samples were properly dipped into solution. Sections of the plant leaves were cut by using surgical blades. Then with the help of autoclaved forcipes the cut section of plant were picked and washed in autoclave distilled water for one minute. Later on the sample was dipped into another petri plate of autoclave distilled water for two minutes.

2.4 Inoculation of plant

The samples were put on autoclave filter paper in Bio-safety Cabinet for few minutes, the purpose was to dry the sample. The cut portion of leaves was exposed to media. 5-6 pieces of plant material were inoculated per petri plates containing Agar in plate. Each Petri plates were sealed with parafilm. All the plates were wrapped in paper and incubated at 27 ^{o}C in dark for 4-5 days.

2.5 Growth of Endophytic fungi

The endophytic fungi start emerging from the cut ends of leaf sections. The fungus was cultured on new plates for isolation and identification.

2.6 Calculation of Colonization frequency

Frequency of Colonization was calculated for each fungus species growing there by formula.

$$\text{Colonization Frequency} = \frac{\text{No of Segments colonized by Fungus}}{\text{Total No of Observed Segments}} \times 100$$

2.7 Identification of fungi

Endophytic fungi were identified on the basis of morphology, colony presentation and microscopy.

2.8 Enzyme Assay

The ability of enzyme production (outside the cell) of endophytic fungi was checked by growing fungi in different media. The goal was achieved by digestion of dissolved substrates in media and zone of clearance was checked around the mycelium plug [13-14].

2.8 (a) Lipase

For lipase test the peptone agar medium was used (peptone 5.g, sodium chloride 2.5g, agar 7g, distilled water 500 mL and pH 6) with Tween 5ml. Fungus was inoculated on peptone agar medium and incubated at 25 ^{o}C for five days. The zone of clearance appeared around the inoculated hyphae indicating the secretion of lipase.

2.8 (b) Amylase

The enzyme activity of fungus was observed by inoculating fungi on glucose yeast extract peptone agar medium (GYP) (glucose-0.5g, yeast extract 0.05g, peptone 0.25g, agar 8g, distilled water 500mL and pH 6) containing 1% soluble starch. The incubation period of plates was five days at 25^{o}C. After five days the plates were flooded with Gram iodine solution. Clear zone was observed around the colony.

2.8 (c) Protease

For protease test fungus was grown on glucose yeast extract peptone agar medium (glucose-1g, yeast extract 0.1g, peptone 0.5g, agar 16, distilled water1000mL and pH 6). The fungus was inoculated on plates at 25 ^{o}C.The Plates were flooded with ammonium sulphate solution after 3-5 days of incubation. Formation of clear zones around protease was considered positive for protease enzyme.

3. Results

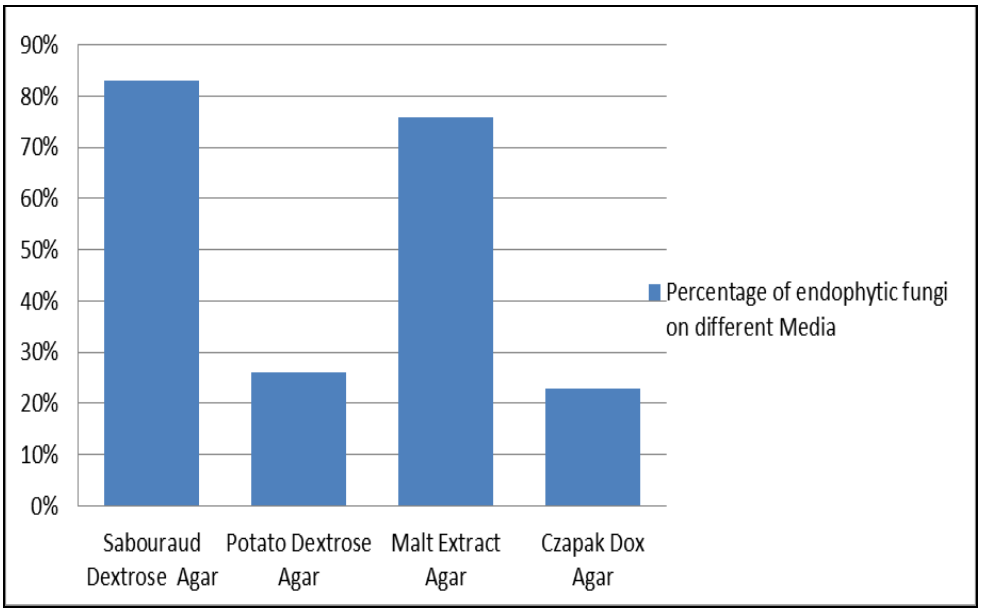
3.1 Colonization frequency

Colonization frequency of endophytic fungi was calculated on different media. The results indicate highest colonization frequency at Sabouraud Dextrose Agar media that was 83%, 76% on Malt Extract Agar, 26% on Potato Dextrose Agar and 23% on Czapak Dox Agar. Also showed in Table 1 and graph 1.

Table 1: Colonization frequency of endophytic fungi on different media

S No	Media	Colonization frequency	Results
1	Sabouraud Dextrose Agar	25/30×100	83%
2	Potato Dextrose Agar	8/30×100	26%
3	Malt Extract Agar	23/30×100	76%
4	Czapak Dox Agar	7/30 ×100	23%

Based on the findings of colonization frequency further work was proceeded with Sabouraud Dextrose Agar as it supported maximum number of endophytes growth.



Graph 1: Percentage of endophytic fungi

3.2 Isolation and identification of Endophytes

As a result of inoculation of plant material on Sabaroud Dextrose Agar media six different types of fungi were isolated from leaves of *Ziziphus nummularia*. (Table 2).

Table 2: Colony Morphology of isolated Endophytic fungi.

S. No	Code	Media	Microscopy	Colony presentation	Background of fungus	Isolated fungal species
1	W1	SDA	Septate,with sessile stalk club shape microconida along hyphae	Light yellow with white margin	Yellow colony on full plate	Fungal species *Microsporum gypseum*
2	W2	SDA	Septate hyphe,smooth, short conidiophore, round conidia	Dark green with with white margin raised colony	Mid brown with green and creamy margin	*Aspergillus fumigatus*
3	G1	SDA	Septate hyphe,smooth conidiophore and brown,conidia spherical, biserate	Dark green with golden creamy margin	Black white white margin	*Aspergillus calidoustus*
4	G5	SDA	Globose conidia,hayline conidiophore,branched metulae	Green with white margin	Yellow in mid, white green margin	*Penicillium viridicatum*
5	G6	SDA	Septate and hyaline hyphae,conidia are in chain,conidia round	Pale red diffusing pigment of stock culture	Yellow in mid, white green margin	*Penicillium marneffei*
6	S6	SDA	Septate, microconidia sessile, of pyriform, cylinder to clavate	Surface of colony white and yellow strain	Brownish with yellow margin on full plate	*Trichophyton tonsurans*

Fig 1: Colony of *Trichophyton tonsurans* fungus on petri plate

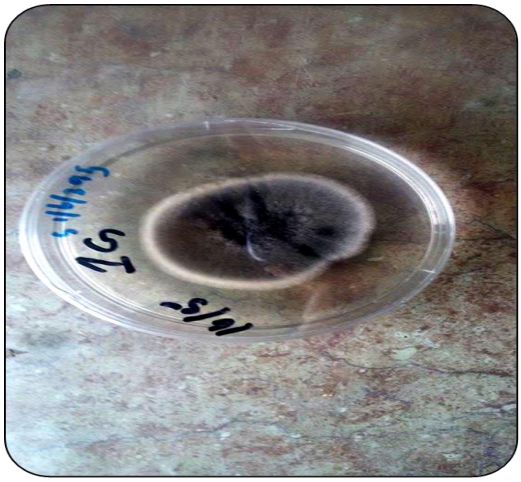


Fig 2: Colony of *Aspergillus calidoustus* on petri plate of Sabouraud Dextrose agar

Fig 3: Colony of Penicillium marneffei on petri plate of Sabouraud Dextrose agar

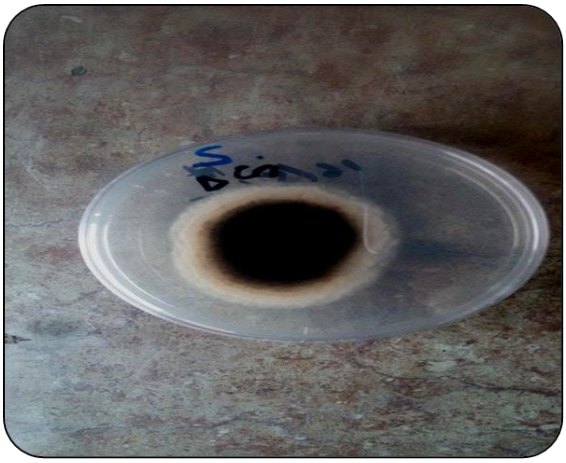


Fig: 4: *Aspergillus fumigatus* on petri plate of Sabouraud Dextrose agar

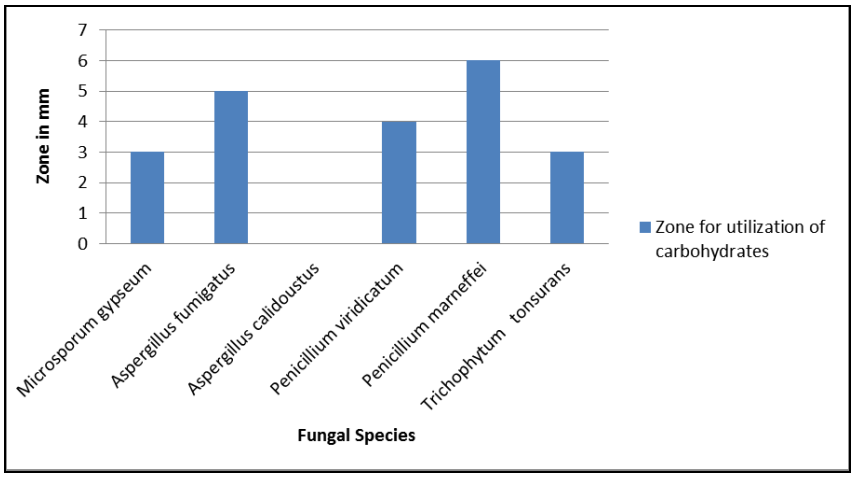
3.3 Enzyme activity

The results of enzyme activity indicates that isolated endophytic fungus has better ability to produce amylase and protease enzyme, while fungi are less active in producing lipase enzyme. *Microsporum gypseum, Aspergillus fumigates, Aspergillus calidoustus, Penicillium viridicatum, Penicillium marneffei and Trichophyton tonsurans* shows 4mm, 0mm, 5mm, 3mm, 0mm and 3mm zones for production of lipase enzyme. This may be due to the fact that stored food material in plants is starch and more production of amylase enzyme is linked with availability of its substrate. While due to less availability of substrate for lipids, less production of lipase was observed. Results for the production of various enzymes by fungal species are indicated in Tables 3 and graph 2.

Table 3: Production of Lipase enzyme by endophytic fungus

S. No	Fungal Species	Production of Lipase enzyme	
		Zone for utilization of carbohydrates (mm)	Lipase activity
1	*Microsporum gypseum*	4mm	Present
2	*Aspergillus fumigatus*	0	Absent
3	*Aspergillus calidoustus*	5mm	Present
4	*Penicillium viridicatum*	3mm	Present
5	*Penicillium marneffei*	0	Absent
6	*Trichophyton tonsurans*	3mm	Present

The fungi isolated from the leaves of plant shows different zone of utilization of carbohydrates, such zones are the indication of enzymatic activity. Fungi isolated from plant *Microsporum gypseum, Aspergillus fumigates, Aspergillus calidoustus, Penicillium viridicatum, Penicillium marneffei and Trichophyton tonsurans* shows 0mm, 2mm, 6mm, 8mm, 6mm and 4mm zones respectively.

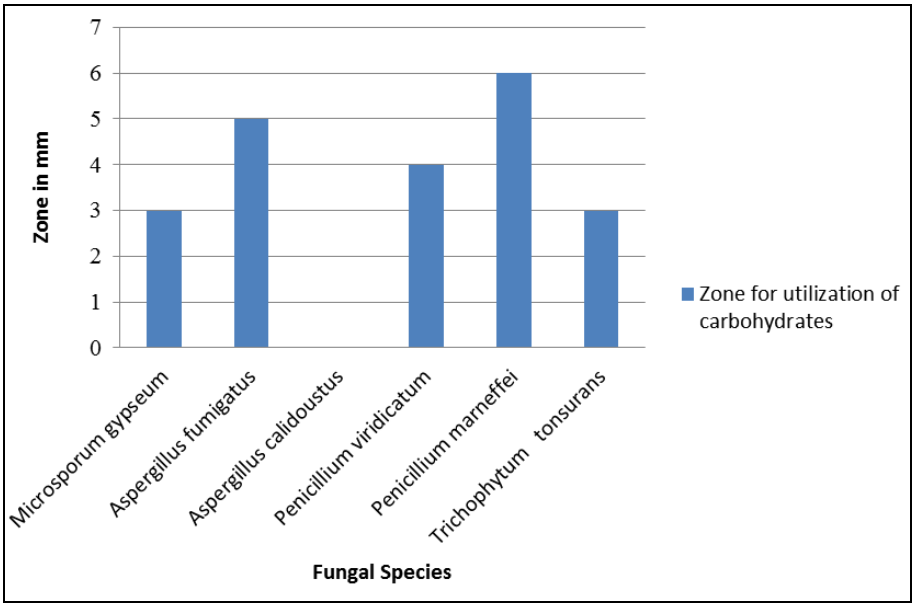


Graph 2: Lipase Activity

Table 4: Production of Amylase enzyme by endophytic fungus

S. No	Fungal Species	Production of amylase enzyme	
		Zone for utilization of carbohydrates(mm)	Amylase activity
1	*Microsporum gypseum*	0	Absent
2	*Aspergillus fumigates*	2mm	Present
3	*Aspergillus calidoustus*	6mm	Present
4	*Penicillium viridicatum*	8mm	Present
5	*Penicillium marneffei*	6mm	Present
6	*Trichophyton tonsurans*	4mm	Present

The fungi isolated plant shows different zone of utilization of proteins, such zones are the indication of enzymatic activity. Fungi isolated from plant *Microsporum gypseum, Aspergillus fumigates, Aspergillus calidoustus, Penicillium viridicatum, Penicillium marneffei and Trichophyton tonsurans* shows 3mm, 5mm, 0mm, 4mm, 6mm and 3mm zones respectively. zones were showed in graph 3.



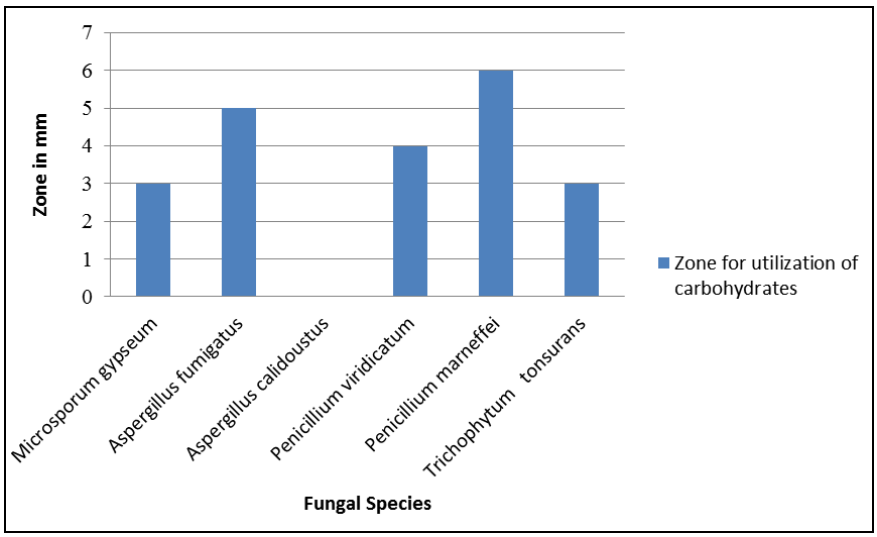
Graph 3: Amylase Activity

Our study showed production of protease enzyme by different fungal species. Fungi isolated from plant *Microsporum gypseum, Aspergillus fumigates, Aspergillus calidoustus, Penicillium viridicatum, Penicillium marneffei and Trichophyton tonsurans* shows 3mm,5mm,0mm,4mm,6mm and 3mm respectively. Represented on Table 5 and graph 4.

Table 5: Production of Protease enzyme by endophytic fungus

S. No	Fungal Species	Production of protease enzyme	
		Zone for utilization of protein (mm)	protease activity
1	*Microsporum gypseum*	3mm	Present
2	*Aspergillus fumigates*	5mm	Present
3	*Aspergillus calidoustus*	0	Absent
4	*Penicillium viridicatum*	4mm	Present
5	*Penicillium marneffei*	6mm	Present
6	*Trichophyton tonsurans*	3mm	Present

Production of protease activity is presented in graph 4.



Graph 4: Protease Activity

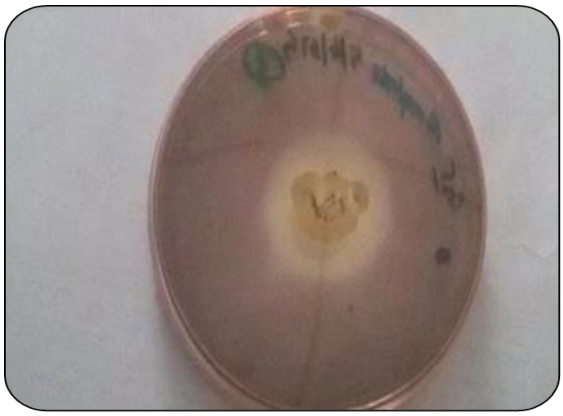


Fig 5: Clear zone of amylase test of *Penicillium viridicatum*

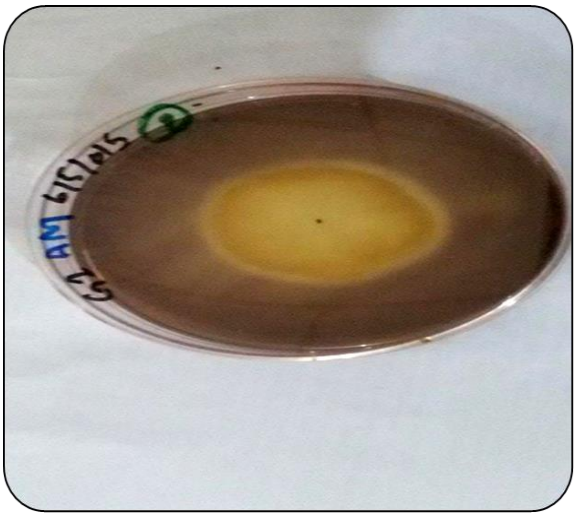


Fig 6: Clear zone of amylase test of *Aspergillus calidoustus*

The result of the research work showed that the *Ziziphus nummularia* plant contains endophytic fungi. These endophytic microorganisms showed some biological properties. Different tests like amylase test, protease test and lipase test were performed on endophytic fungi of Ziziphus *nummularia*. *Aspergillus calidoustus fungi* show clear zone and highest amount of lipase enzyme and *Penicillium marneffei* and *Aspergillus fumigatus* have no production of enzyme.

Production of amylase enzyme was high in *Penicillium viridicatum* and it showed clear zone of carbohydrate utilization. *Trichophyton tonsurans* show very small amount of amylase enzyme production. High amount of protease enzyme is produced in *Penicillium marneffei.* Enzymes produced from *Ziziphus nummularia* are available to use in different industries. There is need to isolate the enzyme from other medicinal plants and use them as a cheap source in industries.

4. Discussion

Isolation of endophytic fungi correlates with the previous studies where endophytic fungi have been isolated from medicinal plants [15].isolated endophytes from *Brucea javanica* that is tropical 3 meter heights woody shrub. The seeds of plant are used in treatment of dysentery, malaria and cancer. Approximately 21 endophytic fungi are isolated from single plant were tested to produced extracellular cellulase, amylase, ligninase, pectinase and xylanase. All of the fungi produces huge amount of amylase and cellulase enzyme.

Endophytic fungi are isolated from nine different medicinal plants. These plants are *Azadirachta indica, Citrus limon, Gossypium hirsutum, Magnolia champaca, Datura stramonium, Piper betle, Phyllanthus emblica* collected from the K.T.H.M college, India. The Endophytic fungi were subculture on Potato dextrose medium. Nine Endophytic fungi are isolated which includes *Cladosporium sp, Rhizoctonia sp, Aspergillus sp, Chaetomium sp, Biosporus sp, Fuzarium sp, Curvularia sp, Cladosporium sp, Colletotrichum sp*. These endophytes have property to produce qualitatively and quantitatively for the production of extracellular enzymes.

Endophytic fungi were isolated from leaves of *Ziziphus nummularia* in this study because of abundance of such fungi in the leaves. Ong and Nordiana 1998; 1999 study also show that most of endophytic fungi was found in leave. The study was carried out on 121 plants out of which 90 percent of endophytes were isolated from leaves of plants as compared to other branches [16-17].

Endophytic fungi can produce enzymes which may helpful in other functions for plants and as well as for humans. Endophytic fungi which can act as parasites for plants but not cause severe diseases can produce proteinase and pectinase [18-19], if such fungi living in mutualistic relations with plant can produce cellulase, mannanase and xylanase [20]. Same results were found in our study when finding the endophytic fungi and enzymes produced by them.

Ethnomedicinal plants namely *Potentilla fulgens, Osbeckia stellata, Osbeckia chinensis, Camellia caduca* and *Schima khasiana* were collected from the sohra in india, screened form ability to produce amylase, cellulase, protease, lipase and xylanase [21].

From plant *Centella asiatica*, Penicillium spp was isolated. The fungus produced large quantity of cellulase enzyme [22].

5. Conclusion

Endophytic fungi are an unwell examined group of Microorganisms. That produced different types of compound. These compounds have the potential for utilization in a wide variety of medical, agricultural, and industrial applications. In the current study endophytic fungi isolated from *Ziziphus nummularia plant*. Which showed that the *Aspergillus calidoustus* fungi have highest production of lipase enzyme and *Penicillium marneffei and Aspergillus fumigatus* have no production of enzyme.

6. Acknowledgement

I am immensely grateful to my friends and teachers for their constant support and guidance at each and every step throughout the completion of this thesis.

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7. References

1 Smith AL. Oxford Dictionary of Biochemistry and Molecular Biology, Oxford University Press, 1997.
2 Arnold AE, Mejía L, Kyllo D, Rojas EI, Maynard Z, Robbins N *et al*. Fungal endophytes limit pathogen damage in atropical tree. Proceedings of the national academy of sciences USA. 2003; 100:15649-15654.
3 Konsoula Z, Liakopoulou-Kyriakides M. Co-production of α-amylase and β-galactosidase by Bacillus subtilis in complex organic substrates, Bioresource Technology.

2007; 98(1):150-157.
4 Pandey A, Nigam P, Soccol CR, Soccol VT, Singh D, Mohan R. Advances in microbial amylases, Biotechnology and Applied Biochemistry. 2000; 31(2):135-152, 2.
5 Higgins RC. Bio Factsheet, The Economic Importance of Enzymes Number 47 Unit 305B, Birmingham. B18 6NF Bio Factsheets. 1999; ISSN 1351-5136
6 Petrini O. Fungal endophytes of tree leaves. Microbial Ecology of Leaves, Springer, New York. 1991, 179-197.
7 Huang Y, Wang J, Li G, Zheng Z, SU W. Antitumor and antifungal activities in endophytic fungi isolated from pharmaceutical plants. 2001, 163-167.
8 Dreyfuss M, Chapela I. Potential of fungi in the discovery of novel, low- molecular weight pharmaceuticals.1994; 26(5):49-80.
9 Schulz B, Boyle C, Draeger S, Römmert A, Krohn K. Endophytic fungi: a source of novel biologically active secondary metabolites. 2002; 106(9):996-1004.
10 Warris HM, Wang H, Ahmad S, Alam K. Taxonomic studies of trees of the cholistan desert, Pakistan Journal of Biodiversity and Environmental Science. 2015; 6(1):1-8.
11 Chopra RN, Nayar SC, Chopra IC. Glossary of Indian Medicinal Plants, Council of Industrial and Scientific Research, New Delhi, 1986.
12 Sharma BM, Rathore SS. Compatibility studies on Acacia tortilis and *Ziziphus rotundifolia*, Indian Forester. 1994; 120(5):423-429.
13 Pavithra N, Sathish L, Ananda K. Antimicrobial and enzyme activity of endophytic fungi Journal of pharmaceutical and biomedical sciences. 2012; 16(16):1-6.
14 Nameirakpam Nirjanta Devi1 J, John P, Femina W. Asian Pacific Journal of Tropical Biomedicine Phytochemical analysis and enzyme analysis of endophytic fungi from Centella asiatica. 2012; 1280-1284.
15 Adaskaveg JE, Gilberton RL, Dunlap MR. Effects of incubation time and temperature on in vitro selective delignification of silver leaf oak by Ganoderma colossum. Applied and Environmental Microbiology. 1995; 61:138-144.
16 Ong HC, Nordiana J. Malay herbal medicine in Gemencheh, Negri Sembilin, Malaysia. Fitoterapia.1998; 70:10-14.
17 Ong HC, Nordiana J. Malay ethno-medico botany in Machang, Kelantan, Malaysia. Fitoterapia. 1999; 70:503-513
18 Brett CT. Cell wall degradation. In: Physiology and Biochemistry of Plant Cell Walls (eds. C.T. Brett and K. Waldron). Unwin, Hyman, London. 1990, 169-179.
19 Reddy OVS, Basappa SC. Preparation of sweet potato flour and its fermentation to ethanol. Journal of Food Science and Technology. 1997; 34:108-112.
20 Pointing SP. Qualitative methods for the determination of lignocellolytic enzyme production by tropical fungi. Fungal Diversity. 1999; 2:17-33.
21 Bhagobaty RK, Joshi SR. Enzymatic Activity of Fungi Endophytic on Five Medicinal Plant Species of the Pristine Sacred Forests of Meghalaya, India Biotechnology and Bioprocess Engineering. 2012; 17:33-40.
22 Nameirakpam Nirjanta Devi1 J, John P, Femina W. Phytochemical analysis and enzyme analysis of endophytic fungi from Centella asiatica. Asian Pacific Journal of Tropical Biomedicine. 2012, 1280-1284.