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STUDIES ON EXTRACELLULAR ENZYMATIC ACTIVITIES OF ENDOPHYTIC FUNGI ISOLATED FROM *PHYLLANTHUS AMARUS* AND *ADHATODA ZEYLANICA*

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ABSTRACT
Endophytic microorganisms are those organisms that dwell within intercellular spaces, tissue cavities or vascular bundles without harming the host plants and often benefit the host. Endophytic fungi are also the store house of naturally occurring bioactive compounds. The endophytic fungi associated with medicinal plants are diverse and potential to produce many bioactive substances including extracellular enzymes. So in this work endophytic fungi were subjected for extracellular enzyme production. The purpose of this investigation was to detect the capability of isolated endophytic strains to produce extracellular hydrolytic enzymes. In present investigation, a total of 12 endophytic fungi representing 10 different fungal species were isolated from roots, stems and leaves of two medicinal plants namely *Phyllanthus amarus* and *Adhatoda zeylanica*. Seven endophytic fungi, out of 12 strains isolated from medicinal plants were screened qualitatively for production of extracellular enzymes such as amylase, lipase, cellulase, pectinase and protease. All strains showed positive result for lipase activity while only some fungal strains showed positive result for amylase, cellulase, pectinase and protease. These isolates indicated promising potential for deployment in biotechnological processes involving production of amylases, cellulases, pectinases, proteases and lipases.

KEYWORDS: Medicinal plants, Endophytic Fungi, Extracellular Enzymatic Activity.

INTRODUCTION

Endophytic fungal communities are microfungi that internally infect living plant tissues without causing any visible manifestation of disease and live in mutualistic association with plants for atleast a part of their life cycle. One or more endophytic organisms are found in nearly every land plant (Strobel *et al.*, 2003). Endophytes have also been coined for their protective role to the host plant during biotic and abiotic stress conditions (Leitao & Enguita, 2016). Medicinal plants as well as their endophytes have been proven as an important source of precious bioactive compounds and secondary metabolites that contribute to more than 80% of the natural drugs available in the market (Singh & Dubey, 2015). Therefore, endophytic microorganisms are being investigated as sources for novel secondary metabolites and as playing functional roles in phytoremediation and adaptation to climate and other agricultural changes (Porras-Alfaro and Bayman 2011). The endophytic fungi improve the resistance of host plants by secretion of different bioactive agents. These metabolites have unique structure such as alkaloids, benzopyranones, chinones, flavonoids, phenolic acids, tetralones, xanthones, quinones, terpenoids and steroids. Endophytes have been reported to produce a number of bioactive metabolites in a single plant or microbe which served as an excellent source of drugs for treatment against various diseases and with potential applications in agriculture, medicine, food and cosmetics industries (Strobel and Daisy, 2003; Jalgaonwala *et al*. (a), 2011; Godstime *et al*., 2014; Shukla *et al*., 2014). The novel natural compounds produced by endophytes have been shown to have important medical applications such as antimicrobial, antiparasitic, cytotoxic, neuroprotective, antioxidant, insulin mimetic and immunosuppressant properties. Endophytes have been shown to have the ability to degrade plastics (Russell *et al.*, 2011; Abdel-Motaal *et al*., 2014). Endophytes also produce planthormones and other bioactive compounds of biotechnological interest such as enzymes and pharmaceutical drugs (Joseph & Priya, 2011; Parthasarathi *et al*., 2012). Extracellular enzymes are the product of microbial cell growth and perform its function outside the cell in many biological or environmental processes (Khan *et al*., 2017).

Currently, fungal endophytes have been explored for diverse applications owning to their production of extracellular enzymes (Pavithra *et al*., 2012). They produce extracellular hydrolases as a resistant agent against pathogenic attack, and these enzymes are lipases, pectinases, proteinases, xylanase, laccase, cellulases, phosphotases. The enzymes function so as to obtain nutrition from their host, hydrolyze food substances and are involved in eliciting defence mechanisms against pathogens (Desire *et al.*, 2014). The extracellular enzymes produced by endophytic fungi are playing importance role in textile, food industry, leather, confectionery, agriculture

and human health and beverages because of their stability at different extreme conditions such as high temperature and pH (Benjamin and Pandey, 1998).

Endophytic fungi are common sources of commercial enzymes due to their high production capability, low cost susceptibility to genetic manipulation, high biotechnological interest, pharmaceutical products and medical therapy (Rao *et al*., 1998). Filamentous fungi are also considered to be an interesting one for the production of enzymes due to their easier cultivation process and large scale production of enzymes for industrial objectives as compared to all nonpathogenic micro-organisms. Enzymes derived from endophytes are more stable than enzyme derived from other sources (Sunita *et al*., 2013). Enzymes play key roles in numerous biotechnology products and processes that are commonly encountered in the production of food and beverages, cleaning supplies, clothing, paper products, transportation fuels, pharmaceuticals and monitoring devices. Amylase is an enzyme that catalyses the breakdown of starch into sugars. Amylase is widely used in industries and has nearly 25% of the enzyme market (Rao *et al*., 1998). The amylase obtained from microorganisms has a broad spectrum of industrial uses as they are more stable than plant and animal amylase. The major advantage of using microorganisms for the production of amylases is the economical bulk production capacity and also microbes are easy to manipulate to derive enzymes of ranging from food, fermentation, textile to paper industries (Pandey *et al.*, 2000). Amylases have a wide range of application in various industries such as in the food (Maarel *et al*., 2002), bread making, paper industries (Maarel *et al*., 2002), textiles (Ahlawat *et al*., 2009), sweeteners, glucose and fructose syrups, fruit juices, detergents (Mitidieri *et al*., 2006), fuel ethanol from starches, alcoholic beverages, digestive aids, spot remover in dry cleaning and in medicine (Das *et al*., 2011).

Like this, lipases serve important roles in human practices as ancient as yogurt and cheese fermentation. High enzyme activity lipase can replace traditional catalyst in processing biodiesel; this enzyme is more environmental and safe. Lipase enzyme is used in textile industry, detergent industry, food industry, diagnosis, in medical applications, cosmetics, biosensor and biodegradation. It is indicated that cholesterol can be utilized as a energy source (Maria *et al*., 2005). Microbial cellulases have shown their potential application in various industries including paper and pulp, textile, laundry, biofuel production, feed industry, food, brewing and agriculture (Kuhad *et al*., 2011). Proteases remain the dominant enzyme type, because of their extensive use in the detergent and dairy products. Various carbohydrases, primarily amylases and cellulases used in industries such as the starch, textile, detergent and baking industries represent second largest group (Underkofler *et al*., 1957).

Pectinases have become one of the upcoming enzymes of fruit and textile industries. Application of pectinases has been reported in the textile industry for the retting and degumming of fiber crops, production of good quality paper, fermentation of coffee and tea, oil extractions and treatment of pectic waste water (Kashyap *et al*., 2001). Various enzymes, specifically microbial proteases are the most essentially used in different corporate sectors such as textile, detergent, leather, feed, waste and others (Razzaq *et al*., 2019).The protease enzyme is also used to produce a therapeutic agent against deadly diseases, such as cancer and AIDS (Rawlings *et al*., 2004). With the growing population and raising need enzymes seem to be one of the most vital molecules that have a great impact in every sector that may dairy, industrial, agriculture or medicine. The current investigation was carried out in finding novel sources of treasured extracellular enzymes from endophytic fungi and understanding their functional role in the host plants.

MATERIALS AND METHODS

A. Sources of Endophytic Fungi

In the present study, endophytic fungi were isolated from fresh and healthy tissues of two medicinal plants identified as *Phyllanthus amarus* and *Adhatoda zeylanica* collected from reserve forest area of Katni District, Madhya Pradesh. The medicinal plants selected for the present investigation were collected from their natural habitats. The plants were identified by SFRI (State Forest Research Institute) Jabalpur

B. Sample Processing for Isolation of Endophytic Fungi

Endophytic isolation was carried out under aseptic conditions. Different symptomless parts of the selected ethano-medicinal plants such as stems, leaves and roots were used for the isolation of endophytes. All selected parts were washed thoroughly in running tap water followed by double distilled water to remove the dust and debris before processing. All plant samples were then thoroughly washed with mild detergent and running tap water and air dried. The collected plant materials were first surface sterilized following the method of Santos *et al.* (2003) with few modifications. Surface sterilization was performed by sequentially rinsing the plant materials with 70% ethanol (C_2H_5OH) for the 2 minutes, then further with 0.01% mercuric chloride ($HgCl_2$) for 3 minutes and finally with sterile distilled water for 2-3 times under laminar air flow. Plant materials were then dried in between the folds of sterile filter papers. After proper drying, the surface sterilized plant materials i.e. leaves were cut into smaller pieces and each piece was placed on different culture media supplemented with penicillin and streptomycin. Similarly, stems and roots were cut vertically into small segments to expose the inner surface and then inoculated on the culture media. Appropriate controls were also set up in which no plant tissues were inoculated. All the plates were incubated at 28^0C to promote the growth of endophytes and were regularly monitored for any microbial growth. On observing the microbial growth, sub culturing was done and the isolated fungi were preserved in PDA slants at 4^0C. The endophytic fungi were identified based on the cultural characteristics, morphology of the fruiting bodies and spores using standard manuals.

C. Screening of Fungal Extracellular Enzymes

The endophytic fungal isolates from selected plants were screened for enzyme production by plate assay method and were assessed by placing 5 mm mycelial plugs on solid media with substrates specific to the respective enzymes. Screening was carried out using qualitative method i.e. agar plate method. The potential of extracellular enzymes secreted by endophytic fungi was

analyzed by growing them on potato dextrose agar for about 6 to 7 days for incubation at 25^0C and placing them on different media used for enzyme activity. After incubation, the clear zones of enzyme activity surrounding the fungal colonies were measured using cm scale. Procedure used for the qualitative analysis of amylolytic, lipolytic ,cellulolytic, pectinolytic and proteolytic activity is as follows.

1. Estimation of Amylolytic Activity

For the amylase activity, The isolates were grown on starch agar medium (starch - 20.0 gm, peptone - 5.0 gm, beef extract - 3.0 gm, agar - 15.0 gm and d/w - 1000 ml, pH 6.0). After incubation at 28^0C for 3-4 days, the surface of the plates was flooded with 1% iodine solution with a dropper for 30 seconds. Starch in the presence of iodine produced a dark-blue colouration of the medium, and a yellow zone around a colony in an otherwise blue medium indicated amylolytic activity (Jalgaonwala, *et al.*,(b) 2011).

2. Estimation of Lipolytic Activity

For the lipase activity, the fungi were grown on peptone agar medium (peptone - 10 gm, NaCl - 5 gm, $Cacl_2.H_2O$ - 0.1gm, agar - 16 gm, d/w - 1000 ml, pH - 6.0) supplemented with tributyrin solution (1%). At the end of the incubation period for 4-5 days, a visible precipitate around the colony due to the formation of calcium salt of the lauric acid liberated by the enzyme indicated positive lipase activity.

3. Estimation of Cellulolytic Activity

Cellulolytic activity was assessed by growing the fungi on Czapek - mineral salt agar medium ($NaNO_3$ - 2.0gm, K_2HPO_4 or KH_2PO_4 - 1.0gm, $MgSO_4.7H_2O$ - 0.5gm, KCI - 0.5gm, Carboxymethyl cellulose - 5.0gm, Peptone - 2.0 gm, Agar - 20.0 gm and d/w - 1000 ml). After incubation, the plates were then flooded with 0.2% aqueous Congo red solution for 10 minutes and destained with 1M NaCl for 15 minutes. Formation of yellow clear zone around the fungal colony was the indication of positive cellulolytic activity of isolated fungi (Sunita *et al*., 2013 and Prabavathy *et al*., 2013).

4. Estimation of Pectinolytic Activity

Pectinolytic activity was examined by growing the isolated endophytes on the Hankin's medium $(NH_4)_2SO_4$ - 2gm, KH_2PO_4 - 4gm, Na_2HPO_4 - 6gm, $FeSO_4,7H_20$ - 0.2 gm, $CaCl_2$ - 1mg, H_3BO_3 - 10 mg, $MnSO_4$ - 10 mg, $ZnSO_4$ - 70 mg, MoO_3 - 10mg, Yeast extract - 1.0 g, Agar-15.0 gm, Pectin-5.0 gm and d/w - 500 ml. After incubation period, the plates were flooded with 1% aqueous solution of hexadecyltrimethylammonium bromide. A clear zone around the colonies in the plates was observed due to the precipitation of intact pectin by the hexadecyl trimethyl ammonium bromide indicating the degradation of pectin due to the secretion of extracellular pectinase enzyme.

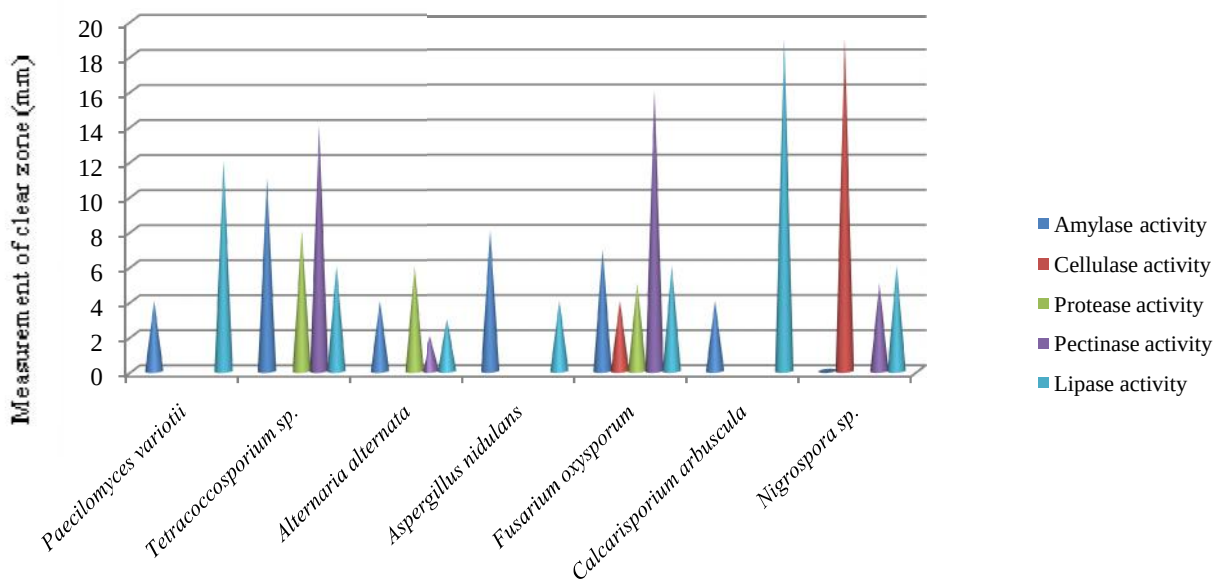
5. Estimation of Proteolytic Activity

Protease activity was assessed by growing the isolated endophytic fungi on the gelatin agar medium (Beef extract - 9gm, Peptone - 2gm, Gelatin - 48gm, NaCl - 2gm, Agar - 6 gm and d/w-500 ml) at pH 6.0. After incubation for the five days at 28^0C in the BOD incubator. The plates were then flooded with saturated 1% aqueous tricarboxyacetic acid which resulted in the formation of a precipitate. This made the agar opaque and developed a clear zone around the fungal colony.

RESULT AND DISCUSSION

A. Enzymatic Activity of Isolated Strains

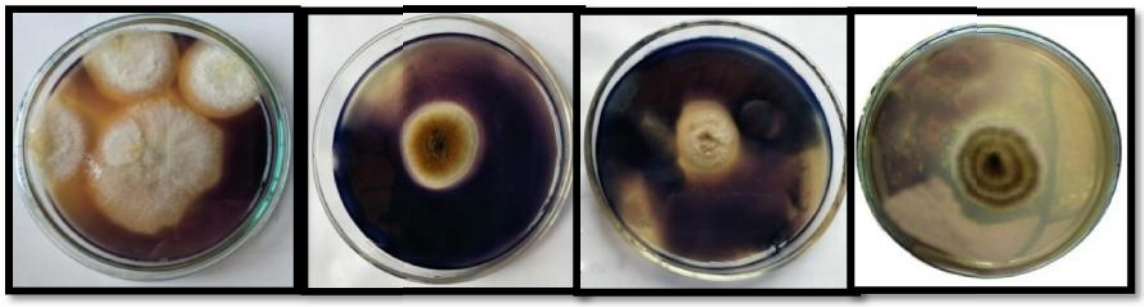
The fungal strains were isolated from two medicinal plants. All strains were subcultured and maintained at 4^0C. Each endophytic fungus was able to produce one or more extracellular enzymes. None of the strain was able to produce all five enzymes. In the present study, isolated strains were capable to produce hydrolytic enzymes. The seven strains of endophytic fungi tested were able to produce one or more extracellular enzymes (Graph 1).



GRAPH 1: Showing enzyme activities by various endophytic fungi

1. Amylolytic Activity

The seven isolates were screened for amylase production, out of which six endophytic fungi were able to degrade starch. The maximum production of amylase was from the *Tetracoccosporium* sp. (11 mm). *Aspergillus nidulans*(8mm) and *Fusarium oxysporum*(7mm) exhibited significant extracellular enzyme activity. However *Calcarisporium arbuscula*(4mm), *Paecilomyces variotii*(4mm) and *Alternaria alternata*(4mm) were weak producers of the enzyme. While *Nigrospora* sp. was unable to produce amylase enzyme (Fig. 1 and Table 1).



(A) *Fusarium* sp. (B) *Alternaria* sp. (C) *Tetracoccosporium* sp. (D) *Calcarisporium arbuscula*

FIGURE 1: Showing amylase activity of isolated endophytic fungi

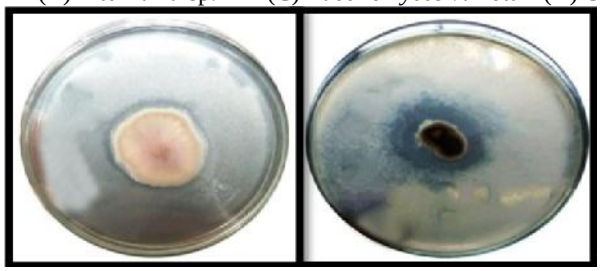
TABLE 1: Showing enzyme activity of isolated endophytic fungi

Enzyme activity	Amylase activity	Cellulase activity	Protease activity	Pectinase activity	Lipase activity
Isolates					
Paecilomyces variotii	4mm	-	-	-	12 mm
Tetracoccosporium sp.	11 mm	-	8 mm	14 mm	6 mm
Alternaria alternata	4 mm	-	6 mm	2mm	3 mm
Aspergillus nidulans	8mm	-	-	-	4 mm
Fusarium oxysporum	7mm	4 mm	5 mm	16 mm	6 mm
Calcarisporium arbuscula	4 mm	-	-	-	19 mm
Nigrospora sp.	-	19 mm	-	5 mm	6 mm

2. Lipolytic Activity

All seven endophytic fungi were able to produce lipase enzyme. In present study, *Calcarisporium arbuscula* (19mm) and *Paecilomyces variotii*(12mm) exhibited maximum lipase activity, *Tetracoccosporium* sp.(6mm), *Nigrospora* sp.(6mm) and *Fusarium oxysporum* (6mm) showed moderate activity, while *Aspergillus nidulans* (4mm) and *Alternaria alternata* (3mm) showed low lipase activity (Fig. 2 and Table 1).

(A)*Aspergillus nidulans* (B) *Alternaria* sp. (C) *Paecilomyces variotti* (D) *Calcarisporium arbuscula*



(E *Fusarium* sp. (F) *Tetracoccosporium* sp.

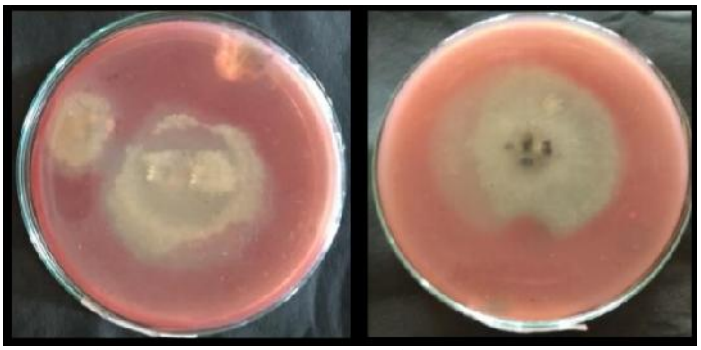
FIGURE 2: Showing lipase activity of isolated endophytic fungi

3. Cellulolytic Activity

Two isolates out of seven endophytic fungi were able to produce cellulase enzyme. In our study *Nigrospora* sp.(19mm) exhibited maximum cellulolytic activity, while *Fusarium oxysporum* (4mm) showed low cellulolytic activity. *Paecilomyces variotii, Tetracoccosporium* sp., *Aspergillus nidulans, Alternaria alternata* and *Calcarisporium arbuscula* were negative for cellulase activity (Fig. 3 and Table 1).

4. Pectinolytic Activity

Maximum pectinase activity was observed in *Fusarium oxysporum*(16mm) and *Tetracoccosporium* sp. (14mm). *Nigrospora* sp.(5mm) was the significant producer of pectinase enzyme. *Alternaria alternata* (2mm) also exhibited moderate pectinase activity, while *Paecilomyces variotii, Calcarisporium arbuscula* and *Aspergillus nidulans* were negative for pectinase activity (Fig.4 and Table 1).

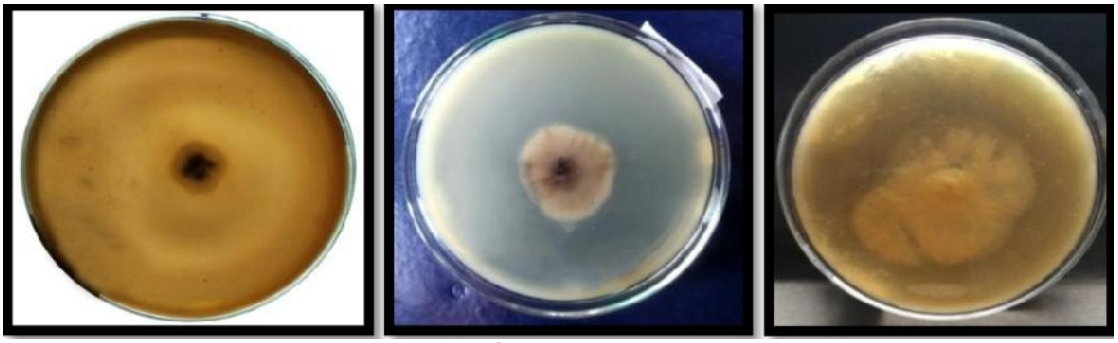


(A) *Fusarium* sp. (B) *Nigrospora* sp.

FIGURE 3: Showing Cellulase activity of isolated endophytic fungi

(A) *Tetracoccosporium* sp. (B) *Nigrospora* sp. (C) *Fusarium* sp. (D) *Alternaria* sp.

FIGURE 4: Showing pectinase activity of isolated endophytic fungi



(A) *Tetracoccosporium* sp. (B) *Alternaria* sp. (C) *Fusarium* sp.

FIGURE 5: Showing protease activity of isolated endophytic fungi

5. Proteolytic Activity

Among the tested organisms, maximum extracellular protease activity was observed in *Tetracoccosporium* sp. (8mm) and moderate activity in *Fusarium oxysporum*(5mm) and *Alternaria alternata* (6 mm), while other isolates like *Calcarisporium arbuscula, Paecilomyces variotii, Nigrospora* sp.and *Aspergillus nidulans* indicated negative result (Fig. 5 and Table 1).

The usefulness of endophytic fungi has been reported to industries by the production of various extracellular enzymes and bioactive agents (Toghueo *et al*., 2017). Apart from these, they are also being used as a biocontrol agents against pathogens. In previous studies, The endophytic fungi have been screened for their ability to produce ligninase, cellulase, lignocellulase, amylase, pectinase and xylanase by Choi *et al.* (2005). Uzma *et al*. (2016) screened fungal isolates for the production of extracellular enzymes, of which 29% were positive for amylase, 28% for cellulase, 18% for pectinase and 40% for asparaginase activity and mani *et al*. (2018) studied on

enzymatic and phytochemical analysis of endophytic fungi on *Aegle marmelos* from western ghats of tamil nadu. The representative potent stains *Curvularia australiensis* and *Alternaria citrimacularis* produced extracellular enzymes in different pH at different incubation period in a considerable range. Hence, this study explains the production and biological activity of enzymes in endophytic fungi. Alberto *et al*. (2016) worked on extracellular enzymatic profiles and taxonomic identification of endophytic fungi isolated from four plant species. In their study, several fungal strain from four plants namely *L. divaricata, T. elegans, S. saponaria* and *Saccharum* sp. were identified by ribosomal DNA typing and evaluated semi-quantitatively for their enzymatic properties, including amylase, cellulase, pectinase and protease activity. 62% of the isolates exhibited amylase, 93 % cellulase, 50% pectinase and 64% protease activity. Amirita *et al*. (2012) also reported that *Cladosporium cladosporioides, Curvularia brachyspora, C.verruciformis, Drechslera hawaiiensis, Colletotrichum carssipes, Colletotrichum falcatum, Colletotrichum gleosporioides, Lasiodiplodia theobromae, Nigrospora sphaerica, Phyllosticta* sp. and *Xylariales* sp. were tested for their ability to produce extracellular enzymes i.e. amylase ,cellulase, laccase, lipase and protease by the qualitative assays, majority of the endophytic fungi showed the positive results.

In our investigation, seven various endophytic fungi were screened qualitatively for the presence of extracellular enzymes such as amylase, cellulase, protease, pectinase and lipase. Out of seven test strains, six endophytic fungi showed amylase activity and two strains showed cellulase activity while four and three endophyitc fungi were positive for pectinase and protease activity respectively. All endophytic fungi were positive for lipase activity. This study reported that *Tetracoccosporium* sp. showed maximum amylolytic activity, pectinolytic activity and proteolytic activity out of all the isolates. The highest cellulolytic activity was demonstrated in *Nigrospora* sp. While *Paecilomyces variotii* was found to be maximum lipase producer. Plant is known to be a source of starch which can be utilized by endophytes after the plant host dies (Choi *et al*., 2005).The concept that amylase of fungal origin is more stable than bacterial amylase reported by Duochuan *et al*. (1997). Sunita *et al.*, 2013 also reported that cellulases, amylases and pectinases are major enzymes involved in plant polysaccharide degradation along with protease. It has been reported by Patil *et al*. (2015) that the strong enzymatic activities of the endophytic extracts show a high potential for clinical microbiology and therapeutic applications. Fungal enzymes are more stable than enzymes obtained from plants and animals. They are used in food processing industries, production of beverages, textile and leather industries (Maria *et al.*, 2005). There is a doubtless necessity of future scrutiny in these biomolecules which will later be remunerative for the humankind in their relevance.

CONCLUSION

The aim of present study is to identify the kind, sources of enzymes and perspectives for further investigation in their application in endophytic based extracellular enzymes resources. An additional resource are provided by endophytic microorganisms to host due to presence of nutrients, secondary metabolites and beneficial enzymes, which help the host plant to combat stressful conditions of biotic as well as abiotic exertions. Besides, the extracellular enzymes can be harnessed in fermentation industries where endophytes derived from plants living in extreme environmental conditions possess higher potential to produce higher quantities of extracellular enzymes. Thus, the extracellular enzymes produced by endophytic fungi are playing vibrant and potent role in textile, food industry, leather, confectionery, agriculture, ecology, biotechnology, industry, human health and beverages because of their stability at different extreme conditions. The concluding remark assumed the future profitable role of endophytic organisms by using new technology in different fields.

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