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Original Article

SCREENING OF ENDOPHYTIC FUNGI FOR THEIR ABILITY TO PRODUCE EXTRACELLULARCELLULASES

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ABSTRACT

Objective: Screening endophytic isolates from woody perennial medicinal plants of Western Ghats for production of extracellular cellulases

Methods: Endophytes were isolated using normal microbiological methods and their colonization frequency and dominance were calculated using statistical methods. Efficiency of growth on complex cellulosic substrates was evaluated on media supplemented with specific substrates. Enzyme assays with identified endophytic fungi were carried from their secretome.

Results: Forty endophytic fungal isolates were obtained using standard isolation methods from different medicinal plants from a biodiversity hotspot in the Western Ghats region of Karnataka, India. The isolated endophytic fungi were then identified based on their morphological characters. The percentage of dominant endophytes (*D*) was calculated based on the colonization frequency. Among the isolated fungi, colonization frequency of *F. solani and Talaromyces*sp. was found to be highest, at5.5 and 5.6 respectively. Each of the identified fungi was grown on (Carboxyl Methyl Cellulase) CMC and seven among the 40 isolates were found to grow luxuriantly as measured by radial growth. The identities of these fungi were morphologically re-confirmed and were completely carbon drained by growing them on a low nutrient medium. These fungi were later evaluated for their growth on avicel and microcrystalline cellulose. *Fusariumsolani* and *Talaromyces*sp. were significantly better in their growth when compared to other endophytes tested. Further, the cellulosome complex of enzymes was analysed in the secretome of *Fusariumsolani* and *Talaromyces*sp. Total filter paper activity of *Fusariumsolani* was found to be FPU/ml, 76 FPU/ml and 70 FPU/ml at 24, 48 and 72 h respectively. Similarly, Filter paper activity of *Talaromyces*sp. was found to be 89, 86 and 78 FPU/ml at 24h, 48h and 72h respectively. Endogluconase activity of *Fusariumsolani* was found to be 63CMCase, 60and 61CMCase at 24, 48 and 72hours of incubation respectively, which was greater than *Talaromyces*sp. Similarly, Exogluconase and Beta-glucosidase activities were also found to be high in *Talaromyces*sp. when compared to *Fusariumsolani* at all the time intervals tested.

Conclusion: The results from the present study reveals that *Fusariumsolani* and *Talaromyces*sp. are extremely potent producers of cellulases and can thus be used for eco-friendly and economic hydrolysis of biomass for biofuel purposes.

Keywords: Cellulases, Endophytes, Hydrolysis and enzyme assays.

INTRODUCTION

Biofuels are widely considered to be a major renewable energy source that has the advantage of mitigating climate warming [1-3]. Conversion of abundant lignocellulosic biomass to biofuels as transportation fuels presents a viable option for improving energy security and reducing greenhouse emissions [4, 5].

Biofuelsare cleaner-burning than fossil fuels, and the short cycle of growing plants and burning fuel made from them does not add CO_2 to the atmosphere. Lignocellulosic materials, such as agricultural residues (e. g., wheat straw, sugarcane bagasse, corn stover), forest products (hardwood and softwood), dedicated crops (switch grass, salix), *Misthanthussinesis* are extensively used for the production second generation of biofuel. Overall, the efficiency of biomass conversion to sugars and to fuel and chemicals is still a challenge and demands more research including the search for more efficient microbes, enzymes and pre-treatment methods. For the conversion of biomass to fuel, the cellulose and hemicelluloses must be broken down by hydrolysis into their corresponding sugar monomers. However, the primary obstacle in conversion of biomass to biethanol is deconstructing the complex cellulosic architecture which is recalcitrant to hydrolysis due to low availability of microcrystalline fiber and presence of lignin and hemicelluloses on the surface of cellulose. Therefore, pre-treatment of biomass is an absolute pre-requisite in order to achieve high yield and make the second generation of bioethanol cost competitive [6].

Among the methods available for hydrolysis of biomass in enzymatic method has the potential for high yields, and mild operation condition. Cellulose can be hydrolyzed by a class of enzymes called cellulases composing of endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91), exoglucohydrolases (EC 3.2.1.74), and β-glucosidases (EC 3.2.1.21). Cellulases allow the bioconversion of such energy biomass to biofuel and mitigate depends on fossil oil [4, 7].

Efficient enzymatic hydrolysis or saccharification is central to production of renewable biomass chemicals and fuel. Fungi are the primary sources of lignocellulose hydrolyzing enzymes. Endophytic fungi due to their unique life style interact biochemically with host tissues for survival of symbiotic relationship. Their physiological adaptation depends upon the ability of the organism to secrete hydrolytic enzymes like cellulases at the surface of the host tissue where they compete for substrates during the process of establishment of the symbiosis. The exploration of the biotechnological potential of woody perineal plants of Western Ghats, as a source for the discovery of new or improved enzymes and microorganisms for second-generation biofuel production, would have several simultaneous benefits. It could result in the increased protection and preservation of these ecosystems, leading to the maintenance of their ecological and biotechnological potential. Thus, screening for cellulolytic enzymes among the endophytic community may lead to the potential breakthrough in cellulose conversion in the form of highly specific enzymes [4].

In this context, the present work reports systematic screening of endophytic fungal isolates from woody perineal plants of Western Ghats that are able to utilize/hydrolysis complex cellulosic substrate and quantify the cellulases from the secretome of such endophytic fungi.

MATERIALS AND METHODS

Plant materials and study site

Plant parts such as stem, root, and leaves were collected from ten woody perineal plant species namely: *Tylophoraasthmatica*, *Rubiacordifolia*, *Plumbagozeylanica*, *Phyllanthusamarus*, *Eryngiumfoetidum*, *Centellaasiatica* , *Zingiber*sp., *Azadirachtaindica, Ocimumtenuiflorum and Alfalfa*. These plants were inhabiting the natural vegetation of the Koosalli falls near Byndoor of Kundapur taluk (13 °57′46 ″N-74 °40′58 ″E coordinates), Karnatakasituated in the Western Ghats. The plants were collected during the monsoon (July 2nd week) of 2013. The natural vegetation is a semi evergreen type of forest. The mean temperature was 23 °C and means annual precipitation is 3525 mm. The samples were placed in polyethylene bags, labelled, transported in ice box to the laboratory, and placed in a refrigerator at 4 °C until isolation. All samples were processed within 24 h of collection.

Isolation and identification of endophytic fungi

Samples were washed thoroughly in distilled water, blot dried, and first immersed in 70% ethanol (v/v) for one min followed by second immersion in sodium hypochlorite (3.5%, v/v) for three minutes. They were rinsed three times in changes of sterile distilled water and dried on sterile blotters under the airflow to ensure complete drying. Bits of 1.0 × 0.1 cm sizes were excised with the help of a sterile blade. A total of 90 segments from stem, roots and leaves of plant species were placed on (Potato dextrose agar) PDA (2.5%) supplemented with the antibiotic streptomycin sulphate (100 mg/l). The plates were wrapped in clean wrap film and incubated at 37 °C with 12h light and dark cycles for up to 6 to 8 w. The effectiveness of surface sterilization of tissues was checked by placing the aliquots of sterilants on (Potato dextrose agar) PDA plates and observing fungal colonies if any for two weeks. Periodically the bits were examined for the appearance of fungal colony and each colony that emerged from segments was transferred to antibiotic-free potato dextrose agar medium (PDA, 2%) to aid identification. The morphological identification of the isolates was done based on the fungal colony morphology and characteristics of the reproductive structures and spores (Watanabe, 2002). All fungal mounts were made on microscopic glass slides in lacto phenol cotton blue and sealed. For long-term storage, the colonies were preserved in the vegetative form in 15% (v/v) glycerol at-80 °C.

Calculation of colonization frequency and % dominance

Isolation rate (IR), the measure of fungal richness of a sample, was calculated as the number of isolates obtained from tissue segments, divided by the total number of segments, and expressed as fractions but not as percentages. The colonization frequency (CF), expressed as the percentage, was calculated according to (Nalini *et al.*2014) as follows:

$$\%CF = \frac{\text{Number of tissue segments colonized by a fungus}}{\text{Total number of tissue segments plated}} \times 100$$

.The percentage of dominant endophytes (*D*) was calculated based on the % CF divided by the total number of endophytes × 100.

Test for cellulose degradation on CMC

To test the efficiency of the identified endophytic fungi, they were cultured on PDA (Potato dextrose agar*)* media supplemented with 3% (Carboxy Methyl Cellulose) CMC. Endophytes cultured on PDA without CMC served as control. They were incubated for seven days and the efficiency of utilization of cellulose was quantified by measuring the zone of hydrolysis. The zone of hydrolysis was obtained using 1N Nacl and Congo red dye. *Trichodermareesei* isolated from the rhizosphere soil of *Azadirachtaindica* served as the positive control.

Growth on low nutrient media for draining carbon

Seven isolates which recorded zone of hydrolysis of more than 5 cm (Similar to that of the positive control *Trichodermareesei*) on the (Potato dextrose agar) *PDA* media supplemented with the (Carboxy Methyl Cellulose) CMC were selected for all future experiments. In order to the test the efficiency of utilization of complex cellulose substrates, the seven isolates were cultured on low carbon, nitrogen and MMN (Modified Melin Norkrans) agar media for seven days. *Trichodermareesei* was also cultured on MMN agar media as a positive control. The cultures were incubated at dark at 37 °c for seven days the radial growth was recorded from the leading edge of the colonies [7].

Utilization of complex cellulosic substrates by carbon drained endophytes

5 mm plugs from the leading edge of the seven endophytic fungi (carbon drained) cultured on low nutrients MMN agar media culture inoculated on MMN agar with the supplemented with 1% microcrystalline cellulose and avicel. This was done in order to test the ability for the endophytes to utilize complex cellulosic substrate as sole source of carbon. *Trichodermareesei* served as positive control. After 21 d of incubation at 20 ° c under dark condition, the radial growth was recorded [7].

Harvesting the secretome

Seven endophytic fungi along with the positive control were cultured on PDB (potato dextrose broth). 5 mm plugs of seven endophytic fungi were inoculated on PDB and incubated in the shaker at 25 °C at 150 rpm for 3days. Cell free medium was harvested at 24 h, 48hr and 72hr of incubation under aseptic condition for enzyme assay. *Trichodermareesei* served as positive control. The following enzyme assays were conducted according to Zhang *et al.* 2009 for the quantification of secreted cellulosome complex by endophytic fungi.

Cellulase assays

a) Total filter paper assay

Total filter paper assay was conducted according to the protocol of [8]

FPU was calculated according to the formula below and expressed as FPU/ml.

$$FPU = \frac{0.37}{EDR}$$

b) Beta glucosidase assay

Total beta glucosidase assasy was conducted according to the protocol of [8]

Beta Glucosidase assay was conducted using Cellobiase as the substrate and expressed in cellobiose Units/ml and were calculated according to the formula.

$$Cellobiase = \frac{0.0926}{EDR}$$

c) Endoglucanase assay using CMC/DNS method

Total endoglucanase assay was conducted according to the protocol of [8]

Endoglucanase assay was conducted using CMC as the substrate and expressed as Endoglucanase Units/ml.

$$CMCase = \frac{0.185}{EDR}$$

d) Exogluconase assay using avicel method

Total exoglucanase assay was conducted according to the protocol of [8]

Exogluconase assay was conducted using Avicel as the substrate and expressed in Exogluconase Units/ml. Enzyme activity was calculated according to formula.

$$Avicel = \frac{0.185}{EDR}$$

Statistical analysis

Differences in the extent of colonization of the samples were analysed by differences in the extent of colonization. The samples were analysed by un-variant analysis of variance (one-way ANOVA) and Tukey's honestly significant difference (HSD) as post hoc test using the statistical software SPSS16.0. Values are the mean±SE of radial growth in cm from three independent experiments for each of the plant and vegetative tissue tested. * symbol represents statistical significance, * P<0.05, **P<0.001 when compared with the un-

inoculated control. For, the enzymes assays the results are a mean of three separate independent repeats harvested from newly inoculated samples. The samples were analysed by uni-variant analysis of variance (one-way ANOVA) and Tukey's honestly significant difference (HSD) as post hoc test using the statistical software SPSS16.0. Values are the mean±SE of enzyme units. Letters represent statistical significance, [a]P<0.05, [b]P<0.001 when compared with the un-inoculated control.

RESULTS

Colonization frequency and % dominance of isolated endophytic fungi

A total of 40 isolates were obtained from 90 tissue fragments. All the 40 isolates were catalogued and preserved for future work. The extent of isolation varied from 80% on *Azaridicataindica* to 5.5% on *Tylophoraasthmatica*. The 40 isolates after identification could be grouped into 23 genera. The most dominant genera were *Fusariumsolani* and *Talaromyces*sp. *Cladosporium and Aspergillus* sp. were also isolated quite often and colonization frequency was found to be highest on the stem (80%) followed by root (17.5%) and leaves (2.5%). Among 23 genera identified *Talaromyces*sp. and *Fusariumsolani* were found to be highly dominant with the percentages 5.5% and 5.6% respectively. Among the identified fungal isolates 96% belonged to Ascomycota followed by Zygomycota3%. Few endophytes like *Aspergillus* sp. were isolated from most of the plants; Talaromycessp. and *Fusariumsolani* were isolated from the few plant species. A detailed account of colonization frequency is presented in (table 1).

Growth of the isolated endophytic fungi on soluble CMC (3%)

All the 40 endophytic fungi obtained in the study were cultured on PDA (Potato dextrose agar) media supplemented with 3% cellulose and radial growth was recorded after 7 d of incubation. Endophytic fungi demonstrated differential ability in terms of utilization of cellulose. Zone of hydrolysis of 5.9 and 5.7 cm was obtained for the *Talaromyces*sp. and *Fusariumsolani* respectively. *Trichodermareesei* used as positive control demonstrated a clearance zone of 5 cm. Other fungi recorded radial growth between 2.03 cm to 4.7 cm which was significantly lesser when compared to that of the control *Trichodermareesei* (table 2). Seven different fungal isolates which had radial growth of 5 cm and above (Similar to that of positive control) were selected for further analysis. The seven isolates were confirmed morphologically at Agarkar research institute, Pune and accession numbers were obtained.

Table 1: Colonization frequency and % dominance of the isolated endophytic fungi

Host plant	Endophyte isolated	Colonization frequency on vegetative tissue			% of dominance
		Stem	Root	Leaf	
Tylophoraasthmatica(W. and A.)	*Aspergillus terreus*	6.67±0.03*	-	-	2.04
	*Phomopsis*sp.	2.47±0.03*	-	-	1.2
	Chaetomiumglobosum	3.37±0.03*	-	-	1.6
	Aspergillus terreus	3.10±0.06*	-	-	1.54
	Cladosporiumherbarum	1.00±0.06*	-	-	1.31
RubiacordifoliaL	*Alternariaalternata*	8.37±0.03*	-	-	3.02
	*Talaromyces*sp.	5.87±0.03*		-	1.1
	Aspergillus ornatus	3.87±0.03*	-		2.15
PlumbagozeylanicaL.	*Aspergillus ornatus*	-	1.47±0.03*	-	2.0
	Talaromyces species	-	2.83±0.03*	-	.9
	Alternariaalternata		1.50±0.06*	-	0.5
Phyllanthusamarus(Schum. andThonn.)	*Cladosporiumherbarum*	2.50±0.06*	0.53±0.03*	-	0.020
	*Phomopsis*sp.	1.10±0.06*	1.47±0.07*	-	0.039
	Cladosporiumherbarum	1.87±0.03*	1.87±0.09*	2.10±0.06*	
EryngiumfoetidumL.	*Tricodermareessei*	2.13±0.09*	-	-	1.2
	Colletotrichumdematium	-	2.30±0.06*	-	1.3
	*Talaromyces*sp.	-	3.10±0.03*	-	1.0
CentellaasiaticaL	*Chaetomiumglobosum*	1.10±0.03*	-	-	2.0
Zingibersp.	*Aspergillus terreus*	2.80±0.06*	1.80±0.03*	-	2.0
	*Phomopsis*sp.	-	0.97±0.03*	1.87±0.03*	0.5
	Curvularialunata	-	1.10±0.03*	4.80±0.06*	2.3
Azadirachtaindica	*Non sporulatingdematiaceous form*	4.10±0.06*	3.80±0.06*	-	2.5
	Non sporulatingdematiaceous form	1.80±0.06*	1.03±0.03*	-	0.09
	*Talaromyces*sp.	7.70±0.06*	3.10±0.03*	--	2.6
	non sporulatingdematiaceous+other fungi	1.13±0.06*	3.80±0.06*	-	1.8
	*Phomopsis*sp.	2.76±0.06*	2.10±0.06*	-	1.0
	Pestalopsis	3.80±0.06*	1.13±0.06*	--	1.25
	Trichodermareesei	3.23±0.03*	1.83±0.03*	2.10±0.06*	1.34
	F. solani	3.10±0.06*	5.10±0.06*	-	3.2
	F. oxysporum	0.87±0.03*	1.03.±0.03*	-	0.03
	*Penicillium*sp.	2.03±0.03*	0.60±0.06*	1.80±0.06*	0.09
	F. solani	4.40±0.06*	3.10±0.06*	-	2.2
	Trichodermareesei	0.87±0.03*	1.03±0.03*	-	0.02
	Trichodermaharzianum	2.67±0.03*	4.37±0.03*	-	2.4
	Trichodermareesei	3.80±0.06*	1.10±0.06*	-	1.9
Ocimumtenuiflorum	*F. oxysporum*	1.87±0.03*		-	0.02
	*Penicillium*sp.	-	1.70±0.06*	-	0.01
	Aspergillus terreus	-	2.77±0.06*	-	0.09
Alfalfa	*F. solani*	2.80±0.06*	-	-	0.08
	F. solani	-	2.13±0.09*	-	0.07
	Trichodermareesei	-	2.70±0.06*	-	0.9

Table 2: Growth of the isolated endohytic fungi on soluble CMC (3%)

Endophyte isolated	Zone of Hydrolysis on soluble CMC (in cms)
Trichodermareesei (Control)	4.67±0.15
Aspergillus terreus	4.30±0.06
Alternariaalternata	4.07±0.03
Aspergillus ornatus	5.17±0.03
Cladosporiumherbarum	3.07±0.22
Colletotrichumdematium	3.10±0.10
Chaetomiumglobosum	3.93±0.07
Curvularialunata	4.13±0.07
Curvalariaprotureberete	3.83±0.07
F. oxysporum	4.53±0.07
F. solani	5.67±0.03
F. pseudonygamai	3.67±0.13
F. ciliata	2.03±0.07
*Gelasinospora*sp.	2.47±0.03
Non sporulating dematiaceous form	4.93±0.07
Non sporulating dematiaceous form	4.87±0.09
Talaromyces species	5.83±0.07
Non sporulating dematiaceous+other form	4.97±0.03
*Phomopsis*sp.	5.07±0.03
*Penicillium*sp.	4.07±0.03
Pestalopsis	3.90±0.10
Rhizopusoryzae	3.20±0.10
Trichodermaharzianum	4.13±0.07

Values are the mean±SE of radial growth in cm from three independent experiments for each of the plant and vegetative tissue tested. * Symbol represents statistical significance, * P<0.05, **P<0.001 when compared with the un-inoculated control.

Growth of the selected endophytes on low carbon media (Carbon Drainage)

Seven fungal isolates which showed radial growth similar to that of the positive control were cultured on low carbon, nitrogen and MMN agar media in order to drain their carbon sink for seven days at 20 ° C. It was found that the radial growth on the low nutrient medium had significantly reduced for all the fungi tested including the positive control.

However, Talaromycessp. and *Fusariumsolani* showed the highest growth at 1.4 and 1.2 cm respectively which was still lesser than that of the control(table **3)**. The decrease in biomass could be therefore attributed to absence of carbon and nitrogen source.

Table 3: Growth of the selected endophytic fungi on low carbon/nitrogen media

Endophytes	Radial growth in (cm)
Non sporulating dematiaceous form	0.87±0.03
Non sporulating dematiaceous form	0.57±0.09
*Talaromyces*sp.	1.30±0.06
Non sporulating dematiaceous+other form	0.60±0.17
Aspergillus ornatus	0.30±0.06
*Phomopsis*sp.	0.50±0.60
Fusariumsolani	1.10±0.06
Trichodermareesei (Control)	1.83±0.12

Values are the mean±SE of radial growth in cm from three independent experiments for each of the plant and vegetative tissue tested. * Symbol represents statistical significance, * P<0.05, **P<0.001 when compared with the un-inoculated control.

Growth of endophytic fungi on avicel and microcrystalline cellulose

All the seven isolates, including positive controls which were carbon drained were later grown on media containing 1% Avicel and microcrystalline cellulose. Since the endophytic fungi were devoid of any internal carbon source, they showed little growth in the first week of the incubation. There was a very marginal grow in the second week followed by substantial increase in the growth after21 d of incubation. The endophytic fungi demonstrated differential ability of growth on avicel and microcryatalline cellulose. Higher growth was found on microcrystalline cellulose when compared to Avicel. *Trichodermareesei* recorded growth of 4.4 and 4.8 cm on avicel and microcrystalline cellulose respectively followed by *Fusariumsolani* and *Talaromyces*sp. which recorded as 5.5 and 5.6 cm respectively. Other fungi were found to have very little radial growth. Similarly, *Fusariumsolani, Talaromyces* sp. recorded growth of 3.93, 5.60 and 4.30. 5.70 on avicel and mycorystalline cellulose respectively. Similar trend was also followed by *Phomopsis* sp. and *Aspergillus ornatus* though the radial growth was lesser than that of the positive control (table 4).

Table 4: Growth on endophytic fungi on avicel and microcrystalline cellulose

Endophytes	Avicel 1%	Microcrystalline Cellulose 1%
Non sporulating dematiaceous form	2.90±0.06	4.77±0.09
Non sporulating dematiaceous form	3.47±0.15	4.63±0.12
Talaromyces species	4.30±0.06	5.70±0.06
Non sporulating dematiaceous+other form	3.77±0.30	4.87±0.09
Aspergillus ornatus	1.87±0.20	4.40±0.21
Phomopsis species	2.90±0.06	4.70±0.12
Fusariumsolani	3.93±0.07	5.60±0.06
Trichodermareesei	3.73±0.12	4.60±0.12

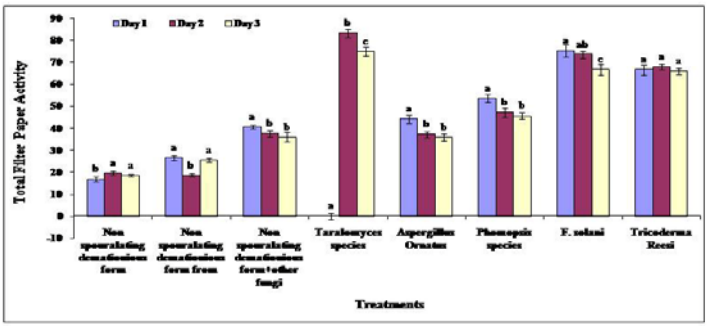

Fig. 1: Total filter paper activity of seven selected endophytic fungi at 24, 48 and 72 h

The results are a mean of three separate independent repeats harvested from newly inoculated samples. Values are the mean±SE of enzyme units. Letters represents statistical significance, [a]P<0.05, [b]P<0.001 when compared with the un-inoculated control.

Cellulase assays

Total filter paper assay

Filter paper assay (FPA) was carried out with the secretome of the seven selected endophytic fungi at 24, 48 and 72 h. It was found their enzymatic activity was highest at 24 h compared to 48 and 72 h of incubation. The total filter Paper activity of *Talaromyces*sp. was found to be highest at 89, 86 and 78 units/ml after 24, 48 and 72 h followed by *Fusariumsolani* at 70, 76 and 60 units/ml at the time intervals mentioned above. Both the fungi recorded FPA activities above that of the positive control. *Phomopsis*sp. and *Aspergillus ornatus* were found to have activities less than that of the control as well as the fungi mentioned above (fig. 1).

Beta glucosidase assay

Beta glucosidase assay was carried out with the secretome of the seven selected endophytic fungi at 24, 48 and 72 h. It was found their enzymatic activity was highest at 24 h compared to 48 and 72 h of incubation. The Beta glucosidase assay of *Talaromyces*sp. was found to be highest at 236, 234 and 228 units/ml after 24, 48 and 72 h followed by *Fusariumsolani*at 197.3, 187.6 and 194.6 units/ml at the time intervals mentioned above. Both the fungi recorded Beta-glucosidase activities above that of the positive control. *Phomopsis*sp. and *Aspergillus ornatus* were found to have activities less than that of the control as well as the fungi mentioned above (fig. 2).

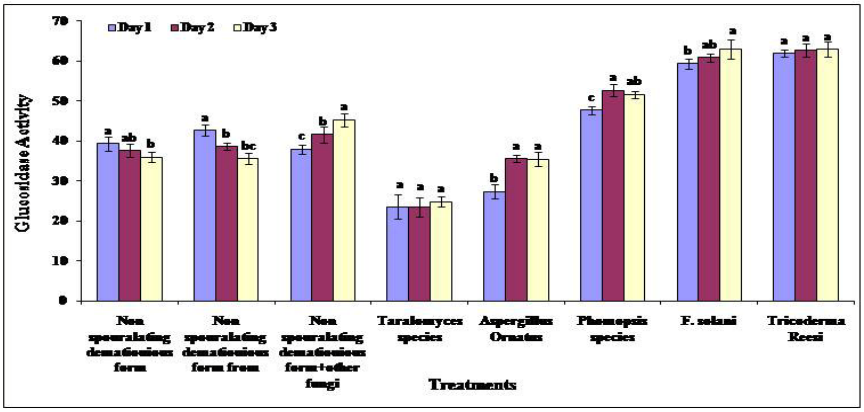

Fig. 2: Beta glucosidase assay activity of seven selected endophytic fungi at 24, 48 and 72h

The results are a mean of three separate independent repeats harvested from newly inoculated samples. Values are the mean±SE of enzyme units. Letters represents statistical significance, [a]P<0.05, [b]P<0.001 when compared with the un-inoculated control.

Endoglucanase assay

Endoglucanase assay was carried out with the secretome of the seven selected endophytic fungi at 24, 48 and 72 h. It was found their enzymatic activity was highest at 24 h compared to 48 and 72 h of incubation. The Endoglucanase assay activity of *Talaromyces*sp. was found to be highest at 57.6, 56.0 and 53.0 units/ml after 24, 48 and 72 h followed by *Fusariumsolani* at 51.3, 57.6 and 56.6 units/ml at the time intervals mentioned above. Both the fungi recorded endoglucanase activities above that of the positive control. *Phomopsis*sp. and *Aspergillus ornatus* were found to have activities less than that of the control as well as the fungi mentioned above (fig. 3).

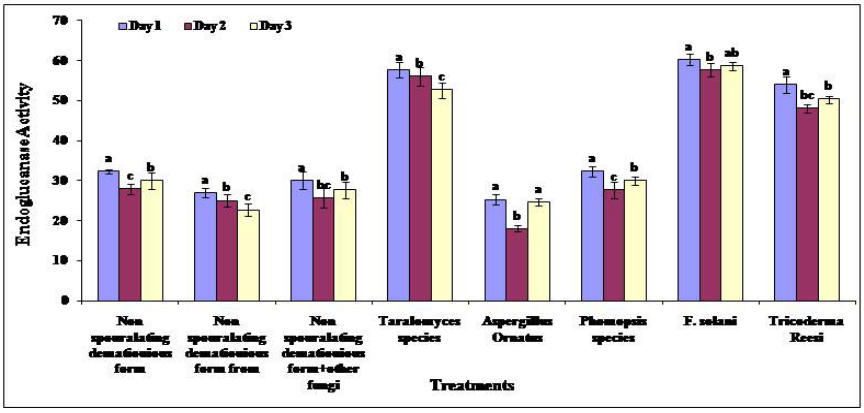

Fig. 3: Endoglucanase assay activity of seven selected endophytic fungi at 24, 48 and 72h

The results are a mean of three separate independent repeats harvested from newly inoculated samples. Values are the mean±SE of enzyme units. Letters represents statistical significance, [a]P<0.05, [b]P<0.001 when compared with the un-inoculated control.

Exoglucanase assay

Exoglucanase assay was carried out with the secretome of the seven selected endophytic fungi at 24, 48 and 72 h. It was found their enzymatic activity was highest at 24 h compared to 48 and 72 h of incubation. The Exoglucanase assay activity of Talaromycessp. was found to be highest at 47.6, 42.6 and 43.3 units/ml after 24, 48 and 72 h followed by *Fusariumsolani* at 52.6, 51.3 and 47.6 units/ml at the time intervals mentioned above. Both the fungi recorded Exoglucanase activities above that of the positive control. *Phomopsis*sp. and *Aspergillus ornatus* were found to have activities less than that of the control as well as the fungi mentioned above (fig. 4).

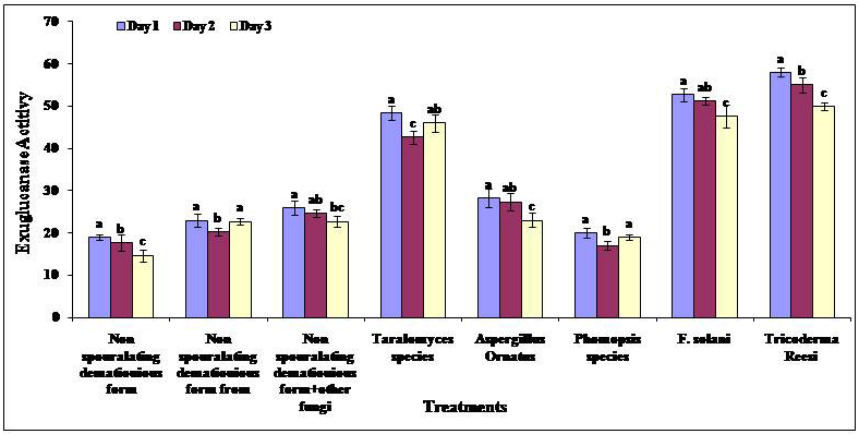

Fig. 4: Exoglucanase assay activity of seven selected endophytic fungi at 24, 48 and 72h

The results are a mean of three separate independent repeats harvested from newly inoculated samples. Values are the mean±SE of enzyme units. Letters represents statistical significance, [a]P<0.05, [b]P<0.001 when compared with the un-inoculated control.

DISCUSSION

Woody perineal plants are considered as a repository of "endophytic microbes" living in the internal tissues of the plant. The quest for identifying novel bio-actives from the endophytic fungi has resulted in the sampling of host plants such as herbs, shrubs, tree species, and vines in unique places of ecological adaptations around the rainforests of the world. Such niches harbour great species diversity, un-intervened by human activities. Efforts in this direction to sample plants located in the rainforests around the world with potential ethno medicinal values have resulted in the isolation of fungal endophytes, unique to a particular plant species with distinct bioactivity [9].

Many studies have identified endophytes isolated from neotrophic hot spots like Western Ghats. Nalini *et al.* (2014) have isolatedone thousand five hundred and twenty-nine fungal isolates from 5200 fragments. Stem fragments harbored more endophytes (80.37%) than roots (19.22%). 31 fungal taxa comprised of coelomycetes (65%), hyphomycetes (32%), and ascomycetes (3%). *Fusarium, Acremonium, Colletotrichum, Chaetomium, Myrothecium, Phomopsis,* and *Pestalotiopsis* sp. were commonly isolated. They also showed that Diversity indices differed significantly between the seasons and also between various vegetative tissues tested. In the present study, also differences in the colonization frequencies of endophytes on various host tissues were observed (table 1). This could be because the isolation was done during the monsoon season the slimy conidia of fungal spores are dispersed better by rain splashes and germination of conidia is influenced by climatic factors.

Fungi are the primary microorganisms that break down plant biomass in nature where they occur as saprophytes colonizing plant biomass in soil. Enzyme complexes produced by endophytes can help to create economically sustainable non-food based energy sources. Endophytes display a vast diversity in the range of substrates that can be utilized. Previously studies have shown that endophytes are capable of metabolizing *in vitro* most substrates found in plants and produce enzymes including proteases, amylases, phenol oxidases, lipases, laccases, polyphenol oxidases, cellulases, mannanases, xylanases, and pectin lyase [10]. It can be hypothesized that due to their ecologically unique lifestyle, endophytes might have evolved to possess the necessary enzymatic machinery to breakdown and utilize lignocellulosic biomass *in situ*. In our study, we have isolated 40 different endophytic fungal strains with differential ability to utilise complex cellulosic substrates like soluble CMC, avicel and microcrystalline cellulose (table 2, 3 and 4). We have used an extensive screening method where the isolated endophytic fungi were grown on CMC followed by carbon drainage and were finally tested for their ability to utilise complex cellulosic substrates like avicel and microcrystalline cellulose. Our results reveal that among the fungi tested *Fusariumsolani* and *Talaromyces*sp. recorded significantly higher growth on both avicel and microcrystalline cellulose when compared with the positive control *Trichodermareesei* (table 2, 3 and 4). From present data it was found that, endophytic fungal isolates when grown on different substrates of complexity, cellulolytic the growth pattern was found to differ. Among them, *Taralomyces*sp. and *Fusariumsolani* isolates, regardless of their origin, have broadly similar patterns of growth. Several other endophytic fungi reported in this study to grow on substrates like avicel and microcrystalline cellulose have been previously reported to utilise cellulosic susbtates. The endophytic fungi that have been reported to be cellulase producers include *Alternaria alternate Hymenoscyphusericae* and *Aspergillus terreus* [11].

Endophytic fungi produce many enzyme complexes and bioactive molecules in response to the various ecological stimuli which results in a biochemical and molecular regulation of such responses. [6,4]. The use of these enzymes must be related to the mutualistic relationship with the host plant.

In this study, the total filters Paper activity of Talaromycessp. was found to be highest at 89, 86 and 78 units/ml after 24, 48 and 72 h followed by *Fusariumsolani* at 70, 76 and 60 units/ml at the time intervals mentioned above. Both the fungi recorded FPA activities above that of the positive control. *Phomopsis*sp. and *Aspergillus ornatus* were found to have activities less than that of the control as well as the fungi mentioned above (fig. 1). The results obtained are in line with the study conducted by [12] who showed that FPA activities were higher for *Fusariumsolani* and *Taralomyces*sp. In another study by [13] total FPA activities were determined for endophytic fungi namely *Sclerotinia* sp., *Rhizoctoniasolani, Fusarium* sp., *Penicillium*sp. and *Pythiumultimum;* These fungi not only produced various cellulase enzymes but also produced measurable growth on all carbon sources tested as was the case in the present study.

In addition to total FPA activity *Fusariumsolani* and *Talaromyces*sp. was also found to produce considerable amounts of beta-glucosidase, endoglucanse and exoglucanase when compared to the control *Trichodermareesei* (fig. 2, 3 and 4). The concentrations of the enzymes produced were found to be highest at 24 h which decreased after 48 and 72 h respectively. All the seven endophyticfungi examined were also found to produce extracellular cellulases. However, the concentrations of the enzymes were significantly lower than that of *Fusariumsolani* and *Talaromyces*sp. (fig. 2, 3 and 4).

It has been previously reported that thermphilic fungi like *Talaromycessp* produce a complete cellulose system. Talaromyces cellulolyticus strain Y-94 (CBS 136886, FERM BP-5826), which was isolated in Japan, is one of several promising filamentous fungi for the industrial production of cellulase and hemicellulase to hydrolyze lignocellulosic biomass [14]. In the study, the authors selected strains from the *Acremonium* endophyte species for cellulases production and quantified betaglucosidase, endoglucanase and exoglucanase.

Degradation of cellulose in fungi is facilitated by the synergistic action of endo-acting endoglucanase, exoglucanse and β-1,4 glucosidase activities [12]. These enzyme activities have been previously demonstrated for *C. cellulolyticum* and *C. globosum* [15-17) However, compared to these reports, the FPA, exoglucanase, β-glucosidase and endogluconase activities determined for the isolate in this study were comparatively higher for both *Fusariumsolani* and *Talaromyces*sp. [5] in their paper report endophytic cellulase production by strain FS22A increased from day 2reaching maximum on day 6 and thereafter enzyme production declined.

Whereas FS5A displayed early maximum cellulase production on day 2 and levels of cellulase were only slightly variable between days 2 and 10. Dilution of the enzymes prior to assay revealed that more enzymes are present in the cell-free culture supernatant than required to react with the substrates. This is an indication that lesser enzyme can be used or more substrates can be added to the reaction to maximize saccharide yield.

Similarly, in our study also the celllulase enzyme activities also showed a decreasing trend after 24 h and reached saturation at 72h. This phenomenon was true for all the fungi tested. The decrease could be due to the fact that many fungi repress their cellulolytic and hemicellulolytic systems in the presence of low molecular weight carbon sources such as those found in photosynthate. Among the three enzymes tested beta-glucosidase was found to have the lowest concentrations for all the endophytic fungi tested. It has been proposed that the reason for the low secretion of β-glucosidase is that the major part of this enzyme is tightly bound to the cell wall of the fungus during culturing, and some parts of the enzyme may be found inside the cell [18]. Another reason for the low activity of cellulases is that the cellobiohydrolase does not act synergistically with endoglucanase to hydrolyze crystalline cellulose and produce cellobiose, which β-glucosidase transforms into sugars [18].

Variability in secretion of cellulases by the endophytic fungi tested in this suggests that there is still significant variation among isolates which may reveal better candidates. Since, both *Fusariumsolani* and *Talaromyces*sp. are hypervariable; further studies on the secretomes of these two fungi using proteomics will be the obvious future step in order to understand the dynamic nature of the cellulose production. However, these two fungi could be ideal for future exploitation in biofuel production.

CONCLUSION

Fusariumsolani and *Talaromyces*sp. isolated in this study are an excellent source for extracellular cellulases based on the data from the extensive screen used in this study. These fungi can be used for economical, eco-friendly and efficient deconstruction of lignocellulosic biomass for production of biofuels.

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CONFLICT OF INTERESTS

Declare None

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