

## Full Length Research Paper

# Diversity and extracellular enzymes of endophytic fungi associated with *Cymbidium aloifolium* L.

Shubha J. and Srinivas C.\*

Fungal Metabolites Research Laboratory, Department of Microbiology and Biotechnology, Bangalore University, Jnana Bharathi Campus Bangalore, Karnataka, India.

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*Cymbidium aloifolium* is an epiphytic orchid used in treatment of many human ailments. The endophytic fungi associated with orchids are diverse and have potential to produce many bioactive compounds including extracellular enzymes. A total of 165 endophytic fungi representing 22 different fungal species were obtained from root, leaf and flowers of *C. aloifolium*. The colonization rate (CR) and isolation rate (IR) varied with different plant parts and was highest in root (CR = 40.6%, IR = 0.83) followed by leaf (CR = 32.12%, IR = 0.66) and flower (CR = 27.27%, IR = 0.56). The diversity of isolated endophytic fungi in root, leaf and flower was determined; Shannon-Wiener index ( $H'$ ) was highest in root ( $H' = 2.64$ ) followed by leaf ( $H' = 2.12$ ) and flower ( $H' = 1.5$ ). Simpson diversity index ( $D'$ ) was high in root ( $D' = 0.93$ ) with a maximum of 16 species, followed by leaf ( $D' = 0.88$ ) with 9 species and flower ( $D' = 0.78$ ) with 5 species. Shannon evenness index ( $J'$ ) was highest in leaf ( $J' = 0.96$ ) followed by root ( $J' = 0.95$ ) and flower ( $J' = 0.93$ ). The endophytic fungi subjected for production of extracellular enzymes; 93% produced phosphatase, 80% cellulase, 70% amylase, 63.33% protease, 30% pectinase, 23.33% lipase and 10% laccase.

**Key words:** *Cymbidium aloifolium*, orchid, endophytic fungi, diversity, extracellular enzymes.

## INTRODUCTION

*Cymbidium aloifolium* L. is an herbaceous epiphytic orchid growing on tree trunks at an altitude of 300 to 2900 m (Rajbhandari and Dahal, 2004; Pothangbam and Nirmala, 2011). It is used for ornamental purposes (Subedi and Paudyal, 2001) and also as medicine to cure ear ache, cuts, wounds, paralysis, boils, fever and for joining the fractured bones (Mukul et al., 2007). The endophytic fungi are the important components of

epiphytic orchids which exhibited gamut of fungal dependency for nutrition; the fungi in turn help the orchid to assimilate nutrition from the bark of trees by the production of extracellular enzymes (Benzing and Friedman, 1981).

The study of Orchidaceae family is interesting as its entire species (approx. 25,000 sp.) are heterotrophic and nourished by endophytic fungi during the early stages of

\*Corresponding author. E-mail: [srinivasbub@gmail.com](mailto:srinivasbub@gmail.com), [csrinivas@bub.ernet.in](mailto:csrinivas@bub.ernet.in). Tel: +91-80-22961461/1624 or +91-9972091611.

development (Smith and Read, 1997). The fungal colonization occurs in roots, leaves or other structures and process depends on the genera of the fungus and the orchid species (Rasmussen and Whigham, 2002). Mycorrhizal fungi in orchid promote the germination of seeds, stimulate the development and growth of protocorms, seedlings, adult plants and tubers (Dearnaley, 2007; Takahashi et al., 2007). The orchid and fungus exhibit mutuality, with orchid providing vitamins and sugars to the fungus, while fungus transfers water, mineral salts and up to 85% of orchid's carbon requirement (Pridgeon et al., 2001); hence, the association between endophytic fungi and orchid is symbiotic. The endophytic fungi have been explored for diverse range of valuable compounds like enzymes and secondary metabolites. The production of bioactive compounds in fungi depends on its host habitat (Hyde and Soyong, 2008).

The extracellular enzymes produced by fungi are gaining importance in textile, leather, confectionery, beverage, food industry, agriculture and human health due to its stability at various extreme conditions such as high temperature and pH (Benjamin and Pandey, 1998). The amylases convert starch into sugar syrup and used it in food and pharmaceutical industry. The lignocelluloses abundantly found in nature are degraded by cellulase and hence play an important role in the cycling of carbon and other nutrients. The cellulases along with hemicellulase and pectinase are used in degradation of lignocellulosic materials that serve as raw material for fermentation and paper industries (Dyk and Pletschke, 2012). The protease provides softness to the silk fiber; it is also used in hide dehairing, as contact lens cleaners, healing and management of skin ulcers by removal of necrotic materials. The pectinase is an important enzyme required in food and wine industry. The laccases are used in pulp and paper industry; also it has environmental applications such as bioremediation. The lipase has vast industrial application in detergent, oil, fat, dairy and therapeutic industries (Benjamin and Pandey, 1998). The microbes with phosphate solubilizing ability enhance the availability of soluble phosphate and thus promote the plant growth (Ponmurugan and Gopi, 2006).

The endophytic fungi produce several enzymes to support its host growth and defense against microbial pathogens (Sunitha et al., 2012). The endophytic fungi invade the plant tissues by producing extracellular hydrolases like pectinase, cellulase, lipase, laccase, etc. (Ward, 2012). These extracellular enzymes degrade lignocellulosic fibers; the hydrolytic enzymes like xylanases and cellulases degrade polysaccharides and oxidative lignolytic system produces laccases, ligninases and peroxidases which degrade lignin and opens the phenyl ring system. The enzyme production and regulation by the endophytic fungi is due to its genetic recombination with the host which has evolved with evolutionary time (Priest, 1984). However, the enzymes produced by endophytic fungi have been barely exploited

for industrial interest (Correa et al., 2014).

The endophytic fungi associated with this epiphytic orchid have remained unexplored vicinity for the production of extracellular enzymes and thus may prove to be a possible source in obtaining enzymes with different potentialities (Schulz et al., 2002). Hence, in the present study, an attempt was made to isolate endophytic fungi from different parts of *C. aloifolium* like root, leaf and flower; diversity of endophytic fungi was studied. These endophytic fungal isolates were further evaluated for the production of industrially important extracellular enzymes.

## MATERIALS AND METHODS

### Endophytic fungi isolation and identification

The *C. aloifolium* was collected during the flowering period of March to June, 2015, from different regions of Western Ghats of Karnataka such as Sringeri, Chikkamagaluru, Kemmanagundi and Shivamoga. The samples were collected and brought to laboratory, washed with tap water, cut into 0.5 cm<sup>2</sup> segments and surface sterilized with 95% ethanol (1 min), 4% NaOCl (5 min) and rinsed with sterile distilled water 5 times. The sterilized segments were teased using sterile blade and placed aseptically on potato dextrose agar (PDA) plate amended with 50 µg/ml tetracycline. The plates were incubated in dark conditions at 30°C and observed daily for up to four weeks for the fungal colonies. The endophytic fungi were isolated from root, leaf and flower (80 segments each) of *C. aloifolium* using a modified procedure of Zhu et al. (2008). The pure cultures of endophytic fungi were transferred to PDA slants and then stored at 4°C. The identification of fungi was done by morphological methods (Ellis, 1971; Sutton, 1980; Barnett and Hunter, 1998).

### Data analysis

The frequency of endophytic fungi colonizing different organs of *C. aloifolium* such as root, leaf and flower was determined. The absolute frequency (f) was estimated as the number of each endophytic fungi isolated from each plant organ (Larran et al., 2002). The relative frequencies (fr) of isolation was calculated for each organ; the number of isolates of each endophytic fungal species was divided by the total number of isolates obtained in each organ and expressed as percentage. The isolation rate (IR) was determined by dividing the number of endophytic fungal isolates from an organ divided by the total number of tissue segments. The percentage of colonization rate (CR) was determined by dividing the total number of endophytic fungal isolates obtained from each organ segments divided by the total number of isolates obtained from overall organ segments incubated multiply by 100. The Simpson's diversity index (D'), Shannon-Wiener index (H'), evenness (J) and species richness (S) were determined as described by Magurran (1988).

### Detection of extracellular enzyme production in endophytic fungi

The ability of the endophytic fungi to produce extracellular enzymes: amylase, lipase, pectinase, cellulase, protease, laccase and phosphatase, were screened using different solid media

(Hankin and Anannostakis, 1975; Neha et al., 2015). The production of extracellular enzymes was assessed by growing each of the endophytic fungi on PDA for 1 week and 5 mm of these mycelia plugs were placed on the solid media having different substrates for respective enzymes. After incubation for 5 to 7 days at room temperature, the diameter of hydrolysis and fungal colony were measured; enzyme index was calculated (Florencio et al., 2012).

Enzyme index = Diameter of hydrolysis / Diameter of fungal colony.

### **Amylase**

Amylase production was assessed by growing endophytic fungi on glucose yeast extract peptone agar (GYP: glucose, 1 g; yeast extract, 0.1 g; peptone, 0.5 g; agar, 15 g, distilled water, 1 L) with 0.2% soluble starch; pH 6.0. After incubation, plates were flooded with 1% iodine in 2% potassium iodide. The zone of clearance around the blue background indicated the production of amylase.

### **Lipase**

The lipase production was assessed by growing endophytic fungi on peptone agar (peptone, 10 g; NaCl 5 g; CaCl<sub>2</sub>·2H<sub>2</sub>O 0.1 g; agar, 15 g; distilled water 1 L; pH 6.0) with 1% Tween 20 (sterilized and added separately to the media before pouring). The visible precipitate around the colony indicated the presence of lipase. The precipitate formed was due to the formation of calcium salts of lauric acid liberated by lipase.

### **Pectinase**

The pectinase production was assessed by growing fungi on pectin agar medium (pectin 5 g; yeast extract 1 g; agar, 15 g; distilled water, 1 L, pH 5.0). After incubation, the plates were flooded with 1% aqueous solution of hexadecyl trimethyl ammonium bromide. A clear zone around the colony showed the production of pectinase.

### **Cellulase**

The cellulase production was detected by growing fungi on glucose yeast extract peptone agar with 0.5% carboxy-methyl cellulose. After incubation, the plates were flooded with 0.2% aqueous congo red solution and destained with 1 M NaCl for 15 min. The yellow zone around the colony showed the presence of cellulase.

### **Protease**

Glucose yeast peptone agar with gelatin at pH 6.0, was used to detect the production of protease by endophytic fungi. Gelatin (8%) in water was sterilized separately and added to GYP medium at the rate of 5 ml per 100 ml. The clear zone around the colonies showed the presence of protease which was enhanced when the plates were flooded with saturated ammonium sulphate.

### **Laccase**

Glucose yeast peptone agar with 0.05 g of 1 naphthol L<sup>-1</sup>, pH 6, was used to detect the production of laccase. The laccase production was indicated by change from colourless media to blue colour due to the oxidation of 1-naphthol.

### **Phosphatase**

The phosphate solubilizing ability of the fungal isolates were tested using the modified procedure of Neha et al. (2015) on Pikovskaya's solid agar media (glucose, 10 g; Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub>, 5 g; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.5 g; NaCl, 0.2 g; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.1 g; KCl, 0.2 g; yeast extract 0.5 g; MnSO<sub>4</sub>·H<sub>2</sub>O, 0.002 g, FeSO<sub>4</sub>·7H<sub>2</sub>O 0.002 g and Agar 15 g, distilled water 1 L) with bromo phenol blue at a concentration of 0.003%. After 3 to 5 days of incubation, clear zone around the fungal colony indicated the ability of fungus to solubilize inorganic phosphorus.

### **Statistical analysis**

All the experiments were performed in triplicates and the means of the enzyme index were analyzed statistically and Duncan multiple range test was carried out using SPSS software program version 20 (Duncan, 1955).

## **RESULTS**

### **Colonization of endophytic fungi in *C. aloifolium***

A total of 165 endophytic fungi representing 22 different species were obtained from 240 organ fragments analyzed; 80 fragments were taken from each of the root, leaf and flower of *C. aloifolium*. Sixty seven endophytic fungi were obtained from root, 53 from leaf and 45 from flower; all the isolates belonged to Ascomycota except one isolate which was Basidiomycota and belonged to the groups of Dothideomycetes, Eurotiomycetes, Sordariomycetes, Botryosphaeriaceae, and Leotiomycetes. The root was more frequently associated with *Aspergillus terreus* followed by *Colletotrichum gleosporidioides* and other fungi; leaf was more frequently associated with *Penicillium chrysogenum* followed by *Alternaria alternata*, *Trichoderma* species; in flower, *Fusarium oxysporum* was most frequently isolated followed by *Cyperus rotundus*, *Penicillium purpurogenum* and others. The endophytic fungi were found to be associated with all the parts of *C. aloifolium* analyzed and the extent of colonization varied with different organs; colonization rate and isolation rate was highest in root (CR = 40.6%, IR=0.83) followed by leaf (CR = 32.12%, IR=0.66) and flower (CR= 27.27%, IR=0.56) (Table 1). Shannon diversity index (H') was highest in the root (H'=2.64) followed by leaf (H'=2.12) and lowest in flower (H'=1.5). Simpson diversity index (D') was high in root (D'= 0.93) with a maximum of 16 different species, followed by leaf (D'=0.88) with 9 species and flower (D'=0.78) with 5 species. Shannon evenness index (J') was highest in leaf (J'=0.96) followed by root (J'=0.95) and flower (J'=0.93) (Table 2).

### **Screening of endophytic fungi for extracellular enzyme production**

The 30 different endophytic fungi obtained from root, leaf

**Table 1.** Frequency of Endophytic fungi isolated from different parts of *C. aloifolium*.

S/N	Endophytic fungi	Roots		Leaf		Flower		Total	
		f	fr (%)	f	fr (%)	f	fr (%)	f	fr(%)
1	<i>Aspergillus japonicas</i>	3	4.47	0	0	0	0	3	1.81
2	<i>Curvularia lunata</i>	3	4.47	3	5.66	0	0	6	3.63
3	<i>Nigrospora</i> spp.	1	1.5	0	0	0	0	1	0.60
4	<i>Colletotrichum gleosporidioides</i>	8	11.94	0	0	0	0	8	4.84
5	<i>Rhizoctonia</i> spp.	4	5.97	7	13.20	0	0	11	6.66
6	<i>Xylaria</i> spp.	3	4.47	0	0	0	0	3	1.81
7	<i>Trichoderma</i> spp.	5	7.46	8	15.09	0	0	13	7.87
8	<i>Fusarium chlamydosporum</i>	3	4.47	0	0	0	0	3	1.81
9	<i>Penicillium citrinum</i>	4	5.97	4	7.54	0	0	8	4.84
10	<i>Helminthosporium</i> spp.	2	2.98	0	0	0	0	2	1.21
11	<i>Curvularia</i> spp.	3	4.47	0	0	0	0	3	1.81
12	<i>Aspergillus sydowii</i>	7	10.44	4	7.54	0	0	11	6.66
13	<i>Cladosporium</i> sp.	4	5.97	0	0	8	17.78	12	7.27
14	<i>Aspergillus terreus</i>	10	14.92	0	0	0	0	10	6.06
15	<i>Alternaria alternata</i>	3	4.47	8	15.09	0	0	11	6.66
16	<i>Fusarium oxysporum</i>	4	5.97	0	0	15	33.33	19	11.51
17	<i>Penicillium chrysogenum</i>	0	0	10	18.86	0	0	10	6.06
18	<i>Colletotrichum truncatum</i>	0	0	4	7.54	0	0	4	2.42
19	<i>Bipolaris</i> spp.	0	0	5	9.43	0	0	5	3.03
20	<i>Taloromyces rotundus</i>	0	0	0	0	10	22.22	10	6.06
21	<i>Penicillium purpurogenum</i>	0	0	0	0	9	20	9	5.45
22	<i>Cylindrocephalum</i> spp.	0	0	0	0	3	6.66	3	1.81
Total		67	100	53	100	45	100	165	100
Colonization rate (CR%)		40.6		32.12		27.27		100	
Isolation rate (IR)		0.83		0.66		0.56		0.68	

F, Absolute frequency; fr, relative frequency.

**Table 2.** Diversity, evenness and species richness of endophytic fungi isolated from different organs of *C. aloifolium*.

<i>C. aloifolium</i> part	H'	D'	J'	S
Root	2.64	0.93	0.95	16
Leaf	2.12	0.88	0.96	9
Flower	1.5	0.78	0.93	5

Shannon-Wiener (H'), Simpson (D') diversity indices, evenness (J') and richness (S).

and flower of *C. aloifolium* were screened for the production of extracellular enzymes: amylase, lipase, laccase, protease, pectinase, cellulase, and phosphatase (Table 3). The endophytic fungi producing extracellular enzymes were assessed based on the formation of halo zone around the fungal colony and enzyme index were calculated (Figure 1). The endophytic fungi produced one or the other extracellular enzyme necessary for penetrating and colonizing their host. In the present study, none of the endophytic fungi were able to produce all the seven enzymes screened but, all the fungi

produced more than one enzyme, except *Curvularia* species (CAR11) and *A. alternata* (CAL8) which produced only cellulase enzyme (Figure 2). Ninety nine percent of endophytic fungi produced phosphatase followed by 80% cellulase, 70% amylase, 63.33% protease, 30% pectinase, 23.33% lipase and 10% laccase (Figure 3).

### Production of phosphatase enzyme

Most of the endophytic fungi (93.33%) produced phosphatase enzyme; enzyme index for phosphatase ranged from the highest of 1.58 in *Colletotrichum truncatum* (CAL7) to lowest of 1.04 in *Helminthosporium* species (CAR10) indicating the phosphatase produced was almost similar in all the endophytic fungi screened. *A. alternata* (CAL8) and *Curvularia* spp. (CAR11) from root were negative for phosphatase.

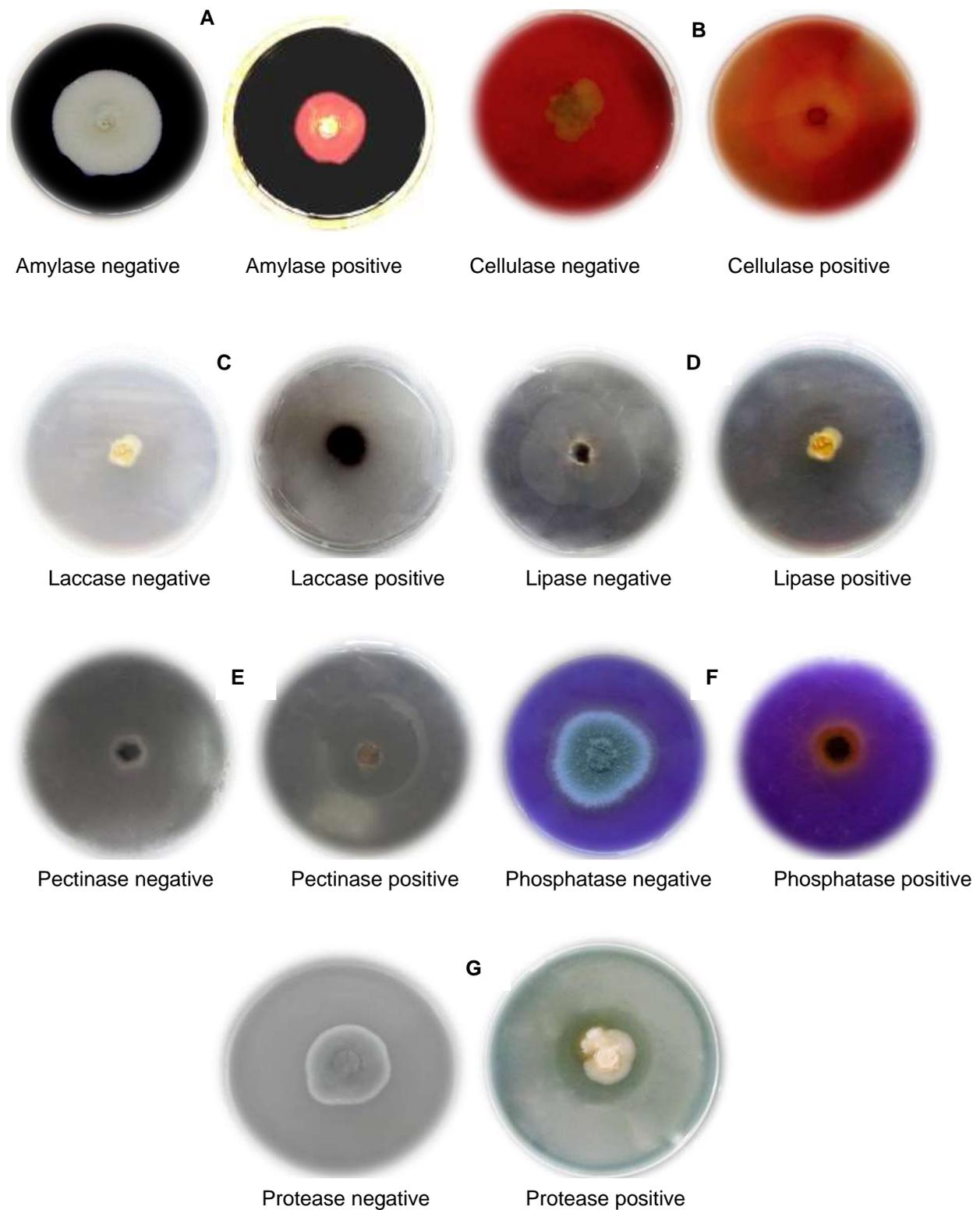
### Production of cellulase enzyme

Eighty percent of endophytic fungi produced cellulase

**Table 3.** Extracellular enzyme production by endophytic fungi isolated from *C. aloifolium*.

S/N	Fungal isolate	Endophytic fungi	Enzyme index							
			Amylase	Lipase	Laccase	Protease	Pectinase	Cellulase	Phosphatase	
1	CAR1	<i>Aspergillus japonicas</i>	2.15±0.11 <sup>hi</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	3.68±0.13 <sup>h</sup>	1.52±0.06 <sup>d</sup>	2.28±0.32 <sup>hi</sup>	1.41±0.15 <sup>k</sup>	
2	CAR2	<i>Curvularia lunata</i>	2.21±0.16 <sup>hi</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	2.43±0.49 <sup>fg</sup>	0.00 <sup>a</sup>	1.45±0.16 <sup>def</sup>	1.1±0.02 <sup>bcd</sup>	
3	CAR3	<i>Nigrospora</i> spp.	2.15±0.1 <sup>hi</sup>	3.46±0.14 <sup>g</sup>	1.41±0.18 <sup>b</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	2.5±0.14 <sup>i</sup>	1.26±0.03 <sup>fghi</sup>	
4	CAR4	<i>C. gleosporidioides</i>	3.07±0.08 <sup>j</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	5.3±0.34 <sup>j</sup>	1.85±0.04 <sup>e</sup>	7.99±0.16 <sup>m</sup>	1.37±0.05 <sup>ijk</sup>	
5	CAR5	<i>Trichoderma</i> sp.	2.15±0.11 <sup>hi</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	4.56±0.38 <sup>i</sup>	1.41±0.04 <sup>cd</sup>	4.5±0.29 <sup>j</sup>	1.24±0.05 <sup>efgh</sup>	
6	CAR6	<i>Xylaria</i> spp.	1.88±0.15 <sup>g</sup>	1.93±0.3 <sup>c</sup>	1.58±0.08 <sup>c</sup>	2.26±0.1 <sup>f</sup>	0.00 <sup>a</sup>	1.33±0.11 <sup>bcd</sup>	1.26±0.13 <sup>fghi</sup>	
7	CAR7	<i>Rhizoctonia</i> spp.	0.00 <sup>a</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	1.73±0.28 <sup>de</sup>	0.00 <sup>a</sup>	1.57±0.15 <sup>ef</sup>	1.07±0.05 <sup>bc</sup>	
8	CAR8	Root	<i>Fusarium chlamydosporum</i>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	1.49±0.35 <sup>ef</sup>	1.33±0.0 <sup>ghijk</sup>
9	CAR9		<i>Penicillium citrinum</i>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	1.51±0.12 <sup>d</sup>	1.36±0.06 <sup>cde</sup>	1.19±0.01 <sup>cdef</sup>
10	CAR10		<i>Helminthosporium</i> spp.	0.00 <sup>a</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	2.42±0.04 <sup>fg</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	1.04±0.01 <sup>b</sup>
11	CAR11		<i>Curvularia</i> spp.	0.00 <sup>a</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	1.64±0.03 <sup>f</sup>	0.00 <sup>a</sup>
12	CAR12		<i>Aspergillus sydowii</i>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	1.73±0.28 <sup>de</sup>	1.41±0.11 <sup>cd</sup>	0.00 <sup>a</sup>	1.28±0.07 <sup>fghij</sup>
13	CAR13		<i>Cladosporium</i> spp.	2.32±0.07 <sup>i</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	2.61±0.5 <sup>g</sup>	0.00 <sup>a</sup>	1.91±0.07 <sup>g</sup>	1.12±0.01 <sup>bcd</sup>
14	CAR14		<i>Aspergillus terreus</i>	1.21±0.02 <sup>b</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	1.24±0.09 <sup>b</sup>	1.15±0.01 <sup>bc</sup>	1.24±0.04 <sup>efgh</sup>
15	CAR15		<i>Alternaria alternata</i>	1.7±0.39 <sup>f</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	1.93±0.2 <sup>e</sup>	0.00 <sup>a</sup>	1.17±0.1 <sup>bc</sup>	1.16±0.02 <sup>bcd</sup>
16	CAR16	<i>Fusarium oxysporum</i>	1.25±0.02 <sup>b</sup>	2.67±0.17 <sup>e</sup>	0.00 <sup>a</sup>	2.23±0.15 <sup>f</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	1.13±0.02 <sup>bcd</sup>	
17	CAL1	Leaf	<i>Penicillium chrysogenum</i>	2.15±0.05 <sup>hi</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	3.68±0.13 <sup>h</sup>	2.02±0.13 <sup>f</sup>	2.42±0.14 <sup>i</sup>	1.42±0.03 <sup>k</sup>
18	CAL2		<i>Aspergillus sydowii</i>	1.35±0.02 <sup>bc</sup>	3.18±0.14 <sup>f</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	1.24±0.01 <sup>efgh</sup>
19	CAL3		<i>Trichoderma</i> spp.	1.37±0.17 <sup>bcd</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	1.73±0.28 <sup>de</sup>	0.00 <sup>a</sup>	1.18±0.04 <sup>bc</sup>	1.22±0.08 <sup>defg</sup>
20	CAL4		<i>Rhizoctonia</i> spp.	1.58±0.08 <sup>ef</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	1.58±0.27 <sup>cd</sup>	0.00 <sup>a</sup>	1.18±0.04 <sup>bc</sup>	1.36±0.03 <sup>hijk</sup>
21	CAL5		<i>Curvularia lunata</i>	2.26±0.13 <sup>j</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	1.46±0.14 <sup>bcd</sup>	0.00 <sup>a</sup>	1.09±0.04 <sup>b</sup>	1.22±0.1 <sup>efg</sup>
22	CAL6		<i>Penicillium citrinum</i>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	1.19±0.05 <sup>bcd</sup>	1.54±0.13 <sup>lm</sup>
23	CAL7		<i>Colletotrichum truncatum</i>	1.45±0.02 <sup>cde</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	1.46±0.14 <sup>bcd</sup>	1.23±0.14 <sup>b</sup>	2.28±0.32 <sup>hi</sup>	1.58±0.1 <sup>m</sup>
24	CAL8		<i>Alternaria alternata</i>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	1.39±0.16 <sup>cdef</sup>	0.00 <sup>a</sup>
25	CAL9		<i>Bipolaris</i> spp.	1.65±0.04 <sup>f</sup>	1.98±0.22 <sup>c</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	1.39±0.09 <sup>jk</sup>
26	CAF1	Flower	<i>Fusarium oxysporum</i>	1.55±0.03 <sup>def</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	1.31±0.24 <sup>bc</sup>	3.6±0.28 <sup>k</sup>	1.57±0.02 <sup>m</sup>
27	CAF2		<i>Talaromyces rotundus</i>	1.45±0.02 <sup>cde</sup>	2.25±0.16 <sup>d</sup>	0.00 <sup>a</sup>	1.66±0.05 <sup>de</sup>	0.00 <sup>a</sup>	2.36±0.16 <sup>j</sup>	1.36±0.01 <sup>hijk</sup>
28	CAF3		<i>Penicillium purpurogenum</i>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	1.79±0.09 <sup>d</sup>	1.32±0.03 <sup>bc</sup>	0.00 <sup>a</sup>	2.79±0.14 <sup>j</sup>	1.46±0.03 <sup>k</sup>
29	CAF4		<i>Cladosporium</i> spp.	2.04±0.03 <sup>gh</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	1.16±0.02 <sup>b</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	1.17±0.08 <sup>cdef</sup>
30	CAF5		<i>Cylindrocephalum</i> spp.	2.26±0.13 <sup>j</sup>	1.61±0.2 <sup>b</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>	2.05±0.1 <sup>gh=</sup>	1.22±0.01 <sup>defg</sup>

The values represent mean of enzyme index ± SD, n=3; Mean values followed by the same letter are not significantly different according to DMRT at p< 0.05.



**Figure 1.** Production of extracellular enzymes by endophytic fungi isolated from *C. aloifolium*.

enzyme and their enzyme index varied with different fungi; the highest enzyme index was 7.99 by

*Colletotrichum gloeosporioides* (CAR 4), followed by 4.5 by *Trichoderma* spp. (CAR5), 3.6 by *F. oxysorum* (CAF

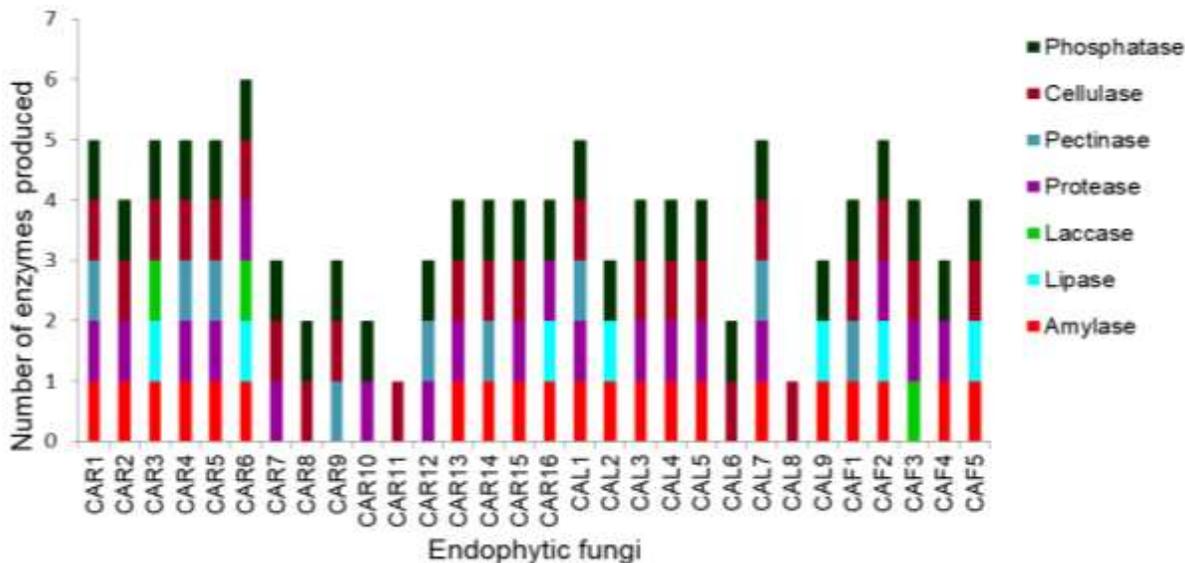


Figure 2. Production of extracellular enzymes by endophytic fungi of *C. aloifolium*.

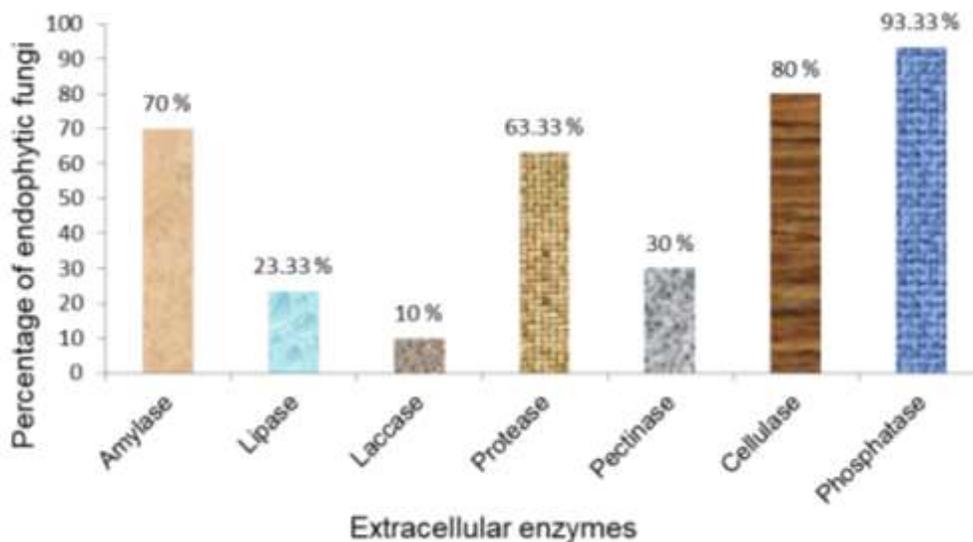


Figure 3. Percentage of endophytic fungi of *C. aloifolium* producing extracellular enzymes.

1) and lowest enzyme index was 1.09 by *Curvularia lunata* (CAL5). The cellulase enzyme was produced by all the fungi screened except CAR10, CAR12, CAR16, CAL2, CAL9, and CAF4 which showed cellulase negative results.

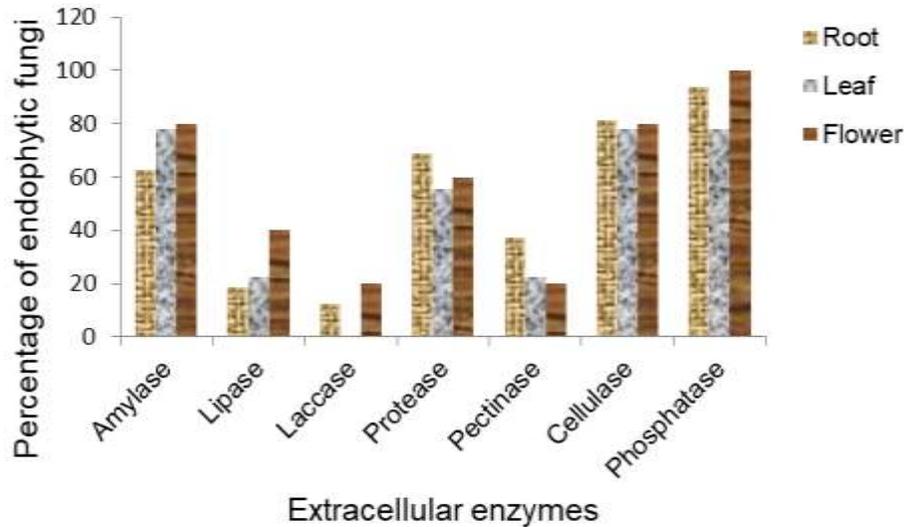
#### Production of amylase enzyme

The 70% of endophytic fungi produced amylase enzyme and enzyme index ranged from the highest which was 3.07 by *C. gloeosporioides* (CAR 4) to the lowest of 1.21

by *A. terreus* (CAR14). The enzyme index varied among the fungi producing amylase. The amylase was produced by all the endophytic fungi except CAR7, CAR8, CAR9, CAR10, CAR11, CAR12, CAL6, CAL8, and CAF3 which were negative for amylase.

#### Production of protease enzyme

The 63.33% of endophytic fungi produced protease enzyme. The highest protease enzyme was produced by *C. gloeosporioides* (CAR4) with enzyme index of 5.3,



**Figure 4.** Percentage of endophytic fungi associated with root, leaf and flower of *C. aloifolium* producing extracellular enzymes.

followed by *Trichoderma* spp. (CAR5) with enzyme index of 4.56, *A. japonicas* (CAR 1) with enzyme index of 3.68 and all the other fungi produced protease with an enzyme index which ranged from 2.43 to 1.16; lowest enzyme index was in *Cladosporium* species (CAF4).

#### **Production of pectinase enzyme**

The pectinase enzyme was produced by 30% of endophytic fungi; significant enzyme index was 2.02 by *P. chrysogenum* (CAL1) and least was 1.23 by *C. truncatum* (CAL7). The endophytic fungi producing pectinase enzyme were CAR1, CAR4, CAR5, CAR9, CAR12, CAR14, CAL1, CAL7, and CAF1.

#### **Production of lipase enzyme**

The lipase was produced by 23.33% endophytic fungi. The prominent enzyme index was 3.46 by *Nigrospora* species (CAR3) followed by 3.18 in *Aspergillus sydowii* (CAL2); 2.67 by *F. oxysporum* (CAR16), 2.25 by *T. rotundus* (CAF2), 1.93 by *Xylaria* species (CAR6), 1.98 by *Bipolaris* species (CAL9) and 1.61 by *Cylindrocephalum* (CAF5).

#### **Production of laccase enzyme**

The laccase was produced only in 10% of the endophytic fungi; three isolates CAF3, CAR6 and CAR3 produced laccase. The significant amount was produced by CAF3 (*P. purpurogenum*) with the enzyme index of 1.79 followed by 1.58 by CAR6 and 1.41 by *Nigrospora* spp. (CAR3).

#### **Comparison of the endophytic fungi from different parts of *C. aloifolium* producing extracellular enzymes**

The production of extracellular enzymes varied with the endophytic fungi obtained from different parts of *C. aloifolium*. Eighty percent of endophytic fungi associated with flower, produced amylase followed by leaf endophytic fungi (77.77%) and root endophytic fungi (62.5%). Forty percent of flower endophytic fungi produced lipase followed by leaf endophytic fungi (22.22%) and root (18.75%). Twenty percent of flower endophytic fungi produced laccase followed by root endophytic fungi (12.5%) but there was no laccase production in the endophytic fungi from leaves. The 68.75% of root endophytic fungi produced protease followed by flower endophytic fungi (60%) and leaf endophytic fungi (55.55%). The root endophytic fungi were significant pectinase producers with 37.5% fungi producing it which was followed by leaf endophytic fungi (22.22%) and flower endophytic fungi (20%). The 81.25% of root endophytic fungi produced cellulase followed by flower endophytic fungi (80%) and leaf endophytic fungi (77.77%). All the endophytic fungi associated with flower produced phosphatase enzyme followed by root endophytic fungi (93.75%) and leaf (77.77%) (Figure 4).

#### **DISCUSSION**

*C. aloifolium* is a potent medicinal epiphytic orchid in the Indian system of medicine, traditionally used in treatment of many human ailments (Radhika et al., 2013). The endophytic fungi associated with this orchid have vital role in its life cycle. In the present study, 165 endophytic

fungi were obtained from different parts of *C. aloifolium*; root represented the maximum number of isolates followed by leaf and stem. All the fungal isolates belonged to *Ascomycota* except one which was *Basidiomycota* and they belonged to the groups of *Dothideomycetes*, *Eurotiomycetes*, *Sordariomycetes*, *Botryosphaeriaceae* and *Leotiomycetes*. Selosse et al. (2004) have also found *Ascomycetes* to be predominating than *Basidiomycete* fungi in roots of *Epipactis microphylla* (Ehrh.). The Bidartondo et al. (2004) have designated *Ascomycetes* as the potential mycorrhizal fungi in orchids. Our findings are in accordance with those of Stone et al. (2004) who have reported that the endophytic fungi of non-grass hosts represent broader range of taxa mainly from several orders and families of *Ascomycetes* and few from *Basidiomycetes* families. The fungi possessing ascospores with thick cell wall and gelatinous sheath or appendages are adapted to launch their spores onto the plants hence are the predominant endophytic representatives, whereas *Zygomycetes* and *Basidiomycetes* are poorly represented in endophytic inventories.

The extent of endophytic fungi colonizing the different organ segments of *C. aloifolium* varied significantly. The colonization rate and isolation rate was maximum in roots (CR = 40.6%, IR=0.83) followed by leaf (CR = 32.12%, IR=0.66) and flower (CR= 27.27%, IR=0.56); root was more frequently colonized with *A. terreus*, *C. gloeosporioides*, *A. sydowii*, *Trichoderma* spp., *Rhizoctonia* spp., *Penicillium citrinum*, *Aspergillus japonicus*, *Curvularia* spp., *Xylaria* spp., *Chlamydosporium* spp., *Curvularia* spp., *A. alternata*, *Helminthosporium* spp., and *Nigrospora* spp. Chen et al. (2013) have found that, endophytic fungi associated with *Dendrobium* spp. were dominated by *Fusarium* spp., *Colletotrichum* species, and *Xylariaceae* fungi. The findings of this study are in agreement with those of Chen et al. (2010) who have reported that significant number of endophytic fungi were isolated from roots when compared with stem and leaves of *Dendrobium loddigessi*; as roots may provide a better niche for endophytic colonization, whereas findings of Uzma et al. (2016) have reported that colonization rates were higher in leaves when compared with roots of medicinal plants. The present results also agrees with those of Bayman et al. (1997) who have reported that the heterogeneity of endophytes in single plants and its organs are greater than between the species.

The diversity indices of the endophytic fungi associated with different *C. aloifolium* parts were analyzed by Shannon diversity index ( $H'$ ) and Simpson diversity index ( $D'$ ) which showed the variations in the endophytic fungal isolates and its species richness within the different organs of this orchid. The highest diversity indices were observed in root followed by leaf and flower; root exhibited maximum species richness with 16 different

species followed by leaf with nine different species and flower with five species, whereas Shannon evenness index was highest in leaf followed by root and flower. The species richness signifies the colonization of different endophytic fungal species within a specific organ, whereas evenness index enumerates the identical abundance of the species in a particular tissue. The present research findings are in agreement with Tao et al. (2008) who have reported that different orchid parts like roots, leaves have different endophytic associations and speculated that could be because of the difference in its tissue texture, physiology and chemistry (Barman and Devadas, 2013).

The endophytic fungi obtained from different parts of *C. aloifolium* produced different extracellular enzymes such as, amylase, lipase, laccase, protease, pectinase, cellulase and phosphatase and are promising source of industrially useful enzymes (Zaferanloo et al., 2013). The present study agrees with those of Bezerra et al. (2012) who have reported the richness of endophytic fungi from *Opuntia ficus-indica* Mill and its capacity to produce extracellular enzymes. In the present study, endophytic fungi were able to produce one or the other extracellular enzymes necessary for penetrating and colonizing their host, but none of them produced all the seven enzymes screened. Petrini et al. (1993) have reported that majority of endophytes utilizes a good number of substrates which are likely to be present on the surfaces and in the host cell wall.

The phosphatase enzyme was produced by 93.33% of the endophytic fungi. The *C. truncatum* (CAL7) isolated from leaf produced significant phosphatase followed by *F. oxysporum* (CAF1) isolated from flower. The phosphatase enzyme produced by the endophytic fungi helps to solubilize the available phosphate which can be easily assimilated by the growing plants. Our findings are in accordance with those of Neha et al. (2015) who have reported production of phosphatase in most of the endophytic fungi. The phosphate solubilization could be due to the various organic acids produced by endophytes which in turn results in reduced pH, thus facilitating phosphate solubilization (Illmer and Schineer, 1995).

Eighty percent of endophytic fungi screened produced cellulase enzyme. The enzyme index varied significantly among the endophytic fungi producing cellulase; *C. gloeosporioides* (CAR4) isolated from root exhibited the highest enzyme index for cellulase. The endophytic fungi associated with *C. aloifolium* help its host to assimilate complex carbohydrates like cellulose present in the tree barks by production of cellulase enzyme (Read et al., 2004). The present findings are supported by the findings of Choi et al. (2005) who have reported that endophytic fungi from *Brucea javanica* produced extracellular cellulase which resulted in weight loss of wood blocks; most of the endophytic fungi are degraders of complex carbohydrates such as cellulose available in dead leaves and wood. The highest cellulase activity have also been

reported from several endophytic fungi of Egyptian marine sponge *Latrunculia corticata* (El-Bondkly and El-Gendy, 2012) and also in endophytic *Xylaria* spp. associated with plant tissues (Wei et al., 1996).

The amylase enzyme was produced by 70% of the endophytic fungi screened. The significant amylase was produced by *C. gloeosporioides* (CAR4), isolated from root with an enzyme index of 3.07. The present findings are in accordance with the findings of Amirita et al. (2012), Choi et al. (2005), and Sunitha et al. (2013) who have reported prominent amylase production by endophytic fungi. The amylolytic activity of endophytic fungi helps to degrade starch into simple carbohydrates which can be later assimilated by both fungi and host (Venkatesagowda et al., 2012).

The 63.33% of endophytic fungi produced protease enzyme and *C. gloeosporioides* (CAR4) from root significantly produced protease. The endophytic fungi produce various enzymes including protease which helps them to overcome the host defense barrier and in turn obtain nutrients for their development (Amirita et al., 2012). The present findings agrees with Boyle et al. (2001) and Sunitha et al. (2013) who have reported the production of protease and other enzymes by endophytic fungi which helps in penetrating and colonizing the host plants. Thirty percent of endophytic fungi produced pectinase enzyme which is lesser than those of Sunitha et al. (2013) who reported 62% endophytic fungi of *Alpinia calcarata*, *Bixa orellana* and *Calophyllum inophyllum* producing this enzyme, whereas Choi et al. (2005) have reported that pectinase production was absent in all the endophytic fungi of *Brucea javanica*.

23.33% of the endophytic fungi produced lipase enzyme and *Nigrospora* spp. (CAR3) isolated from root was major lipase producer. The percentage of endophytic fungi that produced lipase in the present study was greater when compared with 3.12% of the endophytic fungi from oil bearing seeds producing lipase, but Sunitha et al. (2013) have reported that 50% of endophytic fungi of *Alpinia calcarata*, *Bixa orellana* and *Calophyllum inophyllum* produced lipase. The laccase was produced by three endophytic fungi, *P. purpurogenum* (CAF3) from flower being the highest producer. The present findings are in concurrence with those of Sajben et al. (2014) who have reported that the endophytic fungi and marine fungi producing laccase are meagre, but Maria et al. (2005) and Uzma et al. (2016) have reported that the endophytic fungi of medicinal plants from Western Ghats failed to produce laccase.

The production of extracellular enzymes varied with the endophytic fungi associated with different organs of *C. aloifolium* like root, leaf and flower. The highest number of endophytic fungi associated with flower produced amylase, lipase, laccase and phosphatase, whereas more number of root endophytic fungi produced protease, pectinase and cellulase enzyme. The present findings are in accordance with those of Alves et al. (2014) who have reported that the predominance of extracellular enzyme

depends on the type of samples.

The ability of the endophytic fungi to produce the extracellular enzymes like cellulase and laccase establish their functional role as mutualistic partners with its host plant (Pointing, 1999). The endophytic fungi prove it as saprophytes by exhibiting the capability to produce diverse enzymes without degrading living tissue of the host. The endophytes decompose the dead host plant not only as a single species, but as communities, because invariable species are detected on a single decaying plant part (Sun et al., 2011). The genetic recombination of the endophytes with the host during the evolutionary time could have crooked the endophytes to produce various secondary metabolites as those produced by its associated host (Priest, 1984).

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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