Journal of Agricultural Technology

Antimicrobial and enzyme activity of mangrove endophytic fungi of southwest coast of India

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Maria, G.L., Sridhar, K.R. and Raviraja, N.S. (2005). Antimicrobial and enzyme activity of mangrove endophytic fungi of southwest coast of India. Journal of Agricultural Technology 1: xx-xx.

The antimicrobial potential of 14 endophytic fungi isolated from *Acanthus ilicifolius* and *Acrostichum aureum* towards selected bacteria (*Bacillus subtilis, Enterococcus* sp., *Klebsiella pneumoniae, Pseudomonas aerugionsa, Salmonella typhi* and *Staphylococcus aureus*) and fungi (*Candida albicans* and *Trichophyton metagrophytes*) was tested using ethyl acetate extract of fungi cultivated under solid-state fermentation (SSF). *Aspergillus* spp. showed promising antagonistic features. All test bacteria were inhibited by sterile isolate MSI 1. *Cumulospora marina* and *Pestalotiopsis* sp. showed considerable inhibition on Gram-positive and Gram-negative bacteria. Crude ethyl extracts derived from submerged fermentation (SmF) of four endophytes showing positive results were further evaluated. *Aspergillus* sp. 3 and *Pestalotiopsis* sp. inhibited many bacteria and *Candida albicans*, while inhibition by *Aspergillus* sp. 2 and MSI 1 was confined to bacteria. Crude ethyl acetate extracts purified by TLC of four endophytes grown under SmF showed many fluorescent fractions. Two fractions of *Aspergillus* sp. 3 showed high antimicrobial activity. One of the fractions of *Pestalotiopsis* sp. showed considerable inhibition of *Bacillus subtilis, Staphylococcus aureus* and *Candida albicans*. Seven endophytic fungi were assessed for the production of extracellular enzymes (amylase, cellulase, chitinase, laccase, lipase, protease and tyrosinase) by culture plate method. Cellulase and lipase activity was present in all fungi, while amylase and protease in a few. No fungi exhibited chitinase, laccase and tyrosinase activity. Enzyme production of *Pestalotiopsis* sp. (cellulase by SmF; xylanase, pectinase and protease by SSF) at pH 7 and pH 9 during 3-15 days of fermentation was assessed. The Cellulase activity was highest at pH 7 on 6th day, while xylanase activity was highest on 9th day at pH 9. Highest activity of pectinase was seen on 6th (pH 7) and 9th days (pH 9). Protease activity was highest on 6th day at pH 7 and 9.

Key words: antimicrobial activity, endophytic fungi, enzymes, mangroves.

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Introduction

Mangrove plants have adapted to a unique habitat with muddy saline waters, anaerobic soil, brackish tidal activities and high microbial and faunal competition. Endophytic fungal association with mangrove plants confers protection from adverse environmental conditions and allows them to successfully compete with saprobic fungi decomposing senescent parts (Kumaresan and Suryanarayanan, 2002). Leaves, stems, bark, roots and fruits of mangrove plants are a valuable resource for folk medicine (Bandaranayake, 1998). Although several studies on fungi associated with mangrove detritus have been published (see Hyde and Lee, 1995), there are a few studies on the endophytic fungi of mangrove plants and most of them are confined to foliar endophytes (e.g. Suryanarayanan *et al*., 1998; Suryanarayanan and Kumaresan, 2000; Kumaresan and Suryanarayanan, 2001, 2002). A recent study showed that roots of mangrove plants are a rich source of fungal endophytes (Ananda and Sridhar, 2002). Metabolites exploited in pharmaceutical and agricultural industries are widespread among the endophytic fungi (Petrini *et al*., 1992). For example, the anticancer drug, taxol, which was previously thought to be produced only by the plant genus *Taxus* (yew) has been found in many genera of endophytic fungi (*Alternaria, Fusarium, Monochaetia, Pestalotia, Pestalotiopsis, Pithomyces* and *Taxomyces*) (Strobel *et al*., 1996). Marine mangrove fungi have proven to be an important source of new bioactive compounds (Lin *et al*., 2001) and enzymes (e.g. Grant *et al*., 1996; Pointing *et al*., 1998; Pointing and Hyde, 2000). Endophytes might involve in decomposition when the tissue becomes senescent or die. Hence, they elaborate the enzymes necessary for degradation of lignocellulosic materials. In the present study, endophytic fungi from two mangrove associates (*Acanthus ilicifolius* and *Acrostichum aureum*) were investigated for their anti-microbial and extracellular enzyme potential.

Materials and Methods

Fungal strains and source

Fourteen endophytic fungal strains, isolated from mangrove angiosperm associate, *Acanthus ilicifolius* L. and mangrove fern, *Acrostichum aureum* L. of Nethravathi Mangrove, on the southwest coast of India were maintained on 2% malt extract agar and tested for anti-microbial and enzyme activity (Table 1).

Table 1. Endophytic fungi tested for antimicrobial (A) and enzyme (E) potential.

Fungus	Host plant	Tissue	Assay
Acremonium sp.	*Acrostichum aureum*	Pinna	A, E
Alternaria chlamydosporus Mouchacca	*Acanthus ilicifolius*	Leaf	A, E
Alternaria sp.	*Acrostichum aureum*	Pinna	A, E
Aspergillus sp. 1	*Acanthus ilicifolius*	Stem	A
Aspergillus sp. 2	*Acrostichum aureum*	Root	A, E
Aspergillus sp. 3	*Acanthus ilicifolius*	Leaf	A, E
Cumulospora marina Schmidt	*Acanthus ilicifolius*	Root	A
Fusarium sp.	*Acrostichum aureum*	Petoile	A, E
Nigrospora oryzae (Berk. *et* Br.) Petch.	*Acrostichum aureum*	Petiole	A
Penicillium sp.	*Acrostichum aureum*	Rhizome	A
Pestalotiopsis sp.	*Acanthus ilicifolius*	Root	A, E
Trichoderma sp.	*Acrostichum aureum*	Rhizome	A
Tritirachium sp.	*Acrostichum aureum*	Rhizome	A
Sterile isolate (MSI 1)	*Acanthus ilicifolius*	Root	A

Antimicrobial assay

Fourteen fungi were preliminary screened for anti-microbial activity (Table 1) using solid-state fermentation (SSF), comprising wheat bran seawater medium (wheat bran 5 g; 25% seawater, 15 mL in 125 mL conical flasks). Two mycelial plugs (3 mm diam.) from 5-7 day-old cultures were inoculated and incubated for 12 days at 30±2ºC. To extract the active principles from SSF flasks, 25 mL ethyl acetate was added to each flask and shaken for 60 minutes at 125 rpm at 30±2ºC. The extract was filtered through Whatman # 1 filter paper, the residue on the filter paper was further extracted by 25 mL ethyl acetate and pooled filtrates were evaporated to dryness in beaker. Based on initial results, *Aspergillus* sp. 2, *Aspergillus* sp. 3, *Pestalotiopsis* sp. and a sterile isolate (MSI 1) were selected to assess anti-microbial potential by growing under submerged fermentation (SmF). Isolates were cultivated in 500 mL conical flasks containing 200 mL malt extract-peptone-glucose broth (malt extract, 20 g; peptone, 1 g; glucose, 20 g; 25% seawater, 1000 mL) (Rodrigues *et al*., 2000) and incubated for 15 days at 30±2ºC under static condition. The broth of each flask was filtered through Whatman # 1 filter paper, extracted with 100 mL ethyl acetate (60 min, 150 rpm, 30±2ºC) and the upper layer was collected. The broth was re-extracted with 50 mL ethyl acetate and the extracts were pooled and allowed to dry. The dried extracts were eluted with five mL ethyl acetate and spotted on TLC plates for separation in butanol, acetic acid

and distilled water (4:1:5 v/v/v). The fluorescent spots were located on exposure to UV light. Each fraction was scraped, eluted with 10 mL ethyl acetate, filtered and allowed to dry.

Antimicrobial activity was assessed with a disk diffusion assay. Dried crude extracts recovered from SSF, SmF and partially purified fractions were eluted with five mL ethyl acetate. Plain antibiotic disks (6 mm; Hi-Media Laboratories, Mumbai, India) were impregnated with 10 µL crude or purified extract. Four bacteria (*Enterococcus* sp., *Pseudomonas aeruginosa*, *Salmonella typhi* and *Staphylococcus aureus*) and two fungi (*Candida albicans* and *Trichophyton mentagrophytes*) (from the Department of Medical Microbiology, Kasturba Medical College, Mangalore, India) were employed. Purified fractions were further tested against six bacteria (*Bacillus subtilis*, NCIM 2063; *Escherichia coli***,** NCIM 2256; *Pseudomonas aeruginosa*, NCIM 2200; *Staphylococcus aureus*, NCIM 2079; *Streptococcus faecalis*, NCIM 2080) and two yeasts (*Candida albicans*, NCIM 3471; *Cryptococcus albidus*, NCIM 3444) (from National Chemical Laboratory, Pune, India). Sterile nutrient agar (peptone, 5 g; sodium chloride, 5 g; beef extract, 1.5 g; yeast extract, 1.5 g; agar 15 g; distilled water, 1000 mL) served as basal medium. Suspensions of 24 hour bacterial cultures prepared in phosphate buffer mixed with 8 mL nutrient agar at 40ºC and overlaid. The disks impregnated with fungal extracts were placed on the overlaid medium. The plates were incubated at 37ºC for up to 24 hours. Sterile Czapek agar (Sucrose, 3 g; sodium chloride, 2 g; magnesium glycerophosphate, 0.5 g; potassium chloride, 0.5 g; dipotassium sulphate, 0.35 g; ferrous sulphate, 0.01 g; agar 15 g and distilled water, 1000 mL) served as basal medium for fungi. Mycelial macerate of 5 day-old fungal cultures in 8 mL Czapek agar at 40ºC were overlaid and the plates were incubated at 32±2ºC up to 72 hours.

Enzyme assay

Production of extacellular enzymes by the fungal endophytes was assessed by digestion of suspended or dissolved substrate in agar plates after inoculation with 3 mm mycelial plugs and incubation for 3-5 days at 32±2ºC (cellulase: Lingappa and Lockwood, 1962; rest of the enzymes: Hankin and Anagnostakis, 1975). The zone of enzyme activity surrounding the fungal colony was measured. Amylase activity was assessed by growing the fungi on glucose yeast extract peptone (GYP) agar medium (glucose, 1 g; yeast extract, 0.1g; peptone, 0.5g; agar, 16 g; distilled water, 1000 mL; pH 6) with 2% soluble starch. After incubation, the plates were flooded with 1% iodine in 2% potassium iodide. The clear zone formed surrounding the colony was

considered positive for amylase activity. For cellulase, the fungi were cultured on yeast extract peptone agar medium (yeast extract, 0.1g; peptone 0 .5 g; agar, 16 g and distilled water, 1000 mL) supplemented with 0.5% Na-carboxymethyl cellulose (CMC). After incubation, the plates were flooded with 0.2 aqueous Congo Red and destained with 1M NaCl for 15 minutes. The clear zone surrounding the colony indicated the cellulase activity. For chitinase activity, the fungi were grown on colloidal chitin agar medium (colloidal chitin, 2 g; agar, 16 g; distilled water, 1000 mL). The clear zone surrounding the colony after incubation was considered positive for chitinase activity. Laccase activity was assessed by growing the fungi on GYP agar medium amended with 1-naphthol, 0.005% (pH, 6) and incubated. On oxidation of 1-naphthol by laccase, the medium changes from clear to blue. For lipase activity, the fungi were grown on peptone agar medium (peptone, 10 g; NaCl, 5 g; $CaCl_2$ $2H_2O$, 0.1 g, agar, 16 g, distilled water, 1000 mL; pH 6) supplemented with Tween 20 (separately sterilized and added 1 mL to 100 mL medium). A clear zone around the colony indicated lipase-positive fungi. Protease assay was performed by growing the fungi on GYP agar medium amended with 0.4% gelatin (gelatin, 8 g/100 mL distilled water, sterilized separately and mixed with sterile GYP agar medium) adjusted the pH to 6. After incubation, plates were flooded with saturated aqueous ammonium sulphate. The undigested gelatin precipitated with ammonium sulphate and digested area around the colony was clear. For tyrosinase activity, fungi were grown on GYP agar medium. After incubation a mixture of 0.11% *p*-cresol and 0.05 % glycine was overlaid on the surface of the fungal colony. Culture plates were observed after 24 hours for the appearance of red brown colour around the colony which indicated tyrosinase activity.

Pestalotiopsis sp. was assessed further for the production of four extracellular enzymes. Cellulase production was detected after SmF, while xylanase, pectinase and protease after SSF. For cellulase activity, basal medium [KH_2PO_4, 1g; K_2HPO_4, 0.4 g; $MgSO_4$ $7H_2O$, 0.5 g; $CaCl_2$ $2H_2O$, 0.013 g; L-asparagine, 1.5 g; NH_4NO_3, 0.5 g; 1 mL of trace element solution ($ZnSO_4$ $7H_2O$, 2.64 g; $MnCl_2$ $4H_2O$, 2 g; $CaCl_2$ $6H_2O$, 0.4; $CuSO_4$ $5H_2O$, 0.4 g; supplemented with 2% carboxymethylcellulose); 25% seawater, 1000 mL] was adjusted to pH 7 and 9 using 2 M KOH. Twenty mL medium was dispensed in to 125 mL conical flasks and autoclaved. Two 3 mm mycelial plugs were aseptically inoculated to each flask and incubated at 30±2ºC for 15 days under static condition. For xylanase, pectinase and protease activity, 20 mL wheat bran seawater medium (wheat bran, 5 g; 25% seawater, 15 mL; pH was adjusted to 7 and 9) in 125 mL conical flasks were inoculated with two mycelial plugs (3 mm diam.) per flask and incubated under static condition for

15 days at 30±2ºC. Enzyme activity was assessed at 3, 6, 9, 12 and 15 day intervals. Prior to cellulase assay, the fungal mycelia from SmF flasks were removed by filtration through Whatman # 1 filter paper. The filtrate was assessed directly for cellulase activity by Nelson and Somogyi method (Nelson, 1944; Somogyi, 1952) using CMC as polymer, boiled enzyme extract served as a blank and D-glucose as a standard. Reducing sugars was estimated spectrophotometrically at 620 nm. To each SSF flask 25 mL distilled water was added and shaken at 100 rpm for 30 minutes. The extract was then filtered through Whatman # 1 filter paper, filtrate was assessed for xylanase and pectinase by Nelson and Somogyi method using xylan and polygalecturonic acid polymers and D-xylose and D-galacuronic acid as standards respectively. Protease activity was determined by the method outlined by Kunitz (1947) with a slight modification. The reaction mixture consisted of 1 mL 2% casein suspended in 100 mM phosphate buffer (pH 7.6) and 1 mL enzyme filtrate. The reaction was carried out at 35±1ºC and terminated after 20 minutes on addition of 3 mL ice cold 0.306 M trichloroacetic acid (TCA). One mL clear TCA soluble extract was mixed with 5 mL 0.4 M and Na_2CO_3 and 0.5 mL 1 N Folin Ciocalteus reagent and measured the absorbance at 660 nm.

Results and Discussion

Antimicrobial activity

Only eight crude extracts of the 14 endophytes grown on SSF showed inhibitory activity toward the test organisms (Table 2). None of the fungal extracts inhibited *Trichophyton mentagrophytes*, but *Aspergillus* sp. 2 and *Aspergillus* sp. 3 inhibited *Candida albicans*. All bacteria were inhibited by a sterile fungal isolate MSI 1. *Cumulospora marina* and *Pestalotiopsis* sp. inhibited representatives of both Gram-positive and Gram-negative bacteria. Crude extracts derived from SmF of four endophytes were tested against bacterial and fungal cultures (Table 3). *Aspergillus* sp. 3 and *Pestalotiopsis* sp. inhibited many bacteria and *Candida albicans*, while *Aspergillus* sp. 2 and MSI 1 were only effective against bacteria. None of the crude extracts were inhibitory against *Cryptococcus albidus*. Partial purification of crude extracts of four endophytes through TLC and observation under UV light revealed interesting results (Table 4). *Aspergillus* sp. 2 had a single fluorescent spot, *Aspergillus* sp. 3 and *Pestalotiopsis* sp. showed two fluorescent spots (A and B) and MSI 1 revealed three fluorescent spots (A, B and C).

Discovery of endophytic fungi in plant tissues opened up new possibilities in the search for metabolically active compounds. Cuomo *et al*. (1995) examined a large number of terrestrial and marine fungal isolates and

Table 2. Anitmicrobial activity of crude ethyl acetate extracts derived from SSF of endophytic fungi (diameter of zone of inhibition in mm; n = 4, mean ± SD). (Test organisms: Bacteria - Pa, *Pseudomonas aeruginosa*; Es, *Enterococcus* sp.; St, *Salmonella typhi*; Sa, *Staphylococcus aureus*; Yeast - Ca, *Candida albicans*).

Endophytes	**Bacteria**				**Yeast**
	Pa	**Es**	**St**	**Sa**	**Ca**
Alternaria chlamydosporus	0	0	0	10.8±1.4	0
Aspergillus sp. 1	0	0	0	7.5±0.2	0
Aspergillus sp. 2	11.1±0.3	11±0.1	9.6±0.2	11.3±0.3	12.3±1.5
Aspergillus sp. 3	0	0	14.4±0.9	10.7±0.7	9.7±0.5
Cumulospora marina	15.2±1	0	0	10.2±0.1	0
Nigrospora oryzae	0	0	0	2.2±0.1	0
Pestalotiopsis sp.	14.2±1.4	0	0	10.5±0.7	0
Sterile isolate (MSI 1)	9.4±0.3	8.8±0.2	8.3±0.4	9±0.1	0

found a higher number of anti-microbially active species among marine isolates. According to Dreyfuss and Chapela (1994), about 4000 secondary metabolites of fungal origin have been described as biologically active.

Table 3. Anitmicrobial activity of crude ethyl acetate extracts derived from SmF of endophytic fungi (zone of inhibition in mm; n = 4, mean ± SD). (Test organisms: Bacteria - Bs, *Bacillus subtilis* NCIM 2063; Ec, *Escherichia coli* NCIM 2256; Kp, *Kebsiella pneumoniae* NCIM 2719; Pa, *Pseudomonas aeruginosa* NCIM 2200; Sa, *Staphylococcus aureus* NCIM 2079; Sf, *Streptococcus faecalis* NCIM 2080; Yeast - Ca, *Candida albicans* NCIM 3471).

Endophytes	**Bacteria**						**Yeast**
	Bs	Ec	Kp	Pa	Sa	Sf	Ca
Aspergillus sp. 2	9.8±1	0	13.5±1	8.8±0.6	13.2±0.6	8.2±0.6	0
Aspergillus sp. 3	8.4±0.4	0	7.1±0.1	10±0.2	12.2±1.1	7.1±0.1	9.1±0.1
Pestalotiopsis sp.	12.6±0.3	0	12.7±1.1	9.4±0.4	15.1±0.8	8.6±0.4	8.3±0.2
Sterile isolate (MSI 1)	8.4±0.4	7.6±0.04	11.3±0.3	7.3±0.2	10.8±0.7	8.6±0.4	0

Mangrove fungi are known as sources of new bioactive compounds (Lin *et al*., 2001). However, investigations on mangrove endophytic fungal metabolites are scarce. In our study, more than 50% of the fungi tested showed anti-microbial activity, emphasizing the potential of endophytic fungi of mangrove

Table 4. Antimicrobial activity of partially purified ethyl acetate extracts derived from SmF of selected endophytic fungi (zone of inhibition in mm; n = 4, mean±SD). (Test organisms: Bacteria - Bs, *Bacillus subtilis* NCIM 2063; Ec, *Escherichia coli* NCIM 2256; Pa, *Pseudomonas aeruginosa* NCIM 2200; Sa, *Staphylococcus aureus* NCIM 2079; Sf, *Streptococcus faecalis* NCIM 2080; Yeast - Ca, *Candida albicans* NCIM 3471; Cra, *Cryptococcus albidus* NCIM 3444) (*Single fraction).

Endophytes	Fractions (RF)	Bacteria					Yeasts	
		Bs	Ec	Pa	Sa	Sf	Ca	Cra
Aspergillus sp. 2	* (0.54)	9±2	0	8.3±0.2	10.4±1.6	0	0	0
Aspergillus sp. 3	A (0.88)	8.1±0.2	0	0	9.5±1.7	0	10.9±0.1	13.8±1.5
	B (0.65)	8.6±0.5	0	0	7.5±0.6	0	8.1±0.1	10.1±0.1
Pestalotiopsis sp.	A (0.91)	15±1.2	0	0	13.2±2.5	0	10.9±0.1	0
	B (0.71)	8.2±1.4	0	0	0	0	0	0
Sterile isolate (MSI 1)	A (0.95)	8.7±1.4	8.9±0.2	9.7±0.9	9.4±1.3	0	0	0
	B (0.9)	8.5±0.8	8.6±0.5	10±1.3	8.6±0.7	0	0	0
	C (0.77)	8.6±0.4	9.4±0.3	8.8±0.4	8.4±0.6	8.3±0.2	0	7.3±0.4

plants as producers of novel metabolites. Crude extract and purified fractions of *Aspergillus* spp. showed a wide spectrum of anti-microbial activity. Similarly, several metabolites of the marine isolate, *Aspergillus niger* showed anti-bacterial and anti-fungal potential (Bugni *et al*., 2000). Crude extracts of *Aspergillus* sp. 3 and MSI 1 did not inhibit *Cryptococcus albidus*, while partially purified extracts did. This suggests possible interference between different active principles of these fungi. Crude and purified extracts of *Pestalotiopsis* sp. also showed a wide spectrum of anti-microbial activity in the current study. Endophytic *Pestalotiopsis* spp. are known to generate anti-fungal, anti-oxidant and anti-cancer metabolites (Li *et al*., 1996, 1998; Li and Strobel, 2001; Strobel *et al*., 2002). Dreyfuss and Chapela (1994) considered the fungal genera *Acremonium, Aspergillus, Fusarium* and *Penicillium* as 'creative species' based on the production of several bioactive metabolites. In our study, all three species of *Aspergillus* showed anti-microbially active, which supports their characterization by Dreyfuss and Chapela (1994) as 'creative species'. Based on a large number of biologically active HPLC peaks of *Pestalotiopsis guepinii*, Rodrigues *et al*. (2000) considered it as another 'creative species'. Clearly *Aspergillus* spp. and *Pestalotiopsis* spp. of mangrove endophytic origin deserve further study as potential sources of novel metabolites. Many endophytes of mangrove plants do not sporulate under

cultivation, and are therefore classified as ‘sterile fungi’ (Suryanarayanan *et al*., 1998; Kumaresan and Suryanarayanan, 2001; Ananda and Sridhar, 2002). These fungi are likely to possess novel metabolites. In our study, sterile fungus, MSI 1 showed potent anti-bacterial activity. Medicinal properties of different parts of mangrove plants (Bandaranayake, 1998) may be fully or partially dependent on the endophytic fungi. Shaking the broth inoculated with endophytic fungi suppressed growth in our study. Possibly culture conditions (e.g. still and variable shaking) influence fungal growth, which in turn affect production of secondary metabolites. Other factors to be considered when looking for anti-microbial compounds by endophytic fungi include, nature of fermentation (SmF/SSF), inoculum levels, extent of mixing, period of incubation and solvents used for extraction.

Enzyme activity

Cellulase and lipase were produced by seven of the fungi tested, while amylase and protease were produced by a few of them. None showed chitinase, laccase and tyrosinase activity (Table 5). Figure 1 shows the enzyme potential of *Pestalotiopsis* sp. at pH 7 and pH 9. Cellulase activity was highest at pH 7 on the 6^{th} day, with xylanase on the 9^{th} day at pH 9. Highest activity of pectinase was seen on the 6^{th} day at pH 7 and the 9^{th} day at pH 9. Protease activity was highest on the 6^{th} day irrespective of pH.

Fungal enzymes are gaining importance in agriculture, industry and human health, as they are often more stable (at high temperature and extreme pH) than the enzymes derived from plants and animals. Fungal enzymes are used in manufacturing food, beverages, confectioneries, textile and leather and help simplifying the processing of raw materials. Wood-inhabiting marine fungi serve as a potential source of exoenzymes (e.g. Rohrmann and Molitoris, 1992; Raghukumar *et al*., 1994, 1999; Pointing *et al*., 1998, 1999). Kumaresan and Suryanarayanan (2002) studied the extracellular enzyme production by the foliar endophytic fungi of *Rhizophora apiculata* and demonstrated their

Table 5. Enzyme activity of endophytic fungi on agar plates (n = 3; mean ± SD).

Endophytes	Diameter of enzyme activity (cm)			
	Amylase	**Cellulase**	**Lipase**	**Protease**
Acremonium sp.	3.5±0.4	3.8±0.02	2.8±0.02	0
Alternaria chlamydosporus	0	3.2±0.3	6.2±0.2	3±0.5
Alternaria sp.	2.9±0.1	3.1±0.3	4.9±0.1	3.5±0.2
Aspergillus sp. 2	0	4.2±0.02	6.9±0.1	3.8±0.2
Aspergillus sp. 3	0	4.9±0.01	7.1±0.1	0
Fusarium sp.	3.3±0.2	4.5±0.2	6.7±0.2	0
Pestalotiopsis sp.	2.9±0.4	4.1±0.04	3.4±0.3	2.8±0.3

involvement in litter degradation after senescence. All the endophytes tested in our study showed cellulase activity similar to that of leaf inhabiting salt marsh fungi (Gessner, 1979). The high lipase activity suggests their ability to use fats as energy source. Gessner (1979) also demonstrated lipase activity in 20 higher marine fungi from salt marshes. Pisano *et al*. (1964) reported protease activity in 13 out of 14 marine fungi.

Protease activity was seen in some endophytes of current study. None of the fungi showed laccase activity although laccase activity is seen in many marine fungi (Raghukumar *et al*., 1994, 1999; Kumaresan and Suryanarayanan, 2002; Bucher *et al*., 2004). The endophytic nature of these fungi might be the reason for the lack of laccase activity, since an active enzyme might damage the host plant. Tyrosinase is a common enzyme participating in lignin degradation by detoxifying the breakdown products. No chitinase and tyrosinase activity was evident in endophytes tested in our study. Cellulase and xylanase were produced by the *Pestalotiopsis* sp. at different incubation periods (6 and 9 days). Neutral (pH 7) and alkaline (pH 9) conditions stimulated higher production of these enzymes respectively. It is interesting to note the highest production of xylanase at pH 9, which deserves further exploration for their utility in paper and pulp industries. The current study indicates that when xylanase activity was highest at pH 9, cellulase activity declined. Cellulase-free xylanase or xylanase with low cellulase activity have been regarded as environment-friendly alternatives for effective bleaching of paper pulp without employing toxic chlorine compounds (Srinivasan *et al*., 1999). Cellulase-free xylanase selectively hydrolyse xylan without degrading cellulose of pulp fibres. Highest pectinase production was not affected by pH alterations in our study, its highest production was achieved after 6 days (at pH 7) and 9 days (at pH 9) of fermentation. Neutral and alkaline pH did not influence the highest protease activity after 6 days fermentation.

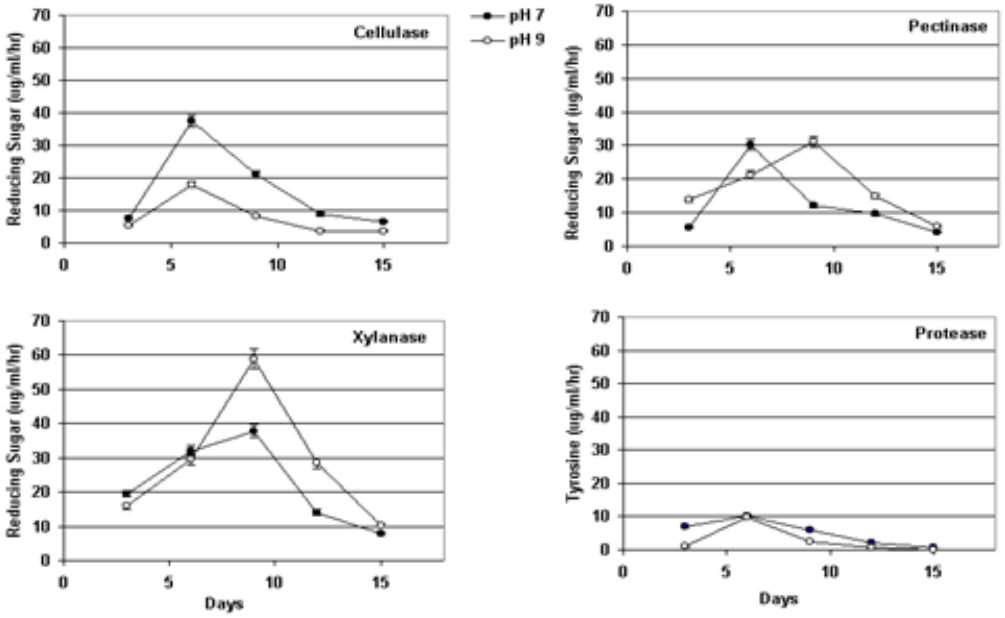

Fig. 1. Enzyme activity of endophytic fungus *Pestalotiopsis* sp. at pH 7 and pH 9: Cellulase (CM cellulose seawater medium, SmF), xylanase, pectinase and protease (wheat bran seawater medium, SSF) (mean; n = 5 ± SD).

Fungi have proven themselves invaluable sources of natural products for agriculture as well as biomedical development for over a half century (Turner, 1971; Turner and Aldridge, 1983; Biabani and Laatsch, 1998). As fungi thrive in competitive environments, it is hypothesised that their metabolic compatibility has been strongly influenced by natural selection (Glover, 1995). Bioactive product discovery depends on the knowledge of habitats where fungi are abundant and the strength of culture collection (Hyde, 2001). Studies on mangrove endophytic fungi were initiated recently and it has been realised that mangrove plants harbour an extremely diverse endophytic fungal flora (e.g. Petrini *et al*., 1992; Suryanarayanan and Kumaresan, 2000; Ananda and Sridhar, 2002). Screening this fungal resource for novel metabolites and enzymes and their application are major goals of current research to accomplish environment-friendly technological development.

Acknowledgements

We are grateful to Mangalore University for permission to carry out this research at the Department of Biosciences. One of us (GLM) thank the Principal, St. Agnes College for approval of study leave during the tenure of this study.

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(Received 25 October 2005; accepted 29 March 2005)