J Biochem Tech (2012) 3(5):S138-143
ISSN: 0974-2328

Isolation, production and partial purification of fungal extracellular pectinolytic enzymes from the forest soils of Bhadra Wildlife Sanctuary, Western Ghats of Southern India

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Received: 25 August 2012 / Received in revised form: 5 December 2012, Accepted: 11 December 2012, Published online: 29 December 2012
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Abstract

Isolation of effective industrially important extracellular pectinolytic enzyme producing fungi was done from the forest soil samples, using nutrient enrichment method. Among them, five fungal strains were selected for the production studies, based on the rate of zone of clearance on the pectin agar plates by using Congo red test. Production of pectinase and exo-polygalacturonases were studied under submerged fermentation, pectin broth supplemented with 1% pectin at an optimal condition of temperature 28 ± 1 ºC, pH 7.0 for 5 days of incubation under shake culture condition. Enzyme activity and specific activities were measured at an interval of 24 h. during the time of incubation. Maximum pectinase activity was showed by *Mortierella* sp. (5.38 U/mL) followed by *Syncephalastrum recemosum* and *Aspergillus fumigatus*, maximum polygalacturonase activity was by *Mortierella* sp. (2.22 U/mL) followed by *Aspergillus fumigatus* and *Trichosporiella cerebriformis* than the other isolates at 5th day of incubation. Maximum soluble crude and partially purified proteins were produced by *Trichosporiella cerebriformis* and *Syncephalastrum recemosum* at 5th day of incubation.

Keywords: Enrichment, Filamentous fungi, Forest soil, Pectinase, Exo-Polygalacturonase, Submerged fermentation

Introduction

Forest soil is the major source for the isolation of industrially important enzyme producing fungi than the other type of soils, forest environments having different type of vegetation, climatic condition and nutrient composition of the plants tissues that favors the fungal growth. The 95% of the plant tissues are composed of carbon, nitrogen, phosphorous and sulfur. Saprophytic fungal species mainly involved in the decomposition activities and involved in the nutrient cycling in the environment. The fungi were indigenous to the forest environment are more potent to produce industrially important enzymes than the other isolates.

Pectinases are frequently using in fruit and vegetable industry, employed widely in textile and food industries. Pectic enzymes were commonly used in fruit juice processing, the advantages of pectic enzymes are, produced a more rapid flow of juice, improved juice yields, facilitated filtering and gave greater clarity (Alkorta et al. 1998; Naidu and Panda 1998). Pectinases are mainly using in wine industry for decreasing astringency by solubilizing anthocyanins without leaching out procyadin polyphenols and they increase pigmentation by extracting more anthocyanins (Tucker and Woods 1991).

Pectinases are group of enzymes that attack pectin and depolymerise it by hydrolysis and transelimination as well as by deesterification reactions. Endo-polygalacturonase (EC: 3.2.1.15), Exo-polygalacturonase (EC: 3.2.1.67), Exo-poly α-galactouronosidase (EC: 3.2.1.82), Endo-pectatelyase (EC: 4.2.2.2), Exo-pectatelyase (EC: 4.2.2.9), Oligo D-galactosiduronate lyase (EC: 4.2.2.6) and Endopectinylase (EC: 4.2.2.10) are depolymerizing enzymes that cleaves glycosidic bonds of pectins by means of hydrolysis and transelimination (Jayani et al. 2005; Patil et al. 2012). Pectinesterase (EC: 3.1.1.11) is the pectolytic enzyme that catalyzes the hydrolysis of ester links between carboxyl and methyl groups of pectin (Ceci and Loranzo 1998). These enzymes act on pectin, a class of complex polysaccharides found in the cell wall of higher plants and cementing material for the cellulose network, but is most concentrated in citrus fruits and apples (Whitaker 1984; Thakur et al. 1997). Action of both Polygalacturonase and Pectinesterase enzymes needed in order to obtain complete hydrolysis of the pectin molecule (Taragano and Pilosof 1999). Pectic enzymes account for about 25% of the world's food enzyme production (Kashyap et al. 2001). Crude enzymes normally used for industrial purposes, since purification may make the product expensive, so the commercial pectic enzymes used in the food industry normally contain a mixture associated with hemicellulases, cellulases, glycosidases, proteases and oxidoreductases. However, in some food processes it is convenient to use only one type of pectic enzyme (Mantovani et al. 2005).

Microorganisms are currently the primary sources of industrial enzymes, originated from fungi and yeasts is of 50 %, from bacteria 35 %, while the remaining 15 % are either of phytopathogenic fungi (Cabanne and Doneche 2002) and some plant parasitic nematodes or animal origin. Filamentous microorganisms are most widely used in submerged and solid-state fermentation for pectinase production.

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More than 30 different genera of bacteria, yeasts and moulds have been used for the production of PGases.

Many plant-pathogenic bacteria and fungi are known to produce pectolytic enzymes useful for invading host tissues and nonpathogenic microorganisms are essential for the decay of dead plant material by producing these enzymes and thus assist in recycling carbon compounds in the biosphere (Patil et al. 2012). However, *Aspergillus, Penicillium, Fusarium, Pythium, Colletotrichum, Aureobasidium pullulans, Peacilomyces clavisporus, Phytophthora, Rhizoctonia solani, Neurospora crassa, Rhizopus stolonifer, Thermomyces lanuginosus, Alternaria mali, Thermoascus auratniacus, Saccharomyces cerevisiae, Lachnospira pectinoschiza* and *Erwinia, Agrobacterium, Bacteroides thetaiotamicron, Ralstonia, Bacillus* sp., *Pseudomonas, Lactobacillus* have been the genera most frequently studied from the past 15 years (Jayani et al. 2005). Major commercial preparations of pectinases were produced from the fungal sources and strains of *Aspergillus*, *Penicillium* and *Erwinia* were mainly used for the industrial production of pectinases (Gummadi and Panda 2003; Jayani et al. 2005).

Present study was taken up to isolate and characterize the more efficient pectinolytic fungi from forest soils, screen them for pectinolytic enzyme production and selection of more efficient isolates for industrial applications. The selected strains were studied for the production of pectinase and Exo-polygalacturonase under submerged fermentation (SmF) at different time of incubation and they were partially purified.

Materials and Methods

Study area and sample collection

Bhadra Wildlife Sanctuary (492.46 sq km) is a hot spot of biological diversity in the Western Ghats of southern India, with a wide range of tree vegetation such as dry and moist deciduous, semi-evergreen and evergreen forests (Champion and Seth 1968). Temperatures in the sanctuary ranges between 9 °C (mean minimum temperature in December) and 36 °C (mean maximum temperature in March), the mean annual precipitation of 2000 - 2500 mm most of which occur during the southwest monsoon (July to September) (Raju and Hegde 1995). Organic soil sample was collected from the study area in a depth of 5-10 cm by random mixed sampling method in a sterile polyethylene cover, carried to the laboratory, preserved at 4 ºC and used for further analysis.

Isolation and characterization of more efficient pectinolytic fungal species

Enrichment technique

Isolation of pectinolytic fungi was done by stepwise enrichment technique in a 250 mL Erlenmeyer's flask containing 100 mL of enrichment medium containing 0.5 and 1% pectin as a carbon source. Enrichment broth was prepared by adding 1 g of forest soil sample to 100 mL of pectin broth, containing 5% w/v yeast extract, 1 mL of 0.1% v/v Bromothymol blue, 0.6 mL of 10% v/v $CaCl_2.2H_2O$ pH 6.0 and supplemented with 0.5% w/v pectin. The flask was incubated in a shake culture condition at 28 ± 1 ºC for one week. After the one week of incubation, the enriched inoculum (1 mL) was transferred to the fresh sterile pectin broth supplemented with 1% w/v pectin and incubated for further one week by maintaining similar conditions (Patil and Chaudhari 2010). After the incubation it was used as inoculum for the screening of effective pectinolytic fungal species.

Qualitative screening

Selection and isolation of effective pectinolytic fungi were done by using 0.1 mL of inoculums from the enriched medium, they were plated on pectin agar contains 0.2%, $NaNO_3$; 0.1%, K_2HPO_4; 0.05%, $MgSO_4$. $7H_2O$; 0.05%, KCl; 10mg, $FeSO4.7H_2O$; 3%, Sucrose; 0.001%, $ZnSO_4$ and 0.001%, $CuSO_4$ supplemented with 1% pectin (HiMedia) and streptomycin sulphate (35 µg/mL) to control the bacterial contamination (pH 7.0), incubation was done at 28 ± 1ºC for 4-5 days. After the incubation, the characteristic fungal colonies were selected, subcultured on pectin agar and Czapek agar plates, used for the subsequent purpose. Secondary screening was done for the selection of more potent colonies for the production of extracellular pectinase by culturing on pectin agar plates supplemented with Congo red solution at pH 7.0. Actively growing mycelium's (3 days old) were removed from the growing edge of the fungal isolates by using sterile cork borer of 6 mm dia., the discs were inoculated to the pre-welled pectin agar plates and incubated at 28 ± 1 ºC for 3-5 days, after the incubation plates were observed for the zone of clearance around the colony. Pectinase producing colonies were seemed to be surrounded by the pale orange to clear zone against the dark red background (Teather and Wood 1982).

Characterization of pectinolytic fungi

The fungal species were grown on the CzA plates, identified based on Cultural and morphological characteristics using standard fungal identification manuals (Pitt 1979; Domsch et al. 1980; Ellis and Ellis 1987; Gilman 2001; Nagamani et al. 2006).

Extracellular enzyme production

Submerged fermentation (SmF)

Cultures were grown in 250 ml Erlenmeyer flask containing 100 ml of Pectin broth (pH 7.0), contains 0.2%, $NaNO_3$; 0.1%, K_2HPO_4; 0.05%, $MgSO_4.7H_2O$; 0.05%, KCl; 0.01%, $FeSO_4.7H_2O$; 3%, Sucrose; 0.001%, $ZnSO_4$; 0.001%, $CuSO_4$ and 1% of pectin were used for pectinase and exo-polygalacturonase enzyme production. After the sterilization of the Erlenmeyer flasks containing fermentation medium, young fungal myceliums of 3 day old cultures at the growing edges were used to inoculate aseptically. Culture flasks were incubated in the incubator shaker operating at 120-180 rpm at 28 ± 1 ºC for 5 days. Aseptically transferred 10 ml of incubated broth from the culture flasks at different time intervals of incubation (24, 48, 72, 96 and 120 hrs), mycelia and spores were harvested by centrifugation, under 4 ºC at 10000 rpm for 10 min. The supernatants obtained from the centrifugations were used as crude enzyme sources for assaying purpose and quantification of protein content.

Protein estimation

The protein concentration was determined by the Lowry's method, as described by Lowry's (1951) using bovine serum albumin (BSA) as a standard, absorbance was read at 660 nm using JENWAY-6305 UV-VIS Spectrophotometer. The culture filtrates from the culture flasks at different time of incubation were used as crude proteins and ammonium sulfate precipitated and dialyzed proteins were used as partially purified proteins for the quantification.

Estimation of reducing sugars

The reducing sugar (Galacturonic acid) concentration was determined by DNS method, as described by Miller (1959) using D-

Galacturonic acid as a standard. The color developed was measured at 540 nm using JENWAY-6305 UV-VIS Spectrophotometer.

Enzyme Assay

Determination of Pectinase activity

Supernatants from the incubated shake culture flasks at different time intervals (24, 48, 72, 96 and 120 hrs) were used as crude pectinases, activity was assayed using a method described by Okafor et al. (2010). The pectinase activity was determined using 1% pectin as substrate. Reaction mixture containing equal amounts of 1% pectin (0.5 mL) prepared in Citrate buffer (0.05 M; pH 5) and crude enzyme (0.5 mL). Blank solution maintained by using an enzyme (0.5 mL) with buffer (0.5 mL) instead of substrate was incubated at 50 ºC in water bath for 30 min. in a water bath, optical density was read at 540 nm against blank using JENWAY-6305 UV-VIS Spectrophotometer. The amount of reducing sugar released was quantified using Galactouronic acid as standard. A standard curve of D-Galactouronic (1 mg/mL) was developed under identical conditions to determine the reducing sugars formed. The enzymatic activity of filtrate was expressed as Unit per ml (U/ml), which was defined as the amount of enzyme, which liberates 1 µmole of reducing sugar per mL per minute under assay conditions.

Determination of Exo-Polygalacturonase (Exo-PGase) activity (Galacturan 1,4 α- galactouronidase) (E.C.3.2.1.67)

Supernatants from the incubated shake culture flasks at different time intervals (24, 48, 72, 96 and 120 hrs) were used as crude exo-polygalacturonases, activity was assayed according to the method of Cassanco et al. (1997). The Exo-PGase activity was determined using 1% polygalacturonic acid (PGA) as substrate, prepared in Citrate buffer (0.05 M; pH 5.0). The reaction mixture (1 mL) containing equal amounts of crude enzyme (0.5 mL) and substrate (0.5 mL) by maintaining a blank containing crude enzyme (0.5 mL) with buffer (0.5 mL) instead of substrate was incubated at 50 ºC for 30 min in a water bath, its optical density was determined at 540 nm against blank using JENWAY-6305 UV-VIS Spectrophotometer. A standard curve of D-Galactouronic acid (1 mg/mL) was developed under identical conditions to determine the reducing sugars formed. The enzymatic activity of filtrate was expressed as Unit per ml (U/ml), which is defined as the amount of enzyme, which liberates 1 µmole of reducing sugar per mL per minute under assay conditions.

Partial purification of proteins

After the incubation of 5th day, the contents of the culture flasks were centrifuged under refrigerated (4 ºC) condition at 10000 rpm for 10 min. and supernatants were filtered through Whatman filter paper to remove the fungal cells and spores. The crude enzyme was precipitated with different concentrations of ammonium sulfate solution up to the saturation level from 20-100% under magnetic stirrer at 4 ºC for overnight, the protein precipitate was collected by refrigerated centrifugation at 10000 rpm for 10 min. The pellet was suspended in Citrate buffer (pH 5.0) and dialyzed against 2-3 changes of buffer during the process in order to obtain proper purification under magnetic stirrer at 4 ºC for 24 hr. An aliquot of partially purified proteins were quantified (Hara et al. 1984).

Results

Isolation and characterization of more efficient pectinolytic fungal species

Isolation of more efficient pectinolytic fungal species were done from the inoculums of forest soil samples inoculated nutrient enriched culture on the Congo red agar plates. From the cultural and morphological characters of the isolates, fungal species were identified as, *Paecilomyces marquandii, Aspergillus flavus, Mucor hiemenis, Penicillium* sp., *Aspergillus fumigatus, Cladosporium herbarum, Trichosporiella cerebriformis, Mortierella* sp., *Syncephalastrum recemosum* and *Cunninghamella echinulata*. Further screening of qualitative pectinolytic activity on CzA plates supplemented with pectin and Congo red, based on the rate of zone of clearance around the fungal colony (Fig 1), (Table 1). The five effective pectinolytic fungal strains were selected among the others, like, *Paecilomyces marquandii, Aspergillus fumigatus, Trichosporiella cerebriformis, Mortierella* sp. and *Syncephalastrum recemosum* and they were used for extracellular pectinase and exo-polygalacturonase production studies under submerged fermentation.

Table 1: Isolated fungal species showed different rate of pectinolytic activity on Pectin agar by using Congo red test.

Sl. No.	Fungal Isolates	Pectinolytic activity	
		Fungal Colony (dia. in mm)	Clear zone (dia. in mm)
1	*Paecilomyces marquandii*	30	13
2	*Aspergillus flavus*	10	3
3	*Mucor hiemenis*	8	2
4	*Penicillium* sp.	20	5
5	*Aspergillus fumigatus*	16	9
6	*Cladosporium herbarum*	12	4
7	*Trichosporiella cerebriformis*	14	14
8	*Mortierella* sp.	15	6
9	*Syncephalastrum recemosum*	17	17
10	*Cunninghamella echinulata*	6	2

Enzyme assays

Fungal extracellular crude enzyme preparations were assayed for Pectinase and Exo-Polygalacturonase activity at different time of incubation (24, 48, 72, 96 and 120 hrs) and they were showed varied levels of enzyme activities among the species investigated. All the five investigated species were showed very high pectinase and exo-polygalacturonase activities at 5th day of incubation (Table 2), (Fig. 2).

Pectinase activity

Culture filtrates were assayed for pectinase activity, the following fungal species shows different rate of activities under assay conditions. *Mortierella* sp. showed maximum activity (5.38 U/mL) at 5th day of incubation followed by *Syncephalastrum recemosum* (4.95 U/mL), *Aspergillus fumigatus* (4.94 U/mL), *Trichosporiella cerebriformis* (4.61 U/mL) and *Paecilomyces marquandii* (2.78 U/mL), (Fig. 2(a), & 3), (Table 2). Specific activity of Pectinase at 5th day of incubation from *Mortierella* sp. was maximum (8.15 U/mg) at 5th day of incubation followed by *Syncephalastrum recemosum* (6.93 U/mg), *Aspergillus fumigatus* (6.48 U/mg), *Trichosporiella cerebriformis* (5.83 U/mg) and *Paecilomyces marquandii* (4.18 U/mg), (Fig. 4(a)), (Table 2).

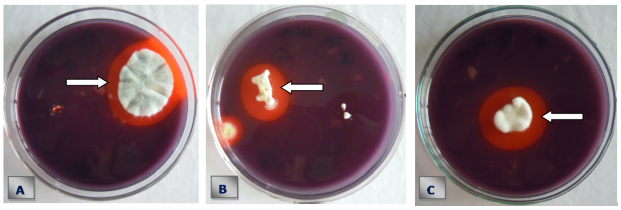


Figure 1: Fungal species showed the rate of pale orange to clear zone around the colony with dark red background on pectin agar supplemented with Congo red solution (A- *Paecilomyces marquandii*, B- *Mortierella* sp. and C- *Syncephalastrum recemosum*).

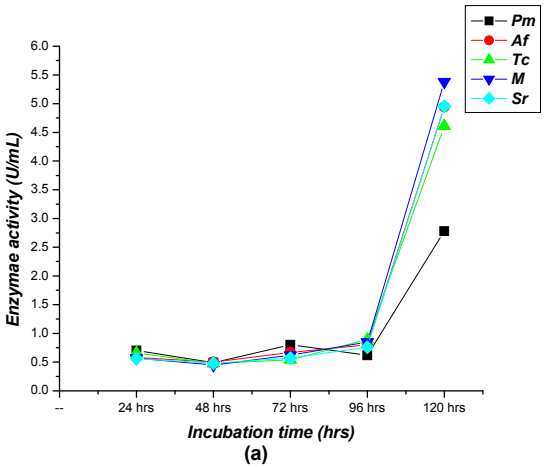


Figure 2: Pectinase (a) Exo-PGase (b) activity of fungal species at different time of incubation (Pm – *Paecilomyces marquandii*, Af - *Aspergillus fumigatus*, Tc – *Trichosporiella cerebriformis*, M – *Mortierella* Sp., and Sr – *Syncephalastrum recemosum*).

Exo-Polygalacturonase (Exo-PGase) activity

Culture filtrates were assayed for Exo-PGase activities, the following fungal species shows different rate of activities under assay conditions. *Mortierella* sp. showed maximum activity (2.22 U/mL) at 5th day of incubation followed by *Aspergillus fumigatus* (2.16 U/mL), *Trichosporiella cerebriformis* (1.99 U/mL), *Syncephalastrum recemosum* (1.70 U/mL) and *Paecilomyces marquandii* (1.1 U/mL) (Fig. 2(b) & 3) (Table 2). Specific activity of Exo-PGase at 5th day of incubation from *Mortierella* sp. was maximum (3.36 U/mg) at 5th day of incubation followed by *Aspergillus fumigatus* (2.84 U/mg), *Trichosporiella cerebriformis* (2.52 U/mg), *Syncephalastrum recemosum* (2.38 U/mg) and *Paecilomyces marquandii* (1.65 U/mg) (Fig. 4(b)), (Table 2).

Protein Content

Soluble crude proteins were more in *Trichosporiella cerebriformis* (790 µg/mL) followed by *Aspergillus fumigatus* (762 µg/mL), *Syncephalastrum recemosum* (714 µg/mL), *Paecilomyces marquandii* (666 µg/mL) and *Mortierella* sp. (660 µg/mL), they were obtained from the culture filtrates of fungal species grown at 5th day of incubation (Fig. 5) (Table 2). Partially purified and concentrated proteins were obtained from the fungal culture filtrates at 5th day of incubation, maximum concentration of protein was showed by *Syncephalastrum recemosum* (80 µg/mL) followed by *Mortierella* sp. (62 µg/mL), *Trichosporiella cerebriformis* (38 µg/mL), *Aspergillus fumigatus* (18 µg/mL) and *Paecilomyces marquandii* (16 µg/mL), (Table 2).

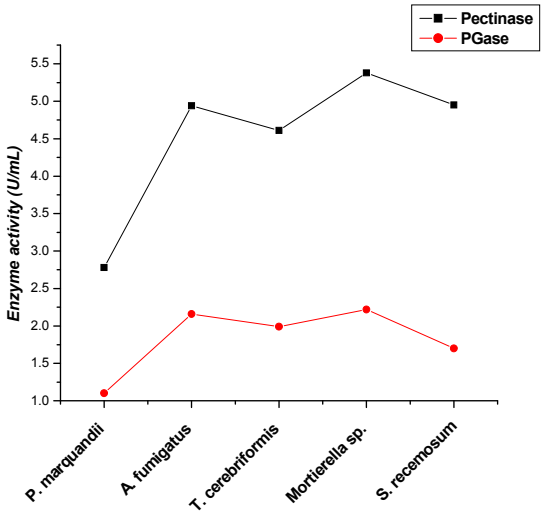


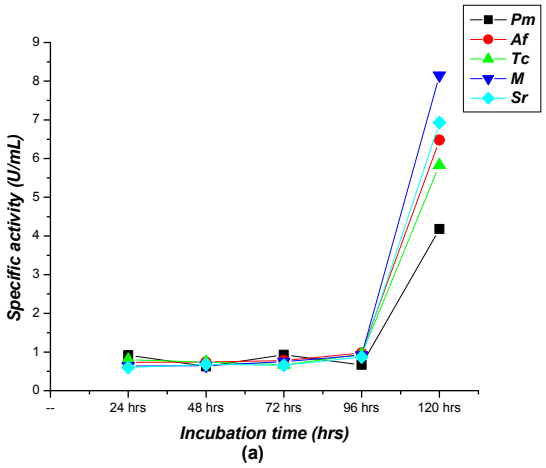
Figure 3: Pectinase and Exo-PGase activity of selected fungal species at 5th day of incubation.

Discussion

The present study showed that forest soil is rich source of biodiversity of pectinolytic fungi was played an important role in the biogeochemical cycles in the environment. The indigenous fungi from the forest soils were more potent for the production of industrially important extracellular pectinolytic enzymes.

Nutrient enrichment method and semi-quantitative plate assay approach providing rapid selection, it was very helpful for the direct enumeration and isolation of more efficient extracellular pectinase and exo-polygalacturonase producing fungal strains from the natural environments (Hankin and Anagnostakis 1977). During our study, many filamentous fungal species showed highest pectinase and exo-polygalacturonase activity among the other species. Maximum pectinase activity was observed in *Mortierella* sp. followed by *Syncephalastrum recemosum*. *Aspergillus fumigatus, Trichosporiella cerebriformis* and *Paecilomyces marquandii*. Maximum Exo-Polygalacturonase activity was showed by *Mortierella* sp. followed by *Aspergillus fumigatus*, *Trichosporiella*

cerebriformis, *Syncephalastrum recemosum* and *Paecilomyces marquandii.*



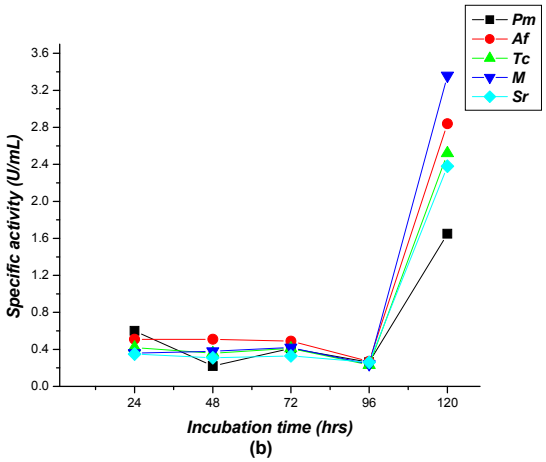


Figure 4: Specific activity of Pectinase (a) and Exo-PGase (b) at different time of incubation (Pm – *Paecilomyces marquandii*, Af - *Aspergillus fumigatus*, Tc – *Trichosporiella cerebriformis*, M – *Mortierella* Sp., and Sr – *Syncephalastrum recemosum*).

Filamentous fungi are considered to be, one of the most efficient hyperactive producers of pectinases and exo-polygalacturonases, those have much importance in the industrial processes. *Aspergillus niger* is a GRAS microorganism and a well-known pectinase producer (Naidu and Panda 1998). Mantovani et al. (2005) reported the strains of *A. niger* had showed similar levels of pectin esterase activity as earlier reported by Villarino et al. (1993) but it showed elevated pectinlyase activities during his study.

The synthesis of extracellular enzymes by microorganisms is highly influenced by the components of the growth medium, whereas pectolytic enzymes induced by several substances, in many cases pectin itself has been used (Alkorta et al. 1998; Palaniyappan et al. 2009).

Ogunlade and Oluwayemisi (2012) reported in their study as Pectinase production from Solid state fermentation culture of *Aspergillus niger* was significantly higher than that obtained by submerged fermentation. Banana peels were identified and used as a suitable low-cost substrate for the pectinase production by the strains of *A. niger.* Phutela et al. (2005) and Fujio et al. (1993)

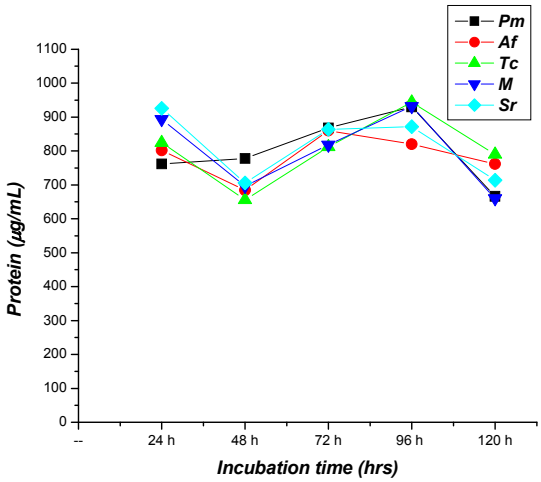


Figure 5: Soluble protein content at different time of incubation (Pm – *Paecilomyces marquandii*, Af - *Aspergillus fumigatus*, Tc – *Trichosporiella cerebriformis*, M – *Mortierella* Sp., and Sr – *Syncephalastrum recemosum*).

reported that the wheat bran, which is a cheap and readily available carbon source, supported the production of pectinases by the *Aspergillus fumigatus* TF3 and *Rhizopus* sp., the pectinase production was enhanced by supplementing of sucrose (Crotti et al. 1998). The use of banana peels, wheat bran and soy bran for pectinase production will not only reduce the production costs of the enzyme but also helps to decrease the pollution-load generated from the agriculture and agro-industrial wastes (Castilho et al. 2000; Ogunlade and Oluwayemisi 2012).

Table 2. Pectinase (PLase), Polygalacturonase (PGase) and protein content of more efficient fungal species on 5th day of incubation.

Fungal Species	Pectinase*		Polygalacturonase*		Protein content *	
	Activity (IU/mL)	Specific Activity (IU/mL)	Activity (IU/mL)	Specific Activity (IU/mL)	Crude (µg/mL)	Partially purified (µg/mL)
Paecilomyces marquandii	2.78± 0.06	4.18± 0.08	1.1± 0.1	1.65± 0.05	666± 22.54	16±2
Aspergillus fumigatus	4.94± 0.05	6.48± 0.13	2.16± 0.05	2.84± 0.13	762± 17.09	18±0.87
Trichosporiella cerebriformis	4.61± 0.1	5.83± 0.15	1.99± 0.12	2.52± 0.11	790±1 0	38±3.61
Mortierella sp.	5.38± 0.08	8.15± 0.13	2.22± 0.07	3.36± 0.05	660±1 2	62±2
Syncephalastrum recemosum	4.95± 0.05	6.93± 0.11	1.70± 0.1	2.38± 0.07	714±1 0	80±6

*Mean of three replicates ± standard deviation (SD)

Pectinases have been extensively used in several conventional industrial processes, such as textile, fruit juice processing, plant fiber processing, tea, coffee, oil extraction, treatment of industrial wastewater containing pectinacious material, etc.

Conclusion

The results from our study indicated that the nutrient enrichment, screening, isolation, production and partial purification of pectinolytic enzyme studies of indigenous microorganisms were yielded maximum quantity of extracellular enzymes at optimum conditions. Forest soil is rich source for the isolation of industrially

important pectinolytic microorganisms. Among the five potential extracellular fungal pectinase and exo-polygalacturonase enzyme producers, the *Mortierella* sp. showed maximum pectinase and exo-polygalacturonase activity. Further investigations will be required to achieve a maximum amount of pectinase and exo-polygalacturonase enzymes by optimization and enzyme kinetic studies.

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