International Journal of Advanced Biotechnology and Research
ISSN 0976-2612, Online ISSN 2278–599X,
Vol5, Issue2, 2014, pp188-201
http://www.bipublication.com

PRODUCTION AND PARTIAL PURIFICATION OF FUNGAL PECTINASES FROM ARECA NUT (*ARECA CATECHU* L.) HUSK WASTE UNDER SUBMERGED FERMENTATION

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[Received-27/03/2014, Accepted-05/04/2014]

ABSTRACT

The present study was aimed to selection of more efficient strains of fungal species from the areca nut husk waste for the production of industrially important fungal pectinase under submerged fermentation (SmF). The out of 24 fungal species, 20 species showed the pectin degradation ability and remain four fungal species not showed any zone of clearance around the colony. More efficient ten fungal species were selected based on the rate of zone of clearance on the Czapek Dox agar (CDA) plates supplemented with pectin and Congo red at an optimal conditions of temperature 28±1ºC, pH-7.0 and 5th days of incubation under shake culture. Maximum pectinase activity was showed by *Penicillium canescens* (2.192±0.20 IU/ml) followed by *Rhizopus stolonifer* (2.134±0.23 IU/ml), *Aspergillus candidus* (2.133±0.19 IU/ml), *Gliocladium viride* (2.13±0.22 IU/ml), *Penicillium* spp1 (1.852±0.21 IU/ml) than the other isolates at 3rd day of incubation. Exo–polygalacturonase activity was showed by *Rhizopus stolonifer* (1.553±0.11 IU/ml) and *Aspergillus candidus* (1.55±0.08 IU/ml) followed by, *Penicillium brevicompactum* (1.251±0.12 IU/ml), *Penicillium canescens* (1.202±0.13 IU/ml), *Penicillium* spp1 (1.171±0.09 IU/ml) than the other isolates at 2nd day of incubation. Soluble crude proteins were maximum by *Aspergillus terreus* (650±9.12 µg/ml) followed by *Penicillium brevicompactum* (530±6.23 µg/ml), *Rhizopus stolonifer* (500±7.56 µg/ml), *Aspergillus niger* (360±3.91µg/ml) and *Gliocladium viride* (350±3.46 µg/ml) at 4th day of incubation. Partial purified proteins were maximum by *Penicillium brevicompactum* (620±7.47 µg/mg) followed by, *Gliocladium viride* (490±6.23 µg/mg), *Aspergillus terreus* (470±6.98 µg/mg), *Fusarium chlamydosporum* (370±7.12 µg/mg), *Penicillium* spp1 (330±5.91 µg/mg) than the other isolates at 5th day of incubation.

Keywords: Areca nut husk waste, Exo–polygalacturonase, Filamentous fungi, Pectinase, Screening, Submerged fermentation.

[1] INTRODUCTION

Pectinolytic enzymes are one of the several extracellular enzymes produced by fungi that break down pectin, a polysaccharide substrate that is found in the cell walls of plants [8]. This enzyme splits polygalacturonic acid into monogalacturonic acid by opening glycosidic linkages. Through this process, it

softens the cell wall and increase the yield of juice extract from the fruits. The two major sources of the enzyme pectinase are plant and microorganism. But for both technical and economic point of view microbial source of pectinase has become increasingly important [4, 38]. A great variety of strains of bacteria [20, 42], yeast [26, 39], actinomycetes [6, 19] and mold [4] are capable of producing pectic enzymes.

Areca nut (*Areca catechu* L.) is one of the most important commercial crops in Shivamogga district, Karnataka state, India [29]. The area under areca nut cultivation has increased more rapidly in Shimoga district. The area under Shivamogga district areca nut is 94, 077.50 hectares with a production of around 52,781 Metric tonnes [36, 37]. The areca nut husk fibers are predominantly composed of cellulose and varying proportions of hemicelluloses, lignin, pectin and protopection. The total hemicellulose content varies with the development and maturity, the mature husk containing less hemicellulose than the immature ones. The lignin content proportionately increases with the development until maturity [34].

Microbial pectolysis is important in plant pathogenesis, symbiosis and decomposition of plant deposits [22]. Thus, breaking down pectin polymer for nutritional purposes, microbial pectolytic enzymes play an important role in nature [9]. The composition of pectic enzymes varies among species of microorganisms. Many studies have been reported that the enzyme preparations used in the food industry are of fungal origin because fungi are the potent producers of pectic enzymes [1, 3, 24]. *Apergillus niger* pectinases are most widely used in industries because this strain posses GRAS (Generally Regarded As Safe) status so that metabolites produced by this strain can be safely used. This fungal strain produces various pectinases including polymethyl-galacturonase (PMG), polygalacturonase (PG), and pectin esterase (PE) [33, 38, 43].

Now a day's pectinase is one of the most important enzymes in food processing industries mainly for extraction and clarification of fruit juices and wines. Pectinases have been used in several conventional industrial processes, such as textile, plant fiber processing, tea, coffee, oil extraction, and treatment of industrial wastewater [4]. Evidence showed that pectinases are inducible and they can produce from different carbon sources. Numerous reports have appeared on the optimization of fermentation and microbiological parameters and different fermentation strategies for the production of pectinases. Submerged fermentation (SmF) is important fermentation method employed for the production of microbial enzymes [26, 30]. Submerged fermentations (SSF) were used for the production of fungal extracellular pectinolytic enzymes.

Present study was taken up to isolate and characterize the fungi from areca nut husk waste, screen them for pectinase enzyme production and selection of more efficient isolates for industrial applications. The selected strains were studied for the production of pectinase at different time of incubation and they were partially purified by submerged fermentation.

[II] MATERIALS AND METHODS

2.1. Study area

Shivamogga is a district in the Karnataka state of India. A major part of Shivamogga district lies in the Malnad region of the Western Ghats. Shimoga lies between the latitudes 13°27' and 14°39' N and between the longitudes 74°38' and 76°04' E at a mean altitude of 640 meters above sea level. As the district lies in the tropical region, rainy season occurs from June to October. The average annual temperature of Shimoga District is around 26 °C. The average temperature has increased substantially over the years. In some regions of the district; the day temperature can reach 40 °C during summer. This has led to water crisis and other problems (Fig. 1).

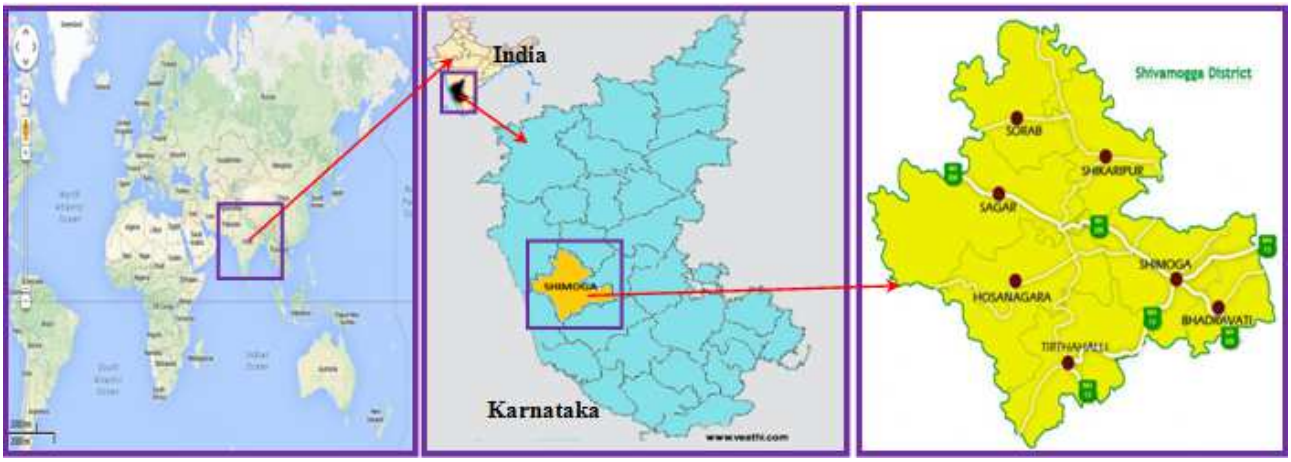

Figure 1. Study location and map of Shivamogga district, Karnataka state, India.

2.2. Collection of areca nut husk waste

Partially degraded areca nut husk waste was collected from the local farmer in Shivamogga region, Karnataka, India during the 2012-2013. The samples were taken by means of sterilized spatulas and collected in sterile sealed polythene bags. The sample was brought to the laboratory and was maintained at room temperature for microbiological study.

Figure 2. Partially degraded areca nut husk waste.

2.3. Isolation of fungi by serial dilution method

One gram of areca nut husk waste sample was taken in a conical flask containing nine milliliters of sterile distilled water and shaken well in vortex mixer for 30 minutes. From this stock, various dilutions were prepared from 10^{-1} to 10^{-7}, using sterile distilled water. One milliliters of the diluted sample was poured into Petri plates containing the Martin Rose Bengal agar medium and Potato Dextrose agar medium. Streptomycin was added to the molten medium after autoclave and the plates were incubated at 28 ± 2^0 C for 4 to 5 days to identify the fungi. Distinct fungal colonies grown on Martin Rose Bengal agar medium and Potato Dextrose agar medium were isolated from repeated plating [7, 28, 31].

2.4. Isolation of fungi by warcup (direct soil plate) method

Areca nut husk waste sample was collected in sterilized polythene bag. Then, 0.15 g of soil sample was added to sterile plates with the help of a sterilized cooled loop or transfer needle. Then, 15-20 ml of melted, cooled (45°C) sabouraud's agar media was added supplemented with Streptopenicillin and Rose Bengal, to each soil inoculated Petri plate. Dispense the soil particles throughout the medium by gentle rotation of the Petri dishes and allowed the plates to solidify. Plates were incubated at room temperature (28°C) in an inverted position for 15 days. After incubation, observed the different fungal colonies grown on sabouraud's agar media [7, 28].

2.5. Identification of fungi

Fungal morphology was studied macroscopically by observing colony features (colour and surfaces) under stereo binocular microscope (Magnus BQ0004) and microscopically by staining with lacto phenol cotton blue and observe under binocular compound

microscope (LABOMED Vision 2000) for the conidia, conidiophores and arrangement of spores [11, 14, 16, 17, 40].

2.6. Screening of more potential pectinolytic fungal species

Screening was done for the selection of more efficient pectinolytic fungi for the production of extracellular pectinase. Discs of actively growing mycelium's (3 days old culture) were removed from the growing edge of the fungal isolates by using sterile cork borer of 6 mm diameter, the discs were inoculated to the pre-welled pectin agar plates supplemented with Congo red solution at pH 7.0 incubated at 28 ± 1^0 C for 4−5 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 [41].

2.7. Submerged fermentation (SmF)

Cultures were grown in 250 ml Erlenmeyer flask containing 100 ml of Czapek Dox Broth (CDB) of pH 7.0 supplemented with 1% of pectin were used for pectinase and exo-polygalacturonase enzyme production. After the sterilization of the Erlenmeyer flasks containing fermentation medium, cooled and aseptically inoculated, discs of more efficient fungal mycelium of 3 days old cultures from the growing edge. Cultures were incubated in the incubator shaker operating at 120−160 rpm at 28 ± 1^0 C 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 4^0 C at 10,000 rpm for 10 min. The supernatants obtained from the centrifugations were carried to filtration through sterile whatman filter paper; they were used as crude enzyme sources for assaying purpose [10].

2.8. Estimation of protein

The protein concentration was determined by the Lowry's method, as described by Lowry, [23] using using bovine serum albumin (BSA) as a standard (0.2 mg/ml), absorbance was read at 660 nm using JENWAY−6305, UV−VIS Spectrophotometer and plotted the standard protein calibration curve. The culture filtrates from the culture flasks at different days of incubation were used as crude proteins and ammonium sulfate precipitated and dialyzed proteins were used as partially purified proteins and they were quantification.

2.9. Estimation of reducing sugar

The reducing sugar (Galacturonic acid) concentration was determined by Dinitrosalicylic acid (DNS) method, as described by Miller, [27] using D-galacturonic acid as a standard (1 mg/ml). The colour developed was measured at 540 nm using JENWAY-6305 UV−VIS Spectrophotometer and plotted the standard galacturonic acid calibration curve.

2.10. Determination of Pectinase activity

Culture filtrates from the incubated shake culture flasks at different time intervals (24, 48, 72, 96 and 120 hrs) were used as crude pectinase, activity was assayed using a method described by Okafor et al., [30]. The pectinase activity was determined using 1% pectin as substrate prepared in citrate buffer (0.05 M, pH 5). The reaction mixture containing equal amounts of 1% pectin (0.5 ml) and crude enzyme (0.5 ml) by maintaining a blank containing enzyme (0.5 ml) with buffer (0.5 ml) instead of substrate was incubated at 50^0 C in water bath for 30 min. The reactions were stopped by the addition of 1 ml of DNS reagent followed by keeping in boiling water bath for 5 min. and cooled in ice cold water then added 10 ml of distilled water and its optical density was read at 540 nm against blank using spectrophotometer. A standard curve of D−galacturonic 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 1µmol of reducing sugar per ml per minute under assay conditions.

2.11. Determination of Exo-Polygalacturonase (Exo-PGase) activity

Culture filtrates from the incubated shake culture flasks at different time intervals (24, 48, 72, 96 and

120 hrs) were used as crude Exo-Polygalacturonase (Exo-PGase), activity was assayed according to the method of Cassanco et al., [15]. The Exo-PGase activity was determined using 1% polygalacturonic acid (PGA) as substrate, prepared in citrate buffer (0.05 M, pH 5). 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 50^0 C for 30 min. in a water bath. The reactions were stopped by the addition of 1 ml of DNS reagent followed by keeping in boiling water bath for 5 min. and cooled in ice cold water then added 10 ml of distilled water and its optical density was determined at 540 nm against blank using spectrophotometer. A standard curve of D−galactouronic acid (1 mg/ml) was developed under identical conditions to determine the reducing sugars formed. The exo-polygalacturonase activity of culture filtrate was expressed as unit per ml (U/ml), which is defined as the amount of enzyme which liberates 1 µmol of reducing sugar per ml per minute under assay conditions.

2.12. Partial purification of Enzymes

After the incubation of 5^{th} day, the contents of the culture flasks were filtered through Whatmann's filter paper after the incubation and centrifuged under refrigerated condition at 8000 rpm for 10 min to remove the fungal cells and spores. The crude enzyme was precipitated with different concentrations of ammonium sulfate up to the saturation level from 20-100%, kept under magnetic stirrer at 4ºC for 24 hrs**,** protein precipitate was collected by refrigerated centrifugation at 10,000 rpm for 10 min. The pellet was suspended in citrate buffer (pH 5.0) and dialysed against 2─3 changes of buffer during the process under magnetic stirrer at 4ºC for 24 hrs. An aliquot of partially purified proteins were quantified (U/ml) [10].

2.13. Statistical analysis

Data presented on the average of three replicates as means ± standard error obtained from independent experiments.

[III] RESULTS

3.1. Isolation and identification of fungi

Isolation and screening of fungal species were done from the inoculums of partially degraded areca nut husk waste by serial dilution method and Warcup (direct soil plate) method. Cultural and morphological characters of the isolates were examined and identified as *Aspergillus niger, Aspergillus terreus, Aspergillus fumigatus, Aspergillus flavus, Aspergillus clavatus, Aspergillus wentii, Aspergillus candidus, Aspergillus* sp1., *Aspergillus* sp2., *Penicillium chrysogenum, Penicillium brevicompactum, Penicillium canescens, Penicillium* sp1., *Penicillium* sp2., *Penicillium* sp3., *Gibberella avenacea, Fusarium chlamydosporum, Rhizopus stolonifer, Paecilomyces carneus, Trichoderma viride, Humicola fuscoatra, Exophiala jeanselmei, Gliocladium viride* and *Lacellina graminicola.*

3.2. Screening of more potential pectinolytic fungal species

The strains were furthered screened qualitative and semi-quantitatively for the selection of more efficient pectinolytic activity by the fungus on Czapek Dox agar (CDA) plates supplemented with pectin and Congo red, based on the rate of zone of clearance around the fungal colony. The out of 24 fungal strains, 20 strains showed the pectin degradation ability and remain four fungal strains not showed any zone of clearance around the colony. The more efficient ten pectinolytic fungal strains were selected among the others like, *Penicillium brevicompactum* (20±0.23 mm), *Fusarium chlamydosporum* (18±0.28 mm), *Penicillium canescens* (17±0.24 mm), *Gliocladium viride* (15±0.09 mm), *Aspergillus terreus* (14±0.12 mm), *Humicola fuscoatra* (13±0.12 mm), *Penicillium* sp1 (12±0.16 mm), *Rhizopus stolonifer* (11±0.27 mm), *Aspergillus niger* (10±0.11 mm) and *Aspergillus candidus* (10±0.19 mm) and they were used for extracellular pectinase and exo-polygalacturonase (Exo–PGase) enzyme production studies under submerged fermentation (Table 1).

Table-1. Screening of extracellular pectinolytic fungi from areca nut husk waste

Sl. No.	Fungi	Pectinolytic activity at 5th day of incubation	
		Fungal Colony (dia. in mm)	Zone of Clearance (dia. in mm)
1	*Aspergillus niger*	40±1.1	10±0.11
2	*Aspergillus terreus*	32±0.8	14±0.12
3	*Aspergillus fumigatus*	33±1.1	9±0.08
4	*Aspergillus flavus*	32±0.9	2±0.09
5	*Aspergillus clavatus*	25±1.2	7±0.06
6	*Aspergillus wentii*	28±1.4	0.0±0.0
7	*Aspergillus candidus*	49±2.1	10±0.19
8	*Aspergillus* sp1.	22±1.0	0.0±0.0
9	*Aspergillus* sp2.	26±1.3	4±0.01
10	*Penicillium chrysogenum*	28±0.9	8±0.09
11	*Penicillium brevicompactum*	43±1.6	20±0.23
12	*Penicillium canescens*	39±1.1	17±0.24
13	*Penicillium* sp1.	23±1.4	12±0.16
14	*Penicillium* sp2.	38±2.1	0.0±0.0
15	*Penicillium* sp3.	34±1.8	3±0.02
16	*Gibberella avenacea*	31±0.8	7±0.09
17	*Fusarium chlamydosporum*	36±1.0	18±0.28
18	*Rhizopus stolonifer*	36±2.0	11±0.27
19	*Paecilomyces carneus*	37±1.2	3±0.03
20	*Trichoderma viride*	42±2.0	6±0.01
21	*Humicola fuscoatra*	29±1.7	13±0.12
22	*Exophiala jeanselmei*	30±1.3	5±0.06
23	*Gliocladium viride*	40±0.9	15±0.09
24	*Lacellina graminicola*	23±1.1	0.0±0.0

Note: Results are mean ± S.E of three replicates (n=3)

3.3. Enzyme assay

Culture filtrates were used as fungal extracellular crude enzyme preparations for the assays of 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 ten investigated species were showed very high pectinase and exo-polygalacturonase activity at 72 hrs of incubation and some species were showed slight increased or decreased rate of activity at 4th and 5th day of incubation.

3.3.1. Pectinase activity

The culture filtrates were assayed for pectinase activities, the following fungal species showed different rate of enzyme activities under assay conditions. Maximum pectinase activity was observed at 3rd day of incubation by *Penicillium canescens* (2.192±0.20 IU/ml) followed by *Rhizopus stolonifer* (2.134±0.23 IU/ml), *Aspergillus candidus* (2.133±0.19 IU/ml), *Gliocladium viride* (2.13±0.22 IU/ml), *Penicillium* sp1 (1.852±0.21 IU/ml), *Humicola fuscoatra* (1.851±0.09 IU/ml), *Aspergillus terreus* (1.851±0.08 IU/ml), *Fusarium chlamydosporum* (1.79±0.19 IU/ml), *Penicillium brevicompactum* (1.792±0.24 IU/ml) and *Aspergillus niger* (1.696±0.13 IU/ml). (Table 2). Specific activity of pectinase was maximum by *Gliocladium viride* (11.83±0.34 IU/mg) at 3rd day of incubation followed by, *Rhizopus stolonifer* (10.67±0.24 IU/mg), *Humicola fuscoatra* (8.04±0.30 IU/mg), *Aspergillus terreus* (8.04±0.14 IU/mg), *Aspergillus niger* (6.784±0.25 IU/mg), *Aspergillus candidus* (6.454±0.36 IU/mg) and *Penicillium canescens* (5.763±0.27 IU/mg) (Table 3).

Table 2. Pectinase activity at different time of incubation

Sl. No.	Fungal species	Pectinase activity (IU/mL)				
		24h	48h	72h	96h	120h
1	*Penicillium brevicompactum*	1.171±0.09	0.945±0.08	1.792±0.24	1.251±0.09	0.927±0.02
2	*Fusarium chlamydosporum*	0.942±0.01	1.068±0.13	1.79±0.19	1.492±0.21	0.518±0.01
3	*Penicillium canescens*	0.958±0.02	0.785±0.06	2.192±0.20	1.463±0.24	0.785±0.05
4	*Rhizopus stolonifer*	0.675±0.04	0.817±0.04	2.134±0.23	1.466±0.13	0.675±0.03
5	*Aspergillus terreus*	1.64±0.08	1.601±0.15	1.851±0.08	1.09±0.08	0.895±0.08
6	*Penicillium* spp1	1.463±0.10	1.642±0.04	1.852±0.21	1.571±0.09	0.927±0.07
7	*Aspergillus candidus*	0.942±0.08	1.037±0.13	2.133±0.19	1.463±0.20	1.037±0.12
8	*Gliocladium viride*	0.785±0.03	0.817±0.08	2.13±0.22	1.681±0.26	0.67±0.01
9	*Humicola fuscoatra*	1.25±0.11	1.383±0.07	1.851±0.09	1.605±0.19	1.084±0.13
10	*Aspergillus niger*	0.455±0.04	0.397±0.02	1.696±0.13	1.694±0.18	1.021±0.09

Note: Results are mean ± S.E of three replicates (n=3)

Table 3. Pectinase specific activity at different time of incubation

Sl. No.	Fungal species	Pectinase specific activity (IU/mg)				
		24h	48h	72h	96h	120h
1	*Penicillium brevicompactum*	5.57±0.26	2.231±0.22	5.114±0.33	3.906±0.12	1.749±0.10
2	*Fusarium chlamydosporum*	3.364±0.16	3.814±0.28	4.475±0.18	5.321±0.25	1.231±0.09
3	*Penicillium canescens*	5.043±0.21	4.61±0.34	5.763±0.27	5.619±0.28	2.803±0.14
4	*Rhizopus stolonifer*	3.375±0.13	4.085±0.31	10.67±0.24	2.932±0.34	1.771±0.11
5	*Aspergillus terreus*	3.64±0.12	2.640±0.21	8.04±0.14	1.67±0.11	3.891±0.24
6	*Penicillium* spp1	3.946±0.11	3.64±0.16	5.285±0.29	6.038±0.21	2.207±0.21
7	*Aspergillus candidus*	4.952±0.18	4.938±0.38	6.454±0.36	4.427±0.16	2.116±0.18
8	*Gliocladium viride*	4.617±0.19	5.106±0.29	11.83±0.34	5.09±0.42	2.04±0.19
9	*Humicola fuscoatra*	2.242±0.09	3.285±0.26	8.04±0.30	5.01±0.33	5.161±0.30
10	*Aspergillus niger*	0.771±0.01	0.651±0.03	6.784±0.25	4.70±0.39	3.403±0.22

Note:
Results are mean ± S.E of three replicates (n=3)

Pectinase enzyme was partially purified by ammonium sulphate precipitation of culture filtrates at 5th day of incubation followed by dialysis and they were showed maximum pectinase activity by *Aspergillus candidus* (0.597±0.05 IU/ml) followed by *Aspergillus niger* (0.565±0.06 IU/ml), *Aspergillus terreus* (0.565±0.04 IU/ml), *Rhizopus stolonifer* (0.434±0.02 IU/ml), *Gliocladium viride* (0.414±0.04 IU/ml), *Penicillium brevicompactum* (0.370±0.01 IU/ml), *Humicola fuscoatra* (0.314±0.02 IU/ml), respectively. Specific activity of pectinase was observed at 5th day of incubation from *Aspergillus candidus* (7.462±0.16 IU/mg) followed by, *Aspergillus terreus* (6.277±0.34 IU/mg), *Humicola fuscoatra* (5.233±0.03 IU/mg), *Aspergillus niger* (5.136±0.37 IU/mg), *Gliocladium viride* (4.870±0.07 IU/mg), *Rhizopus stolonifer* (4.34±0.14 IU/mg), *Penicillium canescens* (3.525±0.01 IU/mg), respectively (Table 7).

3.3.2. Exo–Polygalacturonase activity

The culture filtrates were assayed for exo–polygalacturonase activities, the following fungal species showed different rate of enzyme activities under assay conditions. Maximum exo–polygalacturonase activity was observed at 2nd day of incubation by *Rhizopus stolonifer* (1.553±0.11 IU/ml) and *Aspergillus candidus* (1.55±0.08 IU/ml) followed by, *Penicillium brevicompactum* (1.251±0.12 IU/ml), *Penicillium canescens* (1.492±0.13 IU/ml), *Penicillium* spp1 (1.171±0.09 IU/ml), respectively. (Table 4). Specific activity of exo-polygalacturonase was maximum by *Rhizopus stolonifer* (9.70±0.21 IU/mg) at 2nd day of incubation followed by, *Penicillium canescens* (8.776±0.24 IU/mg), *Aspergillus candidus* (7.380±0.19 IU/mg), *Gliocladium viride* (7.131±0.22 IU/mg) and *Fusarium chlamydosporum* (6.905±0.15 IU/mg)

Table 4. Exo-Polygalacturonase (Exo─PGase) activity at different time of incubation.

Sl. No.	Fungal species	Exo-Polygalacturonase activity (IU/mL)				
		24h	48h	72h	96h	120h
1	*Penicillium brevicompactum*	0.474±0.04	1.251±0.12	0.781±0.01	0.644±0.01	0.597±0.05
2	*Fusarium chlamydosporum*	0.958±0.11	1.381±0.08	0.911±0.04	0.597±0.03	0.707±0.06
3	*Penicillium canescens*	1.202±0.12	1.492±0.13	1.132±0.08	0.345±0.01	0.738±0.06
4	*Rhizopus stolonifer*	1.461±0.10	1.553±0.11	1.133±0.09	0.392±0.02	0.581±0.04
5	*Aspergillus terreus*	1.091±0.09	0.534±0.02	0.781±0.02	0.345±0.01	0.675±0.03
6	*Penicillium* spp1	0.911±0.04	1.171±0.09	1.068±0.09	0.644±0.04	0.597±0.02
7	*Aspergillus candidus*	1.068±0.08	1.55±0.08	0.970±0.03	0.392±0.02	0.518±0.04
8	*Gliocladium viride*	0.581±0.01	1.141±0.10	1.22±0.11	0.644±0.05	0.392±0.02
9	*Humicola fuscoatra*	0.675±0.03	0.623±0.01	0.628±0.02	0.424±0.03	0.707±0.06
10	*Aspergillus niger*	1.084±0.09	0.581±0.02	0.691±0.03	0.424±0.02	0.817±0.07

Note: Results are mean ± S.E of three replicates (n=3)

Table 5. Exo-Polygalacturonase (Exo─PGase) specific activity at different time of incubation

Sl. No.	Fungal species	Exo-Polygalacturonase specific activity (IU/mg)				
		24h	48h	72h	96h	120h
1	*Penicillium brevicompactum*	2.251±0.15	2.978±0.09	2.22±0.17	2.012±0.22	1.126±0.09
2	*Fusarium chlamydosporum*	4.79±0.28	6.905±0.15	2.277±0.18	2.13±0.20	1.683±0.13
3	*Penicillium canescens*	6.32±0.34	8.776±0.24	2.973±0.15	1.326±0.09	2.635±0.26
4	*Rhizopus stolonifer*	7.30±0.31	9.70±0.21	2.26±0.21	1.507±0.11	1.528±0.11
5	*Aspergillus terreus*	2.422±0.12	1.089±0.08	1.21±0.09	1.52±0.10	2.934±0.18
6	*Penicillium* spp1	2.461±0.14	2.61±0.11	3.051±0.27	2.47±0.19	1.421±0.10
7	*Aspergillus candidus*	5.623±0.28	7.380±0.19	2.93±0.20	1.187±0.18	1.057±0.09
8	*Gliocladium viride*	3.221±0.18	6.705±0.22	3.69±0.36	3.577±0.29	1.18±0.08
9	*Humicola fuscoatra*	1.923±0.11	1.476±0.08	1.962±0.09	1.834±0.14	3.366±0.31
10	*Aspergillus niger*	1.832±0.13	0.966±0.01	3.94±0.24	1.696±0.08	2.723±0.27

Note: Results are mean ± S.E of three replicates (n=3)

Exo–polygalacturonase enzyme was partially purified by ammonium sulphate precipitation of culture filtrates at 5th day of incubation followed by dialysis and they were showed maximum exo–polygalacturonase activity by *Gliocladium viride* (0.628±0.05 IU/ml) followed by *Humicola fuscoatra* (0.597±0.04 IU/ml), *Penicillium canescens* (0.515±0.04 IU/ml), *Rhizopus stolonifer* (0.502±0.05 IU/ml), *Aspergillus candidus* (0.471±0.02), *Aspergillus terreus* (0.439±0.02), respectively. Specific activity of exo-polygalacturonase was obtained at 5th day of incubation from *Humicola fuscoatra* (9.95±0.09 IU/mg) followed by, *Gliocladium viride* (7.388±0.19 IU/mg), *Penicillium canescens* (6.437 ±0.05 IU/mg), *Aspergillus candidus* (5.887±0.23 IU/mg), *Rhizopus stolonifer* (5.02±0.17 IU/mg), *Aspergillus terreus* (4.877±0.08 IU/mg), respectively (Table 7).

3.4. Protein content

Soluble crude protein was more in *Aspergillus terreus* (650±9.12 µg/ml) followed by *Penicillium brevicompactum* (530±6.23 µg/ml), *Rhizopus stolonifer* (500±7.56 µg/ml), *Aspergillus niger* (360±3.91µg/ml) and *Gliocladium viride* (350±3.46 µg/ml) and they were obtained from the culture filtrates of fungal species grown at 4th day of incubation (Table 6). Partially purified proteins were obtained by ammonium sulphate precipitation of culture filtrates at 5th day of incubation followed by dialysis and they were showed maximum protein content from *Penicillium brevicompactum* (620±7.47 µg/mg) followed by, *Gliocladium viride* (490±6.23 µg/mg), *Aspergillus terreus* (470±6.98 µg/mg), *Fusarium chlamydosporum* (370±7.12 µg/mg), *Penicillium* spp1 (330±5.91 µg/mg), *Rhizopus stolonifer* (250±5.42 µg/mg) and *Humicola fuscoatra* (250±6.16 µg/mg), respectively (Table 7).

Sl. No.	Fungal species	Protein content (µg/mL)				
		24h	48h	72h	96h	120h
1	*Penicillium brevicompactum*	210±4.08	420±7.12	350±6.51	530±6.23	320±7.23
2	*Fusarium chlamydosporum*	280±4.12	200±3.14	400±6.12	280±4.08	420±5.82
3	*Penicillium canescens*	190±3.81	170±3.75	380±6.05	260±3.62	280±3.01
4	*Rhizopus stolonifer*	200±3.15	160±6.23	200±3.65	500±7.56	380±5.96
5	*Aspergillus terreus*	450±6.78	490±7.12	230±3.12	650±9.12	230±3.46
6	*Penicillium* spp1	370±6.12	450±6.91	320±5.83	260±3.85	420±6.10
7	*Aspergillus candidus*	190±3.91	210±4.46	330±5.91	330±6.13	490±7.13
8	*Gliocladium viride*	170±3.08	160±3.86	180±5.42	350±3.46	330±5.51
9	*Humicola fuscoatra*	350±5.63	420±6.81	230±5.36	320±3.62	210±4.13
10	*Aspergillus niger*	590±6.02	600±8.14	250±6.13	360±3.91	300±5.13

Table 6. Soluble crude pectinase protein content at different time of incubation
Note: Results are mean ± S.E of three replicates (n=3)

Table 7. Pectinase and Exo-Polygalacturonase (Exo–PGase) activity, specific activity, crude and partially purified protein content at 5th day of incubation

Sl. No.	Fungal species	Pectinase		Exo-Polygalacturonase (Exo–PGase)		Protein content	
		Activity (IU/mL)	Specific activity (IU/mg)	Activity (IU/mL)	Specific activity (IU/mg)	Crude (µg/mL)	partially purified (µg/mL)
1	*Penicillium brevicompactum*	0.370±0.01	3.083±0.09	0.157±0.02	1.308±0.02	530±10.16	120±7.47
2	*Fusarium chlamydosporum*	0.226±0.02	1.614±0.05	0.251±0.01	3.585±0.05	420±8.12	140±7.12
3	*Penicillium canescens*	0.282±0.01	3.525±0.01	0.515±0.04	6.437 ±0.05	280±6.15	80±4.91
4	*Rhizopus stolonifer*	0.434±0.02	4.34±0.14	0.502±0.05	5.02±0.17	380±7.41	100±5.42
5	*Aspergillus terreus*	0.565±0.04	6.277±0.34	0.439±0.02	4.877±0.08	230±5.89	90±6.98
6	*Penicillium* spp1	0.282±0.01	2.563±0.11	0.439±0.03	3.990±0.16	420±6.43	110±5.91
7	*Aspergillus candidus*	0.597±0.05	7.462±0.16	0.471±0.02	5.887±0.23	490±7.12	80±4.52
8	*Gliocladium viride*	0.414±0.04	4.870±0.07	0.628±0.05	7.388±0.19	330±7.0	85±6.23
9	*Humicola fuscoatra*	0.314±0.02	5.233±0.03	0.597±0.04	9.95±0.09	210±5.91	60±6.16
10	*Aspergillus niger*	0.565±0.06	5.136±0.37	0.439±0.03	3.990±0.34	300±6.0	110±5.16

Note: Results are mean ± S.E of three replicates (n=3)

[IV] DISCUSSION

The present study showed that partially degraded areca nut husk waste is rich source of biodiversity of pectinolytic fungal species, those were plays an important role in the biogeochemical cycles in the environment. The indigenous microflora from the partially degraded areca nut husk waste was more potent for the production of industrially important pectinolytic enzymes.

During our study semi-quantitative plate assay approach providing rapid selection of fungal species, it was very helpful for the enumeration and isolation of more efficient extracellular pectinase and exo-polygalacturonase producing fungal species from the partially degraded areca nut husk waste, from that selection of more potent ten fungal species were used for the enzyme production studies. Culture filtrates were assayed for pectinase and exo–polygalacturonase activities under assay conditions. In the present study, maximum pectinase activity was observed at 3rd day of incubation by *Penicillium canescens* (2.192±0.20 IU/ml) followed by *Rhizopus stolonifer* (2.134±0.23 IU/ml) and *Aspergillus candidus* (2.133±0.19 IU/ml). Pectinase enzyme was partially purified by ammonium sulphate precipitation of culture filtrates at 5th day of incubation followed by dialysis and they were showed maximum pectinase activity by *Aspergillus candidus* (0.597±0.05 IU/ml) followed by *Aspergillus niger* (0.565±0.06 IU/ml) and *Aspergillus terreus* (0.565±0.04 IU/ml). Banakar, [10] reported that the maximum pectinase activity was observed at 5th day of incubation by *Mortierella* sp. (5.38 IU/mL) followed by *Syncephalastrum recemosum* (4.95 IU/mL) and *Aspergillus fumigates* (4.94 IU/mL). Banu et al., [12] reported that moulds isolated from municipal waste soil sample were screened for pectinolytic enzyme production when grown on pectin containing (YPSS) solid media. *Penicillium chrysogenum* was selected based on clearance zones and pectinase enzyme production was carried out in submerged fermentation.

In the present study, maximum exo–polygalacturonase activity was observed at 2nd day of incubation by *Rhizopus stolonifer* (1.553±0.11 IU/ml) followed by *Aspergillus candidus* (1.55±0.08 IU/ml) and *Penicillium brevicompactum* (1.251±0.12 IU/ml). Exo–polygalacturonase enzyme was partially purified by ammonium sulphate precipitation of culture filtrates at 5th day of incubation followed by dialysis and they were showed maximum exo–polygalacturonase activity by *Gliocladium viride* (0.628±0.05 IU/ml) followed by *Humicola fuscoatra* (0.597±0.04 IU/ml) and *Penicillium canescens* (0.515±0.04 IU/ml).

Polygalacturonase is hydrolytic pectin depolymerise produced by both microorganisms and plant tissues [6]. Banakar, [10] reported that the maximum exo-polygalacturonase activity was observed at 5th day of incubation by *Mortierella* sp. (2.22 IU/mL) followed by *Aspergillus fumigates* (2.16 IU/mL) and *Trichosporiella cerebriformis* (1.99 IU/mL). Amande and Adebayo-tayo, [6] reported that the polygaluronase production ranged from 0.115-5.885 U/ml in which *Aspergillus tamari* produced the highest at 3rd day by submerged cultivation on medium with citrus pectin. Fungal polygalacturonase are used in industrial processes for juice clarification [19]. The production of polygalacturonase (PG) by *Aspergillus, Fuasrium, Penicillium, Thermoascus, Lentinus* species on various substrates during solid substrate fermentation and submerged fermentation (21) are strong evidences of the hydrolysis of pectin and pectin containing materials for the growth of the fungi. Pasha et al., [32] reported that the fungal Polygalacturonase from *Aspergillus foetidus* MTCC10367 capable of utilizing citrus pectin as substrate. Polygalacturonase activity for purified enzyme was found to be 40.1 U/ml, protein concentration was 0.5 mg/ml and specific activity was determined as 80.2 U/mg.

In the present study, the yield of soluble crude protein was more in *Aspergillus terreus* (650±9.12 µg/ml) followed by *Penicillium brevicompactum* (530±6.23 µg/ml) and *Rhizopus stolonifer* (500±7.56 µg/ml) and

they were obtained from the culture filtrates of fungal species grown at 3rd day of incubation. Partially purified proteins were obtained at 5th day of incubation and they were showed maximum protein content from *Penicillium brevicompactum* (620±7.47 µg/mg) followed by, *Gliocladium viride* (490±6.23 µg/mg) and *Aspergillus terreus* (470±6.98 µg/mg). Amande and Adebayo-tayo, [6] reported that the yield of extracellular protein released on medium containing banana peels ranged from 0.9247 – 4.0108 mg/ml in which *Mucor piriformis* had the highest biosynthesis potential on 3rd day of submerged cultivation. Banakar, (10) reported that the soluble crude proteins were more in *Trichosporiella cerebriformis* (790 µg/ml) followed by *Aspergillus fumigates* (762 µg/ml) and *Syncephalastrum recemosum* (714 µg/ml) at 5th day of incubation.

Pectinase are constitutive or inducible enzymes that can be produced either by submerged or solid state fermentation [2]. Various factors related to environment affect the production of pectinase. Some of them are concentration of nutrients, pH, temperature, moisture content, influence of extraction parameters on recovery of pectinases and the effects played by the inducers. Both carbon and nitrogen sources show overall effect on the productivity of pectinases [5]. Microbial Pectinase can be stated as the most important enzyme for the juice industry. Although pectinase production is an inherent property of most all organisms, only those microbes that produce a substantial amount of extracellular pectinase are of industrial importance and have been exploited commercially [38]. The applications of pectinases in various fields is increasing therefore, the search for discovering new strains with novel properties and obtaining new enzymes with desirable biochemical and physico-chemical properties is necessary [25].

Environmental pollution due to toxic wastes and the advancement in biotechnology are cause of increasing demand to improve and to replace traditional chemical processes with biotechnological processes [13, 25]. Because of their physiological, enzymological and biochemical properties of fungi and other reasons they are the most important group of microorganisms used to industrial production of pectinolytic enzymes: Although bacteria are known as industrial enzymes producer, fungi are desired for the production of enzymes such as pectinases because of their nature that is generally regarded as safe (GRAS) [35]. The comparison with prokaryotes fungi are eukaryotic organisms and have broad range of genetic information and are able to perform microbial conversions. Almost all the commercial preparations of pectinases are produced from fungal sources. In order to enhance the pectinase production it may be feasible to select fungal strains that are genetically more productive, or modified fungi, which are not subjected to catabolite repression or generate high levels of the enzymes without require to an inducer [25].

Data from this study could be considered very promising due to the potential of pectinolytic fungi isolated from partially degraded areca nut husk waste. These pectinase producing fungi may also serve better in enzyme industries. We consider that these new isolates may have more potential for industrial uses.

[V] CONCLUSION

The results from our study indicated that the screening and isolation of indigenous microorganisms will yields maximum quantity at optimum condition. Areca nut husk waste is rich source for the isolation of industrially important microorganisms. The out of 24 fungal strains, 20 strains showed the pectin degradation ability and remain five fungal strains not showed any zone of clearance around the colony. The more efficient ten fungal strains were selected as potential extracellular pectinolytic enzyme producers by screening and isolation methods. Among them *Penicillium canescens* showed maximum pectinase activity followed by *Rhizopus stolonifer*, *Aspergillus candidus* and *Gliocladium viride*. Maximum exo–polygalacturonase activity showed by *Rhizopus stolonifer* followed by *Aspergillus candidus*,

Penicillium brevicompactum and *Penicillium canescens*. Further investigations will be required to achieve a maximum amount of pectinolytic enzymes by providing optimum conditions.

ACKNOWLEDGEMENT

The authors are gratefully acknowledge the Department of Microbiology for providing laboratory facilities and also grateful to other backward class and minority cell, Kuvempu University for their inspiring help and providing financial support to carry out the research work.

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