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Isolation, Identification and Screening of Pectinolytic Fungi from Vegetable Waste Dump Yards, Warangal (Telangana State, India)

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ABSTRACT

The aim of the investigation was to isolate, identify and screen pectinase producing fungi from vegetable waste dump yards of different regions of Warangal district of Telangana state. Fungal cultures were isolated from vegetable waste dumped soil using potato dextrose agar and sabourauds agar media. Purified fungal isolates were identified based on morphological, microscopic and molecular characteristics. Identified fungal isolates were screened for pectinase production. Fungal isolates showed pectinolytic activity as they produced zone of hydrolysis on pectin agar media. Production of pectinases was studied under submerged fermentation, pectin broth supplemented with 1% pectin at an optimal conditions of temperature 28°C, pH 5 and 16 days of incubation under shake culture conditions. Exopectinase and endopectinase activities were assayed using DNS method and viscometrical methods respectively. Enzyme activity was measured at an interval of 4 days. Quantitative estimation of proteins was also done by using Lowry's method at 660 nm. Out of 30 fungal isolates isolated only two strains were found to be efficient in pectinolytic activity. These two strains were identified as *Aspergillus niger* and *Aspergillus flavus.*

Key words: Aspergillus species. Pectinases, Protein, Submerged fermentation

Pectin is the versatile and structurally a homopolysaccharide present in the middle lamella and primarycell walls of terrestrial plants, occupying one-third of the dry weight of plant tissue (Gupta *et al.* 2008). Pectin is made up of long galacturonic acid chains withresidues of carboxyl groups and with varying degree of methyl esters. Primarily, these are made up of α (1-4) linked D-galacturonic acid residues (Favela *et al.* 2005). Depending on their mode of catalysis, the enzymes hydrolyzing pectin are broadly categorized into endo-polygalacturonase (E.C. 3.2.1.15), exo-polygalacturonase (E.C.3.2.1.6.7), pectin lyase (E.C. 4.2.2.10) and pectin esterase (E.C. 3.1.1.11). Pectinases, a heterogeneous group of related enzymes that hydrolyze the pectin substances, are widely distributed in higher plants and microorganisms (Fogarty *et al*. 1983, Whitaker *et al*. 1991). Microbial pectinases account for 10-25% of the global food and industrial enzyme sales (Singh *et al*. 1999) and their market is increasing day by day.

Pectinolytic enzymes find an important place in the current biotechnological era with their wide applications. Pectinase have cholesterol-lowering effect, fruit extraction and clarification and making biodegradable films (Fernandez *et al*. 1994). Pectinases are used in the textile industry as are capable of depolymerising the pectin breaking it into low molecular water soluble oligomers

improving absorbency and whiteness of textile material and avoiding fiber damage (Sonia *et al*. 2009). They are also used in coffee and tea fermentation by breaking pectin present in tea leaves, oil extraction by avoiding emulsification formation, improvement of chromaticity and stability of red wines (Botella *et al*. 2005). They also help in the maintenance of ecological balance by decomposition and recycling of waste plant materials (Hagerman and Austin 1986). Pectinases from food and food bio products processed waste alone account to a total of one-third quarter of world's food enzyme production.

Pectinase have been reported to be produced by a large number of bacteria and fungi such as *Bacillus* spp., *Clostridium* spp., *Pseudomonas* spp., *Aspergillus* spp., *Monillalaxa*, *Fusarium spp.*, *Verticillium* spp., *Penicillium*s pp., *Sclerotinia libertiana*, *Coniothyrium diplodiella*, *Thermomyces lanuginosus*, *Polyporus squamosus*, nematodes, yeasts and protozoa (Jayani *et al*. 2005). However, the filamentous fungi are most often used in the commercial production of pectinases. Microbial production of pectinases has been extensively studied (Kashyap *et al*. 2001, Torres *et al*. 2006). Keeping in view the importance of pectinases in the food processing industry and the problems associated with the disposal of food processing industry waste, the present study was undertaken to isolate and screen pectinase producing microorganism with an ultimate objective of identifying potential fungi for industrial application.

MATERIALS AND METHODS

Sample collection

Soil samples, collected from the site where the vegetable wastes were dumped, were placed in sterile polythene bags and were immediately transferred to the laboratory for microbial assessment and analysis.

Isolation and screening of fungi

One gram of soil sample collected from each collection site was suspended in sterile distilled water and 10-fold serial dilutions were made. 1 ml from each dilution was inoculated by spread plate method on to the sterile Petri plates containing potato dextrose agar (PDA) medium and incubated in an inverted position at28°C for 7 days (Kaur *et al*. 2014). Pure cultures were transferred onto slants media and maintained for identification and enzyme studies.

Selection and isolation of potential pectinolytic fungi were assessed by using 0.1 mL of inoculum 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, $FeSO_4.7H_2O$; 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), incubated at 28 ± 1°C for 4-5 days. After 5 days of incubation, plates were flooded with iodine- potassium iodide solution (5.0 g potassium iodide and 1.0 g iodine in 330 ml of distilled water) and observed for zone of hydrolysis around the wells (Khairnar *et al*. 2009, Reddy *et al*. 2012). This solution gives colour to medium containing pectin resulting in translucent halo round the wells.

Extracellular enzyme production

Submerged fermentation (SmF)

Cultures grown in 250 ml Erlenmeyer flask containing 100 mlof 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 assay of pectinase and exo-polygalacturonase enzymes. After the sterilization of the Erlenmeyer flasks containing fermentation medium, young fungal mycelia of 3 day old cultures at the growing edges were used to inoculate aseptically. Inoculated flasks were incubated in the orbitolshaker operating at120-180 rpm at 28 ± 1°C for 16 days. 10 ml of incubated broth from the culture flasks was withdrawn at different time intervals. The supernatants obtained from the centrifugations were used as partially purified enzyme sources for assay and quantification of protein content.

Estimation of reducing sugars

The reducing sugar (galacturonic acid) was determined by DNS method as described by Miller (1959) using galacturonic acid as a standard. The color developed was measured at 540 nm using spectrophotometer (ELICO SL 171 UV-VIS spectrophotometer).

Enzyme assay

Assay of Exo-Pectinase

The Exopectinase activity was assayed by DNS method (Miller 1959) using 1% pectin as substrate. Reaction mixture containing equal amounts of 1% pectin (1.0 mL) prepared in citrate buffer (0.05 M; pH 5.0) and partially purified enzyme (1.0 mL) was incubated at 50°C in water bath for 30 min. The reaction was terminated by addition of 3ml of 3,5- dinitrosalicilic acid DNS reagent and the contents were boiled for 15 minutes. After cooling the color developed was read at 540 nm. The amount of reducing sugar released was quantified using galactouronic acid as standard. Standard galacturonic was prepared by taking 100mg galacturonic acid in 100ml standard flask and made up the volume to 100ml. A standard curve of D-galactouronic (1 mg/mL) was prepared 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 µ mole of reducing sugar per mL per minute under assay conditions.

Assay of Exo-Polygalacturonase (Exo-PGase)

Exopolygalacturonases, activity was assayed by quantifying reducing sugars using DNS method (Miller 1959). The Exo-PGase activity was determined using 1% polygalacturonic acid (PGA) as substrate, prepared in sodium acetate buffer (0.1M; pH 4.5). The reaction mixture (2 mL) contained equal amounts of enzyme (1.0 mL) and substrate (1.0 mL) and incubating at 50°C for 30 min in a water bath. The reaction was stopped by addition of 3ml of

3,5- dinitrosalicilic acid DNS reagent and the contents were boiled for 15 minutes. The color developed was read at 540 nm. A standard curve of D-galactouronic acid (1 mg/mL) was developed under identical conditions to determine the reducing sugars released. The enzymatic activity was expressed as unit per ml (U/ml), which is defined as the amount of enzyme, which liberates 1µ mole of galacturonic acid (reducing sugar) per mL per minute under assay conditions.

Assay of Endo polygalacturonase (Endo-PG)

Wood's viscometric method (1955) was employed to estimate the endo-PG. Polygalacturonic acid (0.5%) was prepared by dissolving 0.5g of polygalacturonic acid in 100 ml citrate buffer (pH 5.5). The reaction mixture for the estimation of endo PG contained polygalacturonic acid (0.5%) substrate, citrate buffer (pH 5.5) and enzyme source in 4:1:2 ratio. The reaction mixture consisting of 12ml of substrate, 4ml of enzyme and 1ml of citrate buffer. The loss of viscosity was measured for every 10 minutes over a period of 30 minutes. The reaction mixture with heat killed (inactivated) enzyme and distilled water served as control. The percentage loss of viscosity was calculated by using the following formula:

$$V = \frac{t_i - t_a}{t_1 - t_0} \times 100$$

Where,

V = percentage of loss of viscosity

t_i = flow time of reaction mixture + inactive enzyme.

t_a = flow time of reaction mixture + active enzyme

t_0= flow time of distilled water+ active enzyme at ''O'' time

The Relative Enzyme Activity (REA) of endo PG was calculated by dividing 1000 with time required for 50% loss of viscosity (t 50) and in Relative Viscometric units (RVU).

$$REA = 1000 / tv50$$

Where,

tv50 = time required in minutes taken for 50% loss of initial viscosity

Pectin methyl esterase activity (PME)

Pectin methyl esterase activity was estimated by the method suggested by Kertez (1937). PME activity can be measured either by measuring the amount of methanol released or increase in free carboxyl group by monitoring pH changes.

Pectin esterase activity was measured by increase in free carboxyl group by titrating against NaOH in the presence of a pH indicator like phenolphthalein. For assaying PME activity, 20ml of 1% pectin (dissolved in 0.15M NaCl, pH - 7.0) and 4ml of enzyme extract were taken in a beaker and incubated for 1 hour. After incubation, the solution was titrated against the 0.02 N NaOH to reach pH 7 using phenolphthalein as indicator (colour change from colourless to pink). The heat killed enzyme extract was used as control.

$$\text{Pectin esterase activity} = Vs - V\,b\ (\text{Normality of NaOH}) \times 100/Vt$$

Where,

Vs-volume of NaOH used to titer sample (ml),

V b-volume of NaOH used to titer blank (ml),

V-volume of incubation mixture (ml),

t-Reaction time (min).

PME activity was expressed as milli equivalents of NaOH consumed min^{-1} ml^{-1} of enzyme extract under the assay conditions.

Endo pectin lyase (Endo-PL)

Endo-PL activity was assayed viscometrically as suggested by Wood (1955). 1% pectin was used as substrate in this assay. 4ml of culture supernatant and 0.8 ml of trisHCl buffer pH (8.0) were added to 12ml of pectin solution. Viscosity changes of reaction mixture were determined by using Ostwald viscometer. Initial reading time and the reading after 30 minutes of incubation were determined. The loss of viscosity was measured for every 10 minutes over a period of 30 minutes. The reaction mixture with heat killed (inactivated) enzyme and distilled water served as control. Enzyme activity was expressed in RVU units (relative viscometric units).

Protein estimation

The protein content of enzyme source 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 UV-VIS spectrophotometer.

Statistical analysis

The enzyme activities are presented as Mean ± SE of all values. Results obtained in this study were subjected to analysis of variance using one way ANOVA and difference between means were separated by Duncan Multiple Range Test using SPSS software 17.0 version.

RESULTS AND DISCUSSION

The enzyme activity of 30 fungal isolates was determined on 4^{th}, 8^{th}, 12^{th} and 16^{th} day of incubation at 28°C. Among these, isolates BF1 and WV have shown the high levels of enzyme production in the (Table 1) as well as in (Fig 1). These two isolates have shown high pectinolytic activity as evident from the zone of clearance. Through 18s rRNA based molecular characterization isolates BF1 and WV1 are identified as *Aspergillus niger* and *Aspergillus flavus* respectively. The cultures which were isolated soil sample were tested for pectinolytic enzymes and shown in (Table 1). The other parameter for calculating the enzyme activity of 30 isolates were the measurement of the enzymatic index (EI) by the given formula:

$$EI = \frac{\text{Diameter of hydrolysis zone}}{\text{Diameter of colony}}$$

The isolates with hydrolysis zone>1.0 cm are considered significant because it reflects the pectinolytic potential. The greater is the halo zone, the higher is the EI value. The results of the EI values of all the 30 fungal strains are summarized in (Table 1).

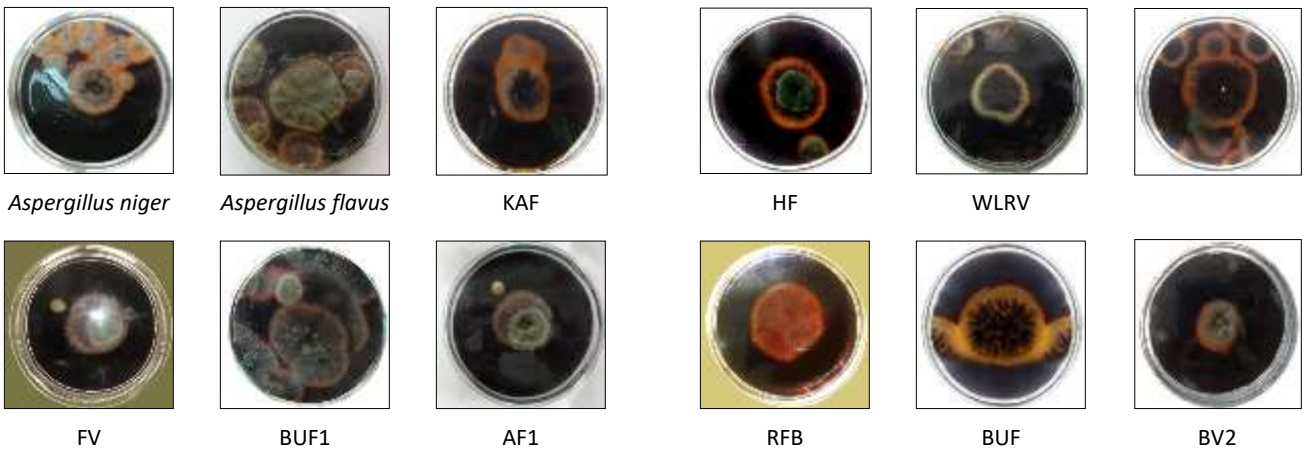

Fig 1 Culture plates showing clearance zone around isolated fungi

Table 1 Zone of hydrolysis and pectinase activity of pectinolytic fungal isolates

S. No	Source vegetable waste soil	Site of sample collection	Strains	Halozones (mm)	Halo hydrolysis (mm)	EI Enzyme index	Pectinase activity
1	Soil	Muluguroad	KF	1.1	1.3	1.11	+
2	Soil	Muluguroad	*A. flavus*	1.6	3.0	1.87	++
3	Soil	Muluguroad	BV1	1.2	2.0	1.66	++
4	Soil	Muluguroad	BUF	1.4	2.0	1.71	++
5	Soil	Muluguroad	KUF	1.4	2.4	1.71	++
6	Soil	Hasanparthy	*A. niger*	1.1	2.0	1.81	++
7	Soil	Hasanparthy	WL	2.0	1.6	0.8	+
8	Soil	Hasanparthy	KV1	2.2	1.0	0.45	-
9	Soil	Hasanparthy	KV2	1.8	1.2	1.1	+
10	Soil	Hasanparthy	HF1	1.5	1.6	1.06	+
11	Soil	Hasanparthy	KAF	1.8	1.2	1.1	+
12	Soil	Narsampet	BV2	1.0	1.4	1.4	+
13	Soil	Narsampet	WV1	1.9	1.2	0.63	-
14	Soil	Narsampet	YV	1.8	2.2	1.22	+
15	Soil	Narsampet	BF3	1.4	2.5	1.78	+ +
16	Soil	Narsampet	RV	1.5	2.3	1.53	++
17	Soil	Narsampet	AF1	1.4	2.0	1.42	+
18	Soil	Kazipet	RF	2.3	2.2	0.95	+
19	Soil	Kazipet	WV2	1.5	2.1	1.4	++
20	Soil	Kazipet	RFB	1.3	1.4	1.07	+
21	Soil	Kazipet	CV	1.8	2.0	1.1	+
22	Soil	Kazipet	FV	1.0	1.5	1.5	++
23	Soil	Kazipet	BF2	1.9	3.2	1.68	++
24	Soil	Kazipet	HF	1.7	2.8	1.64	++
25	Soil	Janagoan	HV1	1.3	1.5	1.52	+
26	Soil	Janagoan	HV2	1.4	2.0	1.42	+
27	Soil	Janagoan	NV	1.8	2.0	1.1	+
28	Soil	Janagoan	NF	1.6	1.2	0.75	-
29	Soil	Janagoan	KBV	1.4	1.1	0.78	-
30	Soil	Janagoan	KBF	2.0	1.5	0.75	-

++Optimum activity, +Good activity, -No activity

Molecular identification of fungi

Efficient pectinase producing isolates were sequenced by Macrogen laboratories, South Korea (www.macrogen.com). The resultant fragment was searched against gene bank data base with nucleotide BLAST (Fig 2, 3). Search option available through National center for biotechnology information (NCBI). Website: htpa://www.ncbi.n/m.nih.gov. Phylogenetic tree was constructed using MEGA-6 (Molecular Evolutionary Genetics Analysis) software. The stains were identified as *Aspergillus niger* and *Aspergillus flavus.*

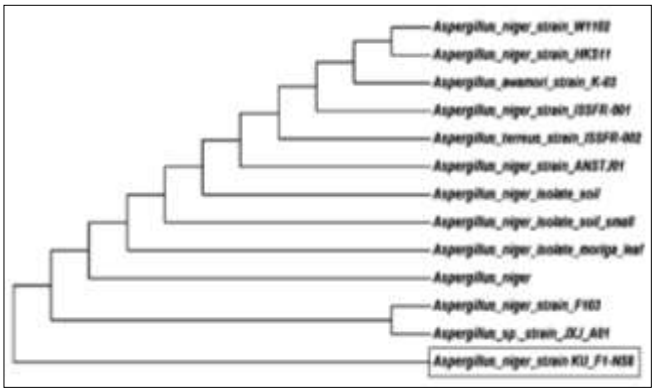

Fig 2 Phylogenetic tree of isolated strain of *A. niger*

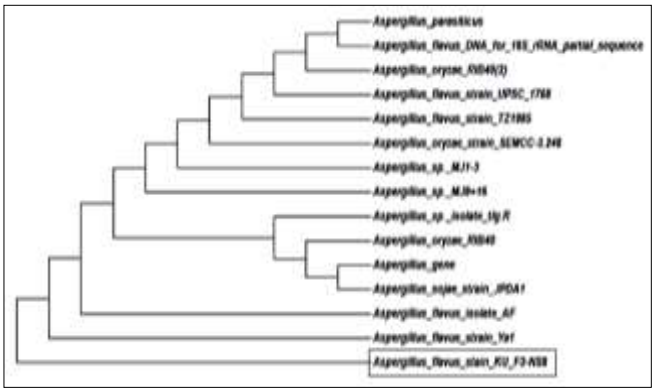

Fig 3 Phylogenetic tree of isolated strain of *A. flavus*

Table 2 Production of Exo-pectinase activity by different isolated fungal strains

S. No	Strain	Exopectinase activity (U/mL)			
		4th day	8th day	12th day	16th day
1	KF	$0.018^{j} \pm 0.008$	$0.373^{no} \pm 0.008$	$1.336^{d} \pm 0.008$	$1.033^{c} \pm 0.003$
2	*A. flavus*	$0.709^{a} \pm 0.005$	$1.507^{a} \pm 0.008$	$1.540^{b} \pm 0.011$	$0.750^{d} \pm 0.011$
3	BV1	$0.456^{abc} \pm 0.008$	$1.293^{b} \pm 0.008$	$1.283^{e} \pm 0.008$	$1.093^{c} \pm 0.008$
4	BUF	$0.066^{fgh} \pm 0.008$	$1.026^{de} \pm 0.0081$	$1.540^{b} \pm 0.011$	$1.370^{ab} \pm 0.011$
5	KUF	$0.018^{j} \pm 0.008$	$0.270^{o} \pm 0.011$	$0.440^{h} \pm 0.011$	$1.523^{a} \pm 0.008$
6	*A. niger*	$0.540^{ab} \pm 0.011$	$1.353^{b} \pm 0.008$	$1.593^{a} \pm 0.008$	$0.533^{e} \pm 0.176$
7	WL	$0.183^{j} \pm 0.008$	$1.100^{cde} \pm 0.090$	$0.423^{mn} \pm 0.008$	$0.100^{h} \pm 0.030$
8	KV1	$0.016^{j} \pm 0.008$	$0.270^{o} \pm 0.011$	$0.200^{q} \pm 0.011$	$0.100^{h} \pm 0.030$
9	KV2	$0.196^{bcde} \pm 0.545$	$0.853^{ghij} \pm 0.008$	$0.440^{h} \pm 0.011$	$0.280^{gh} \pm 0.050$
10	HF1	$0.130^{cde} \pm 0.011$	$0.716^{jk} \pm 0.008$	$0.340^{o} \pm 0.011$	$0.376^{efg} \pm 0.216$
11	KAF	$0.090^{ef} \pm 0.245$	$0.720^{jk} \pm 0.020$	$0.406^{mn} \pm 0.008$	$0.166^{h} \pm 0.008$
12	BV2	$0.064^{fghi} \pm 0.241$	$0.340^{no} \pm 0.011$	$0.340^{o} \pm 0.011$	$0.200^{gh} \pm 0.011$
13	WV1	$0.086^{ef} \pm 0.263$	$0.750^{ijk} \pm 0.011$	$0.390^{h} \pm 0.011$	$0.166^{h} \pm 0.008$
14	YV	$0.034^{hij} \pm 0.011$	$0.373^{no} \pm 0.008$	$0.340^{o} \pm 0.011$	$0.166^{h} \pm 0.008$
15	BF3	$0.016^{j} \pm 0.008$	$0.270^{o} \pm 0.011$	$0.280^{p} \pm 0.015$	$0.166^{h} \pm 0.008$
16	RV	$0.523^{ab} \pm 0.008$	$1.146^{cd} \pm 0.061$	$1.456^{c} \pm 0.0081$	$0.166^{h} \pm 0.008$
17	AF1	$0.028^{ij} \pm 0.057$	$0.560^{lm} \pm 0.011$	$0.923^{g} \pm 0.018$	$1.300^{b} \pm 0.011$
18	RF	$0.051^{ghij} \pm 0.011$	$0.813^{hijk} \pm 0.116$	$1.146^{f} \pm 0.008$	$0.716^{d} \pm 0.008$
19	WV2	$0.023^{j} \pm 0.008$	$0.340^{no} \pm 0.011$	$0.390^{n} \pm 0.011$	$0.166^{h} \pm 0.008$
20	RFB	$0.034^{hij} \pm 0.011$	$0.440^{mn} \pm 0.011$	$0.303^{p} \pm 0.008$	$0.166^{h} \pm 0.008$
21	CV	$0.096^{def} \pm 0.405$	$0.423^{mno} \pm 0.008$	$0.280^{p} \pm 0.015$	$0.166^{h} \pm 0.008$
22	FV	$0.037^{ghij} \pm 0.008$	$0.560^{lm} \pm 0.011$	$0.750^{j} \pm 0.011$	$0.283^{gh} \pm 0.006$
23	BF2	$0.284^{bcd} \pm 0.010$	$1.011^{de} \pm 0.011$	$0.340^{o} \pm 0.011$	$0.200^{gh} \pm 0.011$
24	HF	$0.540^{ab} \pm 0.011$	$0.373^{no} \pm 0.008$	$0.200^{q} \pm 0.011$	$0.166^{h} \pm 0.008$
25	HV1	$0.523^{ab} \pm 0.008$	$0.666^{kl} \pm 0.008$	$0.406^{mn} \pm 0.008$	$0.243^{gh} \pm 0.037$
26	HV2	$0.283^{bcd} \pm 0.008$	$1.116^{cde} \pm 0.011$	$0.340^{o} \pm 0.011$	$0.200^{gh} \pm 0.011$
27	NV	$0.099^{de} \pm 0.008$	$0.750^{ijk} \pm 0.011$	$0.666^{k0} \pm 008$	$0.460^{ef} \pm 0.011$
28	NF	-	-	-	-
29	KBV	-	-	-	-
30	KBF	-	-	-	-

Values are significant at P< 0.005

Exo-Pectinase activity

Culture filtrates of 30 different isolates were assayed for exo-pectinase activity, the fungal species showed different rate of activities under assay conditions have shown in the (Table 2). Fungal isolates have been successfully used for the production of pectinase by researches (Silva *et al*. 2002, Suresh and Viruthagiri 2010, Banu *et al*. 2010). In fact the most common source of microbial pectinase is from *Aspergillus niger* (Castilhoa *et al*. 1999). Results of Naderi *et al*. (2012) demonstrated that the maximum exopectinase enzyme production was achieved on second day. *A. niger* showed optimum activity at 12th day of incubation ($1.593^{a} \pm 0.008$ U/mL) and *A. flavus* showed optimum activity at 12th day of incubation ($1.540^{b} \pm 0.011$ U/mL). Ahmed *et al.* (2006) found that fungal species are responsible for the production of pectinase had maximum activity at 48 hours

after which the activity gradually decreased. According to Khairnar *et al*. (2009) maximum pectinase production was obtained in between 24 hours to 72 hours from *Aspergillus* species. RV and BV1 isolates also shown maximum enzyme production on 12th day of incubation (1.456c ± 0.008 U/m Land 1.283e±0.008 U/mL). Minimum production of enzyme was observed in KV1 (0.200q ± 0.011 U/mL). No enzyme production was observed in NF, KBV and KBF isolates.

Endo-polygalacturonase (Endo-PG)

The results obtained on endopolygalacturonase activity of different fungal isolates under study at different incubation periods are presented in (Table 3). From the (Table 3) it is evident that highest endopolygalacturonase production was shown on at 8th day of incubation. *A. niger* showed 262.60a ± 1.452 RVU and *A. flavus* showed 182.0b ± 1.154 RVU.BV1 and RV isolates also shown maximum enzyme production on 8th day of incubation (180.00c ± 1.154 RVU and 96.00d ± 0.000 RVU). Minimum production of enzyme was observed in KV1 (22.80n ± 0.000RVU). No enzyme production was observed in NF, KBV and KBF isolates.

Table 3 Production of Endo-polygalacturonase activity by different isolated fungal strains

S. No	Strain	Endo polygalacturonase activity (RVU)			
		4th day	8th day	12th day	16th day
1	KF	12.80l ±0.416	58.80i ±0.400	21.60n±0 0.577	12.20gh± 0.057
2	*A. flavus*	49.00b±0.577	182.0b± 1.154	11.30q ±0.577	7.366i ±0.033
3	BV1	58.20a ±0.577	180.0c± 1.154	23.50kl ±0.577	13.10ef± 0.577
4	BUF	13.00l ±0.577	36.60m ±0.577	15.10p ±0.000	7.30i ± 0.000
5	KUF	13.00l ±0.000	37.00m±0.000	15.76p±0.333	23.50c± 0.577
6	*A. niger*	57.80a± 0.577	262.60a ±1.452	92.36a± 0.333	23.60c± 0.577
7	WL	24.70g ±0.577	48.00k ±0.000	39.00g± 0.000	22.60d ±0.000
8	KV1	13.00l ±0.000	22.80n± 0.000	11.30q ±0.577	7.30i ± 0.000
9	KV2	13.40l ±0.577	58.86i ±0.333	39.00g± 0.000	12.80fg±0.000
10	HF1	22.80hi ±0.577	58.53j ±0.666	39.00g ±0.000	12.80fg ±0.000
11	KAF	25.03g ±0.333	58.80j ±0.000	37.60f± 0.000	11.70h ±0.000
12	BV2	24.00gh ±0.000	58.80i ±0.000	37.60f±0.000	11.70h ±0.000
13	WV1	27.20f± 0.100	37.60m ±0.000	21.13o± 0.333	7.30i ± 0.000
14	YV	15.23j± 0.333	48.00k ±0.000	39.00g± 0.000	28.40a ±0.000
15	BF3	58.20a ±0.577	37.60m±0.000	22.60lm± 0.000	12.11gh± 0.010
16	RV	58.20a ±0.577	96.00d ±0.000	59.20b± 0.000	27.73a ±0.333
17	AF1	43.96c ±0.333	74.06g± 0.033	42.33f±0.881	22.93cd ±0.333
18	RF	39.30d ±0.000	95.76d±0.333	44.00e ±0.000	22.93cd ±0.333
19	WV2	39.00d ±0.000	71.76h ±0.881	48.13d± 0.333	24.70b ±0.000
20	RFB	21.80i± 0.000	74.10g±0.000	57.80c± 0.577	11.70h ±0.000
21	CV	48.40b±0.000	90.46f ±0.233	15.10p ±0.000	12.13gh ±0.333
22	FV	13.00l ±0.000	59.20i ±0.000	15.76p±0.333	12.13gh ±0.000
23	BF2	14.00kl± 0.577	22.80n± 0.000	23.73kl ±0.333	12.13gh±0.000
24	HF	22.80hi±0.577	59.13i ±0.066	24.50j ±0.000	11.76h ±0.000
25	HV1	22.80hi±0.000	58.53j ±0.333	27.63i ±0.333	11.76h ±0.000
26	HV2	14.90jk ±0.000	59.20i ±0.000	28.30i ±0.000	13.80e± 0.000
27	NV	37.60e ±0.000	93.03e± 0.333	24.00jk± 0.000	11.66h ±0.033
28	NF	-	-	-	-
29	KBV	-	-	-	-
30	KBF	-	-	-	-

Values are significant at P< 0.005

Exo-Polygalacturonase (Exo-PGase)

Culture filtrates were assayed for Exo-PGase activities and the fungal species showed different rates of activities under assay conditions have shown in the (Table 4). The exploitation of pectinases mainly exopolygalacturonase have been well established in a variety of fruit juice and wine processing industries to increase the juice yield clarification, promoting antioxidant formation and juice concentrate production.

A. niger showed optimum activity (1.490a ± 0.011 U/mL) at 12th day of incubation followed by *A. flavus* (1.440b ± 0.011 U/mL). RV and BV1 isolates also shown maximum enzyme production on 12th day of incubation (1.043c ± 0.008 U/mL and 1.040d ± 0.008 U/mL). Minimum production of enzyme was observed in KV1 (0.010r ± 0.000 RVU). No enzyme production was observed in NF, KBV and KBF isolates.

Endopectin lyase (Endo-PL)

Results in (Table 5) reveals that highest production of endo pectin lyase recorded at 8th day of incubation. *Aspergillus niger* showed 190.00a ± 0.577 RVU and

Aspergillus flavus showed 177.60^{b} ± 0.577 RVU.RV and BV1 isolates also shown maximum enzyme production on 8th day of incubation (172.60^{c} ± 0.577 RVU 164.30^{d} ± 0.577 RVU). Least production of enzyme was observed in KV1 (22.00^{l} ± 0.577RVU). No enzyme production was observed in NF, KBV and KBF isolates.

Table 4 Production of Exo-polygalacturonase activity by different isolated fungal strains

S. No	Strain	Exo- polygalacturonase activity (U/mL)			
		4th day	8th day	12th day	16th day
1	KF	0.250^{hi} ±0.000	0.407^{j} ±0.008	0.584^{j} ±0.009	0.063^{gh} ±0.008
2	*A. flavus*	1.093^{a}± 0.008	1.266^{b}± 0.008	1.440^{b}±0.011	0.923^{a}± 0.008
3	BV1	0.723^{bcd}± 0.203	0.870^{d} ±0.011	1.040^{d} ±0.008	0.520^{bc} ±0.010
4	BUF	0.006^{l} ±0.003	0.016^{q}±0.006	0.010^{r} ±0.000	0.010^{h} ±0.000
5	KUF	0.200^{ij} ±0.011	0.373^{jk} ±0.008	0.543^{jk} ±0.008	0.030^{h} ±0.011
6	*A. niger*	1.150^{a} ±0.005	1.322^{a}± 0.011	1.490^{a} ±0.011	0.670^{b} ±0.011
7	WL	0.150^{ijkl}± 0.011	0.326^{l} ±0.006	0.526^{kl} ±0.008	0.010^{h}± 0.000
8	KV1	0.006^{l} ±0.003	0.016^{q} ±0.000	0.010^{r} ±0.000	-
9	KV2	0.050^{jkl} ±0.005	0.220^{n}±0.011	0.423^{o} ±0.024	0.010^{h} ±0.000
10	HF1	0.010^{l} ±0.000	0.133^{p} ±0.008	0.303^{p}± 0.008	0.010^{h} ±0.000
11	KAF	0.080^{jkl}± 0.011	0.030^{q} ±0.011	0.200^{q} ±0.011	0.043^{h} ±0.006
12	BV2	0.010^{l} ±0.000	0.016^{q}±0.006	0.173^{q} ±0.003	0.013^{h} ±0.003
13	WV1	0.183^{ijk}± 0.008	0.526^{h} ±0.008	0.440^{no}±0.011	0.073^{gh}± 0.021
14	YV	0.006^{l} ±0.003	0.010^{q} ±0.000	0.010^{r} ±0.000	0.023^{h}± 0.008
15	BF3	0.106^{ijkl} ±0.006	0.270^{m} ±0.011	0.853^{f} ±0.008	0.676^{b} ±0.288
16	RV	0.743^{b} ±0.005	1.010^{c}± 0.011	1.043^{c} ±0.008	0.676^{b} ±0.288
17	AF1	0.723^{bcd}± 0.203	0.630^{f}± 0.060	0.837^{f} ±0.008	0.973^{a}± 0.008
18	RF	0.636^{bcde}±0.073	0.870^{d} ±0.011	0.650^{i} ±0.011	0.320^{def} ±0.020
19	WV2	0.363^{ef} ±0.063	0.666^{f} ±0.008	0.750^{g} ±0.011	0.156^{fgh} ±0.006
20	RFB	0.366^{gh} ±0.056	0.476^{i} ±0.008	0.906^{e} ±0.008	0.223^{efg} ±0.014
21	CV	0.383^{gh} ±0.031	0.580^{g}±0.011	0.700^{h} ±0.000	0.403^{cd} ±0.006
22	FV	0.006^{l} ±0.003	0.010^{q} ±0.000	0.010^{r} ±0.000	0.023^{h}± 0.008
23	BF2	0.086^{jkl} ±0.006	0.166^{0p}± 0.008	0.010^{r} ±0.000	0.046^{gh} ±0.003
24	HF	0.050^{jkl} ±0.000	0.126^{p} ±0.003	0.176^{q}± 0.003	0.010^{h}± 0.000
25	HV1	0.166^{ijkl} ±0.008	0.340^{kl} ±0.011	0.493^{lm} ±0.008	0.400^{cd}± 0.010
26	HV2	0.586^{cdef}±0.006	0.750^{e} ±0.011	0.340^{p} ±0.011	0.053^{gh}±0.003
27	NV	0.080^{jkl} ±0.000	0.166^{op} ±0.008	0.340^{p}±0.011	0.360^{cde} ±0.015
28	NF	-	-	-	-
29	KBV	-	-	-	-
30	KBF	-	-	-	-

Values are significant at P< 0.005

Table 5 Production of Endopectinlyase activity by different isolated fungal strains

S. No	Strain	Endopectinlyase activity (RVU)			
		4th day	8th day	12th day	16th day
1	KF	27.00^{h}± 0.577	42.00^{ij}± 0.577	22.00^{h} ±0.577	16.60^{d} ±0.577
2	*A. flavus*	59.80^{d} ±0.577	177.60^{b} ±0.577	42.00^{e}± 0.577	13.86^{fg}±0.333
3	BV1	45.30^{d} ±0.577	164.30^{d} ±0.577	50.30^{b} ±0.5777	13.56^{fg} ±0.317
4	BUF	21.20^{k} ±0.577	41.00^{j} ±0.577	34.00^{f} ±0.577	14.20^{efg}±0.000
5	KUF	13.26^{m}±0.606	23.00^{l} ±0.577	16.60^{i} ±0.577	13.30 ^{fg}±0.000
6	*A. niger*	54.30^{b}± 0.577	190.00^{a} ±0.577	54.30^{a} ±0.577	16.60^{d} ±0.577
7	WL	23.00^{ij}± 0.577	43.50^{i} ±0.577	32.70^{f} ±0.577	22.00^{c} ±0.000
8	KV1	13.26^{m}±0.606	22.00^{l} ± 0.577	16.60^{i} ±0.577	13.30 ^{fg}±0.000
9	KV2	27.36^{h} ±0.333	47.30^{h}± 0.000	33.70^{f} ±0.577	13.30^{fg} ±0.000
10	HF1	23.00^{ij} ±0.577	41.00^{j} ±0.577	16.60^{i} ±0.577	14.30^{efg} ±0.577
11	KAF	14.43^{lm}± 0.296	22.00^{l} ±0.577	22.20^{h}± 0.577	13.30^{fg}±0.000
12	BV2	23.00^{ij}± 0.577	32.66^{k} ±0.881	16.60^{i} ±0.577	13.30^{fg} ±0.000
13	WV1	14.43^{lm}±0.577	22.00^{l} ±0.577	34.00^{f}±0.577	16.60^{d} ±0.000
14	YV	22.00^{jk}±0.577	47.30^{h} ±0.577	43.10^{de}± 0.577	22.20^{c} ±0.000
15	BF3	45.00^{d} ±0.000	32.66^{k} ±0.881	16.60^{i} ±0.577	13.30^{fg} ±0.000
16	RV	50.50^{c}± 0.577	172.60^{c} ±0.577	45.00^{cd}± 0.577	14.30^{efg}± 0.577

17	AF1	15.00^{l} ± 0.577	22.00^{l} ± 0.577	54.00^{a}± 0.577	41.73^{a} ± 0.577
18	RF	27.70^{h} ±0.577	43.50^{i}± 0.577	33.00^{f} ±0.577	13.30^{fg}± 0.000
19	WV2	45.00^{d} ±0.577	47.30^{h} ±0.577	22.00^{h}± 0.577	16.93^{d} ±0.333
20	RFB	13.30^{m}± 0.577	33.00^{k}± 0.577	47.30^{c}±0.577	14.20^{efg} ±0.000
21	CV	45.00^{d} ±0.577	47.30^{h} ±0.577	41.40^{e} ±0.577	14.53^{ef}±0.333
22	FV	34.00^{f}±0.577	54.00^{f} ±0.000	23.66^{h} ±0.666	13.20^{g}±0.577
23	BF2	32.20^{g} ± 0.577	50.00^{g} ±0.000	22.00^{h} ±0.577	14.20^{efg} ± 0.577
24	HF	15.20^{d}± 0.577	41.40^{j} ±0.577	22.33^{h}± 0.333	24.00^{b} ±0.000
25	HV1	42.00^{e} ±0.577	50.00 ^{g} ±0.577	42.33^{e}±0.333	13.66^{fg} ±0.881
26	HV2	22.00^{jk} ±0.577	43.50^{i} ±0.577	22.66^{h}± 0.333	15.33^{e} ±0.333
27	NV	15.30^{l}± 0.577	41.40^{j} ±0.577	29.66^{g}±3.844	13.33^{fg} ± 0.666
28	NF	-	-	-	-
29	KBV	-	-	-	-
30	KBF	-	-	-	-

Values are significant at P< 0.005

Table 6 Production of pectin methyl esterase by different isolated fungal strains

S. No	Strain	Pectin methyl esterase activity (meq. of NaoH consumed /min/ml)			
		4th day	8th day	12th day	16th day
1	KF	0.040^{ef} ± 0.000	0.009^{d} ±0.000	0.015^{a}± 0.000	-
2	*A. flavus*	0.090^{a}± 0.000	0.040^{a}± 0.000	0.011^{b} ±0.000	0.004^{c}±0.000
3	BV1	0.080^{b} ±0.000	0.036^{b} ±0.000	0.004^{f} ±0.000	0.004^{c}±0.000
4	BUF	0.036^{f} ±0.000	-	-	-
5	KUF	0.030^{g} ±0.000	0.009^{d} ±0.000	0.003^{gh} ±0.000	0.002^{d} ±0.000
6	*A. niger*	0.080^{b} ±0.000	0.036^{b} ±0.000	0.005^{de}± 0.000	0.002^{d} ±0.000
7	WL	-	-	-	-
8	KV1	0.006^{h} ±0.000	0.003^{e} ±0.000	0.001^{k}± 0.001	-
9	KV2	-	-	-	-
10	HF1	0.036^{f} ±0.000	0.009^{d} ±0.000	0.001^{k}±0.000	-
11	KAF	0.030^{g} ±0.000	0.008^{d}± 0.000	0.006^{de} ±0.000	-
12	BV2	0.030^{g} ±0.000	0.030^{c}± 0.000	0.002^{j} ±0.000	0.010^{b}± 0.000
13	WV1	0.070^{c} ± 0.000	-	0.001^{k} ±0.000	-
14	YV	0.050^{d} ±0.000	-	0.001^{k} ±0.000	-
15	BF3	0.070^{c} ±0.000	0.030^{c}± 0.000	0.001^{k}± 0.000	-
16	RV	0.050^{d} ±0.000	0.030^{c}± 0.000	0.001^{k}± 0.000	-
17	AF1	0.043^{e} ±0.000	0.030^{c} ±0.000	0.008^{c} ±0.000	-
18	RF	0.036^{f} ±0.000	0.009^{d} ±0.000	0.002^{ij} ±0.000	-
19	WV2	0.040^{ef} ±0.000	0.008^{d}± 0.000	0.003^{gh} ±0.000	0.001^{e} ±0.000
20	RFB	0.040^{ef} ±0.000	-	-	-
21	CV	0.043^{e} ±0.000	0.009d ±0.000	0.003^{gh} ±0.000	0.002^{d}± 0.000
22	FV	0.006^{h} ±0.000	0.010^{d} ±0.000	0.003^{hi} ±0.000	0.001^{e} ±0.000
23	BF2	0.036^{f} ±0.000	0.003^{f} ±0.000	0.006^{d} ±0.000	-
24	HF	0.030^{g} ±0.000	0.009^{d}± 0.000	0.004^{fg} ±0.000	0.002^{d}±0.000
25	HV1	0.030^{g} ±0.000	0.003^{e} ±0.000	0.002^{j} ±0.000	0.002^{d} ±0.000
26	HV2	0.006^{h} ±0.000	0.003^{e} ±0.000	0.001^{k}± 0.001	0.001^{e} ±0.000
27	NV	-	-	-	-
28	NF	-	-	-	-
29	KBV	-	-	-	-
30	KBF	-	-	-	-

Values are significant at P< 0.005

Pectin methyl esterase (PME)

Pectin methyl esterase activity was optimum at 4th day of incubation for *A. flavus* (0.090^{a} ± 0.000 meq of NaOH consumed/ min/ ml) followed by *A. niger* (0.080^{b} ± 0.000 meq of NaOH consumed/ min/ ml) have shown in the (Table 6). Mantovani *et al*. (2005) reported the strains of *A. niger* have shown the similar level of pectin esterase activity as earlier reported by Villarino *et al*. (1993) but it showed elevated pectin lyase activity during his study. BV1 and RV isolates have also shown maximum enzyme production on 4th day of incubation (0.080^{b} ± 0.000 and 0.050^{d} ± 0.000 meq of NaOH consumed/ min/ ml). Minimum production of enzyme was observed in KV1 (0.006^{h} ± 0.000 meq of NaoH consumed/ min/ ml). There is no enzyme production in WL, KV2, NV, NF, KBV, KBF.

In remaining isolates there was continuous increase in activity up to 8 days of incubation. It was interesting to note that subsequent incubation of the fungal culture resulted in

gradual increase in activity except in few isolates. The present data demonstrates that maximum exopectinase and exopolygalacturonase activities were exhibited at 12th day of incubation period and maximum endopolygalacturonase and endo pectin lyase activity shows at 8th day incubation and PME showed maximum activity at 4th day of incubation. It was observed that maximum activity varied significantly from one isolate to other. Calculated ANOVA value is p=0.00 which is <0.05 & thus there is significance in values.

Pectinase activity was found to increase with incubation time and was optimum after incubation of 8th day and later on started depleting when the incubation time was increased. The decrease in the activity can be due to the depletion of nutrients in the medium. The present study indicates that *Aspergillus* sp. might bean efficient and economical source of pectinase enzyme.

The synthesis of extracellular enzymes by microorganisms is highly influenced by the components of growth media, whereas pectinolytic enzymes induced by several substances, in many cases pectin itself has been used (Alkarto *et al.* 1998, Palaniyappan *et al*. 2009). Further studies on optimization of pectinase production by using natural substrates, purification and characterization of pectinase by potential pectinolytic fungal strains are in progress. The present study has shown that dump vegetable waste soil is rich source of biodiversity of pectinolytic fungi that play an important role in the biogeochemical cycles in the environment.

Table 7 Production ofprotein by different isolated fungal strains

S. No	Strain	Extra cellular protein (µg/ml)			
		4th day	8th day	12th day	16th day
1	KF	590^{h}±5.773	810^{de}± 5.773	900^{b}±5.773	520^{e} ±5.773
2	*A. flavus*	850^{a}±5.773	900^{b} ±3.333	973^{a}± 5.773	920^{b}±5.773
3	BV1	810^{cd}±5.773	800^{e}± 5.773	950^{ab}± 5.773	420^{h}± 5.773
4	BUF	610^{g}±5.773	810^{de}± 5.773	900^{b}±5.773	500^{f} ±5.773
5	KUF	730^{e}±5.773	220^{p}± 5.773	520^{gh} ±5.773	400^{i} ±5.773
6	*A. niger*	735^{b}±5.773	910^{a}± 5.773	993^{a} ±3.333	986^{a}± 6.666
7	WL	210^{n}±5.773	410^{mn} ±5.773	610^{f} ±5.773	310^{j}± 5.773
8	KV1	210^{h}±5.773	220^{p}± 5.773	408^{h} ±5.773	220^{m} ±5.773
9	KV2	823^{c}±12.018	720^{f} ±5.773	810^{c}± 5.773	420^{h} ±5.773
10	HF1	540^{i}±5.773	640^{h} ±5.773	830^{c} ±5.773	610^{d}± 5.773
11	KAF	630^{g}±5.773	840^{c}± 5.773	946^{ab}± 6.666	420^{h} ±5.773
12	BV2	710^{ef}±5.773	800^{e}± 5.773	950^{ab}± 5.773	300^{k} ±5.773
13	WV1	210^{h}±5.773	610^{i}± 5.773	850^{c} ±5.773	620^{d} ±5.773
14	YV	810^{cd}±5.773	910^{a}± 5.773	740^{e} ±5.773	250^{l} ±5.773
15	BF3	800^{d}±5.773	800^{e}± 5.773	730^{e} ±5.773	510^{ef} ±5.773
16	RV	823^{c}±12.018	840^{c}± 5.773	946^{ab}± 6.666	420^{h}±5.773
17	AF1	250^{m}±5.773	550^{j}± 5.773	826^{c} ±5.773	310^{jk} ±5.773
18	RF	710^{ef}±5.773	900^{b} ±3.333	993^{a} ±3.333	620^{d}± 5.773
19	WV2	800^{d}±5.773	400^{n} ±5.773	760^{de} ±5.773	910^{b}± 5.773
20	RFB	610^{g}±5.773	810^{de}± 5.773	973^{a}± 5.773	310^{jk} ±5.773
21	CV	710^{ef}±5.773	420^{m}± 5.773	408^{h} ±5.773	220^{m} ±5.773
22	FV	490^{j}±5.773	720^{f}± 5.773	813^{c}± 3.333	420^{h}±5.773
23	BF2	800^{d}±5.773	900^{b}±5.773	993^{a} ±3.333	810^{c} ±5.773
24	HF	420^{k}±5.773	700^{g}± 5.773	800^{cd} ±5.773	510^{ef}±5.773
25	HV1	520^{i}±5.773	610^{i} ±5.773	850^{c} ±5.773	620^{d} ±5.773
26	HV2	320^{l}±5.773	420^{m}± 5.773	550^{g}± 5.773	320^{j} ±5.773
27	NV	700^{f}±5.773	800^{e}± 5.773	950^{ab} ±5.773	240^{l} ±5.773
28	NF	-	-	-	-
29	KBV	-	-	-	-
30	KBF	-	-	-	-

Values are significant at P< 0.005

Protein content

Data depicted in (Table 7) reveals that different isolates showed varying levels of protein production. Among 30 isolates soluble proteins, were more in *Aspergillus niger* (993^{a} ± 5.333 µg/mL) followed by *Aspergillus flavus* (973^{a} ± 5.773 µg/mL) at 12th day of incubation. Maximum protein production was observed in RV (946^{ab} ± 6.666 µg/mL) followed by BV1 (950^{ab} ± 5.773 µg/mL) at 12th day of incubation. Least protein production was seen in KV1 (408^{h} ± 5.773 µg/mL). There is no protein production in NF, KBV and KBF isolates.

Most of the microorganisms are capable of producing various extracellular and intracellular enzymes using different substrates. Pectinase is an extracellular enzyme, which is produced from various organisms including bacteria, fungi, and some actinomycetes. In the current work

pectinase enzyme was produced by *Aspergillus niger* and *Aspergillus flavus* which in turn was obtained from the dump waste soil. Strain improvement method will be applied to increase the potential enzyme secretion is the subject for further research. *Aspergillus niger* and *Aspergillus flavus*can be used for further scaling up process for pectinase production by SSF. Many studies have been conducted on the production, isolation and characterization of pectinases from various microorganisms. In this way it is important to investigate the production conditions and physicochemical characteristics of new enzymes. These studies will provide valuable tools to manipulate microorganisms making them able to produce efficient enzymes in high amounts.

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