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PECTINOLYTIC POTENTIAL OF INDIGENOUS *TRICHODERMA* STRAINS ORIGINATED FROM THE NORTH WESTERN REGIONS OF PAKISTAN

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Abstract

Pectinases are used extensively in food, paper and pulp processing industry with a myriad of potential applications particularly in the synthesis of a variety of biochemicals. Here we screened the indigenous strains of *Trichoderma* spp. previously isolated from the North Western regions of Pakistan for their pectinolytic activity using the standard cup plate method of depolymerized pectin assays. *Trichoderma longibrachiatum* strains TKK3 and TKK6 isolated from the Western parts and *T. longibrachiatum* strains TMK19, TMK21 and TMK22 isolated from the Northern parts of Pakistan showed potential pectinolytic activities with zones of clearance >37mm. An exogenous *T. harzianum* strain TH also showed zone >37mm. All the other strains including *T. virens* strain TMK08 and *T. brevicompactum* strains TMK15, TMK16, TMK17 and TMK18 showed a zone of clearance <37mm or no zone at all. Polygalacturonases are the important commercial pectinases that depolymerize the pectin by hydrolysing the alpha-1,4 glycosidic bonds. The strains TKK3, TKK6, TMK19, TMK21, TMK22, and TH showed polygalacturonase activity > 20 IU/ml/min. The highest specific pectinase activity (498.5 IU/mg of protein) and specific polygalacturonase activity (1008.5 IU/mg of protein) were shown by *T. longibrachiatum* strain TKK6. The pectinolytic potential of the indigenous *Trichoderma* strains using pectin extracted from the orange peels was also evident in the present study. The pectinases produced by these strains may be further purified for their possible use in the industrial processes which is an important perspective of this study

Key words: Pectinases; Polygalacturonases; *Trichoderma*; protein estimation; Enzyme biotechnology.

Introduction

Enzymes are frequently used in industrial applications for efficient processing and output of a variety of products (Shet *et al*., 2018). Production of stable, active and specific enzymes with less costly sources are highly desired (Sudeep *et al*., 2000). Microorganisms are considered as one of the most convenient sources of enzymes. Various microorganisms have vast potential of producing different commercial enzymes due to their genetic makeup and environmental stimuli that trigger the extant microbial machinery (Bills & Gloer, 2017). Therefore, the microbial strains that can efficiently proliferate in a limiting environment producing the maximum amounts of quality enzymes have always been a matter of extensive investigation.

Pectinases are the enzymes extensively utilized for extraction of juices from plant tissues. These enzymes are also used in bio-polishing of textiles, manufacturing of washing powders, preparation of animal feeds, and processing of pulp and manufacturing of paper. The pectin enzymes are generally classified into polygalacturonase, pectin esterase, pectin lyase and pectate lyase depending on their modes of action (Alkorta *et al*., 1998). Polygalacturonases are of industrial importance because they depolymerize pectin and hydrolyse the α-(1-4) glycosidic bonds present among the non-esterified galacturonic acid units (Mohamed *et al*., 2006). The two enzymes namely pectin lyase and pectate lyase break down α- (1-4) glycosidic linkages through transelimination and formation of galacturonide having unsaturated bonds.

The composition of microbial pectinases varies with the microbial species from which they originate. Around 25% of the global sale of food enzymes virtually comprise microbial pectinases (Jayani *et al*., 2005; Sanchez & Demain, 2017). A variety of microorganisms has been reported to synthesize different types of pectinases, and fungi, being potent producers, have been found ideal on industrial scale (Amin *et al*., 2019). The filamentous fungi, because of their physiological and biochemical properties, may have extensive mechanisms for production of pectinases. Blandino *et al*., (2001) reported that major portions (90%) of fungal enzymes are extracellular and secreted directly in the medium of their culture. A good amount of pectinases was produced by *Aspergillus niger*, an ascomycetous fungus, and the United States Food and Drugs Administration (USFDA) regarded as a safe (GRAS) microorganism (Blanco *et al*., 1999). Another ascomycetous fungus, *Trichoderma reesei*, has also been used extensively for production of pectinases (Laothanachareon *et al*., 2015).

Trichoderma species are considered safe for the industrial use and can be safely applied for production of food additives and associated products (Blumenthal, 2004). Therefore, different *Trichoderma* enzymes were applied including pectinases, beta-glucanases, cellulases, hemicellulases to improve the brewing processes and fruit juice production (Galante *et al*., 1998). The present study was aimed to investigate the *Trichoderma* strains previously collected from the Northern and Western areas of Pakistan (Anees *et al*., 2018) for the production of pectinolytic enzymes using synthetic as well as locally extracted pectin from the orange peels. Furthermore, the pectinolytic activity of an efficient strain was optimized using different culture conditions.

Materials and Methods

***Trichoderma* and culture maintenance:** *Trichoderma* strains previously isolated and identified (Anees *et al*., 2018) from Karak and Malakand districts of Khyber Pakhtunkhwa were used in the present study along with a strain of *T. harzianum* TH that was obtained from the Fungal Culture Bank of the University of Punjab, Lahore, Pakistan (Table 1). The cultures were maintained on slants of potato dextrose agar (PDA; Oxoid) and stored in a refrigerator at 4°C for further studies.

Table 1. Screening of *Trichoderma* strains by the cup-plate method using Czapek's agar for measuring pectinase activity and the zone formation. The experiment was replicated thrice.

S. No.	*Trichoderma species*	Strain id	Zone diameter (mm)
1.	*Trichoderma harzianum*	TH	37.50±0.50
2.	*Trichoderma longibrachiatum*	TKK1	26±00.00
3.	*Trichoderma longibrachiatum*	TKK3	37.50±0.50
4.	*Trichoderma longibrachiatum*	TKK4	36.00±1.00
5.	*Trichoderma longibrachiatum*	TKK6	37.00±1.00
6.	*Trichoderma longibrachiatum*	TKK7	20.50±0.50
9.	*Trichoderma longibrachiatum*	TKK8	27.50±2.50
7.	*Trichoderma longibrachiatum*	TKK9	29.00±1.00
8.	*Trichoderma longibrachiatum*	TMK1	33.50±2.50
12.	*Trichoderma longibrachiatum*	TMK2	16.00±1.00
9.	*Trichoderma longibrachiatum*	TMK3	32.50±2.50
10.	*Trichoderma longibrachiatum*	TMK4	21.00±1.00
11.	*Trichoderma longibrachiatum*	TMK5	32.50±2.50
12.	*Trichoderma longibrachiatum*	TMK6	25.00±0.00
13.	*Trichoderma virens*	TMK8	20.50±0.50
14.	*Trichoderma longibrachiatum*	TMK9	36.00±1.00
15.	*Trichoderma longibrachiatum*	TMK10	25.00±1.00
16.	*Trichoderma longibrachiatum*	TMK11	No zone
17.	*Trichoderma longibrachiatum*	TMK12	26.50±0.50
18.	*Trichoderma longibrachiatum*	TMK13	25.00±0.00
19.	*Trichoderma longibrachiatum*	TMK14	No zone
20.	*Trichoderma brevicompactum*	TMK15	35.00±1.00
21.	*Trichoderma brevicompactum*	TMK16	No zone
22.	*Trichoderma brevicompactum*	TMK17	No zone
23.	*Trichoderma brevicompactum*	TMK18	29.00±1.00
24.	*Trichoderma longibrachiatum*	TMK19	40.00±1.00
25.	*Trichoderma longibrachiatum*	TMK20	19.00±1.00
26.	*Trichoderma longibrachiatum*	TMK21	39.50±2.50
27.	*Trichoderma longibrachiatum*	TMK22	38.50±1.50

Screening assays for pectinolytic activity of *Trichoderma*: The *Trichoderma* species were investigated using a modified Czapek's agar and the pectinase production was tested at pH 7.2 (Adeleke *et al*., 2012). *Trichoderma* (5 mm disc) culture from the leading edge of actively growing colonies on Sabouraud dextrose agar (SDA) was inoculated onto the Czapek's agar (Pectin 10 g, sodium nitrate 3 g, magnesium sulphate 0.5 g, potassium chloride 0.5 g, dipotassium phosphate 1g, ferrous sulphate 0.01 g and agar agar 20 g at final pH 7.3±0.2 at 25°C) containing pectin as a sole source of carbon and incubated it at 30ºC for 48 h. After 48 h of incubation, the iodine-potassium iodide solution containing 1.0 g of iodine, 5.0 g of potassium iodide and 330 ml of H_2O was added for detecting the clearance zones (Ouattara *et al*., 2008). *Trichoderma* cultures with clear zones were considered to have the pectinase activity. The presence of transparent halos of larger diameters surrounding the colonies of *Trichoderma* were selected as putative producers of pectinases, while the poor producers showed no or smaller clear lysis zones. The experiments were repeated once and the consistency of the results was ascertained.

Extraction of crude pectinolytic enzymes: The *Trichoderma* strains were refreshed on SDA or PDA for further fermentation. The fresh cultures of *Trichoderma* strains were then grown in the Richard modified medium (KNO_3 10 g, KH_2PO_4 5 g, $MgSO_4.7H_2O$ 2.5 g, $FeCl_3$ 2 mg, pectin 10 g, V8 juice 150 ml, polyvinyl pyrrolidine 10 g, 0.1% chloramphenicol and distilled H_2O 850 ml; pH was adjusted to 6). Two discs (5 mm ⌀) from the growing edge of the fungal cultures were transferred to the flasks containing the Richard modified medium and incubated at a temperature of 28°C for a period of 7 days in triplicates. The liquid media and the fungal mats were separated using a double layered Whatman filter paper no.1 through a glass funnel. Then the culture filtrates were centrifuged (5000 rpm) for 10 min and the supernatants were collected as the sources of crude enzymes (Adeleke *et al*., 2012).

Enzyme activity assays

Pectinase activity assays: The pectinase activity was measured using the Dinitrisalicyclic acid (DNS) method as reported elsewhere (Miller, 1959). A solution was prepared containing 1% pectin (2 ml) in Na-acetate acetic acid buffer of pH 5.0 (0.2 M). The enzyme extract (1 ml) was incubated in a water bath for 1 h at 45°C. After incubation, the reducing sugars liberated with the pectin solution (1%) were measured using a standard galacturonic acid. The enzyme activity was expressed as units ml^{-1} (Baracat *et al*., 1989). The assays were done with three replicates and measured in IU/ml/min by dividing the product of absorbance and a standard factor by time of incubation. The standard factor was measured by dividing the galacturonic acid concentration (µg/ml) by the absorbance (575 nm).

Polygalacturonase activity assays: Polygalacturonase activity was assessed using 1% polygalacturanic acid (1 ml; Sigma), acetate buffer (8.5 ml at pH 5) and the culture filtrate (0.5 ml). The samples were incubated for 1 min at 45°C and the reducing sugars were determined by the modified DNS method. One unit of exopolygalacturonase activity (IU) was defined as "the amount of enzyme that produced 1 µg galacturonic acid/min under the conditions specified". Enzyme assays were performed with three repeats and measured in IU/ml/min by dividing the product of absorbance and standard factor by time of incubation. The standard factor was calculated by dividing the concentration of galacturonic acid (µg/ml) by its absorbance at 575 nm.

Specific enzyme activity and protein estimation: Specific activity was measured by dividing enzyme units by estimated protein in mg (Okafor *et al*., 2010).

The enzyme activity assays using orange peels as source of pectin: The pectinase and polygalacturonase activity assays were performed using pectin that was extracted from the orange peels. The pectin was extracted by the method of Rao & Maini (1999) with a slight modification. The orange peel powder (30 g) was taken in an Erlenmayer flask (250 ml), HCl (0.05N; 90 ml) added and placed over night. The mixture after filtration twice, was mixed with absolute ethanol (two volumes) to the precipitated pectin.

Results

Screening assays for pectinase activity: *Trichoderma harzianum* strain TH and *T. longibrachiatum* strains TKK3, TKK6, TMK19, TMK21 and TMK22 produced clear zones > 37 mm (Table 1). All the other strains including *T. virens* strain TMK08 and *T. brevicompactum* strains TMK15, TMK16, TMK17 and TMK18 showed activity <37 mm of zone of clearance or no zone at all. The maximum pectinase clearance zone was produced by *T. longibrachiatum* TMK19 and TMK22 in the screening assays.

Pectinase activity assays: In total, seven strains (*T. harzianum* strain TH, *T. longibrachiatum* strains TKK3, TKK4, TKK6, TMK19, TMK21, TMK22) were used for the pectinase activity assays. All the selected strains showed activity > 9 IU/ml/min (Table 2). A unit was defined as the amount of the enzyme used to catalyse the formation of 1 µg of galacturonic acid per hour at 45°C. Highest pectinase activity was observed in case of *T. longibrachiatum* TMK22 (14.77 IU/ml/min).

Polygalacturonase activity assays: Of the selected *Trichoderma* strains (*T. harzianum* strain TH, *T. longibrachiatum* strains TKK3, TKK4, TKK6, TMK19, TMK21, TMK22), TH showed the highest polygalacturonase activity of 29.12 IU/ml/min followed by polygalacturonase activity of 27.23 IU/ml/min by *T. longibrachiatum* strain TKK6 and 25.66 IU/ml/min by the strain TMK19 (Table 2).

Protein estimation assays: The total proteins of the crude extracts were estimated. The highest protein estimated was 0.888 mg/ml produced by *T. longibrachiatum* strain TMK22 followed by 0.101 mg/ml by *T. longibrachiatum* strain TKK3 (Table 3).

Specific activity assays: The specific activities of pectinase and polygalacturonase were estimated. The highest specific pectinase activity was 1035.38 IU/mg of protein by *T. longibrachiatum* strain TKK6 followed by 923.12 IU/mg of protein by *T. longibrachiatum* strain TMK22. The specific polygalacturonase activity was estimated in which the highest activity was 2017.03 IU/mg of protein by the *T. longibrachiatum* strain TKK6 followed by 1610.32 IU/mg of protein by the *T. longibrachiatum* strain TMK22 (Table 4).

Enzyme activity of *Trichoderma* strains using pectin derived from orange peels: The orange peels were used for the extraction of pectin that was used as a substrate for pectinases and polygalacturonases (Table 5). Only selected strains were used in these assays which showed efficient pectinase production. The strains TKK3 and TMK19 showed higher activities using pectin originated from the orange peels. The highest specific pectinase and polygalacturonase activities were observed i.e., 123 IU/mg and 66 IU/mg protein, respectively, by TMK 19.

Table 2. Pectinase and polygalacturonase activity of *Trichoderma* strains originated from the Northern and Western areas of Pakistan.

Trichodermal strains	Pectinase activity in IU/ml/min	Polygalacturonase activity in IU/ml/min
TH	11.82	29.12
TKK3	11.53	20.75
TKK4	11.39	25.04
TKK6	13.46	27.23
TMK19	9.50	25.66
TMK21	9.09	22.92
TMK22	14.77	24.96

Table 3. Amount of proteins in culture filtrates produced by *Trichoderma* strains isolated from the Northern and Western areas of Pakistan.

Trichoderma strains	Amount of Proteins in mg/ml
TH	0.037
TKK3	0.101
TKK4	0.068
TKK6	0.027
TMK19	0.072
TMK21	0.036
TMK22	0.884

Table 4. Specific activity of pectinase and polygalacturonase of *Trichoderma* isolates originated from the Northern and Western areas of Pakistan.

Trichodermal strains	Specific activity of pectinase in IU/mg of protein	Specific activity of polygalacturonase in IU/mg of protein
TH	319.46	787.02
TKK3	144.16	205.44
TKK4	167.5	368.23
TKK6	498.51	1008.5
TMK19	131.94	356.39
TMK21	252.5	716.25
TMK22	16.7	28.23

Table 5. Enzyme activity assays using orange peels as substrate for *Trichoderma* strains originated from the Northern and Western areas of Pakistan.

Trichoderma strains	Pectinase (IU/ ml/min)	Pectinase spec activity (IU/mg of proteins)	Polyg activity (IU/ ml/min)	Polyg spec activity (IU/mg of proteins)
TH	12.07	45.38	15.29	57.44
TKK3	33.11	110.73	3.60	12.04
TKK6	29.72	67.54	4.20	9.53
TMK19	31.38	123.05	16.96	66.50
TMK21	18.12	47.93	3.85	10.19
TMK22	21.31	71.51	9.61	32.24

Discussion

Pectinases are the enzymes that are used in food and paper industries and pulp processing units. They also have potential applications in biochemical syntheses. Therefore, screening of microorganisms for production of pectinases is considered as a matter of interest for researcher communities. Here, we screened the *Trichoderma* strains for pectinase and polygalacturonase activities that were previously isolated and identified originating from the North Western areas of Pakistan (Anees *et al*., 2018). Different strains of *Trichoderma* were reported elsewhere to have the pectinolytic activities (Isshiki *et al*., 1997; Kapat *et al*., 1998; Laothanachareon *et al*., 2015). In the present study, a total of 27 strains of *Trichoderma* were screened out, of which four strains showed no activity at all. The seven strains i.e. TH, TKK3, TKK4, TKK6, TMK19, TMK21 and TMK22 were selected for further enzyme assay analyses including pectinase and polygalcturonase activities, total proteins and the enzyme specific activities. The strains, TH, TKK3, TKK6, TMK19, TMK21 and TMK22, showed the highest activity with larger diameter zones of more than 37 mm. Khairnar *et al.*, (2009) used similar plate assays to demonstrate hydrolysis of pectin by different strains of *Aspergillus niger* and observed a zone of clearance around the fugal colonies.

The majority of pectinases are inducible, therefore a substrate rich in pectin content stimulated enzyme production and also enhanced the release of pectinases into the fermentation medium (Pandey, 1992). Gomes *et al.*, (2006) reported two newly isolated fungi, viz., *T. reesei* and *T. viride*, used for enzyme production. They observed higher production of pectinases (10.3 U/ml) with *T. viride,* which was in close agreement to our findings. Both of these strains have been currently re-identified as *T. longibrachiatum*. These findings assert that the specific factors may be triggered in *T. longibrachiatum* by specific substrates present in the medium for production of pectinases. Furthermore, the highest specific pectinase activities were observed in the strains TKK6 (1035 IU/mg) and TMK22 (935 IU/mg) in the present study with concurrent results for polygalacturonase activities also showing their potential to produce specific proteins for the subject functions. Sandhu & Kalra (1982) reported that *T. longibrachiatum* utilized glucose, xylan, pectin and carboxymethyl cellulose as carbon sources and produced pectinases on all carbon sources, but least on glucose. Maximum amounts of pectinases were produced on substrates containing pectin as the sole carbon source. The production of pectinolytic enzymes by *Trichoderma* was confirmed by Isshiki *et al.*, (1997) and Kapat *et al.*, (1998). Usha *et al.*, (2014) also reported different fungal strains including *Trichoderma* species for pectinolytic activity by the plate assays. In the present study, the selected *Trichoderma* strains were tested to produce pectinases using a local waste product, i.e. orange peels. Hence, the fruit juice industry may use their own waste material for the production of the enzyme that may be economically feasible.

Anisa & Girish (2014) studied *Rhizopus* spp. and *T. viride* for pectinolytic activity by the plate and broth assays using the pectin agar medium and pectin broth medium, respectively. The polygalacturonic assays of *T. viride* showed the concentration of reducing sugar in pectin as 8.2 (U/ml), which was too low from the polygalacturonase production in our study, but in close agreement with our pectinase production. This might have been due to the reason that in the polygalacturonase assay in this study specifically polygalacturonic acid was used as a substrate, while they used pectin instead of using polygalacturonic acid.

In brief, different indigenous *Trichoderma* strains showed pectinolytic activity. The pectinolytic enzyme production varied among the strains of *T. longibrachiatum* originated from different locations of Malakand and Karak. Highest specific activities were observed in TKK6 and TMK22, the strains isolated from the Karak and Malakand areas. Furthermore, these Trichoderma strains also showed enhanced pectinlytic activities with the orange peels. In order to ascertain the genetic basis of variation for the optimal growth and the molecular mechanisms involved in the regulatory processes of pectinases secretion at the strain level among the *Trichoderma* strains used in the present study, further work needs to be carried out. These studies can provide valuable tools to manipulate *Trichoderma* to produce efficient pectinases in high amounts.

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