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Research Article

ISOLATION AND MOLECULAR CHARACTERIZATION OF PECTINASE PRODUCING FUNGI USING AGRICULTURAL WASTE

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ABSTRACT

Enzymes are macromolecular biocatalysts that are employed in consumer goods, food and beverages, biofuels, and pharmaceuticals. Pectinase is one of the most widely distributed enzymes in bacteria, fungi, and plants. The pectinase enzyme is produced by organisms like fungi. Microorganisms that produce pectinase provide industry with more efficient and affordable production. Complex polysaccharides present in the fruits and vegetables promotes microbial growth and the synthesis of industrially important enzymes, constitute the majority of agro-industrial residues. In the current study, soil samples, decomposing fruits (*Solanum lycopersicum*, *Citrus sinensis*, *Malus domestica*, *Citrus limetta*, *Daucus carota*, *Musa paradisiaca*, and *Ananas comosus*), and vegetables (*Solanum melongena*, *Allium cepa*, and *Solanum tuberosum*) were collected, and fungi were isolated and purified on potato dextrose agar medium. Twenty fungal strains were isolated from samples they were screened for the synthesis of pectinase. Out of 20 isolates, only 12 fungal isolates showed Pectinase activity. The most potential fungus was molecular characterized and identified as *Aspergillus fumigatus* as species exhibited the largest clear zone diameter among the twelve strains. It can produce maximum amounts of pectinase using cheap substrate *A. fumigatus* may be useful in various industrial sectors for the production of enzyme in a large scale by reducing cost of substrate such as the juice and textile industries.

Keywords: Agricultural waste, Aspergillus spp, Orange peel, Pectinase, *Solanum tuberosum*.

INTRODUCTION

Enzymes are substances present in the cells of living organisms in small amounts which are capable of altering chemical reactions without themselves being altered after the completion of reaction. The demand for industrial enzymes has been increasing to the new heights which tends for a constant research and development to optimize their productions and minimize resource costs (Singh R *et al*., 2016). Pectic substances are the complex polysaccharides present in the middle lamella of plants cells and are degraded by a group of enzymes called as pectinases. Pectinases can isolate from both plants and microbes. Out of the total enzymes being used industrially, more than half are extracted from fungi and yeast, one-third are obtained from bacterial systems and the remaining from animal (8 %) and plants (4 %) sources. Industrially important enzymes, pectinases hold a special significance due to its multiple uses in important sectors namely food, textile and bio-fuel industries thus capturing 25 % total enzyme market. Many plants pathogenic bacteria and fungi have long been known to produces pectinolytic enzymes and it is widely accepted that the production of these enzymes is a major means by which these microorganisms invade the host tissue (Rombouts, 1980). Almost all the commercial preparations of pectinases are produced from fungal sources (Singh S.A *et al*., 1999). A significant amount of agricultural waste is produced during the conversion of raw agricultural materials into meals. Thus, coffee, orange, rice, and sugarcane agro-residues offer an appropriate platform for the fermentation process-based bio-production of various enzymes, making these waste products profitable (Giese E.C *et al*., 2008). Orange bagasse has been used as a fermentation product, including enzymes, and significant amounts of soluble carbohydrates, specifically fructose, glucose, sucrose, and pectin, as well as insoluble cellulose (Rosales E *et al*., 2002). The food

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(dairy and meat) and beverage industries have notable collaborations with microorganisms (Buyukkileci A.O *et al*., 2011). Foods and additives produced biologically are more appropriate than those created artificially (Couto S.R. and Sanromán M.A., 2006) Rather than apple pomace, citrus peel is recognized as the primary source of pectinases in several sectors. The main objective of the work is to isolate, screen and molecularly identify fungi from agricultural waste substrates that can efficiently produce pectinase enzyme, paving the way for cost-effective enzyme production using renewable waste. The present investigation was motivated by the prospective uses of pectinases in many sectors. Despite the potential of agricultural waste as a cost-effective substrate for pectinase production, there is a lack of information on the molecular identification of fungi capable of producing pectinase from diverse agricultural waste sources. While several studies have focused on optimizing pectinase production using specific substrate, the exploration of the unexplored agricultural waste substrates and identification of novel fungal isolates with enhanced pectinase production capabilities remains underexplored, highlighting the need for further research in this area.

MATERIAL AND METHODS

Sample Collection and Isolation of fungi

Various samples of soil, decayed fruits, and vegetables were collected from agricultural field and vegetable markets of Mumbai City. Pectinase producing fungi were isolated from these samples. The soil samples were serially diluted and inoculated into a sterile medium containing (g/l): Pectin -10.0, Sucrose-10.0, Tryptone- 3.0, Yeast extract -2.0, KCl 0.5, $MgSO_4$.7H2O 0.5, $MnSO_4$.5H2O 0.01, $(NH_4)_2SO_4$ 2.0, Agar agar-20. Media was supplemented with mineral salt solution. The pH of the media was adjusted to 5.5- 6.0 by using 1.0 N HCL/1.0N NaOH. The inoculated plates were incubated at 30°C for 3-8 days (Singh S. and Sandhu D.K., 1986).

Primary Screening of fungal isolates for the pectinase activity

The Pectinase Screening Agar Medium (PSAM) was used to test the isolates for pectinase activity (Taskin E *et al*., 2008). Before sterilisation, the pH of the medium was adjusted to 5.5, and it was autoclaved for 15 min at 121°C. Media were poured in a Petri dish and allowed to solidify. All isolates were inoculated in this media and incubated at 30°C for 24 h to 2 weeks. At the end of the incubation period the plates were flooded with 50 mM Potassium iodide-iodine solution (Hankin L *et al*., 1971). clear halo zone around the colonies indicates the ability of an isolate to produce pectinase. From twenty isolates one of the best organisms were identified for pectinase enzyme production based on the zone diameter of the clearing zone (Dingle J *et al*., 1953).

Secondary Screening of Isolates for the Pectinase Activity

In 250 ml Erlenmeyer flasks with 15 g of basal medium (g) - Pectin-1.0, Urea-0.3, Sucrose 3.14, $(NH_4)_2SO_4$-1.26, KH_2PO_4-0.65, $FeSO_4$-0.29, and orange peel- 5g the solid-state cultivation was conducted. 2 ml of a spore suspension that was obtained from a five-day agar slant was used to inoculate the flasks. 1.0 N HCl/1.0 N NaOH was used to bring the media pH down to 5.5-6.0 (Acuña-Argüelles M.E *et al*., 1995). 70% will be the final moisture level. For four to five days, the culture was done at room temperature. With the least amount of distilled water possible, the fermented medium was removed. Following an hour of vigorous shaking, the flasks were filtered using cheese cloth. 100 ml of citrate buffer (0.1 M, pH 5.0) was added to extract the crude enzyme.

Enzyme assay

Pectinase activity was assessed by measuring the amount of reducing groups released, as described by Nelson (Nelson N, 1944) and modified by Somogyi (Somogyi M, 1952).

Procedure

The assay mixture was prepared with 0.2 ml of enzyme, 0.1 ml of 0.1M $CaCl_2$, and 0.5 ml of 1 % Polygalacturonase solution. Blanks for each sample were prepared by heating the reaction mixture prior to the addition of a substrate. The solution was incubated at 37 ºC for one hour. The process was stopped by heating at 100 ºC for three minutes. In both the sample and standard tubes, half ml of the solution mixture was added to two ml of distilled water to create the final volume. Alkaline copper tartarate (1.0 ml) was added and allowed to stand for 10 min. Tubes were chilled, and one ml of arsenomolybdate reagent was added to each of the tubes. The final volume of each tube was adjusted to 10 ml with distilled water. After 10 minutes, the absorbance of the blue color was measured at 620 nm in UV spectrophotometer.

Chemical analysis of the substrate

The substrate which was used for testing undergoes for the determination of percent moisture content, ash content, fat content, crude fibre content, and lignin content (Ahmed I *et al*., 2016).

Determination of % moisture content

To determine the moisture content of orange peel, 2 g sample was placed in a sterile bottle (W1) and dried for two hours at 100 °C. The sample was dried once more until it reached a consistent weight (W2) after being cooled and weighed in desiccators. The following formula was used to get the moisture percentage:

$$(\%)\ \text{Moisture} = \frac{(W1)-(W2)}{(W1)} \times 100$$

Determination of percent ash content

The material was heated until char formed using a pre-weight crucible that contained 2 g of substrate (W1) and 5 mL of HNO_3. The sample was then heated to 555 °C for six hours in a muffle furnace and weighed (W2) to determine the sample's percentage of ash content

$$(\%)\ \text{Ash} = \frac{(W1)-(W2)}{\text{Weight of sample}} \times 100$$

Determination of percent crude fat content

Hexane was used to extract the 5 g sample (W1), which was placed in a pre-weighed thimble and kept in an extraction apparatus (Soxhlet) for 16 hours. Then, weigh the dry sample in a thimble to determine its fat content (W2). To determine the sample's crude fat percentage, the following formula was used:

$$(\%)\ \text{Crude Fat} = \frac{(W1)-(W2)}{(W1)} \times 100$$

Determination of percent crude fibre content

Following ether extraction, 2 g of dry material (W1) was boiled in 200 mL of H_2SO_4 (containing 1.25 g of H_2SO_4 per 100 mL) for 30 minutes, followed by a 200 mL NaOH solution (containing 1.25 g carbonate free NaOH per 100 mL) and filtered to determine the crude fibre contents. After being dried in an oven, the material was weighed (W2). It was considered that the weight loss was crude fibre.

$$(\%)\ \text{Crude Fibre} = \frac{(W1)-(W2)}{\text{Weight of sample}} \times 100$$

Determination of percent lignin content

The procedure involved adding of 2 g sample (W1) to a 10 mL solution of 70% H_2SO_4 and boiling it for 4-5 hours. This treatment hydrolysed the cellulose and hemicellulose contents, and the remaining suspension was then filtered with hot water. Following this, 30 mL of 70% H_2SO_4 was added to the mixture, which was then placed in an oven at 105 °C for 24 hour and weighed (W2). The treated sample was transferred to a pre-weighed dry porcelain crucible for high temperature treatment at 650 °C for 4 hours. After allowing the sample to cool down, the lignin content (%) was calculated using a specific formula.

Lignin (%) = (W1) - (W2)

Molecular identification of most potential pectinase producing species

Morphologically scrutinized fungal sample was further subjected to molecular analysis, for confirmation. Fungal isolate was cultured on PDA medium. The plates were incubated at 30°C approximately 3 days. The fungal isolate was used for DNA extraction by using CTAB methods. PCR amplification was carried out using the forward primer ITS FP (GTGAATCATCGAATCTTTGAA) Reverse primer ITS FP (TCCTCCGCTTATTGATATGC). PCR (standard thermal cycler) was performed in a thermocycler using 50 µL reaction following this procedure: initial denaturation at 96°C for 5 min followed by 35 cycles of denaturation at 95°C for 30s, 55°C for 30s, 72°C for 30s, the final extension at 72°C for 5 min and rapid cooling to 4°C before analysis. PCR products were analysed in 1.5 % agarose gels and was stained with 0.1 µ/ml of ethidium bromide (Hasan N.A. and Zanuddin N.A, 2018).

RESULTS AND DISCUSSION

There were twenty isolates were screened for production of pectinase. Out of 20 isolates, twelve isolates showed zone of clearance. They were selected based on their ability to grow on the medium containing pectin as the carbon source. The maximum pectinase activity was shown in the Isolate 4 (Figure 1) (Table 1). The Isolate-4 showed largest clear zone of 1.54 mm size. Its fungal colony morphology can be described as follows: Macroscopic (colony) characteristics Color: Initially white, becoming blue-green to gray-green as conidia develop. Texture: Velvety to powdery surface due to abundant conidia. Topography: Typically, flat to slightly raised with a somewhat granular surface. Margin: Usually regular and well-defined. Growth rate: Rapid growth at 25-37°C and can grow at temperatures up to 50°C (thermotolerant). For Pectinase production 12 isolates chosen after primary screening. Maximum pectinase activity was recorded in Isolate 4 showing 19.79 U/ml followed by Isolate 10 (19.57 U/ml) (Table 2). The most efficient isolate was identified as *Aspergillus fumigatus* by National Facility for Biopharmaceutical (NFB), Mumbai, India. Studies on pectinase production by *Aspergillus* was carried out using orange peel and pectin as the carbon source.

Table 1. Selected strains during the Screening process.

S.No	Isolates	Colony diameter (mm)	Hydrolyse halo diameter (mm)	Pectinase production ratio (Hydrolyse diam./Colony diam.)
1.	Isolate 1	1.6	2.1	1.31
2.	Isolate 2	2.4	3.3	1.37
3.	Isolate 3	2.9	3.8	1.31
4.	Isolate 4	2.2	3.4	1.54
5.	Isolate 5	3.0	4.0	1.33

6.	Isolate 6	1.5	2.1	1.40
7.	Isolate 7	1.4	1.8	1.28
8.	Isolate 8	0.7	0.8	1.14
9.	Isolate 9	1.7	2.0	1.17
10.	Isolate 10	0.8	1.2	1.50
11.	Isolate 11	2.8	3.2	1.14
12.	Isolate 12	4.3	5.4	1.25

Table 2. Pectinase activity of crude enzyme extract of selected isolates.

S. No	Isolates	Pectinase activity (U/ml)
1.	Isolate 1	18.25
2.	Isolate 2	19.35
3.	Isolate 3	17.48
4.	Isolate 4	19.79
5.	Isolate 5	18.69
6.	Isolate 6	19.35
7.	Isolate 7	17.70
8.	Isolate 8	13.86
9.	Isolate 9	13.21
10.	Isolate 10	19.57
11.	Isolate 11	12.77
12.	Isolate 12	17.15

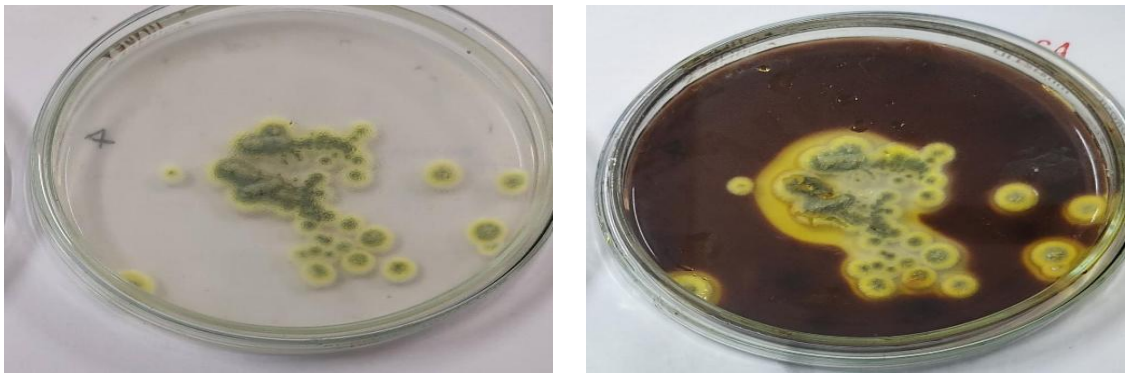


Figure 1. Screening of isolate for pectinase activity. Isolate 4 - *Aspergillus fumigatus*

Physiochemical analysis of substrate was evaluated the chemical composition of orange peel in order to produce pectinase (Table 3). The information in the figure 2 shows the proximally examined orange peel waste contained Moisture, Ash, Fat, Crude Fiber, and Lignin content.

Table 3. Chemical composition of orange peel.

Constituent	Percentage
Moisture	12.5 %
Ash	6.02 %
Fat	1.6 %
Crude Fiber	9.5 %
Lignin	6.4 %

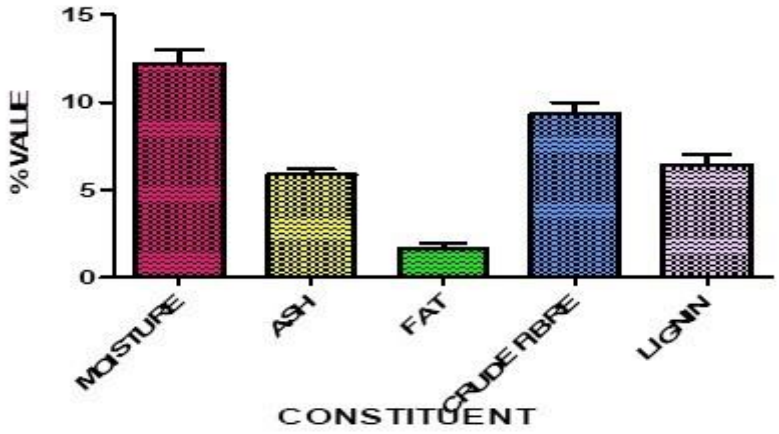


Figure 2. Chemical composition of orange peel waste.

Based on microscopic, colony characteristics and molecular characterization, the fungal strain was confirmed as *Aspergillus fumigatus*. Furthermore, morphologically confirmed *A. fumigatus* strain was further confirmed with molecular identifications using ITS region of the fungi. In figure 3 Agarose gel image showing extracted genomic DNA from samples. After extraction samples were analysed, 0.8% agarose gel was stained with 0.1µg/mL of Ethidium bromide. Lane L shows 10Kb DNA ladder, Lane 1 shows DNA extracted from fungus. In figure 4 Agarose gel image showing PCR amplified gene from DNA Samples. After completing thermal cycler cycles resultant mixture were analysed on 1.5 % agarose gel was stained with 0.1 µ/mL of Ethidium bromide. Lane L shows DNA ladder, Lane 1 shows PCR Amplified product .

Figure 3 - Agarose gel image showing extracted genomic DNA from samples

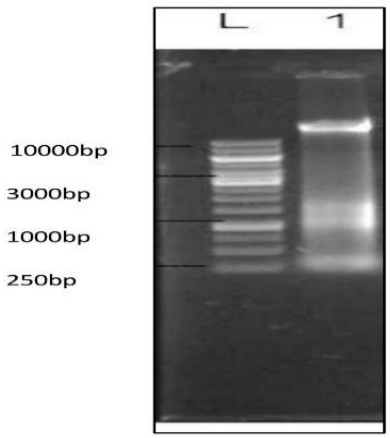
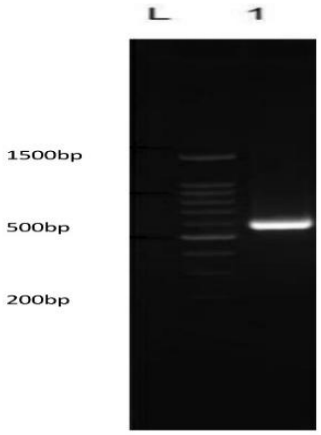


Figure 4 -Agarose gel image showing PCR amplified gene from DNA Samples.



FASTA sequence of *A. fumigatus* deposited to NCBI.

https://www.ncbi.nlm.nih.gov/nucleotide/PQ780056.1?report=genbank&log$=nucltop&blast_rank=1&RID=R3UPPM8R016

>PQ780056.1 *Aspergillus fumigatus* isolate AA1 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

GTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCT
GCGGAAGGATCATTACCGAGTGAGGGCCCTGTGG
GTCCAACCTCCCACCCGTGTCTATCGTACCTTGTTG
CTTCGGCGGGCCCGCCGTTTCGACGGCCGCCGGGG
AGGCCTTGCGCCCCCGGGCCCGCGCCCGCCGAAG
ACCCCAACATGAACGCTGTTCTGAAAGTATGCAGT
CTGAGTTGATTATCGTAATCAGTTAAAACTTTCAA

CAACGGATCTCTTGGTTCCGGCATCGATGAAGAAC
GCAGCGAAATGCGATAAGTAATGTGAATTGCAGA
ATTCAGTGAATCATCGAGTCTTTGAACGCACATTG
CGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGA
GCGTCATTGCTGCCCTCAAGCACGGCTTGTGTGTT
GGGCCCCCGTCCCCCTCTCCCGGGGGACGGGCCCG
AAAGGCAGCGGCGGCACCGCGTCCGGTCCTCGAG
CGTATGGGGCTTTGTCACCTGCTCTGTAGGCCCGG
CCGGCGCCAGCCGACACCCAACTTTATTTTCTAA
GGTTGACCTCGGATCAGGTAGGGATACCCGCTGA
ACTTAAGCATATCAATAAGCGG

Twenty fungal isolates were initially screened for pectinase production. Of which, only 12 isolates exhibited pectinase activity; they were isolated from fruits and vegetables waste. The most of the fungal isolates identified and screened were *Asperg*illus spp. In present study, the strains of *Aspergillus fumigatus* produce pectinase which hydrolyse pectin's. The strains of *A. niger* and *P. chrysogenum* isolates were also reported to produce cellulases and xylanases (Chinedu S.N *et al*., 2008). The strains of *A. niger* and *P. chrysogenum* produced the highest pectinase activity with wheat bran as sole carbon source. They concluded that, wheat bran is the most suitable carbon source when compared to other agro-wastes (pineapple peel, orange peel, sawdust and sugarcane pulp) for pectinase production by the organism. Our results agree with numerous authors who indicated that the orange peel was the best substrate for the maximal pectinase production by *A. niger* (Mrudula S. and Anitharaj R, 2011), *Aspergillus spp* (Camargo L.A *et al*., 2005), *P. oxalicum* (Santos S.F *et al*., 2008).

CONCLUSION

In the present investigation *Aspergillus fumigatus* was found to be potential pectinase producer as it showed highest activity. The results highlight the scope of *A. fumigatus* for maximum pectinase enzyme production using cheaper agro-waste as substrate makes this biosynthesized enzyme useful in textile as well as food processing industries. Further studies are needed for commercial production.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest

ETHICS APPROVAL

Not applicable

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AI TOOL DECLARATION

The authors declares that no AI and related tools are used to write the scientific content of this manuscript.

DATA AVAILABILITY

Data will be available on request

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