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Isolation and chracterization of pectinase producing *Aspergillus Niger* from orange

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Abstract
Pectinases are believed to become important industrial enzymes. These can be produced through different microorganism (bacteria and fungi) Pectinases are very important enzymes in food manufacturing industries. Pectinase shares the total enzyme production of about 25 % on global scale. The current study was focused on isolation and partial characterization of fungus that produces pectinase from orange peel. Samples collected from the fruit shop of Cantonment Lahore, Punjab. Samples collected from the fruit shop of Cantonment Lahore, Punjab. Total ten (10) isolates were identified from six (6) orange peel samples. Based on characterization on the selective growth media, the isolates were grouped as *Aspergillus Niger*. The isolates were further characterized by colony morphology, biochemical and molecular approaches. Four (4) strains were screened for pectinase activity out of which only one (1) harbor the pectinase. Quantitative determination of pectinase was carried out using DNS method using pectin as substrate. Microscopic, biochemical and molecular analysis indicated strains as *Aspergillus Niger*. A 565 amplified DNA confirmed the existence of *Aspergillus Niger*. The presently investigated indigenous *A. Niger* showed incredible potential for industrial use.

Keywords: pectinase, orange peel, *aspergillus Niger*, pectin, pectinase activity, DNS method, amplified DNA

1. Introduction
Enzymes are natural catalysts. They are produced by living organisms to increase the rate of vast and various set of chemical reactions, which are mandatory for life. Enzymes are involved in all processes which is crucial for life such as DNA replication, transcription, metabolism, protein synthesis, and signal transduction. They are able to perform very specific chemical transformation so that they are very useful in industrial processes (Shanley *et al*., 1993 [15]; Nabi *et al*., 2003) [10]. In nature, microorganisms are blessed with immense potentials. They show incredible production of enzymes that's why they have been used commercially over the years. Today's enzyme technology depends on microbes like bacteria and actinomycetes. Potential microorganisms are capable of genetic modification and provide sufficient scope for strain improvement and for further investigation. So biotechnological processes seem to be very important as far as the modern society is concerned for which microbial enzymes are recognized as productive tools. Thus, we attempted a research to screen and report enzymes producing microbes (Nabi *et al*., 2003 [10]; Nadeem *et al*., 2018[11]; Okaiyeto *et al*., 2015) [12].
The biotechnological potential of pectinases from microorganisms has drawn a great deal of attention from various researchers worldwide as likely biological catalysts in a variety of industrial processes. In diverse industrial sectors wherever the breakdown of pectin is required for a particular process, Pectic enzymes can be applied. There are many microorganisms which have been used to produce different types of pectic enzymes. Microbial pectinases have been reported 25% of the global food and industrial enzyme sales and their market value increasing day by day. These are widely used for fruit juice clarification, juice extraction, manufacture of pectin free starch, refinement of vegetable fibers, wastewater treatment, cocoa and tobacco and as an analytical tool in the assessment of plant products. Pectinase treatment increases the rate of tea fermentation and also demolishes the foam forming property of instant tea powders by destroying pectin (Razzaq *et al*., 2019 [13]; Saad *et al*., 2007) [14]. In recent years, Application of enzymes in biotechnological process has grown considerably. In food industries, major importance was being attached to the use of enzymes in upgrading quality, increasing yields of extractive processes, stabilization of product, flavor improvement and utilization of by product (Nabi *et al*., 2003 [10]; Saad *et al*., 2007) [14]. World interest on pectic pectinases has increased because of their industrial value, especially in food and fermentation industries, during the last decade. In food industries pectinases are used in the production of clarified fruit and vegetable juices. The addition of pectinases to the cloudy juice causes a rapid drop in the viscosity as well as in flocculation of the micelles and clear juice can be obtained after filtration. Other industrial uses of pectic enzymes include the extraction of oil, flavors and pigments from plant materials and maceration of vegetables and fruits. Fungal sources do provide a variety of bulk commercial enzymes and have the widest variety of application. The high enzyme production was achieved through strain selection, media development, process development and scale up programs. Therefore, the present research was conducted with the aims of screening pectinolytic microorganisms from orange peel and identifying molecularly using 18S rRNA.

Materials and Methods
Sample collection
Isolation of the Fungi was done from the Fruit Peels: Few orange peels were collected from the dump of fruit wastes near a local fruit market in Lahore Punjab. The peels were kept damp for few days in the Zip Lock Bag with minute openings for aim passage. Once the decaying of the peels was started the growth of fungi started.

Isolation and screening of *Aspergillus Niger*

After this the process of serial dilution was done. The peels were cut into small pieces and ground into fine liquid adding distilled water. This was used as the inoculums for the isolation of the organisms from the peel. Fungal growth obtained from serial diluted samples that were spread on petri plates with minimal media.

Minimal media preparation

Minimal media is used for those microbial organisms which releases pectin. It is known as Pectinase Screening Agar Medium. In this study minimal media were made as per as following composition (Table 1).

Table 1: Minimal Media (Pectinase Screening Agar Medium)

Reagents	Quantity
Na2HPO4	1.2gras/100ro1
KH2PO4	0.6gzus/100rn1
K2HPO4	0.3gansf 100m1.
NaCI	1 grns/100m1
Apr	1.5gms/100m1
Glucose	2gsns/100m1
Pectin and PH. 4.5	1 gm' 100m1

Streaking of samples on minimal media

After preparation of minimal media fungal samples were poured in the Petri plates and were incubated for 72 hours at room temperature

Morphological characterization of fungal isolates

Slides were prepared for 2 different fungal strains and were observed under microscope at 100X.From this we come to know, is there fungal specie present or not.

Biochemical characterization

Starch hydrolysis test

Starch agar medium was inoculated with isolated fungal cultures. The plates were incubated at 25 C 7 days. With iodine solution the surface of plates was flooded. The disappearance of starch from the starch agar media plates was examined by observing the disappearance of clear zones around the fungal growth. It was prepared as per the following composition,

Table 2: Material required for starch hydrolysis test

Reagent	Quantity
Starch	0.2g/100ml
Peptone	0.5g/100m1
Yeast Extract	0.3g/100m1
Agar	1.5g/100m1

Cellulose hydrolysis test

The Czapek-mineral salt agar medium consisted of KCl, K2HPO4, Na NO3. MgSO4.7H2O, peptone, carboxymethyl cellulose (CMC). This medium was complemented with agar 2 % and autoclaved. The autoclaved medium was then poured in petri plates and allowed to solidify than the plates were inoculate with isolated fungal cultures. The plates were incubated at 35 C in for 6 days. 1 % aqueous solution of hexadecyltrimethyl ammonium bromide was used to observed the plates for the formation of a clear zone around the fungal growth (hexadecyltrimethyl ammonium bromide was flooded on the surface of plates) It was prepared as per the following composition.

Table 3: Material Required for cellulose hydrolysis test

Reagent	Quantity
KCL	0.05g/100ml
K2HPO4	0.1g/100ml
NaNO3	0.2g/100ml
MgSO4.7H20	0.05g/100ml
Peptone	0.05g/100ml
Carboxymethyl cellulose	0.2g/100ml
Agar	0.2g/100ml

Enzyme assay

Pectinase enzyme assay was based on the determination of reducing sugars. Different dilution of known concentration of glucose were prepared with DNS method reagent and then the absorbance of those dilutions was measured in spectrometer at 540nm. A Glucose Standard curve will be plotted to measure concentration of pectinase activity. After plotting standard curve graph, pectinase assay of samples containing crude enzyme was performed. For this, solution A was prepared in 5 different test tubes by adding 0.5mL of each supernatant sample and 0.5mL of 1% pectin. Then incubated that solution for 2-3 hrs. at 37°C. After that, reaction mixture was prepared by adding 100µl of solution A, 900µl dH2O, and 3mL of DNS reagent in each test tube. Placed the test tubes in boiling water bath for 15 min and then note the reading at 540nm. After that, compared the OD of pectinase enzyme with glucose standard curve and then multiplied that value with dilution factor to calculate the total crude enzyme production (Mill *et al*., 1996)[9].

Genomic DNA isolation

DNA were isolated from the fungal mycelia**.** Two sets of tubes were prepared and labeled. One set was 0.5ml extraction buffer and the other one was 0.3ml isopropanol. Approximately 50 mg of fungal mycelia were collected with toothpick and were poured into extraction buffer. The fungal mass was than ground in the extraction buffer for 1-2 seconds with the machine. Mycelial tissue was pushed with the plastic pestle. Pestle was dipped into 70% ethanol and machine was operated for another 1-2 seconds. Cell lysate was centrifuged at 5,000 rpm for 15 minutes. Supernatant was directly poured into tubes containing 0.3ml isopropanol. Remaining lysate were poured into another tube. Lysate and isopropanol were mixed by inverting tube several times and this tube was centrifuged at 12,000 rpm for 10minute. Supernatant was discarded. Remaining alcohol was evaporated by incubation at 37°c for 15 minutes. Speed vac were used for drying. 50µl of distilled water and rnase and dissolve DNA pellet was dissolved by tapping.

Polymerase chain reaction

PCR was performed for the amplification of DNA which was isolated from the different species. PCR assays were carried out using primer ITS1 &4. PCR reactions were performed in an Eppendorf follows: one cycle was of 4 min 30 s at 95 C, 25 cycles of 30 s at 95 C (denaturalization), 25 s at 66 C (annealing), 40 s at 72 C (extension) and finally 1 cycle of 5 min at 72 C. In Aspergillus niger except for the 3annealing temperatures which were 62, 65 and 65.5 C, respectively.

Gel electrophoresis

PCR products were separated in 2% agarose ethidium bromide gels in 1 X TAE buffer (Tris- acetate 40 mM and

EDTA 1.0 mM). The DNA ladder was used as molecular size marker. Appearance of bands under UV Trans-illuminator indicated successful amplification of DNA.

Results and Discussion

Rotten oranges were collected from Lahore and were transferred in a sterile plastic bag in aseptic conditions. There were blended colonies of fungal growth obtained from serial diluted samples that were spread on petri plates with minimal media. For morphological characterization of fungal isolates slides were prepared of 2 different fungal strains were observed under microscope at 100X (Figures 1 & 2). Biochemical characterization of fungal isolates by performing two tests i.e. starch hydrolysis test and cellulose hydrolysis test. After this pectinase activity was performed to measure the enzyme activity of fungal isolates (Table 3). Technique of gel electrophoresis was done to confirm genomic DNA isolated from fungal strain. When the Gel was completely run, observed it under UV light. The clear bands were observed under UV light which indicated the Genomic DNA was successfully isolated from fungal strain. After extraction of DNA from the fungus than PCR experiment was performed to amplify the fungal sequencing. The 18s RNA was used for the amplification of desired gene of fungal species and the sequence of primers. PCR was then performed for the amplification of DNA was isolated from the different strains (Iqbal *et al*., 2017 [6]; Zameer *et al*., 2015) [16]. Appearance of bands under UV Trans-illuminator indicated successful amplification of DNA (Figure 3).

Different dilution of known concentration of glucose were prepared with DNS method reagent and then the absorbance of those dilutions was measured in spectrometer at 540nm. A Glucose Standard curve was plotted to measure concentration of pectinase activity. The potentials of using microorganisms as biotechnological sources of industrially relevant enzymes have stimulated interest in the exploration of extracellular enzymatic activity in many microorganisms (Gomes *et al*., 2009 [4]; Makapela *et al*., 2016) [8]. Microorganisms which are capable of producing pectinase have advantage over other sources because they can be subjected to genetic and environmental manipulations to increase yield. In this research, potential pectinase producing micro-organisms were isolated using serial dilution, pour plating, and streak plating techniques from orange peel. Similarly, also followed similar procedures to isolate microorganisms from orange peel. The presence of proteins, pectin, other sugars, and minerals and its high humidity favor the rapid growth of pectinolytic microorganisms. Emerging new applications underline the importance of screening pectinase producing microorganisms with novel properties, greater enzyme activity, and large-scale production of these enzymes (Chouchane *et al*., 2017 [1]; Kashyap *et al*., 2001) [7]. In this research, the isolates were subjected into plate agar and submerged fermentation screening methods to identify potent isolate with highest enzyme activity and enzyme activity with broad pH ranges. The study is in agreement with reporting plate agar screening method used to screen native isolates for pectic enzyme activity. Pectinases are a heterogeneous group of related enzymes and they are classified into three groups: (1) polygalacturonase; (2) pectin lyase and pectate lyase; (3) pectin esteras; Polygalacturonases have been reported in many microorganisms, including *Neurospora crassa, Aspergillus* sp., and *Bacillus* sp. Pectin esterase have been reported in *Pseudomonas solanacearum, Aspergillus niger*, *Lactobacillus lactis*, *Penicillium occitanis*, and *A. japonicus*. Several types of bacteria and some pathogenic fungi produced pectate lyase. They have been reported in Erwinia carotovora, *Pseudomonas syringae*, and Bacillus sp. Pectin lyases have been reported to be produced by *Aspergillus japonicus*, *Penicillium* sp., and Aspergillus sp. In this research, pectinase activity is determined on the basis of measuring the amount of reducing sugar by using 5-dinitrosalicylate reagent method (Deng *et al*., 2003 [3]; Deng *et al*., 2005 [2]; Ishfaq *et al*., 2016). The potential isolates for pectic enzyme production were identified molecularly using the 18S rRNA gene. About 70% of the isolates were under genus of Aspergillus Niger, among the molecular identification of isolates (Shanley *et al*., 1993 [15]; Nabi *et al*., 2003 [10]; Saad *et al*., 2007) [14].

Conclusion

In conclusion, the presently investigated indigenous strain A. Niger showed incredible potential for pectinase synthesis.

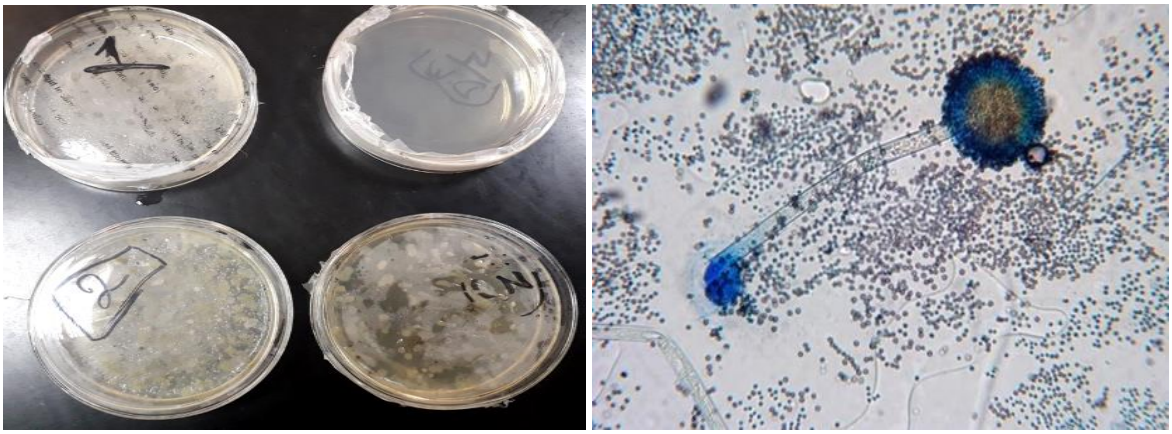

Fig 1: (a) Mixed fungnal colonies of serially diluted samples, (b) Fungal isolates were observed under microscope

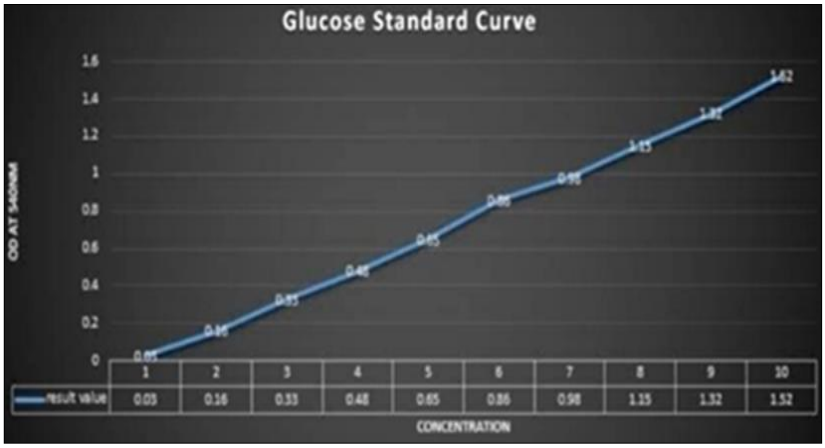

Fig 2: Glucose Standard Curve

Table 3: Detection of highest pectinase activity

No. of Samples	Incubated samples (x)	Without incubation (Y)	Highly active enzymes (X-Y)
1	0.68	0.59	0.09
2	0.72	0.55	0.17
3	0.66	0.60	0.06

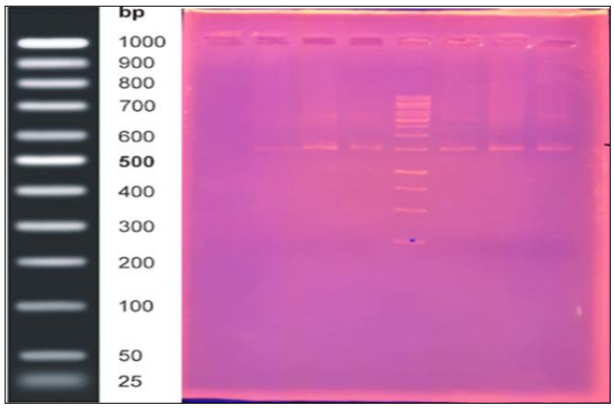

Fig 3: Amplified DNA Bands

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