

Research Article

Production of pectinases towards the extraction of natural pectin from orange peels using fungal sources

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Abstract: Pectin is a polysaccharide, which is present in middle lamella of cell wall of higher plants. Almost all fruits have a part of pectin in their cell wall. Pectinases are used in the degradation of the plant biomass and promptly extract juices from different fruits. For this purpose, in the present study, identified fungi were screened qualitatively and quantitatively for the production of pectinase at 30 °C for 92 hours of incubation time. In liquid state fermentation, identified strains of *Trichoderma asperellum*, *Aspergillus niger* and *Thermomyces lanuginosus* were treated. The results showed that *Trichoderma asperellum* exhibited maximum pectinase activities (9.2 U/mL) after 24 hours of incubation by using orange peels as a sole carbon source. Furthermore, pectinases produced from fungal strains were used to produce natural pectin from fruit waste like orange peels. During fermentation process, pectinase produced from fungal strains were inoculated to dried orange peels for 24 hours of incubation time at 30 °C. The filtered extract was treated with 3 volumes of 80% of ethanol for washing to extract pectin. *Trichoderma asperellum* gave maximum pectin yield (11.7%), while, *Aspergillus niger* and *Thermomyces lanuginosus* gave minimum pectin yield (9.7%, 7.5%) from orange peels respectively and the amount of pectin yield extracted from orange peels with control was (3.5%). Pectinases and pectin have found huge industrial applications in soft drinks, dairy products, pharmaceuticals and food industries, therefore, the present work has focused on the cost effective production of pectinase enzyme and extraction of natural pectin by using potential indigenous fungal strains.

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Introduction:

Pectin is a naturally occurring polysaccharide and have found many applications in food industry. Pectin is present in cell wall of plants and highest concentration of pectin is present in the middle lamella of plant cell wall [6]. Pectin gelling ability depends on its molecular size and degree of esterification therefore, extracted pectin from different sources does not have the same gelling properties [18]. Pectin is widely used throughout the world as an important ingredient to from jellies, fruit drinks, fruit juices, fermented dairy products and jams [17]. Pectin is a polysaccharide that has homogalacturonan and rhamnogalacturonan I and II. Homogalacturonan is (1-4) linked, α -D-galacturonic acid and its methyl ester. Rhamnogalacturonan I is (1, 2) repeating linked, α -L-rhamnose-(1-4) α -D-galacturonic acid disaccharide. Rhamnogalacturonan II has arabinan, galactan and arabinogalactan side chains. Pectin occurs in the form of light brown powder or granular form usually. Natural pectin is easy to purify after isolation and has non-toxic property [8]. Fungi produces various extracellular hydrolytic enzymes used for the decomposition of

organic matter. These include pectinolytic enzymes which degrade pectin present in the middle lamella of plant cell wall to extract nutrients from the plant [4]. Commercial pectinases have been extensively used from fungi including; *Aspergillus spp.*, *Trichoderma spp.*, and *Penicillium spp.* have been extensively used [14].

Pectinases have the main role to catalyze various reactions involved in the preparation of different food products. It is one of the important tools in modern food industry because while processing many intermediate processes are simplified due to use of pectinase in which the complex molecules are broken down. Bulk of the industrial enzymes fall into different groups, the most important group of enzymes are pectinase like protopetinases, pectin methyl esterases and pectin acetyl esterases. These enzymes are used in fruit and vegetable processing industry are pectinases which degrades pectin into galacturonic acid [16]. Pectin degrading enzymes have different properties. In many cases these enzymes are divided into endo and exo- debranching enzymes. Pectin degrading enzyme lyases has an important role in the process of hydrolases additions. There is fact that uronic acid content in the polymer allows its depolymerization by the process of β -elimination [7].

A valuable by product, pectin can be extracted from different fruits wastes. Pectin has important applications at industrial and technological level because of its emulsion stabilization, it is used in the manufacturing of different food products, cosmetics and in medicines. The main aim of pectin extraction is to overcome the increasing demand of pectin in the current economic scenario [1]. Number of methods has been used for the extraction of pectin from fruits. Pectin is extracted from fruit wastes in way with strong acids like oxalic acid [9], sulfuric acid [3] which results into more efficient economical point of view. But the use of all these chemicals for pectin extraction may results into hazardous contaminants. Therefore, new efforts have been developing to minimize the use of chemicals in food processing industries. Therefore, the isolation of pectin introducing environment friendly technology by introduction of enzymatic extraction by introducing microbial enzymes [11].

2. Materials and Methods

2.1. Collection of fungal strains

Fungal strains including *Trichoderma asperellum*, *Aspergillus niger* and *Thermomyces lanuginosus*, were collected from Lahore Garrison University Microbiology lab and further maintained on Potato Dextrose Agar (PDA) media in petri plates by giving the incubation conditions at 30 °C for 48 hours. The fungal strains were screened on pectin screening medium and the composition of the medium was MgSO₄ 4; 4.00 g L⁻¹, K₂ HPO₄; 0.8 g L⁻¹, NaNO₃; 3.00 g L⁻¹, KCl; 8.0 g L⁻¹, FeSO₄; 0.008 g L⁻¹, pectin 8.0 g L⁻¹, agar; 16.00 g L⁻¹, distilled water; 800 mL, pH 5.0 [7]. Qualitative screening was done by the flooding of iodine solution for 15 min and the zone of hydrolysis were formed on fungal strains that was the clear indication of pectinolytic fungi. The strains were further preserved by making agar slants and preserved at -80 °C.

2.2. Pectinase production from Fungal strains

Pectinase activity of *Trichoderma asperellum*, *Aspergillus niger* and *Thermomyces lanuginosus* were observed quantitatively in liquid media which followed the process of liquid fermentation. Orange peels were collected from different local stores in Lahore, Pakistan, in the month of February. The peels were dried in the presence of sun light and shredded in 3 cm in width and length. Fungal strains were examined to observe pectinase activity. Fungal sporulation colonies were obtained from the test tubes by making slants and 0.9%

saline (NaCl) solution was poured on them, by pouring the solution to each test tube contained sporulation colonies and were collected in sterile test tubes. Serial dilutions were made by adding about 1 mL of spore suspension to 9 mL of 0.9% saline solution and then shifted it to the next test tube. The process was repeated until 9th test tube and then 1 mL of discarded from the last one.

2.3. Enzyme production

From each spore suspension, 1 mL culture was taken and added to each flask containing 100 mL of freshly prepared Mandel's media [12]. The composition of the medium was (NH₄)₂SO₄; 0.14 g L⁻¹, KH₂PO₄; 0.2 g L⁻¹, Urea; 0.003 g L⁻¹, CaCl₂; 0.2 g L⁻¹, MgSO₄.7H₂O; 0.03 g L⁻¹, Peptone; 0.1 g L⁻¹, Glucose; 0.1 g L⁻¹, Tween80; 2 mL, Trace metals; 0.1 mL, distilled water; 100 mL and pH; 5. The composition of trace metals was FeSO₄.7H₂O; 0.46 g L⁻¹, MnSO₄.H₂O; 0.93 g L⁻¹, ZnCl₂; 0.08 g L⁻¹, COCl₂.6H₂O; 0.183 g L⁻¹, Distilled water 95 mL and Conc. HCl; 5mL. Then 1% substrates were added separately in separate flasks contained Mendel's medium. Orange peels were used as substrates in liquid state fermentation. The media flasks were incubated for 5 days in a shaking incubator at 30 °C. Pectinase activity was measured after every 24 hours. Pectin (0.5%) was prepared as substrate, by dissolving it in 0.05M citrate buffer at pH 4.8. Enzyme assay for pectinase was performed for screened fungal strains after every 24 hours for 5 days using orange peel as a substrate. Reducing sugars were determined by Dinitrosalicylic acid (DNS) method [2]. Samples were withdrawn from flasks containing substrates (orange peels) and filtered then filtrate was centrifuged at 12000 rpm for 15 min at 4 °C and supernatant was collected for enzyme assay.

2.4. Pectinase assay measurements

Pectinase assay was performed using 0.5 mL of supernatant and 2 mL pectin substrate, both components were added in a test tube and kept in water bath for 60 min at 40 °C. To this tube, 3 mL of DNS reagent was added and boiled for 5 min. O.D values of the samples were then taken at 540 nm wavelength by using spectrophotometer and repeated the process for all fungal isolates. The formula applied for the determination of pectinase activity is $y=x+0.092/0.335$ where x is the OD value and y is the pectinase activity.

Pectin extraction from orange peels using fungal strains

2.5. Preparation of inoculum of fungal strains

Firstly, flasks of 50 mL of Mendel's media were prepared and inoculated the colonies of fungal strains into the flasks and incubated the flasks contained Mendel's media and fungal inoculum into shaking incubator at 150 rpm, 30 °C temperature for 8-10 days.

2.5.1.Procedure

Inoculum of fungal strains containing 5 mL liquid media and 30 g of orange peels were prepared separately in 100 mL of distilled water under sterile conditions. Then covered the flasks with aluminum foil and placed it in shaking incubator at 150 rpm, 30 °C temperature for 24 hours. The process of fermentation took place for the production of pectin from orange peels. After 24 hours each broth was filtered through filter paper. Filtration was very tedious process due to high turbidity of pectin. When the process of filtration was completed then 3 volumes of 90% ethanol was added to each extract and the precipitated pectin from broth was collected by washing at 1500 rpm for 5 min separately. Precipitated pectin was separate from supernatant and dry it hot air oven at 50-55 °C for 10 min.

2.6. Pectin extraction from orange peels control

Pectin was also extracted from orange peels without applying the inoculum of fungus. There was no inoculation of fungal culture in control broth. For pectin extraction, 100 mL of sterile distilled water was used, and 30 grams of orange peel were poured into it. A very small proportion of anti-biotic was added to the broth to prevent from bacterial contamination. The yield of extracted pectin from orange peels was calculated by using following formula:

$$Y_{pec} = [P/Bi] * 100$$

Where P is extracted pectin, B is orange peels used and Y_{pec} is total pectin yield.

3. Results

3.1. Qualitative screening of pectinases

Sub culturing of the fungal strains were done on pectin screening medium. The fungal colony in the plate containing pectin screening media reached about 2 mm in diameter then iodine solution was flooded into the plates and placed it for 15 min. The fungal colonies that had pectinolytic activity showed the clear zone formation or zone of hydrolysis as shown in figure 1.

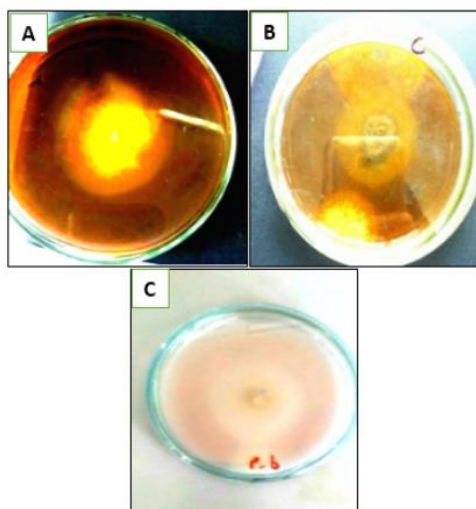


Figure 1. Qualitative screening of pectinases from fungal strains based on zone of hydrolysis (A) *Trichoderma asperellum* (B) *Aspergillus niger* (C) *Thermomyces lanuginosus*.

3.2. Determination of pectinase activity from fungal strains

Fungal strains produced pectinase enzymes to degrade the orange peels and it was confirmed by applying DNS method in which color of the filtrate was changed that indicated the presence of pectinase enzymes as shown in figure 2. Pectinase activity from fungal strains were found out by liquid fermentation having orange peels as sole carbon source at the incubation time period of 24 hours at 30 °C as shown in figure 3.

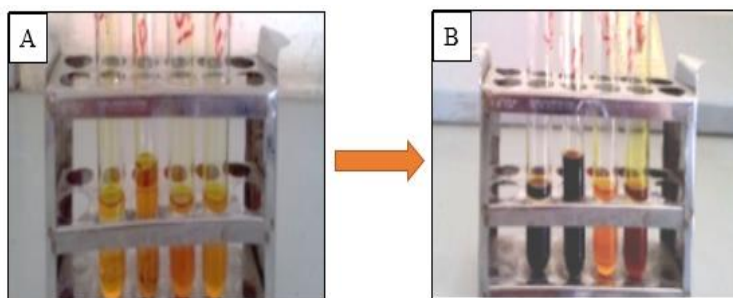


Figure 2. Determination of pectinolytic activity of fungal strain by DNS method. (A): The pouring of DNS reagent before boiling. (B): Change in color after boiling

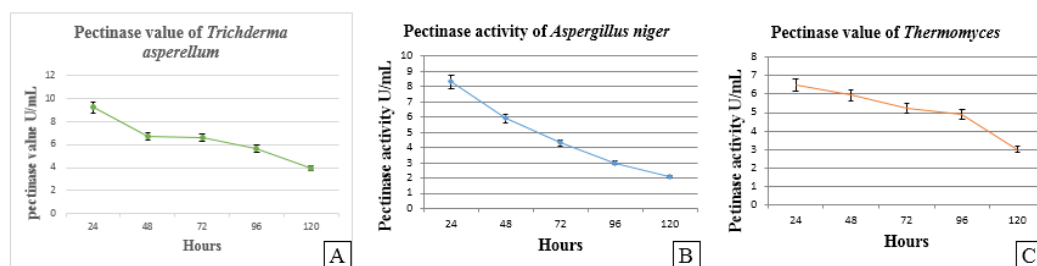


Figure 3. Pectinase activity of fungal strains in different days. (A) Pectinase activity of *Trichoderma asperellum* (B): Pectinase activity of *Aspergillus niger* (C): Pectinase activity of *Thermomyces lanuginosus*

3.3. Extraction of pectin from orange peels by extracellular fungi

Pectin was extracted from orange peels by using pectinolytic fungi which followed the process of fermentation as shown in figure 4.

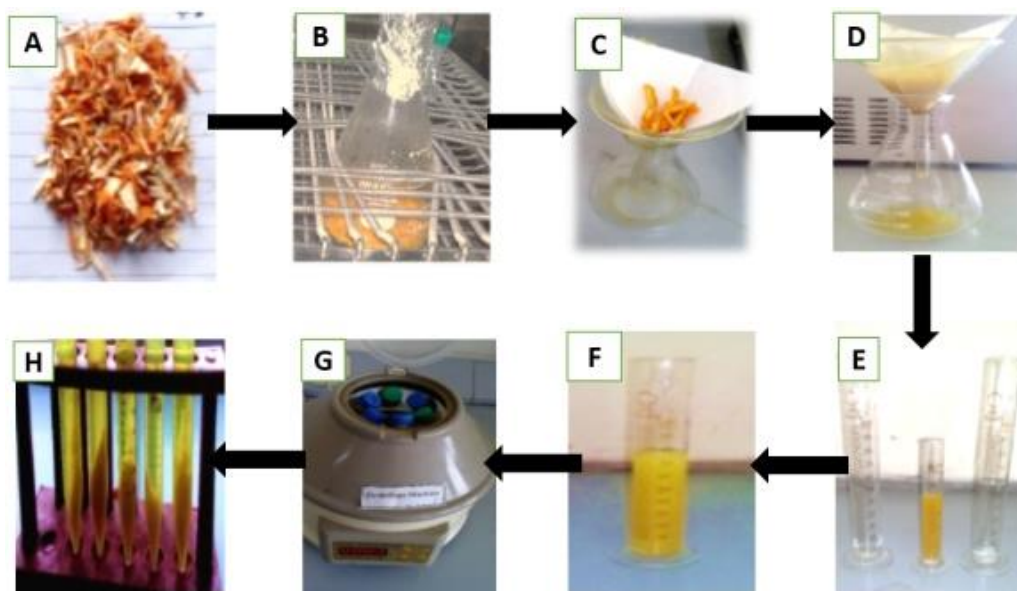


Figure 4. Schematic diagram showing pectin extraction from orange peels by fungal strains. (A): Dried orange peels. (B): Broth containing inoculum of the fungal strains *Trichoderma asperellum*, *Aspergillus niger*, *Thermomyces lanuginosus* and incubate in shaking incubator. (C): Process of broth filtration. (D) Filtrate (E): Adding of 3 volumes of ethanol in filtrate (F) Precipitated filtrate (G): Process of washing by centrifugation (H): Pectin extracted

3.4. Yield of pectin extracted from orange peels

The color of pectin extracted from orange peels was light brown and physical state was jelly like as it is shown in figure 5.

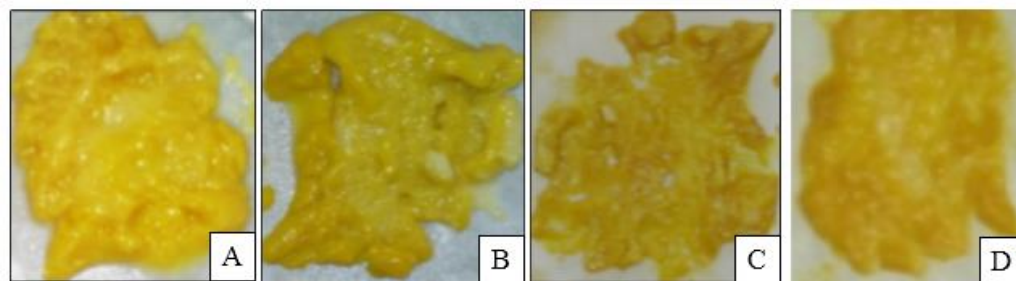


Figure 5. Extracted Pectin: (A) *Trichoderma asperellum* (B) *Aspergillus niger* (C) *Thermomyces lanuginosus* (D) Control from orange peels

3.5. Determination of Pectin yield

The results showed that the yield of the extracted natural pectin from *Trichoderma asperellum* was highest 11.7%, while yield of pectin from *Aspergillus niger* was 9.7% and from *Thermomyces lanuginosus* the extracted pectin yield was 7.5%, respectively. The yield was also calculated for the pectin extracted from orange peels without applying the fungal pectinases, the results showed that about 3.5% of pectin extracted.

4. Discussion

The present investigation was undertaken in order to screen the fungal strains that have pectinolytic activity as well as to obtain the valuable product pectin from fruit wastes like orange peels by the action of pectinolytic fungi. It is also well reported that the fungal strain *Trichoderma asperellum* has maximum pectinolytic activity. Strains formed zone of hydrolysis and was further screened on pectin screening media (the media supplemented with pectin). These results are in correspondence with many studies that have shown *Trichoderma sp.* to be the most communal pectinase manufacturer [11]. In the present study, the fungal strain *Aspergillus niger* also exhibits pectinase activity but its activity was less than *Trichoderma sp.* Pectinase activity of *Aspergillus niger* moved showed maximum 8.2 U/mL activity but less than *Thermomyces lanuginosus* about 6.5 U/mL, respectively. One of the study conducted by Joshi, studied several fungal strains for the production of pectinases [5]. In the present study minimum pectinase activity were shown by the fungal strain *Thermomyces lanuginosus* because it has minimum pectinase producing potential as reported in the literature [19]. Attempts were also made to harness another byproduct pectin from orange peels, peel powder was taken as source for pectin extraction and method described by [15] was followed who recommended 2 volumes of extracting solution, for extracting pectin from mango peel, 5 volumes of extracting solution were used when peel powder was used as source of pectin. In the present study the percentage of pectin that was extracted from orange peels by the action of pectinolytic fungi *Trichoderma asperellum*, *Aspergillus niger* and *Thermomyces lanuginosus* was 11.7%, 9.7% and 7.5% respectively. The amount of pectin obtained from orange peels without introduced the inoculum was only 3.5% and set as a control of experiment.

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