Türk Biyokimya Dergisi [Turkish Journal of Biochemistry–Turk J Biochem] 2014; 39 (1) ; 9–18
doi: 10.5505/tjb.2014.55707

Research Article [Araştırma Makalesi]

Yayın tarihi 30 Mart, 2014 © TurkJBiochem.com
[Published online 30 March, 2014]

Bio-processing of agro-industrial waste orange peel for induced production of pectinase by *Trichoderma viridi*; its purification and characterization

[*Trichoderma viridi* tarafından üretilen pektinaza bağlı tarımsal sanayi atık portakal kabuğu biyoişlenmesi; saflaştırılması ve karakterizasyonu]*

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ABSTRACT

Objective: Agro-industrial residues are primarily composed of complex polysaccharides that strengthen microbial growth for the production of industrially important enzymes. For industrial use, pectinases can be produced from several agricultural pectin-containing wastes like orange peel. The selection of cheap source benefits the production of industrial important enzymes in term to costs-effective production.

Methods: The indigenously produced pectinase was purified to homogeneity level using a combination of ammonium sulphate precipitation and Sephadex G-100 gel filtration chromatography. The molecular weight and properties of the purified enzyme were studied following the standard procedures.

Results: Under optimized solid state fermentation conditions *Trichoderma viridi* exhibited superior enzymatic production. On optimization, the culture showed the maximum enzyme yield (325 U/mL) at 30 °C in an orange peel medium having a pH of 5.5 and a substrate concentration of 4% on the 4th day of fermentation of orange peel based medium that was additionally supplemented with glucose and ammonium chloride as an inexpensive carbon and nitrogen supplements in a ratio of 20:1, respectively. A purification fold of 5.59 with specific activity and percent recovery of 97.2 U/mg and 12.96% was achieved respectively. The molecular weight of purified pectinase from *T. viridi* was 30 kDa evidenced by PAGE analysis. After 6 h incubation the pectinase activity profile showed that the purified enzyme was optimally active and stable at a pH of 5 and at 60°C.

Conclusions: The present study concluded that the indigenous strain *T. viridi* showed incredible potential for pectinase synthesis. The maximum production of pectinase in the presence of a cheaper substrate at low concentrations makes the enzyme useful in industrial sectors, especially for the textile and juice industries.

Keywords: *Trichoderma viridi*, SSF, orange peel, pectinase, purification, PAGE

Conflict of interest: The authors declare that there is no conflict of interest in this work.

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ÖZET

Amaç: Agro-endüstriyel atıklar öncelikle kompleks polisakkaritlerden oluşmaktadırlar. Bu atıklardan mikrobiyal üretimle, endüstriyel açıdan önemli enzimlerin üretimi yapılmaktadır. Pektinazlar endüstriyel kullanım için, portakal kabuğu gibi birçok tarımsal pektin ihtiva eden atıklardan üretilebilir. Ucuz kaynak seçimi maliyetleri düşürerek, vadeli üretimde sanayi de önemli enzimlerin üretiminde etkin olmaktadır.

Metotlar: Yerli üretilen pektinaz, amonyum sülfat çökeltmesi ve Sephadex G-100, jel filtrasyon kromatografisi ile bir kombinasyonla homojen bir seviyeye saflaştırılmıştır. Saflaştırılmış enzimin özellikleri ve molekül ağırlığı standart prosedürler ile belirlenmiştir.

Bulgular: Optimize edilen katı hal fermantasyon koşullarında *Trichoderma viridi* üstün enzimatik üretim sergilemiştir. Optimizasyonda, 5.5 'lik bir pH değerine sahip portakal kabuğu ortamında, 30 °C de en yüksek en yüksek enzim verimi (325 U / ml) elde edildi. Bunun için 4 günlük fermentas sonucu %4'lük portakal kabuğu temelli substrat konsantrasyonu ve karbon ve azot takviyeleri için 20:1 oranında glukoz ve amonyum klorür konsantrasyonu ilave edilmiştir. 5.59'luk bir saflaştırma katsayısı ile spesifik aktivite ve yüzde geri kazanımı, sırasıyla 97.2 U / mg ve % 12.96 olarak elde edilmiştir. PAGE analizi ile saflaştırılmış *T. viridi* pektinazının molekül ağırlığı 30 kDa olarak bulunmuştur. 6 saat inkübasyondan sonra pektinaz aktivitesi profili göstermiştir ki saflaştırılmış enzim pH 5'te ve 60 ° C'de optimum aktif ve stabildir.

Sonuç: Bu çalışma göstermiştir ki yerli *T. viridi* suju pektinaz sentezi için inanılmaz bir potansiyeli vardır. Düşük konsantrasyonlarda, daha ucuz bir substrat varlığında pektinazın maksimum üretimi, özellikle tekstil sanayi ve meyve suyu endüstriyel sektörlerinde enzimı kullanışlı bir hale getirmektedir.

Anahtar Kelimeler: *Trichoderma viridi*, SSF, portakal kabuğu, pektinaz, saflaştırma, PAGE

Çıkar Çatışması: Yazarların çıkar çatışması yoktur.

Registered: 9 December 2012; Accepted: 9 May 2013
[Kayıt Tarihi: 9 Aralık 2012; Kabul Tarihi: 9 Mayıs 2013]

Introduction

Pectic substances are complex acidic structural polysaccharide with high molecular heterogeneous group contain a negative charge, with a backbone of galacturonic acid residues linked by α (1-4) linkage, found in the form of calcium pectate and magnesium pectate [1]. Pectinases are a heterogeneous group of hydrolytic enzymes that degrade pectic substances which produced from variety of microorganisms including filamentous fungi [2]. Fungi considered important source for the production of industrially important enzymes. Pectinase is produced by several fungi including *Aspergillus sp.*, *Botrytis cinerea*, *Fusarium moniliforme*, *Rhizoctonia solani*, *Rhizopus stolonifer*, *Trichoderma sp.*, *Neurospora crassa, Penicillium* and *Fusarium* [3].

Pectinases hydrolyse pectin by different mechanisms, and are divided into de-esterifying enzymes (pectinesterases), depolymerizing enzymes (hydrolases and lyases) [1]. Enzymes like pectinases and ligninases are being used in textile industries, food industries, tea industries, paper and pulp industries, waste management industries, animal feed manufacturing industries, acid fiber to make pectic enzymes, vegetables oil extractions, in the alcoholic beverage [4-6].

Fruit processing industries produce a large amount of waste material leads to disposal problems and pollution. Solid state fermentations (SSF) were used for pectinase production have great advantages over submerged fermentations due to its high productivity, simplicity and concentrated products [5,6]. For industrial use, pectinases can be produced from several agricultural pectin-containing wastes (apple pomace, for example), but the main source remains citrus or orange peel. The selection of cheap source benefits the production of industrial important enzymes in term to reduce the costs and therefore orange peel waste was used as substrates for this process. The present study was conducted keeping in mind the wide range of potential applications for pectinase in different industries.

Materials and Methods

Chemicals and Substrate

All the chemicals used were of analytical grade. Orange peel waste was obtained from a local fruit market in Gujrat, Pakistan. The substrate was sun and oven dried (60 °C), ground to fine mesh size, and stored in plastic jars to avoid free moisture.

Chemical Analysis of the Substrate

Determination of percent moisture content

To determine the percent moisture content of orange peel, 2 g sample was taken in a sterilized bottle (W1) and kept in an oven at 100 ºC for 2 h. It was then kept in desiccators to cool and be weighed. After, it was kept in an oven until constant weight after drying was obtained (W2) and percent moisture was obtained through the following formula.

$$(\%)\ \text{Moisture} = \frac{(W1) - (W2)}{(W1)} \times 100 \qquad (1)$$

Determination of percent ash content

2 g of substrate (W1) was taken in a pre-weight crucible with 5 mL HNO_3 and heated continuously at low flame until the material began to char. After charring the sample, it was kept in a muffle furnace at 555 ºC for 6 hrs and weighed (W2) to determine percent ash content of the substrate sample.

$$(\%)\ \text{Ash} = \frac{(W1) - (W2)}{\text{Weight of sample}} \times 100 \qquad (2)$$

Determination of crude percent fat content

A 5 g sample (W1) was taken in a pre-weight thimble and kept in an extraction apparatus (Soxhelt) and extracted with hexane for 16 hrs. The thimble, along with extracted sample, was dried and from the loss in weight from initial sample the percent of fat was determined (W2).

$$(\%)\ \text{Crude Fat} = \frac{(W1) - (W2)}{(W1)} \times 100 \qquad (3)$$

Determination of percent crude fiber content

For the determination of the crude fiber content, 2 g of dry material (W1), after extraction with ether, was boiled with 200 mL of H_2SO_4, and filtered. The material was dried in an oven and weighed again (W2). The decrease in weight was taken as crude fiber content.

$$(\%)\ \text{Crude Fiber} = \frac{(W1) - (W2)}{\text{Sample weight}} \times 100 \qquad (4)$$

Determination of percent lignin content

2 g (W1) of sample was boiled with 10 mL of 70% (w/w H_2SO_4) solution for 4 to 5 hrs in order to hydrolyze the cellulose and hemicellulose. The remaining suspension after the above treatment was filtered with hot water. Then 30 mL of 70% H_2SO_4 was added into the mixture and the solid residue was dried at 105 °C for 24 h and weighed (W2). The residue was heated at 650 °C for 4 h or until all the carbon is eliminated. After cooling, it was weighed (W3) and lignin content (%) was determined.

$$(\%)\ \text{Lignin} = \frac{(W2) - (W3)}{(W1)} \times 100 \qquad 5)$$

Analytical Methods

Estimation of total sugar

A standard titration method was used to measure the total sugar in the medium. A 25g sample was mixed with 100 mL water and 2 mL of lead acetate solution was added and stayed for 10-15 minutes. Percent total sugar was calculated by using the following formula.

$$\text{Total Sugar (\%)} = \frac{\text{Factor}}{\text{Titer}} \times \frac{\text{Dilution}}{1000} \times 100 \quad (6)$$

Estimation of reducing sugar

Reducing sugar was measured using the 3,5-dinitrosalicyclic acid (DNS) colorimetric method, with glucose as a standard [7]. 1 mL of sample was mixed with 1 mL DNS and boiled for 5 minutes. Optical density was measured at 550 nm to measure color intensity.

Organism and culture maintenance

T. viridi was used for pectinase in SSF. The Potato dextrose agar medium was used to maintain the culture (pH 4.5). The slants of *T. viridi* were incubated for seven days at 30 °C and stored at 4°C in refrigerator.

Vegetative inoculum

The composition of medium for pectinase production is as follow (g/L): glucose 2; $MgSO_4·7H_2O$ 0.05; $CaCl_2·2H_2O$, 0.1; NH_4Cl, 0.12 and thiamine, 0.001 and pH 6.0 with silica gel chips (1.2 mm, dia) in conical flask plugged with cotton wool andsterilized at 15 lbs/in.2 pressure (121°C) for 15 min. Two milliliter of the spores ($1.6×10^6$ conidia/mL) was transferred into growth medium from pre-culture slants. The experiments were performed on orbital shaker by adjusting the speed 100 rpm at 30°C for 96 h.

Fermentation Methodology

During the initial substrate screening trial, 4 g of the above mentioned substrate was taken into 250 mL flasks and inoculated with freshly prepared spore suspension of *T. viridi*. All the inoculated experimental flasks were incubated at 30±1 °C in a shaking incubator at 120 rpm for a 5 day fermentation period. The crude pectinase was extracted by mixing the fermented material with 100 mL of 100 mM citrate buffer followed by keeping them in continuous shaking position (120 rpm) for half an hour. The biomass was then filtered by passing through 125mm filter paper followed by centrifugation (3,000 g, 10 min, and 4˚C) to remove the fungal pellets. The resulting supernatants were collected and used as crude enzyme extract for activity estimation, purification, and characterization. The weight of biomass was determined in pre weighted Petri plates and kept in oven for 24 h at 100°C, till constant weight is obtained.

Determination of pectinase activity

Enzyme assay was based on the determination of reducing sugar produced as a result of enzymatic hydrolysis of pectin by the dinitrosalicylic acid reagent (DNS) method [7]. For this, 0.2 mL of 1% pectin solution, 2.0 mL of sodium citrate buffer of pH 5.0, and 1.0 mL of enzyme extract was added. The reaction mixture was incubated at 35 °C±1 °C for 25 minutes. After 25 min, 1.0 mL of this reaction mixture was withdrawn and added to a test tube containing 0.5 mL of 1 M sodium carbonate solution. 3 mL of DNS reagent was added to each test tube and shaken for 10 min. To mix the contents, test tubes were heated for 10-15 min. After cooling, 20 mL of distilled water was added before measuring the absorbance at 570 nm. The recorded activities were expressed as U/mL while, one unit of pectinase activity was defined as the amount of enzyme required to release 1 μmol of galacturonic acid per mL/minute under standard assay conditions.

Optimization of different Parameters

Different parameters were investigated for optimization, including fermentation period (24-144 h), substrate concentration (1-5%), inoculum size (2-10 %), pH (3.5-7), and temperature (25-45 °C) to achieve maximum yield of pectinase enzyme. All the parameters were run in triplicate.

Enzyme Purification

Crude enzyme was subjected to precipitate after centrifugation at 10,000 g for 20 min at 4 °C by crystals of ammonium sulfate from 30% up to 90% in 5% increments. The resulting precipitate bearing high pectinase activity was suspended in 0.01 M Tris-HCl buffer (pH 6.0) and dialyzed against same buffer. To attain further purification, the collected fractions were subjected to Sephadex G-100 gel filtration column chromatography.

Characterization of Pectinase

Sodium dodecyl-sulphate poly acrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli, [8] to determine the molecular mass of purified pectinase. To 100μL of protein sample, 50 μL of sample buffer (0.05% bromophenol blue, 5% β-mercaptoethanol, 10% glycerol, and 1% SDS in 0.25M Tris–HCl buffer; pH 6.8) was added and boiled for 5 min, cooled at room temperature and loaded onto the 10% SDS-PAGE. Electrophoresis was performed at room temperature for 2.5 h with a 120 Volt and then gel was placed in a fixing solution for 20 min followed by washing with three changes of distal water over 30 min time period. The protein bands were visualized by staining with Coomassie Brilliant Blue G.

The purified pectinase extracted from Sephadex G-100 column was analyzed to define its characterization by studying the effect of different pH and incubation temperatures. An active pH profile of pectinase was determined by studying the effect of different pH buffers ranging from 3 to 9. For pH stability, pectinase was incubated at 35 °C for up to 1 h without its substrate. To find out the thermal stability, pectinase was incubated at varying temperatures ranging from 25-70 °C for up to 1 h in the absence of substrate. Residual activity of enzyme was checked for pH and temperature profiles after an hour using the standard activity assay described above.

Results and Discussion

Chemical Composition of Substrate

Chemical composition of orange peel waste was determined through physiochemical analysis under standard experimental conditions for pectinase production. The proximally analyzed orange peel waste contained 40.7% moisture, 7.39% ash, 1.85% fat, 7.0% pectin, 6.4% lignin, 7.8% crude fiber, 14.08% total sugar, 10.70% reducing sugar, and 3.70% non reducing sugar. The total amount of pectin present in the orange peel waste was 7%, *i.e.* 70 mg/g, according to the investigations. The present reported findings are in accordance with earlier reported data from the scientists [9-11].

Optimization of Fermentation Period

To investigate the effect of incubation time (fermentation period) on the production of pectinase form *T. viridi* using proximally analyzed orange peel waste, experiments were conducted at different time periods, ranging from 24 to 144 h. Data showed that pectinase production increased gradually during the fermentation period and reached to its maximum value (135.5 U/mL) after the 4th day (96 h) of initial incubation (Fig. 1). Any further increase in the fermentation period caused the production of pectinase to decrease. It is also dependent on the nature of fermentation medium, organisms, concentration of nutrients, and the process of physiological conditions [3]. This phenomenon was also observed by Mrudula and Anithrange [12], during pectinase and polygalacturonase production by *Penbicillium sp.*

Optimization of Substrate Concentrations

The effect of different substrate concentrations (1-5%) on production of pectinase by *T. viridi* and the results obtained are presented in the figure 2. The maximum enzyme activity (169 U/mL) was observed under optimum (4%) substrate concentration. After careful analysis during the experiment, it was observed that under optimum substrate (carbon source) concentration, more inducers were available to *T. viridi* that enhance the production of pectinase. The amount of reducing sugars produced by pectinase in fermentation media was 2.92±0.09 mg/mL. On the other hand, the present investigation is in contrary to Naidu and Panda [13], who reported high concentrations of carbon inhibit enzyme synthesis. During the trial it was observed that *T. viridi* used reducing sugars present in the filtrate for its functional activity to produce potential enzymes. Moreover, the culture organism achieved maximum yield of pectinase by hydrolyzing polysaccharides. As a result, the amount of reducing sugar tends to increase when *A. niger* is used as an enzyme-producing microorganism [14].

Optimization of Inoculum Size

Maximum pectinase activity (210 U/mL) was observed with 8% of freshly prepared fungal inoculum size (Fig. 3). Optimum inoculum density is an important consideration for fermentation process since the accumulation of spores can inhibit growth and development of the culture organism [15]. Presently, it was observed that the presence of a large amount of reducing sugars has an inhibitory effect on pectinase production.

Optimization of pH

The effect of initial pH on enzyme production was also investigated and maximum enzyme activity (244 U/mL) was recovered at a pH of 5.5 (Fig. 4). A decline in enzyme activity was observed as the pH level increased from 5.5. The amount of reducing sugars in the medium released was 3.54±0.9 mg/mL. According to Spagna *et al.* [16], maximum pectinase activity occurred at pH 5.0 while, according to Pedrolli et al. [17], a pH of 4.5 was the optimum.

Optimization of Temperature

To elucidate the effect of different temperatures on the production of pectinase, shake flask experiments were run at various temperatures between 25 to 40 °C. Maximum enzyme activity (325 U/mL) and reducing sugars (3.36±3.9 mg/mL) were observed in the batch culture fermented at 30 °C (Fig. 5). Any further increase in temperature suppressed the enzyme activity. These results are in accordance with the data reported earlier by Pedroli *et al.* [17] and Nighojkar *et al.* [15]. Temperature above 45°C results in moisture loss of the substrate which affects metabolic activities of the micro organism that results in reduced growth and enzyme production.

Purification and PAGE

Crude extract of pectinase purified with ammonium sulfate precipitation exhibited 120 mg/mL protein and a specific activity of 23.6 U/mg. After ion exchange column chromatography, the enzyme purity increased 5.59-fold, with a specific activity of 97.2 U/mg (Table 1). Electrophoresis studies revealed that the molecular mass of purified enzyme from *T. viridi* was 30 kDa (Fig. 6), although different molecular weights have been reported for this particular enzyme isolated from different organisms [18]. There was extensive divergence in the molecular mass of exo-PG of different microbial sources, i.e. *Aspergillus kawachii* (60.0 kDa) [19], *Aspergillus japonicum* (38.0 and 65.0 kDa) [20]. The difference has been associated with the type of host cell wall, nature and type of organism used, substrate employed, and analytical methods [18].

Characterization of Pectinase

Effect of pH on activity and stability

Results of an enzyme assay demonstrated that the maximum activity of pectinase enzyme produced from *T. viridi* was observed at pH 5 (Fig. 7). While the

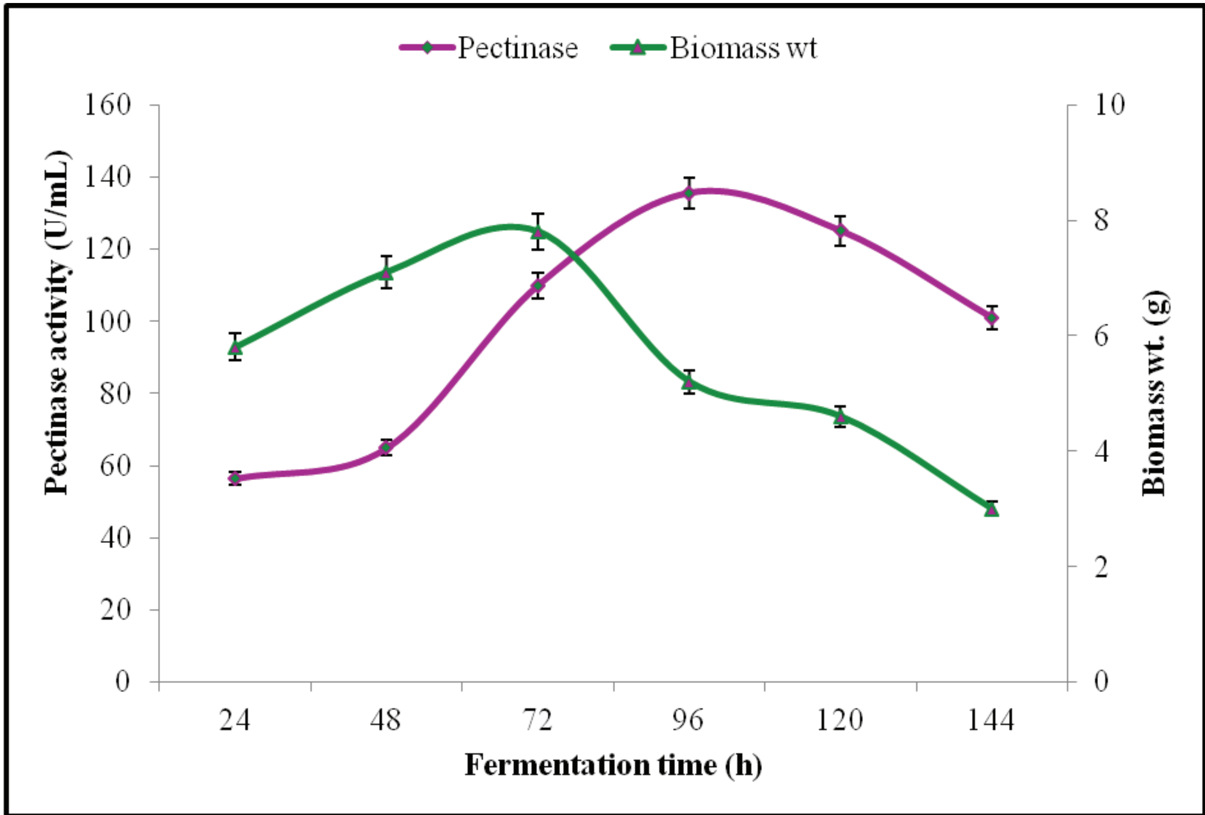

Figure 1. Effect of incubation time on production of pectinase by *T. viridi*.

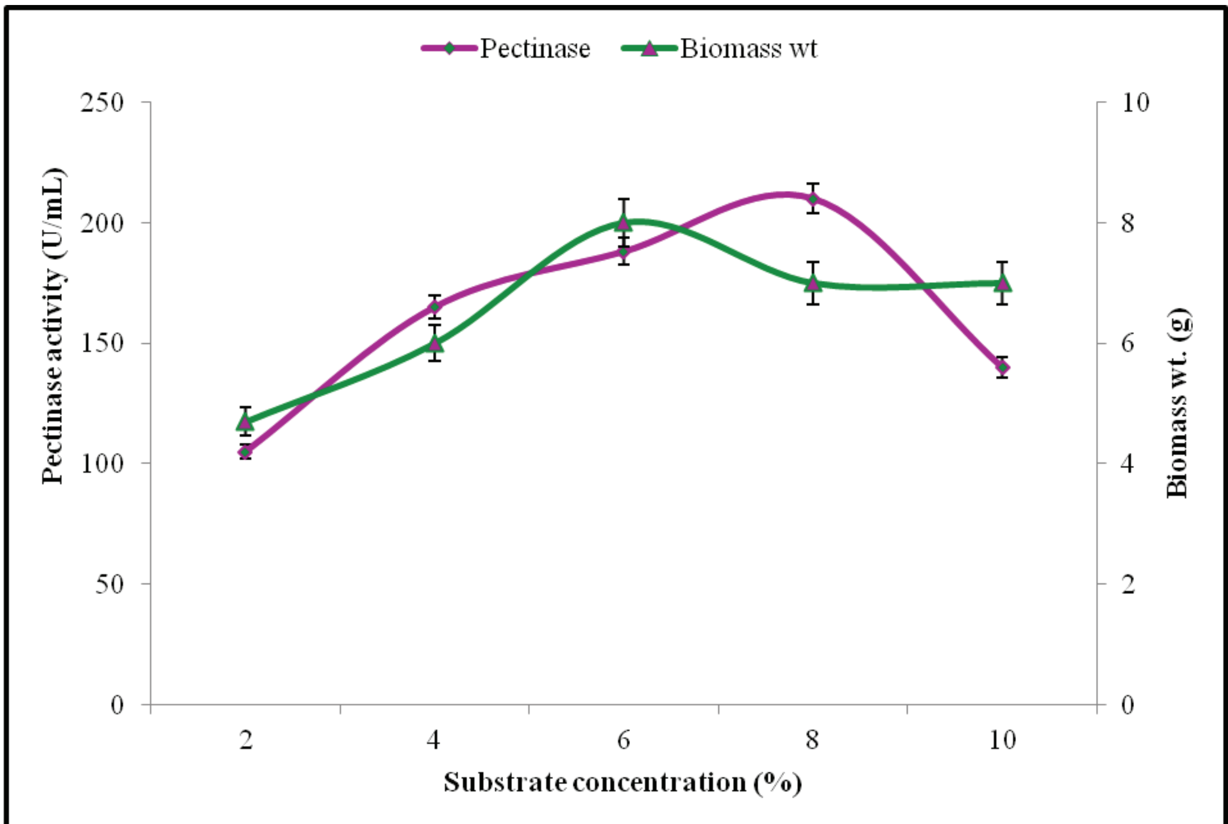

Figure 2. Effect of substrate concentration on production of pectinase by *T. viridi*.

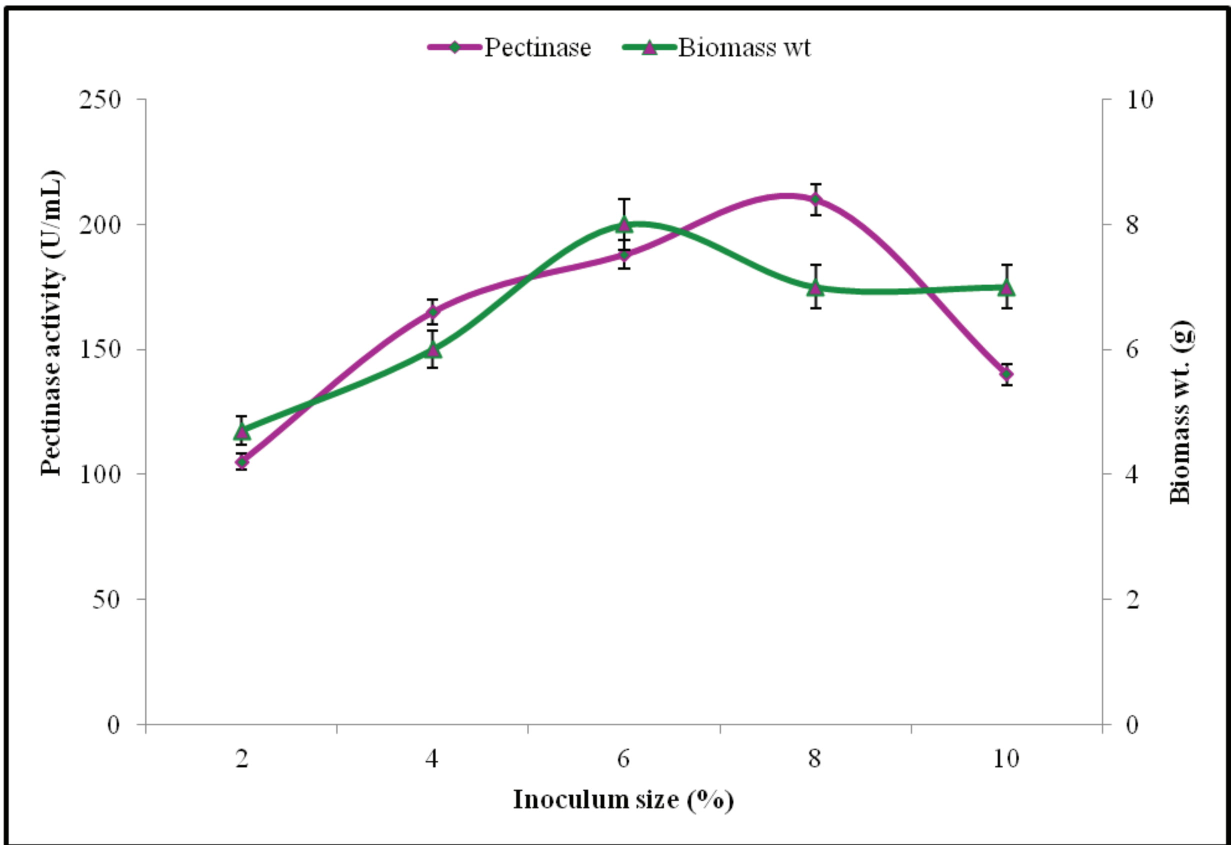

Figure 3. Effect of inoculums size on production of pectinase by *T. viridi*.

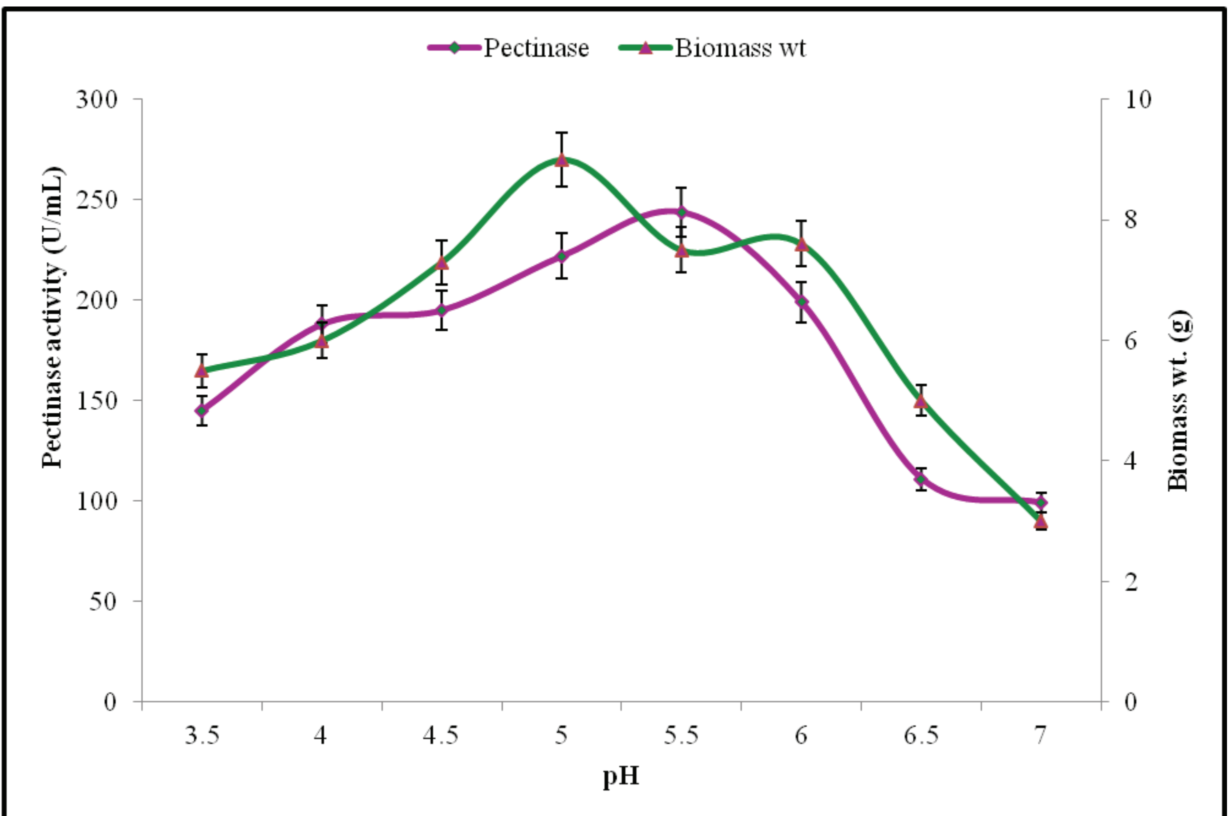

Figure 4. Effect of varying pH levels on production of pectinase by *T. viridi*.

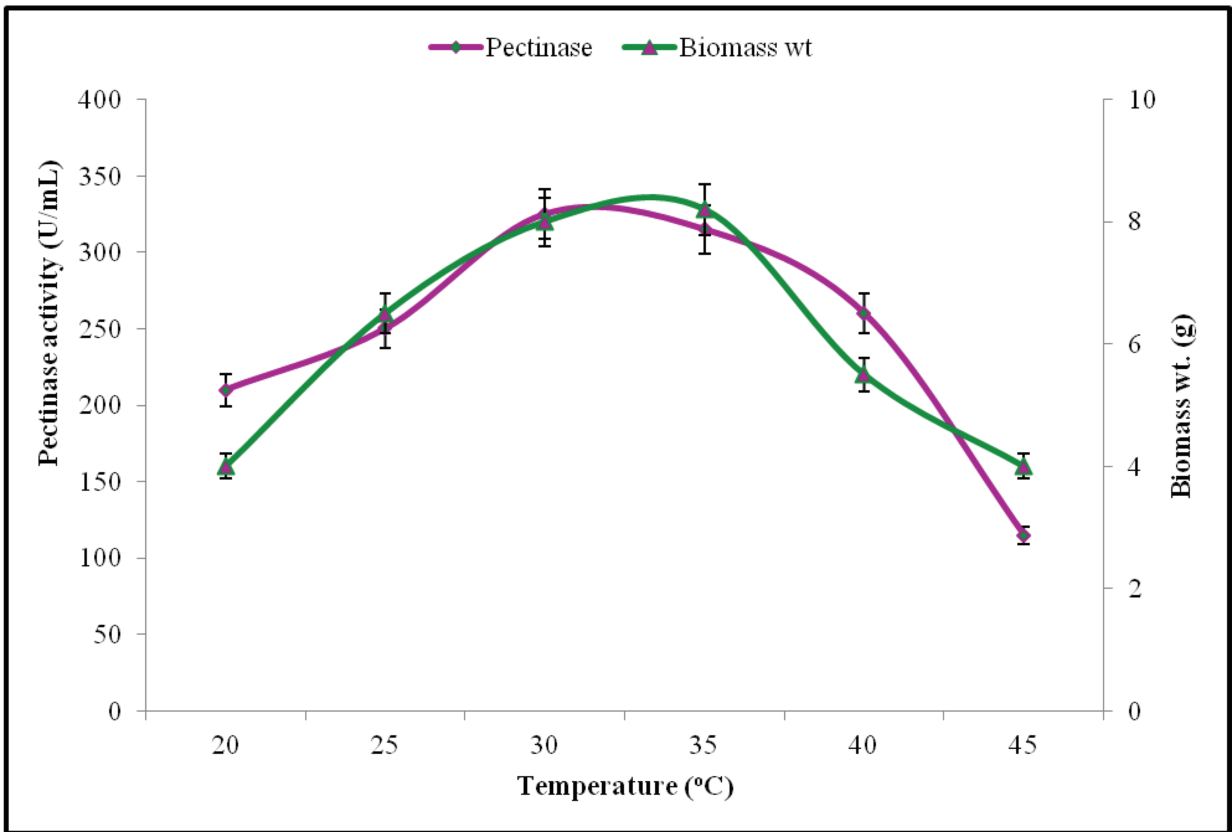

Figure 5. Effect of different temperatures on production of pectinase by *T. viridi*.

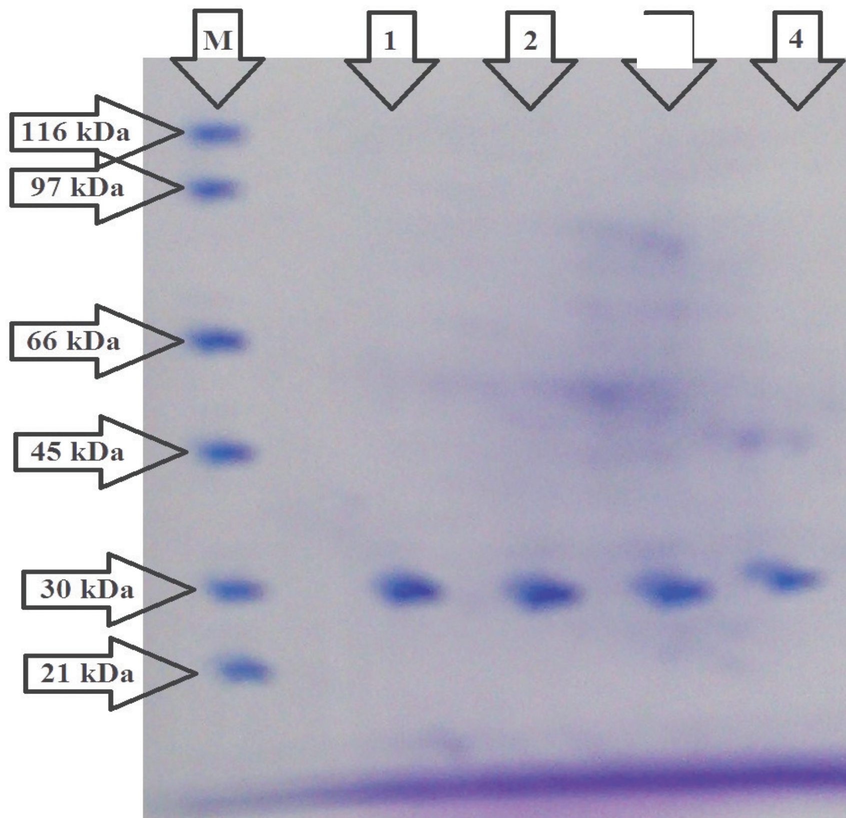

Figure 6. Molecular mass determination of purified pectinase: Lane-M, molecular weights in kDa of standard marker; lane-1, lane-2, lane-3 and lane-4 belong to the repeated samples of the purified pectinase.

Table 1. Summary of Pectinase Purification

Purification Steps	Total Volume (mL)	Total Enzyme Activity (U)	Total Protein Content (mg)	Specific Activity (U/mg)	Purification Fold	% Yield
-------------------------------------	---------------------------------	----------------------	----------	------------	-----------	-------
Crude Enzyme	10-0	13500	775	17.4	1	100
$(NH_4)_2SO_4$ Precipitation	20	2840	120	23.6	1.36	15.11
Dialysis	15	2205	47	46.9	2.87	16.33
Sephadex-G-100	10	1750	18	97.2	5.59	12.96

stability assay profile of pectinase revealed that the present enzyme was more stable over the broad pH range (4-7). The optimum pH of the current findings is comparable to the pectinase of *Penicillium varidictum* and *Penicillium oxalicum*, [21,22] respectively. Frietas *et al.* [23] reported that the optimum pH for pectinase activity from thermo-tolerant *Aspergillus* sp is 5.5.

Effect of temperature on activity and stability

To find out the thermo stability of pectinase, enzyme was incubated at different temperatures ranging from 30-60°C. The results (Fig. 8) revealed that 60 °C was the optimum temperature of purified pectinase, whereby enzyme activity was suppressed at temperatures higher than 60°C. Similar results were reported by Esquivel *et al.* [19] and Chellegatti *et al.* [24] for polygalacturonase. Exo polygalacturaonase from the *Aspergillus* species and endo polygalacturonase from *Mucour rouxxi* exhibited maximum activity at 50 and 35 °C, respectively [23].

Conclusions

The present study concluded that the indigenous strain *T. viridi* showed incredible potential for pectinase synthesis. A purification fold of 5.59 with specific activity and percent recovery of 97.2 U/mg and 12.96% was achieved. The high levels of enzyme secreted by this indigenous strain as well as its thermo-stability suggest that it could be a useful tool for biotechnological applications. The maximum production of pectinase in the presence of a cheaper substrate at low concentrations makes the enzyme useful in industrial sectors, especially for the textile and juice industries.

Acknowledgement

The authors are grateful to the Department of Biochemistry, University of Gujrat, Pakistan for providing financial support and laboratory facilities.

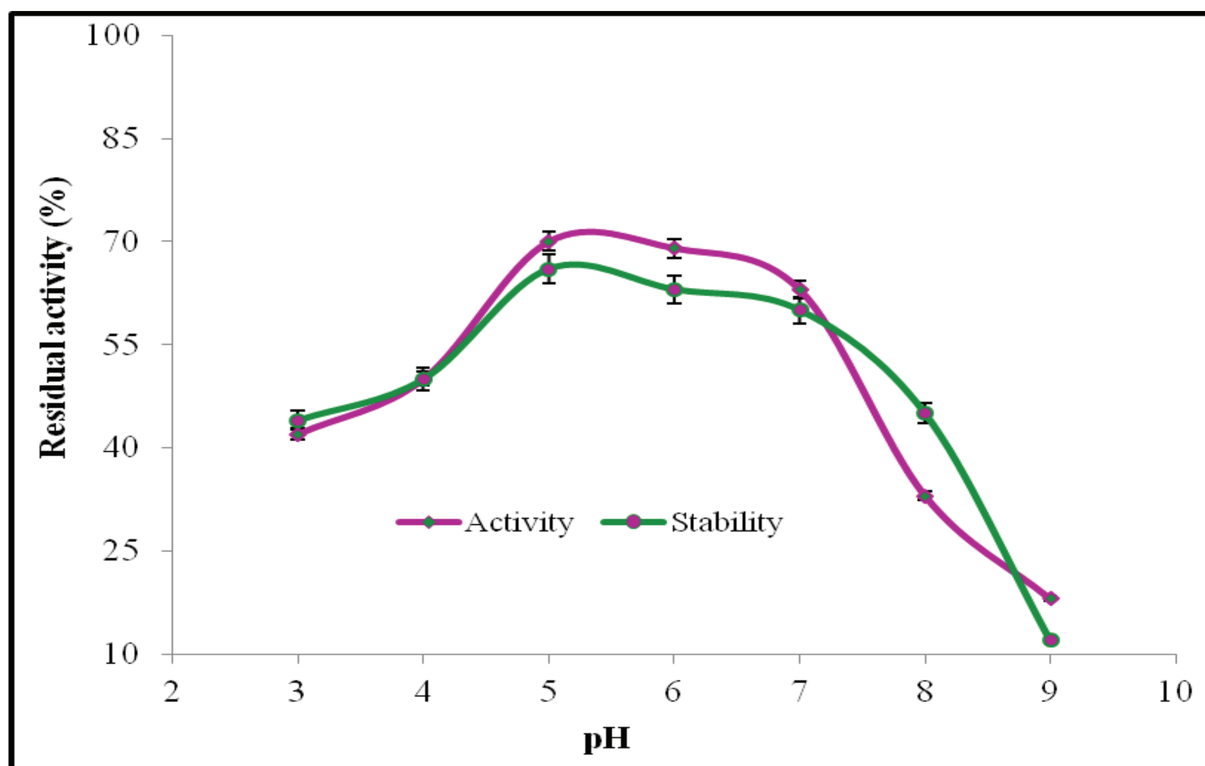

Figure 7. Effect of pH on purified pectinase activity and stability.

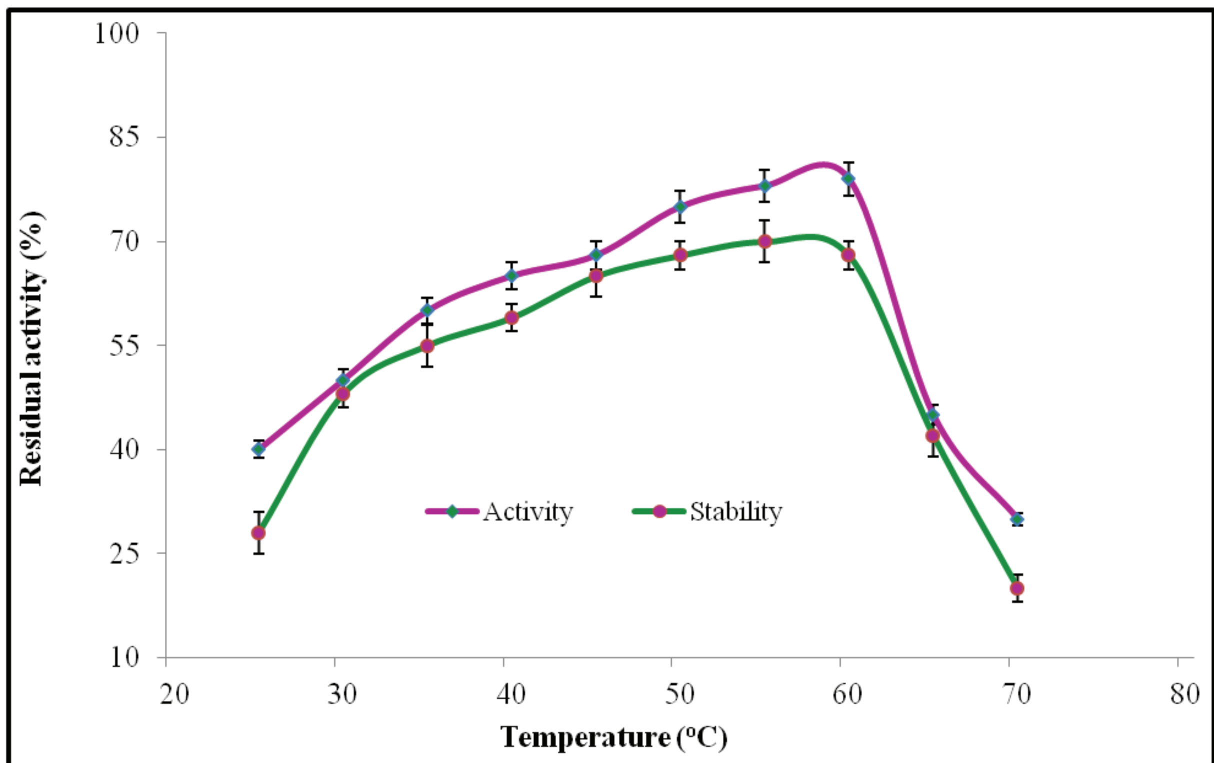

Figure 8. Effect of temperature on purified pectinase activity and stability.

References

[1] Kashyap DR, Chandra S, Kaul A, Tewari R. Production, purification and characterization of pectinase from a *Bacillus sp*. DT7. World J Microbiol Biotechnol 2001; 16:277-282.

[2] Hannan A, Bajwa R, Latif Z. Status of *Aspergillus niger* strains for pectinases production potential. Pak J Phytopathol 2009; 21:77-82.

[3] Joshi VK, Parmar M, Rana NS. Pectin esterase production from apple pomace in solid-state and submerged fermentations. Food Technol Biotechnol 2006; 44:253-256.

[4] Iqbal HMN, Asgher M, Bhatti HN. Optimization of physical and nutritional factors for synthesis of lignin degrading enzymes by a novel strain of *Trametes versicolor*. BioRes 2011; 6: 1273-1278.

[5] Irshad M, Asgher M, Sheikh MA, Nawaz H. Purification and characterization of laccase produced by *Schyzophylum commune* IBL-06 in solid state culture of banana stalks. BioRes 2011; 6 :2861-2873.

[6] Irshad M, Asgher M. Production and optimization of ligninolytic enzymes by white rot fungus *S. commune* IBL-06 in solid state medium of banana stalks. Afr J Biotechnol 2011; 10:18234-18242.

[7] Miller G.L. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 1959; 31:426-428.

[8] Laemmli UK. Cleavage of structural proteins during assembly of head of bacteriophage T4. Nature 1970; 227:680-685.

[9] Wang YC, Chuang YC, Hsu HW. The Flavonoid, carotenoid and pectin content in peels of citrus cultivated in Taiwan. Food Chem 2008; 106:277-284.

[10] Kratchanova M, Pavlova E, Panchev I. The effect of microwave heating of fresh orange peels on the fruit tissue and quality of extracted pectin. Carbohydr Polym 2004; 56:181-185.

[11] Liu LZ, Ahmad H, Lue Y, Gardiner DT. Gunasekera RS. McKeehan WL. Citrus pectin; Characterization and inhibitory effect on fibroblast growth factor receptor. Int J Agri Food Chem 2001; 49:3051-3057.

[12] Mrudula S, Anitharai R. Pectinase production in solid state fermentation by *Aspergillus niger* using orange peel as substrate. G J Biotechnol Biochem 2011; 6:64-71.

[13] Naidu GSN, Panda T. Application of response surface methodology to evaluate some aspects of pectolytic enzymes from *Aspergillus niger*. Biochem Eng J 1998; l2:71-77.

[14] Draginia MP, Madarev SZ, Radulovic LM, Skrinjar M. Production of exo-pectinase by *Penicillium roqueorti* using pumpkin oil cake. Proc Nat Sci 2007; 13:313-320.

[15] Nighoskar S, Phanse Y, Sinha D, Nighojkar A, Kumar A. Production of poly-galacturonase by immobilized cells of *Aspergillus niger* using orange peel as inducer. Proc Biochem 2006; 41:1136-1140.

[16] Spagna G, Pifferi PG, Gillioli E. Immobilization of a pectin lyase from *A. niger* application in food technology. Enz Microb Technol 1995; 17:729-738.

[17] Pedrolli DB, Gomes E, Monti R, Carmona EC. Studies on productivity and characterization of polygalacturonase from *Aspergillus giganteus* submerged culture using citrus pectin and orange waste. Appl Biochem Biotechnol 2008; 144:191-200.

[18] Oyede MA. Studies on cell wall degrading enzymes associated with degradation of cassava (*Manihot esculenta*) tubers by some phytopathogenic fungi. Ph. D Thesis, OAU Nigeria, 1998; pp. 205.

[19] Esquivel JCC, Voget CE. Purification and partial characterization of an acidic polygalacturonase from *Aspergillus kawachii*. J Biotechnol 2004; 110:21-28.

[20] Semenova MV, Grishutin SG, Gusakov AV, Okunev ON, Sinitsyn AP. Isolation and properties of pectinases from the fungus *Aspergillus japonicas*. Biochem 2003; 68:559-569.

[21] Silva D, Martins ES, Silva R, Gomes E. Pectinase production from *Penicillium viridicatum* RFC3 by solid state fermentation using agricultural residues and agro-industrial by products. Braz J Microbiol 2002; 33:318-324.

[22] Zhang CH, Li ZM, Peng XW, Jia Y, Zhang HX, Bai ZH. Separation, purification and characterization of endo-polygalacturonase, from a newly isolated *Penicillium axalicum*. Chin J Proc Eng 2009; 9:242-249.

[23] Freitas PM, Martin N, Silva D, da Silva R, Gomes E. Production and partial characterization of polygalacturonases produced by thermophilic *Monascus sp.* N8 and by thermotolerant *Aspergillus sp.* N12 on solid state fermentation. Braz J Microbial 2006; 37:302-306.

[24] Chellegatti MASC, Fonseca MJV, Said S. Purification and partial characterization of exopolygalacturonase I from *Penicillium frequentans*. Microbiol Res 2002; 157:19-24.