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Pectinase Production in Solid State Fermentation by *Aspergillus niger* Using Orange Peel as Substrate

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Abstract: Optimization of process and leaching conditions for improved production of pectinase by *Aspergillus niger* was carried out using orange peel as substrate under solid state fermentation (SSF). Among 6 substrates tested, orange peel yielded maximum pectinase. The optimum temperature, pH, incubation time, moisture ratio, inoculum size, carbon source and surfactants, were found to be 50°C, 5, 96 h, 1:2 (v/w), 2.5 ml, sucrose and Triton-X-100, respectively. The strain produced maximum pectinase when a combination of yeast extract and ammonium sulfate was added to the medium. The optimum conditions that influence the extraction of pectinase from the fermented substrate were found to be in presence of sodium acetate buffer, with a volume of 1:20 (w/v), at 50°C with a contact time of 90 min. The partially purified enzyme was characterized and it exhibited maximum activity at a temperature of 50°C and pH 5.

Key words: Pectinase · Solid state fermentation · *Aspergillus niger* · Leaching

INTRODUCTION

Pectinases are widely used in food industry especially in the processing of fruits and vegetables, since they decrease the viscosity and facilitate clarification of fruit juices, wines *etc.* [1]. These enzymes not only provide an economically viable alternative, but are also environmental friend [2]. The microbial pectinase accounts approximately for 25% of the total world wide enzyme sale [3, 4].

These pectinases are a part of the group of enzymes involved in pectin degradation. The pectinolytic enzymes act on pectin, a complex polysaccharide which occurs mainly in the middle lamella of higher plants [5]. Due to the great structural diversity of the pectin present in different plant tissues, pectinases have many different mechanisms of action and can be divided into two broad groups: depolymerizing and demethoxylating enzymes. Depolymerising enzymes [6] which break α-1, 4-linkages in the principal pectin chain are polygalacturonase [poly-(1, 4-α-D-galacturonide), glycanohydrolase EC 3.2.1.15] and pectin lyase [poly-(1, 4-α-D-methoxygalacturonide) lyase, EC 4.2.2.10]. Demethoxylating enzyme is a pectinesterase [pectin pectylhydrolase, EC 3.1.1.11] which esterifies pectin to form pectic acid by removing methoxyl residues [4].

The potential synthesis of pectinase is wide spread among the microbial groups including bacteria [7] and fungi [8]. Among fungi, *Aspergillus niger, Penicillium* and *Rhizopus* have many advantages as enzyme producers since they are recognized as GRAS (Generally Regarded As Safe) strains and yield extracellular products which can be easily recovered from fermented medium [9]. Thermophilic fungi are potential sources of various industrially important thermostable enzymes such as lipases, xylanases, proteases, amylases and pectinases [8]. Although a number of pectinases have been studied there are few reports about the production of pectinase by thermophilic fungi [10].

Pectinase production from *Aspergillus niger* was performed in submerged fermentation [11] (SmF) and solid state fermentation (SSF) [12]. The increasing energy demand has been focused worldwide attention on the utilization of renewable agricultural and industrial wastes [10] as their disposal pose environmental problems. The agricultural wastes such as wheat bran, citrus peel, orange peel and orange bagasse have been used for production of pectinase enzymes in SSF [8, 12-14]. Among the most substrates used, orange peel contains an appreciable amount of pectin and hence can be used as the substrate and inducer for the production of polygalacturonase by microorganisms [15].

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In view of the above, the present study was focused on pectinase production by newly isolated strain of *Aspergillus niger* under solid state fermentation using orange peel. Further the partially purified enzyme was characterized.

MATERIALS AND METHODS

Samples Collection and Isolation of Fungi: The fungi were isolated from different soil samples of compost and decomposed matter collected Continue from the vegetable fruit market of Hosur, Krishnagiri District, Tamil Nadu, India. The diluted soil samples were inoculated into a sterile medium of the following composition (g/l): citrus pectin, 10; $(NH_4)_2SO_4$, 1.4; K_2HPO_4, 2; $MgSO_4.7H_2O$, 0.2; nutrient solution, 1ml (the nutrient solution contained the following g/l in distilled water: $FeSO_4.7H_2O$, 5; $MnSO_4.7H_2O$, 1.6; $ZnSO_4.7H_2O$, 1.4; $CoCl_2$, 2); agar agar, 20. The pH of the medium was adjusted to 5 [10]. The inoculated plates were incubated at 30°C for 96 h; the plates were flooded with 1% (w/v) aqueous solution of hexadecyltrimethyl ammonium bromide. The clear zones around the colonies in an opaque white back ground detected pectinase production [27]. The stock culture was maintained on potato dextrose agar (PDA) slants which were inoculated at 30°C for 120 h and then stored at 0-4°C until further use.

Preparation of Spore Suspension: *Aspergillus niger* subcultured on PDA agar slants was used to prepare the spore suspension. The spore crop from each slant was scrapped into 5 ml of sterile water using a sterile glass rod. This suspension was filtered through a thin layer of sterile glass wool placed in a sterile syringe to remove the hyphal filaments. The spore suspension (1×10^7 spores/ml) was used as a source of inoculum.

Submerged Fermentation: In submerged fermentation (SmF), the organism was grown in 250 ml Erlenmeyer flasks containing 100 ml of non-buffered mineral medium (NBMM) with 1% citrus pectin as carbon source, the medium was sterilized by autoclaving at 121°C for 15 min, followed by incubation at 30°C for 96 h on an orbital shaker (150 rpm) [11] . The non-buffered mineral medium contained the following (g/l in distilled water): K_2HPO_4, 4; KH_2PO_4, 1.28; $(NH_4)_2SO_4$, 2; citrus pectin, 10; yeast extract, 0.6; $MgSO_4.7H_2O$, 1.1; pH, 5.

Solid State Fermentation: Solid state fermentation (SSF) was carried out in 250 ml Erlenmeyer flasks that contained 5 g of orange peel and 5 ml of distilled water (moistening agent). The flasks were sterilized at 121°C for 15 min and cooled to room temperature. 1 ml of conidial suspension (1×10^7 spores/ml) was added, mixed well and incubated at 30°C for 96 h. At the end of the incubation period, the flasks were taken out and the content of each flasks were extracted with 25 ml of sterile distilled water [12].

Enzyme Preparation: In SmF, the enzyme was prepared by centrifuging the culture broth at 6000 rpm for 20 min at 4°C in a cooling centrifuge (REMI) [14]. The supernatant was used as a source of extracellular enzyme.

In SSF, the enzyme was extracted from the fermented mycelial substrate by homogenously mixing the entire substrate with distilled water (1:1 w/v) which was agitated on a rotary shaker at 100 rpm with a contact time of 1 h at 30°C. The biomass [16] was separated by filtration through a Whatmann No. 1 filter paper and pooled extracts were centrifuged at 6000 rpm for 20 min at 4°C and the clear supernatant was used as the source of extracellular enzyme.

Pectinase Activity: Pectinase activity was assayed by measuring the reducing sugars released from the action of pectinase on citrus pectin using 3, 5-dinitrosalicylic acid (DNS) reagent [11, 17]. The reaction mixture (3 ml) consists of 0.8 ml of (1.0 % w/v) citrus pectin and 0.2 ml of appropriately diluted enzyme source in 2 ml of sodium acetate buffer (0.1 M, pH 5). The reaction mixture was incubated at 40°C for 10 min followed by addition of 1 ml NaOH and 1 ml of DNS to the tubes and the reaction was stopped by heating the tubes in boiling water bath for 10 min. The reducing sugars released by enzymatic hydrolysis were determined. A separate blank was set up to correct the non-enzymatic release of sugars. One unit of enzyme activity is defined as the amount of enzyme required to liberate 1 μ mole of reducing groups per minute with galacturonic acid as standard under the assay conditions.

Optimization of Process Parameters: The parameters selected for optimization were natural substrate (rice bran, wheat bran, sugarcane bagasse, orange peel, lemon peel and banana peel), temperature (30-70°C), pH (3-8), incubation time (24-120 h), moisture ratio [1:0.5-1:3.5 (v/w)], inoculum size [1-3 ml (1×10^7)],

carbon source (glucose, sucrose, galactose and carboxy methylcellulose), nitrogen sources [urea+$(NH_4)_2SO_4$, yeast extract+$(NH_4)_2SO_4$, peptone+$(NH_4)_2SO_4$, yeast extract+$NaNO_3$, peptone+$NaNO_3$, urea+$NaNO_3$] and surfactants (Triton-X-100, Tween 80, Tween 20 and sodium dodecylsulphate).

Optimization of Leaching Conditions: For the extraction of pectinase, the following parameters were optimized; solvent selection (distilled water, tap water and sodium acetate buffer), solvent volume (1:5-1:25) (w/v), solvent temperature (30-50°C) and contact time (30-120 min).

Characterization of Pectinase Enzyme: The pectinase (0.2 ml) was incubated with varying temperature (40- 60°C) and pH (4-8). The buffers used were: sodium acetate buffer (pH 3.6-5.8), sodium phosphate buffer (pH 5.7-8.0) and glycine-sodium hydroxide-sodium chloride buffer (pH 8.4- 12.8).

RESULT AND DISCUSSION

The fungal strain produced clear zones around the colonies in opaque white back ground and pectinase production was detected. Based on the colony morphology and microscopic observation, the strain used in the present study was confirmed as *Aspergillus niger* [18]. In SmF, the strain produced 232 U of enzyme per ml of culture broth. Selection of a suitable solid substrate for a fermentation process is a critical factor in SSF and this involves the screening of a number of agroindustrial materials for microbial growth and product formation. In the present study, among six substrates screened, orange peel was found to be the most significant for pectinase production by *Aspergillus niger* in 96 h (Fig.1). Similar result was also reported by Phutela *et al*. [8] for pectinase and polygalacturonase production by *Penicillium* sp. Considering the utilization of waste material and cost effectiveness, the use of orange peel powder can prove highly economical at industrial scale. Dhillon [15] reported the use of citrus peel in semisolid fermentation for pectinase production. In contrast, the sugarcane bagasse was suitable for growth and pectinase production by *A. niger* [19] and *Penicillium viridicatum* RFC3 [20]. Silva [20] and Martins [21] reported that the mixture of orange bagasse and wheat bran showed to be the best substrate for the pectinase production in SSF using *Penicillium* sp.

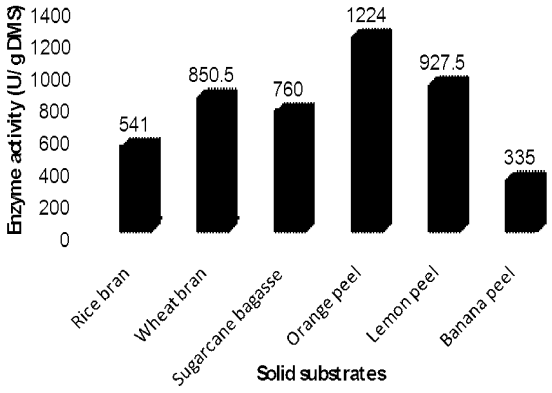

Fig. 1: Effect of solid substrates on pectinase production by *Aspergillus niger* in SSF.

Table 1: Effect of physical parameters on pectinase production by *Aspergillus niger* in solid state fermentation

Physical parameters	Enzyme yield U/g DMS
Temperature (°C)	
30	65.7
40	221.6
50	244.8
60	203.6
70	59.25
pH	
3	36.07
4	48.75
5	70.75
6	56.5
7	52.8
8	32.2
Incubation time (h)	
24	65.7
48	110.8
72	451
96	1211.2
120	529
Inoculum size(1×10^7)	
1	77.33
1.5	83.74
2	100.5
2.5	252.5
3	114.6

Maximum pectinase production by *A. niger* was observed at an incubation temperature of 50°C (Table 1). The incubation temperature has a profound effect on the enzyme yield and the duration of enzyme synthesis phase [22]. Most of the fungi investigated for pectinase production showed optimum growth in the range of 45 to 60°C [23, 24].

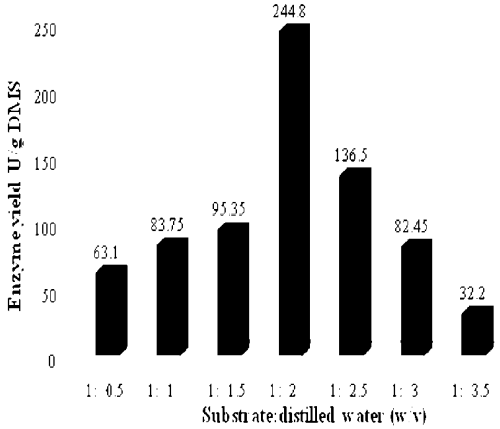

Fig. 2: Effect of moisture levels on pectinase production by *Aspergillus niger* in SSF.

The pH regulates the growth and the synthesis of extracellular enzyme by several microorganisms particularly fungal strains [25]. The ideal pH for pectinase production by *A. niger* has been found to be 5 (Table 1). These results were comparable with the findings of Torres *et al*. [6] for the pectinase production by *A. niger*. The optimum pH of 6 for *A. niger* was reported using citrus peel and sugarcane bagasse, respectively for the production of pectinase in SSF [26].

The period of fermentation depends upon the nature of medium, fermenting organisms, concentration of nutrients and the process physiological conditions [27]. In the present study, accumulation of maximal extracellular pectinase was observed after 96 h of fermentation (Table 1). Similar observation was obtained during poly-galacturonase production in SSF [10]. An 84 h of incubation time was found optimum for production of pectin lyase by *A. niger* in SSF [26].

Maximum pectinase production was obtained at a moisture ratio (1:05 to 1:2) (Fig. 2). The initial moisture content significantly affected hydrolytic enzyme production in SSF, since the moisture content of the medium is a critical factor that determines microbial growth and product yield in SSF [28]. Moisture is reported to cause swelling and thereby facilitates better utilization of the substrate by the microorganisms [29]. It is reported that low substrate moisture in SSF resulted in suboptimal product formation due to reduced mass transfer process such as diffusion of solutes and gas to cell during fermentation. The decrease in moisture level is advantageous since the chance of contamination of fermentation medium is reduced [30].

Table 2: Effect of different supplements on pectinase production by *Aspergillus niger*

Supplements	Enzyme yield U/g DMS
Carbon sources (1% w/w)	
Control	185.5
Sucrose	300
Galactose	280
Carboxy methylcellulose	170
Starch	250
Glucose	180
Nitrogen sources (1%w/w)	
Control	185.5
Urea+$(NH_4)_2SO_4$	208.7
Yeast extract+$(NH_4)_2SO_2$	223.3
Peptone+$(NH_4)_2SO_4$	185.5
Yeast extract+$NaNO_3$	164.9
Peptone+$NaNO_3$	136.5
Urea+$NaNO_3$	126.25

The maximum activity of pectinase was obtained from SSF medium fermented with 2.5 ml (1×10^7 spores/ml) inoculum (Table 1). In contrast, the enzyme synthesis in *A. niger* was best at 4×10^6 to 4×10^7 spores g^{-1} wheat bran but declined at 4×10^8 spores counts [31]. Optimum inoculum density is an important consideration for SSF process since over crowding of spores can inhibit growth and development [32]. Higher inoculum levels besides increasing spores density increase water content of the medium as well.

Addition of different carbon sources to orange peel resulted in induction and repression of pectinase production in SSF. Among the carbon sources tested, sucrose promoted maximum enzyme yield compared to the others (Table 2). Similar results were also reported for pectinase production by fungi in SSF [8, 27]. Increases in enzyme production with additional carbon source have been demonstrated by both SmF and SSF systems [33].

The source of nitrogen in the growth medium has a very important role in microbial growth and enzyme production. The result of the present study showed that addition of a combination of yeast extract and ammonium sulfate enhance pectinase production (Table 2). Our results are in accordance with the observations of Phutela [8]. Yeast extract served as the best inducer of exopectinase by *Aspergillus* sp. [34]. Whereas ammonium sulfate increases the production level of pectinase in SmF and SSF systems using *A. niger* [27]. Banu [35] reported that ammonium persulphate has enhanced the production of *P. chrysogenum*. Results of this investigation have shown that the production of pectinase is highest when Triton-X-100 was supplemented to the orange peel in SSF (Fig. 3).

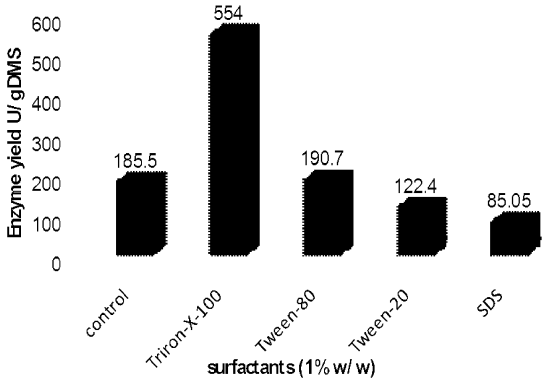

Fig. 3: Effect of surfactants on pectinase production by *Aspergillus niger* in SSF.

Table 3: Effect of leaching conditions on pectinase production from the fermented mycelia substrate

Leaching conditions	Yield
Solvent selection	
Distilled water	1183.26
Tap water	2031
Sodium acetate buffer	9839
Solvent volume (w/v)	
1:5	206.12
1:10	502.5
1:15	850.5
1:20	1185.5
1:25	1185.5
Solvent temperature (°C)	
30	798.75
35	902
40	1005
45	1121
50	1288.5
55	1056.5
60	850.5
Contact time (min)	
30	850.5
60	1069.5
90	1288.5
120	1224
150	1146.75

One of the important steps in leaching of any product in the solid state fermentation system is the selection of a suitable solvent. In the present study, among three solvents tested, the highest enzyme activity was recorded in the presence of sodium acetate buffer (Table 3). All solvents have its own dielectric constant, organic solvents posses lower dielectric constant than inorganic solvents, hence water posses higher dielectric constant than organic solvents. Therefore, from the above theory it can be concluded that force of interaction between pectinase and solvent may have increased due to lowered dielectric constant of the extracting solvent, sodium acetate buffer [36]. Distilled or tap water alone or with glycerin or sodium chloride gave the highest yield in the amyloglucosidase extraction from the fermented mould wheat bran [37].

The solvent volume plays an important role in the extraction of pectinase from the fermented orange peel. In the present study, maximum extraction of pectinase was obtained at a substrate to solvent volume ratio of 1:20 (Table 3).Whereas 1:3 was found the most suitable for maximum extraction of amylase from the fermented bran. Recovery of the low yield of enzyme from the fermented bran with lower volume of solvent might be due to the insufficient solvent volume to penetrate the solid fermented mass [36]. Higher solvent to solid ratios also causes the solute to be more dilute in the final extract [13].

The effect of temperature on the leaching process was studied by varying the temperature of solvent from 30 to 60°C. It was observed that 50°C was the most effective condition for leaching of the pectinase from fermented substrate of *A. niger* (Table 3). At higher temperature, the yield was less. This might be due to the denaturation of the enzyme. In other work, it was reported as 30°C for effective leaching of the α-amylase from the fermented bran [36].

A contact time of 90 min achieved maximum pectinase leaching from the fermented substrate of *A. niger* (Table 3). Similar findings have been reported by Chandra [13] on the extraction of cellulase from fermented bran of *A. niger*. Whereas, 60 min contact time was found most suitable for maximum extraction of cellulase and β- glucosidase [38] and 150 min for extraction of amylase from the fermented bran [36].

The maximal activity was observed at pH 5 (Fig. 4) for pectinase from *A. niger*. The optimum pH found for pectinase activity in this study is comparable to the pectinase of *Penicillium viridicatum* RFC3 [20] and *Penicillium oxalicum* [39]. It was reported that the optimum pH for pectinase activity from thermotolerant *Aspergillus* sp. N12 was 5.5 [23].

The optimum temperature for the activity of the pectinase by *A. niger* was 50°C (Fig. 5). Similar results were also reported for polygalacturonase by *A. kawachii* [40] and *P. frequentans* [41]. Exo-polygalacturonase from *Monascus* and *Aspergillus* sp. [23] exhibited maximum activity at 60 and 50°C, respectively. The endopolygalacturonase from *Mucour rouxii* NRRL 1894 exhibited maximum activity at 35°C [42].

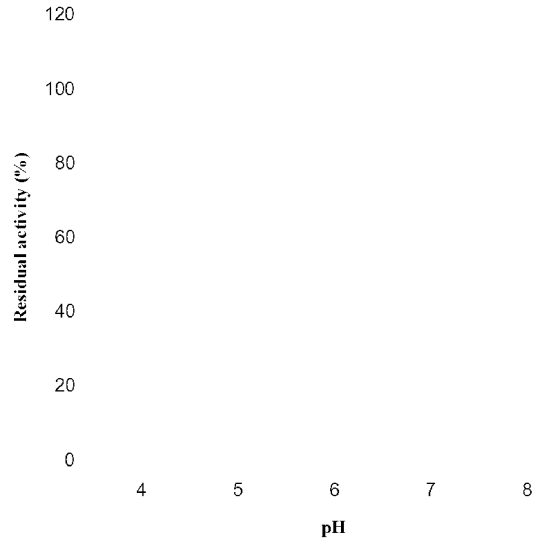

Fig. 4: Influence of pH on pectinase activity of purified enzyme from *Aspergillus niger*.

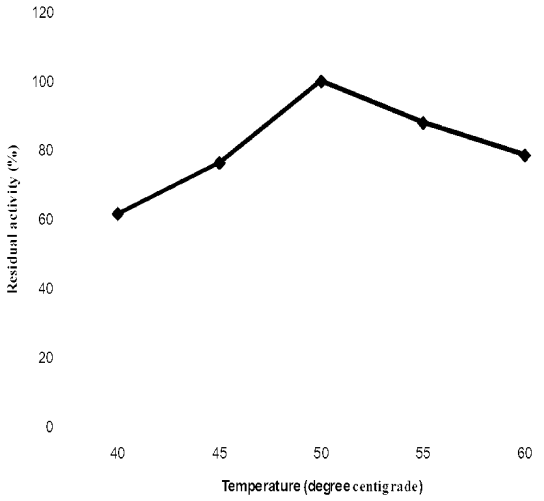

Fig. 5: Influence of temperature on pectinase activity of purified enzyme from *Aspergillus niger*.

In the present study, initially the strain produced 232 U/ml in SmF and 1224 U/g DMS in solid state fermentation. After optimization of culture and leaching conditions in SSF, the strain produced 5283 U/g DMS, which is 23 fold high when compared to the yields obtained in SmF. Therefore, these results clearly indicated the scope for utilization of *Aspergillus niger* for pectinase production through SSF which has wide applications in food processing and textile industries.

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