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Characterization of Pectinase from Cashew Shell Cake Using *Aspergillus niger* with Unique Kinetic Properties

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Abstract

The main purpose of the study was to provide a value addition; to a cashew industry by-product:- Cashew Shell. Production of newly Pectinase enzyme by Solid State Fermentation and the enzyme has been optimized by using Cashew Shell as substrate has been standardized. Strain has been selected by assessing the fungal strains having the better survival capacity in CNSL and based on the Pectinase production studies (MTCC 1344). Optimum Pectinase production by SSF was obtained when incubated for 96 hours at 45ºC and pH 5.The enzyme activity of Pectinase (Polygalactouronase) was determined by measuring the release of reducing groups from polygalactouronic acid. Partially Purified Pectinase powder was obtained by acetone precipitation; followed by filtering the enzyme through Whatman No: 1 filter paper using a vacuum pump. The powdered Pectinase showed more enzyme activity compared to the crude Pectinase enzyme. A 1.78-fold partially purified enzyme and 97% yield was obtained from crude to acetone precipitated. The functional temperature ranged from 10ºC to 60ºC and the functional pH ranged from 3.0 to 8.0 respectively. The kinetic parameters value were found to be Km=1.284 and Vmax=9.901 u/ml. This work clearly proves that Solid state fermentation which is a cheap method was the suitable technique for the production of Pectinase from Cashew Shell Cake using newly identified strains(MTCC 1344). Thus Pectianse enzyme, a very valuable product was obtained from the wasted Cashew Shell cake.

Keywords: Pectinase , *Aspergillus niger*, Solid-State Fermentation (SSF), Cashew Shell Cake, Polygalactouronase.

Pectin is a heteropolysaccharide found in the middle lamella and the primary cell wall of higher plants. Pectins function as a 'glue' that holds the other cell wall polysaccharides, like cellulose and hemicellulose (i.e. xyloglugan or glucuronarabinoxylan), and proteins, such as hydroxyproline-rich glycoprotein extension together (Willats et al., 2001). The molecular structure of pectin is a linear backbone comprised of á-1, 4-linked D-galacturonic acid residues which may be methylated and substituted with l-rhamnose, arabinose, galactose and xylose (Gadre et al., 2003; Kashyap et al., 2001; Nakagawa et al., 2004). *A. niger* produces a number of enzymes active on the homogalacturonan part of the pectin molecule. They include pectin methyl- and acetyl-esterase (EC 3.1.1.11 and EC 3.1.1.6), endopolygalacturonase (EC 3.2.1.15), exopolygalacturonase (EC 3.2.1.67), and pectate lyase (EC 4.2.2.10). While pectin lyase can utilize the naturally occurring methylesterified substrate, the activity of pectin methylesterase is required in order to produce low methylesterified pectin (pectate), the substrate for polygalacturonases and pectate lyase. (Gummadi and Panda, 2003; Soares *et al*., 2001). *Aspergillius* are used by industry for the production of primary metabolites (organic acids, vitamins, fatty acids and amino acids) and enzymes

as well as for bioconversion processes. *A. niger* strains are being used in the production of Citric Acid, a universally accepted additive with a broad range of applications in food, beverage, pharmaceutical and cosmetic industries and for industrial and technical processing (Brooke, A. G.1994;). Two species, viz. *A. niger* and *A. oryzae* (Section Flavi), prevail as enzyme producers (Oxenboll, K 1994). While *A. niger* is mainly exploited in the area of food (bakery, starch industry) and beverages (wine and juice, brewery, distilling industry), *A. oryzae* is applied in fermentation of oriental sauces, such as soya sauce and miso (Aidoo, et al., 1994). Since both species have been used for years, many of their products obtained the GRAS (Generally Regarded As Safe) status. The progress in fungal genetics, gene technology and protein engineering makes the Aspergillus an attractive 'tool' for a number of biotechnological processes in different industries, one of these being the pectin industry. In this industry fungal Pectinase s are applied to generate specific pectic products.

The present study, describes the production, optimization, purification and characterization of Pectinase by using a potent strain *Aspergillus niger* with cashew shell as a substrate.

Materials and Methods

Microorganism and Culture maintenance

Aspergillus niger is a filamentous fungus that commonly occurs in the environment and is considered to be nonpathogenic.10 g of excreta collected from Cashew Shell which was dissolved in 90mL sterile distilled water and serially diluted upto 10^{-4}.From this 1 mL from was inoculated to 10mL of Potato dextrose broth containing 1% Polygalacturonic acid and incubated at 25°C for 3-5 days. Fungal colonies which are able to form clearing zone around the colony due to the hydrolysis of Polygalacturonic acid were selected and purified. Potent strain was then identified. The culture was maintained on PDA broth.

Preparation of Inoculum

Two loops of spores from *Aspergillus niger* culture (6 days old) was transferred on to 5ml of sterile distilled water and was dislodged on to production media under sterile condition. The flasks were then incubated at 30°C for 72-96 hours and the inoculums thus obtained were used for the inoculation of the solid substrate medium.

Solid substrates

Cashew shell used as substrate for Pectinase production, were obtained locally from cashew industry (KSCDC, Kollam) .It was then cleaned, ground and autoclaved at 121°C for 15mins. The Cashew Shell Cake used as the primary carbon source contained a pectin content of 2.3-3.4%.

Culture condition

Solid-State Fermentation was used for the production of Pectinase enzyme. Composition of Culture medium was (in g/L): 0.1%NH_4NO_3, 0.1%$NH_4H_2PO_4$, and 0.1% $MgSO_4.7H_2O$

Solid-State Fermentation

Solid-State fermentation (SSF) was carried out using a 250ml Erlenmeyer flask containing 5g of sterile substrate inoculated with 5ml of spore suspension. After inoculation, 5ml of nutrient solution composed of 0.1%NH_4NO_3, 0.1%$NH_4H_2PO_4$, and 0.1% $MgSO_4.7H_2O$.The final moisture content of the substrate was approximately 60% .After inoculation flasks were incubated at 30°C for 96hrs. At 24-96 h intervals the solid fermented material, corresponding to one Erlenmeyer flask, was mixed with 30 mL of distilled water (5g of fermented material/mL), stirred for 30 min, filtered under vacuum and centrifuged. The supernatant was used as crude enzyme solution.

Downstream processing

Standardization of Partial Purification with Acetone

Extraction of Pectinase was done first by transferring the fermented Cashew Shell Cake into a muslin cloth and pressed manually followed by filtering through Glass fibre filter paper and centrifuged at 9000 rpm for 20mins at 4°C. The supernatant was used as crude enzyme solution and later used for further purification process. Partial Purification of crude Pectinase enzyme was done using acetone precipitation. The acetone rich supernatant was removed using a vacuum pump and the precipitate was collected in the Whatman No.1 Filter Paper. The moisture content was removed by using the inert silica crystals.

Enzyme Activity Measurements

The crude enzyme was separated by filtered through Whatman No.1 filter paper. The filtrate was collected in the sterilized flasks and used for further analysis.

Polygalactouronase (PG) activity was determined by measuring the release of reducing groups from Polygalactouronic acid (Sigma) using the 3,5-dinitrosalicylic acid(DNS) reagent assay (Miller,1959). The reaction mixture containing 0.5ml of 1.0% Polygalactouronic acid in 0.2M Acetate buffer, pH 5.0 and 0.5ml of purified enzyme solution was incubated at 45°C for 30mins. Reducing sugars were determined by dinitrosalicylic acid (DNS) method using galactouronic acid (Sigma) as reference. One exo-P unit (U) was defined as the quantity of enzyme that liberates one micromole of galactouronic acid per minute.

Protein calculation

Total protein of enzyme was determined by Lowry's, et al. (1951) by using standard Bovine Serum Albumin and OD value was taken at 660 nm .

Kinetics

The effect of varying temperature (10ºC to 60ºC), pH (3.0 to 8.0), incubation time (30 minutes), enzyme concentrations (25-200) and substrate concentrations (1% to 5%) were determined for the purified Pectinase enzyme. Based on these kinetic parameters the Km and Vmax was found from the Michaelis-Menten plot using Graph Pad Prism 5 software.

Results and Discussion

Characterization of Pectinase enzyme produced in initial crude form and it's partially purified one are summarized in the Table 1. The acetone precipitated formed at 2:1 ratio, which showed a 1.78-fold increase in specific activity compared to the crude sample.

After the precipitation of acetone 97% Pectinase recovery with a specific activity of 0.374 U/mg protein.

Effect of Temperature on Production of Total Pectinase

The effect of temperature on the Pectinase enzyme production was studied. By conducting experiments at different temperatures namely 10°C, 20 ºC, 30 ºC , 37 °C, 40 ºC, 45 ºC, 50 ºC, 55 ºC and 60 ºC by keeping all other conditions constant for the incubation period of 30 minutes.The results are given in Figure 1; hence optimum temperature was chosen as 45 ºC and was used for further studies. The optimum temperatures for PGs of *A. niger* and *Penicillium dierckii* were shown to be 50 and 60°C, respectively(Shubakov *et al* 2002). Optimum temperature for various Pectinases varied between 20 and 50°C (Kollar, A 1998, Mishra A *et al* 2001; Huang et al 1999.)The decrease in enzyme activity at higher temperature may be due to enzyme denaturation.

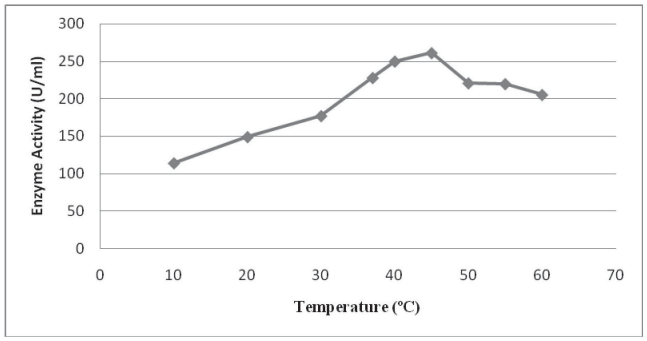

Fig. 1: Temperature vs Enzyme activity

Effect of Initial pH on production of Total Pectinase

The effect of initial pH on the Pectinase production using was studied by conducting experiments at different pH namely pH 3, pH 4, pH 5, pH 5.5, pH 6.0, pH 7.0, and pH 8 by keeping temperature at 45 º C and incubation at 30 minutes. The results are given in Figure 2. As initial pH was increased from pH 3.0 to pH 8.0, the Pectinase activity

Table1: Enzyme activity of Crude and Acetone from Cashew Shell Cake Yield and fold of purification

Sampledescription	OD at 510 nm(mg/ml)	Total Protein (mg/ml)	Total activity (U/ml)	Specific Activity (U/mg)	Purification Fold	Yield for 1 kg of substrate (%)
Crude enzyme	1136.85	1.704	0.36	0.21	1	100
Acetone purified	1144.76	0.937	0.35	0.373	1.78	97

was found to increase, further increase in initial pH beyond pH 5, the Pectinase activity was found to decrease. A maximum Pectinase activity of 0.49 U/ml was obtained with mixed substrate for Incubation period of 30 minutes at temperature 45 ^{o} C and at pH value of 5. Hence optimum pH value was chosen as pH 5. The optimal *p*H for different pectinases has been reported to vary from 3.8 to 9.5, depending upon the type of enzyme and the source. The optimal *p*H of *A. niger* polymethyl polygalacturonase was, however found to be 4.0(Kollar *et al*;1998). While, PGs of *A. niger* were shown to possess maximum catalytic activity at *p*H 5.0(Shubakov et al 2002). Our results are almost in confirmation to these findings.

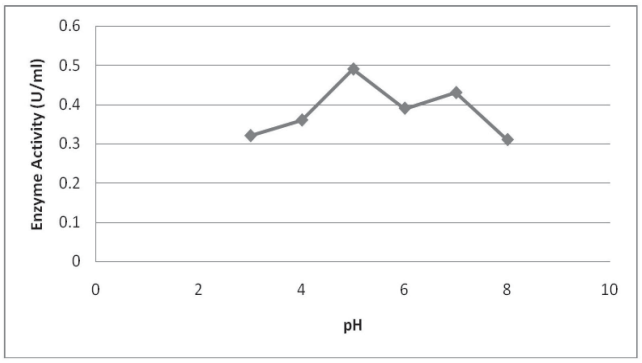

Fig. 2: pH vs Enzyme Activity

Effect Enzyme concentration on production of Total Pectinase

It can be seen from Figure 3 shows the different enzyme concentration on Pectinase activity was observed per ml of substrate (Polygalacturonic acid). The optimum enzyme concentration for Pectinase production was evaluated Pectinase activity increased with increase in concentration was observed at 150 mg/ml.

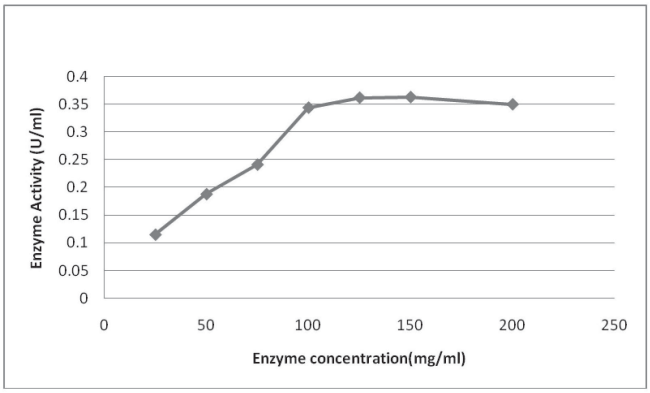

Fig. 3: Enzyme concentration vs Enzyme Activity

Kinetics of Pectinase production from cashew Shell cake

The Kinetic studies of the partially purified and powdered Pectinase revealed that the optimum Pectinase activity from the crude enzyme was 0.21 u/mg (Table 1) at temperature 45ºC, pH 5.0, incubation time of 30 minutes and Enzyme concentration is 150 mg/ml. The Pectinase activity with respect to the substrate concentration is given in the Figure 4 and found that it reaches a maximum of 9.22 u/ml at the concentration of 5% and thereafter it decreased. (Figure 5) The rate of product (dp/dt) formation was found to increase gradually and was maximum at the end of 96 hours and later on it decreased due to non-viability of substrate. The kinetic parameters were determined using Michaelis-Menten plot and the values are given below Km=1.254 and Vmax=9.901 u/ml. Comparing the kinetic parameters and biochemical properties of the purified enzymes, it seems to be equivalent to the endo PG1reported by Benen *et al*.(1999)

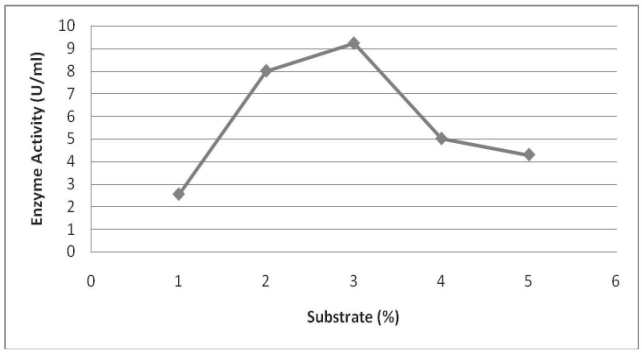

Fig. 4: Subsrate vs Enzyme Activity

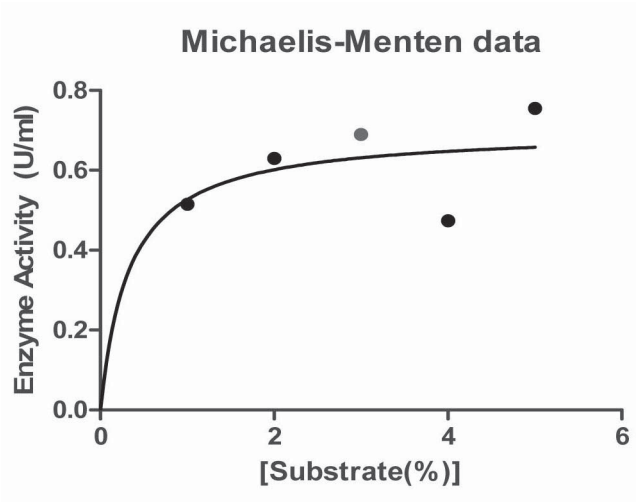

Fig. 5:The Km 1.254 and Vmax 9.901 U/mg for varying concentration of substrate

During the recent years, efforts have been directed to explore the means to reduce the Pectinase production cost through improving the yield, and the use of either cost free or low cost feed stocks or agricultural by products as substrate like cashew shell cake which are highly involved in the Pectinase enzyme production compare than other substrate. Among the tested substrates, the Cashew Shell Cake was the effective inducer for the Pectinase production in fungal strains.

Conclusion

Pectinase enzyme was produced using SSF from cashew shell cake using newly introduced *Aspergillus niger* strain. The enzyme isolated through SSF showed more activity compared to SmF (Submerged fermentation). Pectinase enzyme was produced using solid state fermentation from cashew shell cake (industrial waste) using filamentous fungi like *Aspergillus niger*. Pectinase from cashew shell cake using *Aspergillus niger* showed optimum enzyme activity 0.21 U/mg; under SSF at a pH 5; when incubated at 30°C on the 4th day.

The purification fold of the enzyme increased approximately1.8 times and yield was 97% from crude to acetone precipitated. In this context, the results obtained during the course of study indicate the utilization of filamentous fungi for extracellular Pectinase production through Solid State Fermentation, which is active in pH and temperature owing to its probable application in textile industry.

This work clearly proves that Solid state fermentation which is a cheap method was the suitable technique for the production of Pectinase from cashew shell cake using as identified strains. Thus affecting the recycling and re-usage of the wasted cashew shell cake into a valuable product –Pectianse enzyme.

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