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KINETIC AND THERMODYNAMIC ANALYSIS OF PURIFIED PECTINASES PRODUCED FROM NITROUS ACID MUTANT DERIVATIVE OF *ASPERGILLUS NIGER* (H-97)

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Abstract

The *Aspergillus niger* mutant strain H-97 was used for pectinase production (40.31±0.07 U/ml/min) having specific activity of 12.12 ± 0.01 U/mg. Fermentation was carried out using 5 liters of fermentation medium in 7.5 liter stirred fermenter under controlled condition of temperature (30°C), pH (07), agitation (200 rpm), aeration (1vvm) for an incubation time of 48 h using 1% vegetative inoculum. Purification of enzyme resulted in 44.20% yield with enhancement of specific activity (75.18±0.04 U/mg) by ammonium sulfate precipitation and ion exchange chromatography. Kinetic characterization of enzyme revealed pectin as highly specific substrate for enzyme with Km value of 2.30 mg/ml. Thermodynamic evaluation of enzyme resulted in activation energy (Ea) as -28.95 KJ/mol and enthalpy of activation (ΔH) as -26.73 KJ/mol.

Key words: Fermentation, Thermodynamics, Kinetics, Pectin.

Introduction

Pectinases are complex of enzymes used for the de-branching of pectin substrate into galacturonic acid residues by acting on the glycosidic residues through de-polymerization and esterification reactions (Gregorio *et al.,* 2002). The main components of this enzyme complex are polygalacturonase, pectin methyl esterase and pectin lyase (Voragen &Visser, 2004).

Pectinases can be obtained from various sources i.e. plant, fungi, bacteria. Fungi and yeast accounts for 50%, bacteria for 35% and plant sourcesfor 15% of its production (Phutela *et al*., 2005). Mainly, production of pectinase is carried out by microorganisms to avoid the environmental stresses and changes (Patil & Dayanand, 2006).

Purification of pectinase is an important factor when its application in industry is considered. Salting out or ammonium sulphate precipitation is used to isolate proteins of interest from the solution by aggregating the solutions ionic strength (Bankar *et al.,* 2009). Ion exchange chromatography is usually applied for pectinase purification based on the forces of attraction between particles having opposite charges (Zia *et al.,* 2007). SDS-PAGE is used to check purity and protein's comparative molecular weight (Wilson & Walker, 2010).

Kinetic factors such as Vmax and Km are very important during the characterization of an enzyme. Vmax of an enzyme can be determined by analyzing maximum reaction velocity attained by enzyme when it completely binds with the substrate. In this way it can be measured, to reach the maximum catalytic velocity, how much concentration of the substrate is needed (Nelson & Cox, 2004). This is beneficial for the industry from economical point of view and also its less time requirement (Zia *et al.,* 2007).

The thermodynamic parameters play a major role in catalytic activity of enzymes and the above mentioned parameters must be keep under consideration while analyzing the efficiency of the enzymes. Therefore, in present study after pectinase production and purification its kinetic and thermodynamic characteristics were analyzed.

Materials and Methods

Microorganism: Nitrous acid mutant strain of *Aspergillus niger* H-97 taken from culture bank of Institute of Industrial Biotechnology, GC University Lahore was used for the production of pectinase.

Vegetative inoculums: Slants having profused conidial growth were added with ten ml of sterilized distilled water. The conidia were scratched with sterilized inoculated needle, shaken gently to obtain homogeneous conidial inoculum. One milliliter of this was inoculated in 25 ml sterilized nutrient broth and incubated at 30ºC and 200 rpm for 24 hours to get vegetative inoculums (Malik *et al*., 2016).

Submerged fermentation: Submerged fermentation was carried out in a stirred fermenter of 7.5 liters (Model: BF-110 BioFlo/ CelliGen by New Brunswick, USA). Fermentation medium (5 L) containing K_2HPO_4 (4.0 g), yeast extract (0.6 g), KH_2PO_4 (1.28 g), $(NH_4)_2SO_4$ (2.0 g), $MgSO_4$ (1.1 g) and pectin (10 g) per 1.0 L of distilled water was prepared and autoclaved at 121ºC for 15 minutes. After allowing cooling, the fermentation medium was inoculated with 5% vegetative inoculum and incubated for a period of 96 h at 30ºC and 200 rpm (Iftikhar *et al*., 2015).

Down streaming: After fermentation batch, product was recovered by filtration and centrifugation at 6,000 rpm for 10 min (Model: D-37520, Osterodeam-Harz, Germany). For estimation of pectinase activity, supernatant was used (Iftikhar *et al*., 2015).

Pectinase bioassay: The Pectinase activity was calculated using pectin as substrate by observing the method of Okafor *et al*. (2010). Pectin (1%) was prepared in sodium acetate buffer as substrate and 500 µl of it was incubated with 500 µl of crude enzyme at 50°C in water bath for 30 min. The reaction was stopped with the help of 3 mldinitrosalicyclic acid and was kept in boiling water for about 10 minutes. The change in color of the solution was observed at 550 nm with the help of spectrophotometer (CECIL CE-7200 Series, UK). Pectinase activity was

defined as the quantity of enzyme used to release one microgram (1 µg) equivalent of D-galactouronic acid per minute in assay conditions.

Protein assay: The protein assay was carried out using Bradford Reagent (Bradford, 1976) to determine the specific activity of pectinase.

Ammonium sulphate precipitation: The amount of the salt required for precipitation was analyzed using ammonium sulphate calculator. The salt was added with crude enzyme extract in increments of 10% from 0 to 90% at 4°C. After the mixing of a required salt sample, it was centrifuged at 6000 rpm and 4°C (Saxena *et. al*., 2003). The pellets were isolated and activity assay of both pellet and supernatant was carried out. This procedure was repeated until desired protein was collected. Dialysis of the precipitated enzyme was carried out to isolate any amount of salt present using membrane with 12000-14000 molecular cut off value in phosphate buffer.

Ion exchange chromatography: Pectinase was purified by ion-exchange chromatography (isoelectric homogeneity) on a Bio-Rad Bio-scale Mini Macro-Prep DEAE cartridge column. One ml protein sample was added with a concentration 5mg/ml. The unbounded protein fractions were washed away with the help of Binding Phosphate + buffer. The bound fraction were than eluted by using NaCl concentration gradient between 0-80%. The activity assay of bound and unbound fractions was performed to identify the required protein. The fractions were further used for SDS- PAGE analysis to check the purity of product (Bhatti *et al*., 2006).

SDS- PAGE: Pectinase purity was analyzed using the SDS PAGE by employing 12% gel. The process was carried out after Saxena *et al.* (2003).

Kinetic analysis: Kinetic parameters were evaluated after Line weaver & Burk (1934). Substrate concentration was observed at optimum activity of enzyme after analyzing the enzyme activity for a substrate concentration ranging from 10-80 mg/ml.

Thermodynamic studies: The thermodynamic parameters were examined after Siddiqui *et al.* (1997).

Statistical analysis: The Computer statistical software Costat, cs6204W.exe was used for statistical analysis (Snedecor & Cochrane, 1980).

Results

Submerged fermentation: Mutant strain of *Aspergillus niger* H-97 was used for the production of pectinase using laboratory scale fermenter of 7.5 L. It was observed that Pectinase production was increased gradually from 8 hours of incubation and reached to maximum at 48 hours (40.31±0.11 U/ml/min) under the controlled conditions of temperature (30˚C) and pH (7) as shown in figure 1. However, further increase in the incubation time resulted in decreased product formation be due to depletion of nutrients from medium. Due to the unavailability of nutrients, cells might enter in stationary phase in which production of secondary metabolites occur. These metabolites usually cause inhibition of the enzyme conformation. Similar results were reported by Ray *et al.* (2007) and El-Hadi *et al.* (2014) with maximum production of pectinase after 48 h of incubation.

Purification of pectinase: Pectinase was precipitated between the 50-70% fractions of ammonium sulphate. Pectinase precipitated pellets were re-dissolved in phosphate buffer and subjected to overnight dialysis. This step of fractionation resulted in the increase in specific activity of the enzyme from 12.12 ± 0.01 U/mg to 50.31 ± 0.04 U/mg. After precipitation enzyme fractions collected were passed through anion exchanger using anion exchange chromatography. Pectinase presence was noted in the bound fractions eluted by using NaCl concentration gradient between 0-80% (Fig. 2). Improved pectinase specific activity as 75.18 ± 0.04 U/mg was recorded with 6.20 purification fold. Percentage yield was noted as 44.20% (Table 1). Purified fraction of pectinase was subjected to SDS PAGE to find out the purity of the product. A very clear single band was observed on gel having size of 32 kDa (Fig. 3). The Pectinase molecules have positive charge due to which they get attached to the negatively charged solid support. Elution is due to the change in ionic strength causing the breakage of interactions between oppositely charged substances. Findings of Bhatti *et al*. (2006) can be related to our results who put forward that the yield of 28.43% was obtained by using the anion exchange chromatography with DEAE-Cellulose resin. However, use of hydrophobic interaction chromatography to purify the pectinase was reported by Zakhartsev & Momeu (2007) with percentage yield of 45.32. Similar findings were obtained by Zia *et al.* (2007) for purified pectinase with a single band of 35 KDa size.

Table 1. Summary of pectinase purification.

Sample	Total units	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude extract	200000	16500	12.12	100	1
$(NH_4)_2SO_4$ Precipitation	120750	2400	50.31	60.37	4.15
Ion Exchange Chromatography (Anion)	88,412	1176	75.18	44.20	6.20

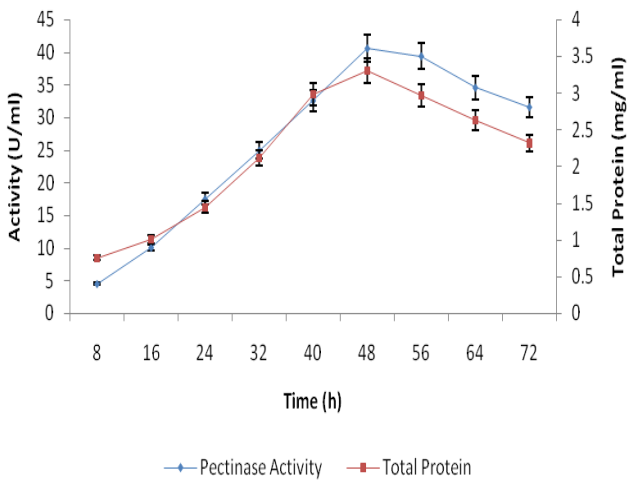

Fig. 1. Production of pectinase using mutant strain of *Aspergillus niger* in a stirred fermenter
Y error bars indicates the standard deviation (=SD) among the three replicates which differ significantly at $p<0.05$

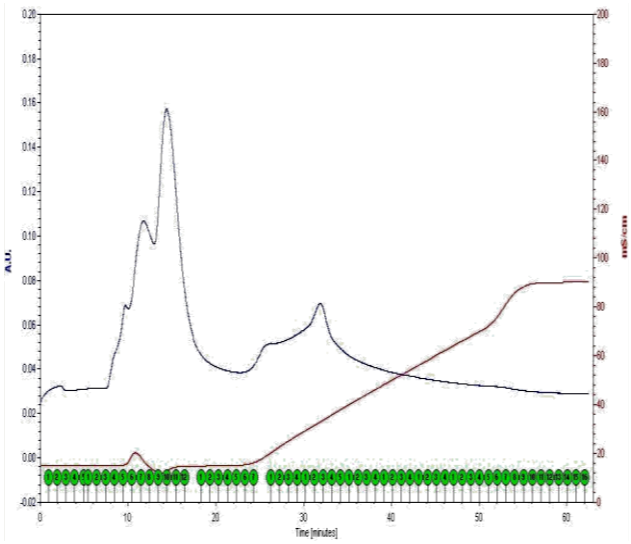

Fig. 2. Purification of pectinase using LPC using Bio-Rad Unosphere ™Q (Bioscale™ mini) column.

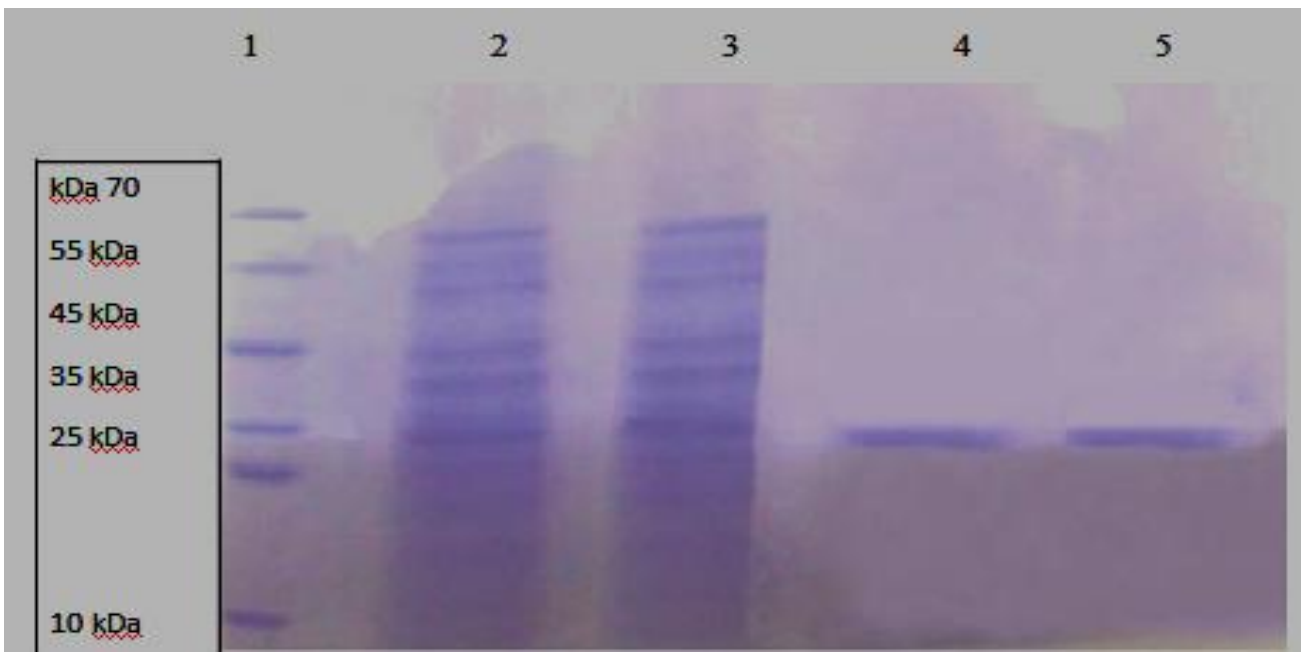

Fig. 3. SDS-Page analysis of purified Pectinase. Lane1: Protein marker (Fermentas SN 0431). Lane 2 and Lane 3: Crude Pectinase. Lane 4 and 5: Purified pectinase (35 KDa).

Kinetic analysis of pectinase: Kinetic parameters i.e., Km and Vmax were evaluated by Lineweaver-Burk double reciprocal plot (Fig. 4). Different concentrations of pectin as substrate (10-80mg/ml) were used. The V_{max} for the enzyme was calculated as 76.98 ± 0.02 U/mg. The K_m for the enzyme for pectin degradation was determined as 2.30 mg/ml. If km value for a particular substrate is low then it is best substrate for the enzyme (Ferri *et al*., 2011). In present study low value of Km suggests the specificity of pectin as a substrate for Pectinase. Zia *et al.* (2007) observed Km values of 10 mg/ml and 20.5 mg/ml, respectively for pectin as a substrate.

hermodynamic analysis: Pectinase activity was analyzed at different temperatures *i.e.,* 15-35°C to find out optimum temperature for activity. Starting from 15°C, enzyme activity increase steadily and reached to maximum (75.18 ±0.12 U/mg) at 25°C as shown in figure 5. The reduced enzyme activity was noticed at 35°C (51.02 ±0.22 U/mg). Arrhenius plot (Fig. 6) revealed activation energy (Ea) and enthalpy of activation (ΔH) as -28.95 KJ/mol and -26.73 KJ/mol respectively. The low values of thermodynamic parameters show that the stable formation of bond has been taken place between the enzyme and the substrate. Enzymes having lower value of the enthalpy form effective activate complex with the substrate (Akolkar and Desai, 2010). Comparable results to the finding of present study were also obtained by Odebunmi & Owalude (2007) with positive value of Ea (26.3 KJ/mol) and negative value of ΔS as -1.48 KJ/mol.

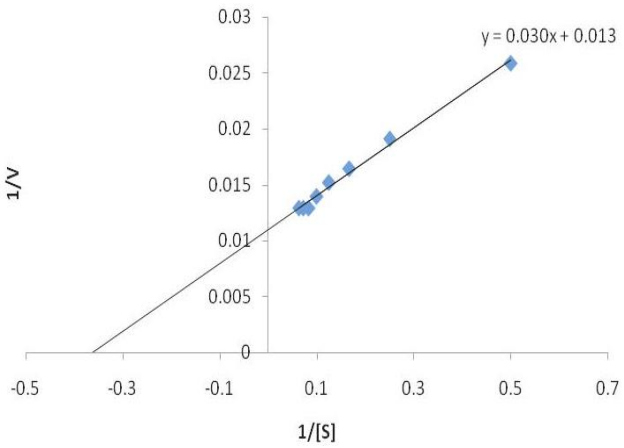

Fig. 4. Line weaver-burke double reciprocal plot to calculate the K_m and V_{max} for Pectinase.

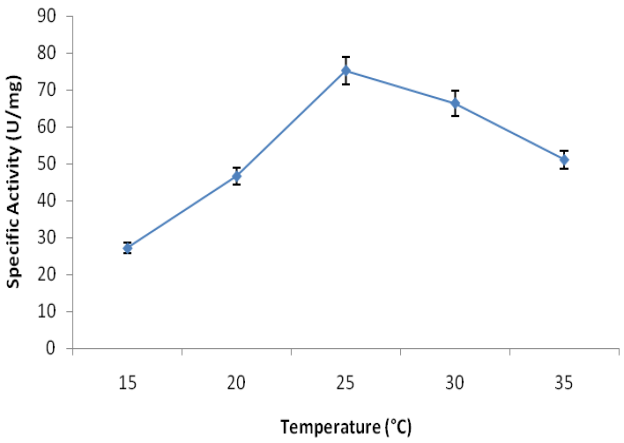

Fig. 5. Effect of temperature on Pectinase activity.

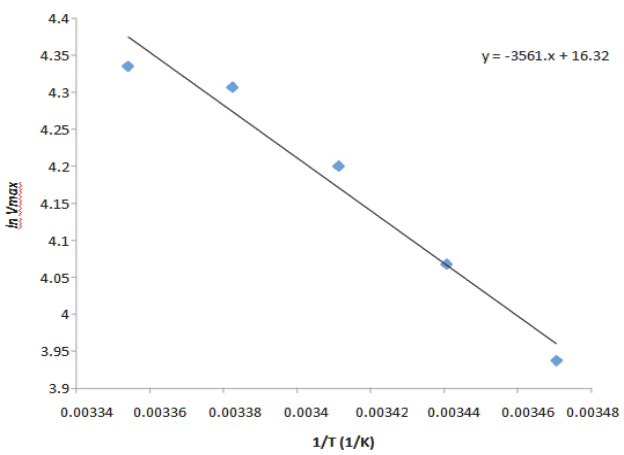

Fig. 6. Arrhenius plot to calculate the activation energy and enthalpy of Activation for pectianse catalyzed enzyme reaction.

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