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Production, Purification and Characterization of Pectinases by *Acremonium sporosulcatum*

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ABSTRACT

The object of the research work was to study Extracellular Pectinases production from *Acremonium sporosulcatum.* The enzyme characterizations, production, purification by ion exchange chromatography and gel filtration were studied. The molecular weight was determined to be 52Kd. The influence of carbon source 1% pectin (w/v) concentration was noticed for maximum productivity 10.23 units/ml and nitrogen source ammonium sulfate was best sources for the yield 9.30 units/ml respectively organic nitrogen peptone was found for the best enzyme synthesis. The influence of metal ions like Ca $^{2+}$and Ni^{2+} were inhibited production, various metal ions Zn^{2+}, Fe^{2+}, Mg^{2+}, and Cu^{2+}, Mn^{2+} were mild inhibitors, whereas K^{2+} 1% concentration increased the synthesis of enzyme activity slightly. The homogeneity of enzyme purification by via ammonium sulfate precipitation, dialysis (10kb), DEAE Sephadex chromatography, Sephadex G 150. The purification was approximately 2.72 fold with 52.90% yield. Optimum pH and temperature for the purified pectinase were found to be 5.0 and 30^{o}C respectively. The enzyme thermo stability gradually decreased 30 ^{o}C to 60 ^{o}C. the fungus appears to be commercial interest in the sense, production of pectinase reasonably good quantities.

Key words: *Acremonium sporosulcatum*, microelements, pectinase, purification factors

INTRODUCTION

Pectinases are the group of enzymes, which hydrolyze pectin. They break the glycosidic bonds of the long chain galacturonic acid residue (linked by 1-4 linkage) of pectin substances (Jayani et al., 2005). These enzymes are classified according to the criteria whether pectin, pectic acid or oligo-D-galacturonate is the preferred substrate,

whether pectinase act by trans-elimination or hydrolysis, or whether the cleavage is random (Pedrolli et al., 2009). They are pectin esterases, depolymerizing enzymes and protopectinases. The enzyme pectinase depolymerising pectin can be divided into hydrolases and lyases (cause depolimerisation). Which results in the formation of unsaturated bond between C4 and C5 at the nonreducing end (Sakai et al., 1993). Pectinolytic enzymes are extremely important in the current biotechnological era with their applications in fruit juice extraction and its clarification, scouring of cotton, degumming of plant fibers, waste water treatment, vegetable oil extraction, tea and coffee fermentations, bleaching of paper, as poultry feed additives and in alcoholic beverages and food industries (Ranveer et al., 2005). They have a share of 25% in the global sales of food enzymes (Ranveer et al., 2005; Rasheedha Banu et al., 2010; zuorro et al., 2011). Degradation of pectin-protein complexes gives viscosity that allow clean filter (Alvarez, 1998; 2000). In literature, information on pectinolytic enzyme on different supports by various methods was available (Vaillant et al., 2000; Rao et al., 2000; Sarioglu et al., 2001; Demirel et al., 2004; Sardar and Gupta, 2005). Among the various commercial pectinolytic enzymes, preparations obtained from the cultivation of *Aspergillus niger* are most popular (Godfrey and West, 1996). Since pectinases are widely used enzymes for different industrial applications, it is necessary to use inexpensive and readily available raw materials for its production. Carbon sources especially of agrarian source are more suitable because they are cost effective, renewable and available in larger quantities.

Extracellular pectinase producing fungi

Pectinase are generally produced by submerged fermentation process employed for different microorganisms (Table 1). Pectinase is incorporated as sole carbon source for pectinase production. Addition of sucrose in Submerged fermentation acted as a catabolite repressor for Polygalacturonas activity (Favela-Torres, 2006). Studies on medium composition and culture conditions using various experimental designs have demonstrated that pectinase production has been considerably improved.

MATERIAL AND METHODS

Microorganism and culture maintenance

Initially in order to assess its ability of the fungus to synthesis pectinase, the fungal strain of *Acremonium sporosulcatum* IMI 393096, Kew, UK and NCIM 1319, Pune, India, was used in this study. Culture was maintained by using was grown in Czapeck agar medium supplement with 1% pectin for 5days at 30°C. a zone of clearance around the colony indicated its ability to produce the enzyme. it was stored at 4°C. These sub cultured fortnightly. Therefore in order to optimize various parameters to maximum the enzyme synthesis fermentation was performed in 2 stages seed stage and fermentation stage.

Preparation of seed culture

A well grown heavily sporulating culture on potato dextrose agar slant was aseptically scraped off after adding 5 ml sterile distilled water. The whole suspension was added

to 25 ml of sterile water present 100 ml Erlenmeyer flask, 5 ml of this suspension was aseptically transferred to the seed medium of the following composition. The culture was grown on a reciprocating shaker with 250 rpm as 2" through maintained at 30°C for 72 h. At the end of which 1.0 ml of the seed culture was aseptically removed to monitor sterility, pH and microscopic characters of the growth.

Production medium

The production medium composition, sucrose 30.0gms, Yeast extract 5.0, $KHPO_43H_2O$ 1.0,$NaNO_3$ 30.0, KCl 5.0, $MgSO_4.7H_2O$ 5.0, $FeSO_4.7H_2O$ 0.1 were dissolved in 1liter of Distilled water. The submerged cultivation was carried out in 500 ml Erlenmeyer flasks containing 100 ml of the sterile fermentation medium. To 2.0 ml of the seed was used to inoculate each flask. Culture was incubated in a rotary shaker with 250 rpm maintained at 30°C for 6 days. The samples were drawn aseptically at every 24 h for analyzing the extracellular enzyme activity. Results were expressed as galacturonic acid equivalents. One Unit (U) of exopectinolytic activity was defined as the amount of enzyme that catalyzes the formation of 1 µmol galacturonic acid under the assay conditions mentioned above (Minijares Carranco et al., 1997)

Purification of pectinase

The pectinase was purified from 100 ml culture broth at optimum growth conditions; initially cell free supernatant was saturated with $(NH_4)_2SO_4$ to cut off 70% saturation. The precipitates were dissolved in the minimum amount of Tris-HCl buffer (0.01M, pH 7.5) and dialyzed against the same buffer.

Ion exchange chromatography

Anion exchanger, DEAE-Sephacel, was packed into a glass column (15 x 0.55 cm, 10ml-bed volume). The column was equilibrated with Tris-HCl buffer (10 mM, pH 7.5) and a 5.0 ml sample was loaded on to it. The column was washed with Tris-HCl buffer containing 0.2, 0.4, 0.6, 0.8 and 1.0 M NaCl concentration. Fractions of 1.50 ml volume were collected. The protein content of the fractions was measured spectrophotometrically at 280 nm and the pectinase activity was assayed by the method described earlier. The fractions showing pectinase activity were pooled, concentrated and saved for further analysis

Gel filtration chromatography

The glass column was packed with Sephadex G 150 (35 X 1.5 cm, bed volume 30 ml. the concentrated sample was loaded on to this column and elution of the proteins was done using Tris-HCl buffer (0.01 mM, pH 7.5). After the void volume (20 ml), fractions of 1.5 ml volume were collected. The absorbance of the samples for analyzing the protein content and pectinase activity was performed.

Gel electrophoresis.

The purity was checked by SDS-PAGE. Polyacrylamide gels were prepared by the method of Laemmli (Laemmli, 1970).

RESULTS

Fermentative production of Pectinases

The physiochemical and nutritional factors that influence the production of pectinase and the optimum conditions required for the maximum synthesis of pectinase have been investigated.

Effect of temperature

The effect of temperature on the production of pectinase was studied at 4 different temperatures i.e. 20°C, 30°C, 35°C, 37°C. It was observed that the maximum activity of pectinase enzyme was observed at 30°C. Further increase in the temperature decreased the enzyme activity. Higher temperatures can affect the production and the activity of the enzyme (Figure-1). Thus a maximum of 9.30 units/ml for pectinase was obtained with *Acremonium sporosulcatum.*

Effect of pH

The optimum pH value for maximum synthesis of pectinase enzyme was found to be 5.5. Decrease or increase in the pH adversely affected the synthesis of the enzyme (Figure-2). The increased high alkaline/acidic conditions will not be suitable for the synthesis of the enzyme by the mould. Therefore all further experiments were carried out at pH 5.5

Effect of carbon source

The addition of carbon source like glucose (monosaccharides) and sucrose (disaccharides) and starch (polysaccharides) with varied concentrations such as 0.025, 0.05 and 0.1% to the production medium and their effects were studied. The substrate was chosen as with 1% of pectin (w/v) concentration based on its maximum productivity 10.23 units/ml when compared to other sources of carbon. The operating variables such as time, temperature, pH and rpm were maintained at its optimal values 96 h, 30°C, 5.5 and 240 rpm, which were observed to be optimum values in the earlier observations (Figure-3).

The nature and concentration of carbon substance is known to influence the synthesis of pectinase. Carbon substances like glucose, sucrose, xylose, wood chip, starch and pectin were incorporated in the fermentation medium at different concentration like 0.025, 0.05, 0.5 and 1.0% (Figure-4). It was observed that in the presence of 1% pectin maximum productivity of the enzyme was observed. And the presence of xylose or wood chips, the enzyme activity was much lower than pectin but better than other carbon substances like sucrose. Glucose was observed to be least preferred as carbon source and the enzyme activities were found to be lowest.

Effect of nitrogen source

Various organic and inorganic nitrogen sources were investigated for their influence on the pectinase production. The organic nitrogen sources were soybean powder, peptone, yeast extract and beef extract while inorganic nitrogen sources include ammonium sulfate, ammonium nitrate, sodium nitrate, ammonium acetate and ammonium hydroxide (Figure-5). The different sources were incorporated in the

growth medium at 1% concentration and fermentation was carried out as described earlier. The results indicate that in the presence of ammonium sulfate broth growth and enzyme activity were found to be the best yield 9.30 units/ml at the end of 144 h of incubation. And ammonium nitrate and ammonium acetate were the next best source of inorganic nitrogen. In the presence of sodium nitrate the enzyme activity was observed to very poor. Therefore ammonium sulfate was selected as the best source of inorganic nitrogen (Figure- 8).

Soybean, peptone, yeast extract and beef extract were incorporated as 1% concentration in the growth medium as organic source of nitrogen and peptone was found the best organic source of nitrogen, yeast extract also gave more or less parallel enzyme activity. With beef extract the enzyme activity was found to be poor (Figure-6). Thus the study envisages that the presence of both organic nitrogen peptone was found essential for the best synthesis of the enzyme (Figure-7).

Effect of Trace elements on pectinase production

Trace elements are known to influence the synthesis of microbial metabolites. Therefore the influence of trace elements on the synthesis of pectinase was investigated by incorporating in the growth medium. Trace elements like Zn^{2+}, Fe^{2+}, Mg^{2+}, and Cu^{2+}, Mn^{2+} were mild inhibitors the synthesis of the enzyme in all the concentration except Potassium chloride, which at 1% concentration increased the synthesis of the enzyme activity slightly; (Figure-9).

Purification of pectinase

Pectin was found suitable as best for purification for protein and pectinase assay. The ammonium sulfate appears to interfere with the determination and forced to lose the samples with high sulfate concentrations. The supernatant after the precipitation with 35% ammonium sulfate and the supernatant with 70% sulfate. The acetone-washing step was necessary. The total protein decreases from 36 mg to 7 mg and the purification factor was increased. The evolution of the purification factor and yield along the purification steps are shown in the table 2.

pH stability of the pectinase

The pectinase activity was determined at 30°C in different pH ranges from 3.0 to 9.0. At pH 5.0 the maximum activity (9.30 units/ml) was observed (Figure-10). Above or below this range the enzyme activity was decreased.

Effect of temperature on the purified pectinase

The purified pectinase activity was determined at pH 5.0 in the different temperatures from 20°C to 60°C. The maximum activity was obtained at 30°C (10.23 units/ml). Above or below the 30°C the activity of the enzyme was observed to decrease gradually (Figure-11). The activity was completely lost in 1 h incubation at 55°C.

Influence of inorganic ions on purified pectinase

In the purification process the activity was observed that in the presence of ammonium sulfate the enzyme concentration has been increased to 30% to 90%. The

enzyme activity decreased gradually at high concentration. At 70% concentration, the enzyme activity has showed maximum 10.23 units/ml (Figure-12).

Discussion

Pectinases are regulated mainly by physicochemical and nutritional factors in *Acremonium sporosulcatum*; the nutritional parameters could be effectively monitored in the process for the maximum production of end product keeping physiochemical parameters as constant. The optimum temperature for the production of pectinase was 30°C. The maximum production of the enzyme is 9.30 units/ml (Figure-1). The pH range between 5.5-5.9 was found to be the best for the production and above or below this range the activity of the enzyme decreased (Figure-2). Among the carbon sources, Sucrose is best source of carbon for the maximum production of the enzyme activity (10.23 units/ml, Figure-3). The studies on the effect various nitrogen sources revealed that the inorganic ammonium sulfate and organic peptone were the best in supporting the enzyme production (Figure-5 and 6). Mineral salts could also influence the enzyme production. Potassium chloride improved the activity of pectinase (Figure-9).

The purification of pectinase using ammonium sulfate precipitation followed by dialysis had resulted in 42.2% purity. The purity could be enhanced to 52.90%, after Sephadex G-50 column chromatography (Table 2). The SDS-PAGE analysis showed that the partially purified enzyme had an apparent molecular weight of 52kDa (Plate). The thermostability and pH stability of the purified enzyme was at pH 5 and 30^{o}C. However, the comparison of the amounts of enzyme produced by *Acremonium sporosulcatum* with other species suggested that maximum production could be achieved using *Acremonium sporosulcatum.*

Table 1: Pectinase production by submerged fermentation

Microorganism	Substrate	Polygalacturonas activity IU/ml	Reference
Aspergillus joponicus	pectin and glucose	500.0	Teixeia et al., 2000
Candida utilis	Apple pomace	239.0	Villas Boas et al., 2002
Penicillium occitanis	Citrus pectin	221.0	Hadj Taieb et al., 2002
Aspergillus oryza	wheat bran & pectin	54.0	Malvessi & Silve, 2004
Penicillium dierckxii	sugar beet pectin	32.0	Shubakov et al., 2002
Aspergillus niger	sucrose	20.0	Friedrich etal., 1992
Aspergillus niger	pectin	14.5	Galiotoupanayotou et al., 1993
Penicillium frequentans	pectin	3.0	Kawano et al., 1999
Penicillium chrysogenum	Sucrose	13.7	Soumita et al., 2014
Penicillium chrysogenum	Glucose, galactose	27.21	Rasheedha banu et al., 2010
Pencillium Oxalicum	Beet pulp	1.39	Diana et al., 2012

Aspergillus niger	wheat bran & pectin	2.4	Taragano & Piloson , 1999
Aspergillus niger	corn meal	1.9	Panda et al., 2004
Polyporus squamosus	pectin	1.5	Antov &Pericin, 2001
Aspergillus niger	corn and glucose	1.3	Panda and Naidu,2000
Trichoderma reesi	sugar beet pulp	1.1	Olsson et al., 2003
Penicillium griseoroseum	pectin	0.7	Pereira et al., 2004
Mucor flavus	sugar beet & citrus pulp	0.4	Kaur et al.,2004
Fusarium moniliforme	citrus pectin	0.3	Niture and Pant, 2004
Aspergillus awamori	pectin	0.046	Blandino et al., 2001
Rhizopus stolonifer	pectin	0.04	Blandino et al., 2001
Acremonium sporosulcatum *	pectin*	9.30*	Present strain

Table 2: Purification steps of pectinase of *Acremonium sporosulcatum*

Steps	Volume (ml)	Total activity Units /ml	Total protein (mg)	Specific activity (U/mg)	Yield %	Purification factor or fold
Culture broth	100	966	36	26.83	100	1
Dialyzed	30	408	13	31.38	42.23	1.16
DEAE Sephadex Chromato--graphy	25	456.25	09	50.69	47.23	1.88
Sephadex G 150	20	511.06	07	73	52.90	2.72

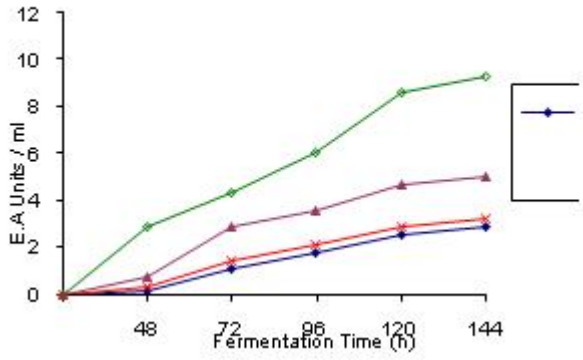


Figure 1: Effect of temperature on the synthesis of pectinase By *Acremonium sporosulcatum*

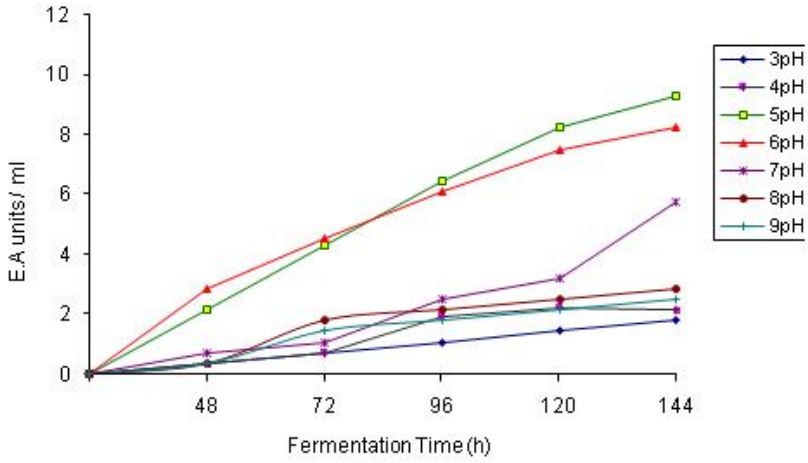


Figure 2: Effect of pH on the synthesis of pectinase by *Acremonium sporosulcatum*

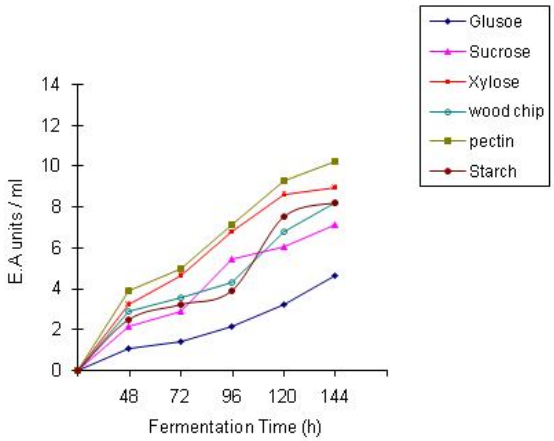


Figure 3: Effect of different carbon substances on the synthesis pectinase by *Acremonium sporosulcatum*

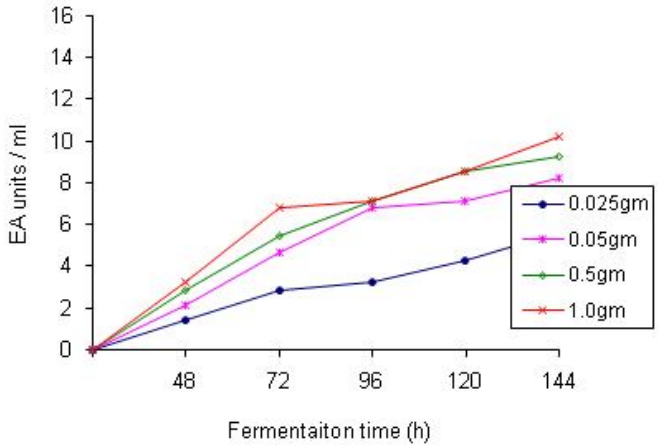


Figure 4: Effect of different concentrations of pectin on the synthesis Pectinase by *Acremonium sporosulcatum*

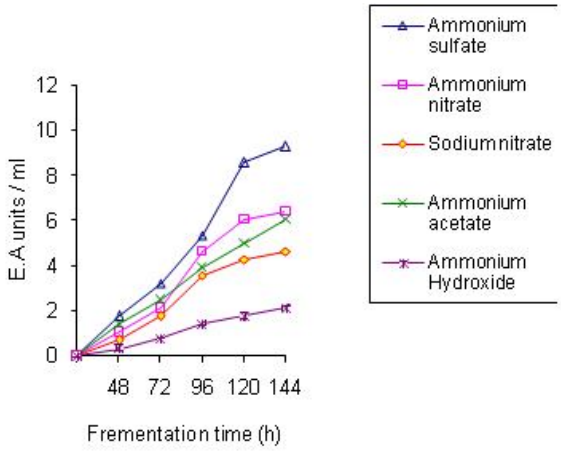


Figure 5: Effect of different inorganic compounds on the synthesis of pectinase by *Acremonium sporosulcatum*

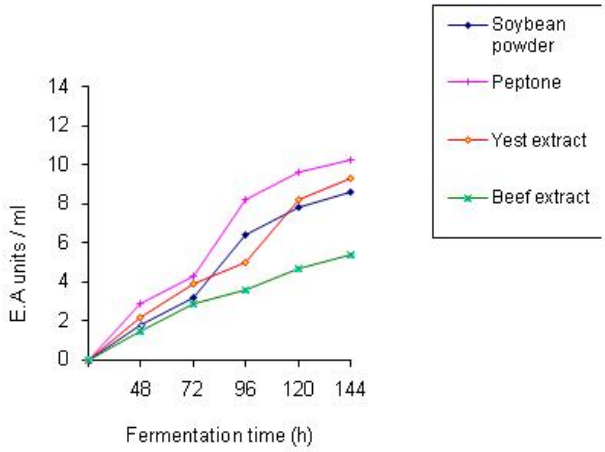


Figure 6: Effect of different organic sources of nitrogen on the synthesis of pectinase by *Acremonium sporosulcatum*

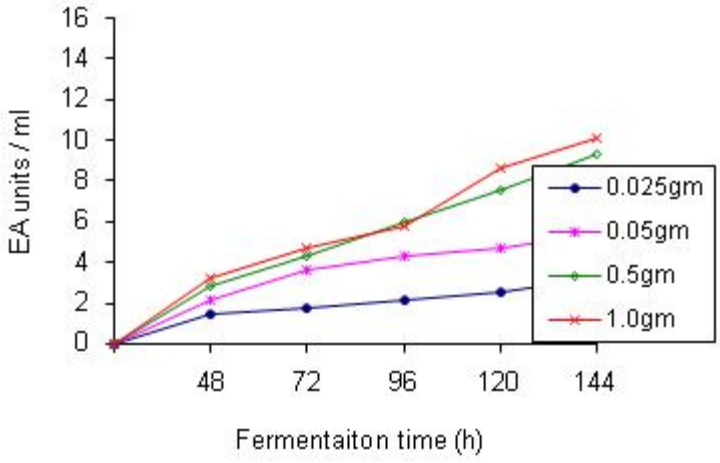


Figure 7: Effect of different concentrations of peptone on the synthesis pectinase by *Acremonium sporosulcatum*

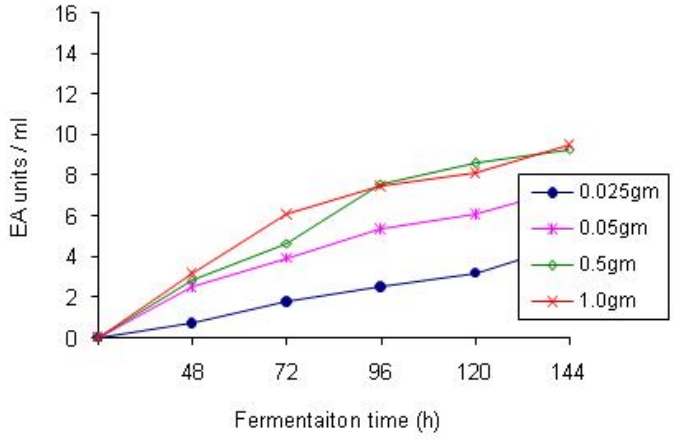


Figure 8: Effect of different concentrations of ammonium sulfate on the synthesis pectinase by *Acremonium sporosulcatum*

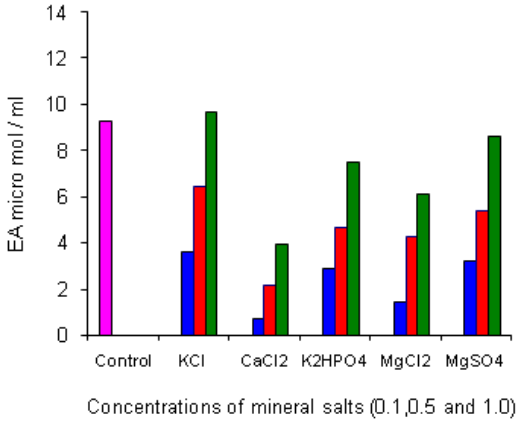


Figure 9: Effect of mineral salts on the synthesis of pectinase by Acremonium sporosulcatum

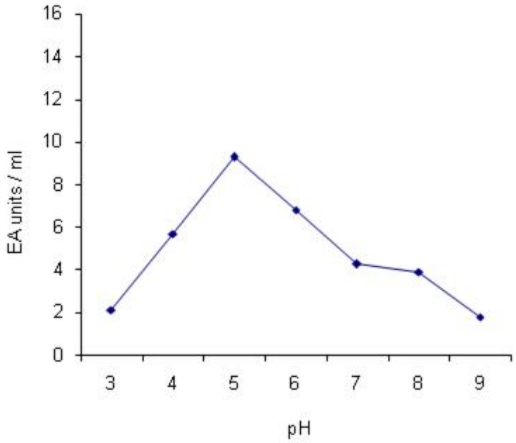


Figure 10: Effect of pH on purified enzyme pectinase

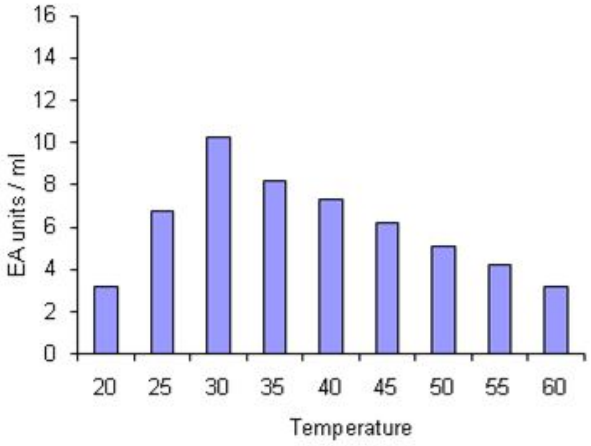


Figure 11: Effect of temperature on the purified pectinase

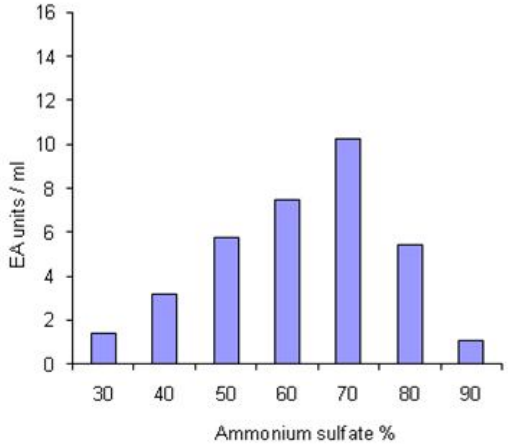
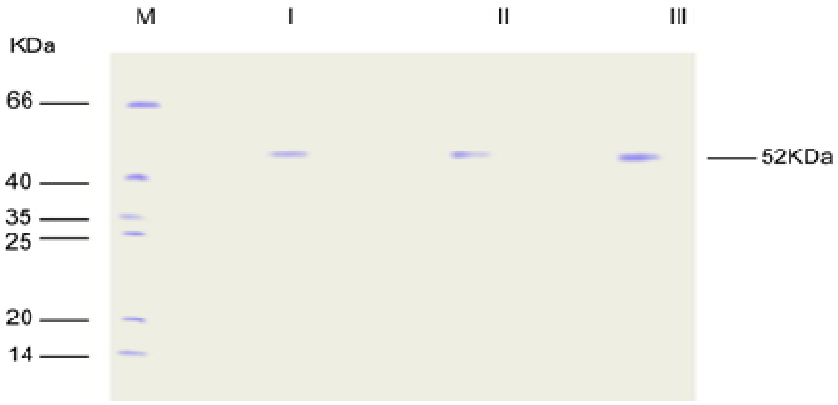


Figure 12: Effect of ammonium sulfate solution on purified pectinase

Plate . SDS - PAGE of samples taken in the purified pectinase
Lane - 1 Crude extract
Lane - 2 Ammonium sulfate precipitation
Lane - 3 After Dialysis



M - Molecular weight marker
I - 30µl sample
II - 30µl sample
III - 30µl sample
Samples were loaded in 1Mm gel

Plate:

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