Gezira j. of eng & applied sci 8 (2): 18 – 35 (2013)

Production and characterization of Pectic enzymes from three fungi

Awad M. Abdel-Rahim[2] and Amina A. Elmustafa[1]

[1]Center of Biosci. And Biotech. Fac. of Eng. & technology

[2] Department of chemistry and Biology, Fac. of Educ. Unv. of Gezira.

ABSTRACT

Pectins are compounds that are resistant to enzymatic cleavage in most organisms and plants, Studies were therefore made towards finding methods for hydrolyzing these compounds. One such approach is enzymatic hydrolysis. Three fungi (Trichoderma viride, Asperigillus niger and penicillum digitatum) are used for production of pectinase enzymes, Two methods (reducing sugar and viscometary) were used for measuring enzymes activities. Pectin and sodium polypectate compounds were used as substrates. The production of pectinases by the three fungi was investigated in culture media. T. viride and P. digitatum gave maximum production of pectinase enzymes after two week and A. niger gave it after one week. Purification by ammonium sulphate precipitation, showed that the maximum pectinase activity was at 80% concentration for both fungi A niger and P. digitatum. By using gel electrophoresis, five bands were found to give pectinase enzymes activities. The present study showed the importance of fungi as sources of enzymes and recommends that more studies must be done in the field of biotechnology to produce glucose from natural products by using fungi.

Key words: Pectic enzymes fungi

Introduction

Braverman (1974) defined enzymes as organic, colloidal Catalysts mostly soluble in water. Their properties are determined by their structure. All known enzymes are proteins. They are high molecular weight compounds made up principally of chains of amino acids linked together by peptide bonds that act as catalysts, speeding up chemical reactions that would take far too long to occur on their own, The protein nature of the enzymes ensures that the catalytic function highly specific, the protein surface providing specific areas for the binding of a single substrate or a small group of similar substrates. Fungi are the main micro - organisms reported to be enzyme producers, including species of the genera. Trirchoderma, Penicillium and Aspergjllus (Coral et al, 2001) . Fungal pectinases are among the most important industrial enzymes and are of great significance with a wide range of applications in several conventional industrial processes, such as textile processing, fruit juice, extraction, degumming of plant fibers, coffee and tea fermentation, paper making and pulp industry, animal feed, treatment of pectic waste waters, purification of plant viruses and oil extraction (Birch, et al, 1981; Bateman and Miller. 1976). They are yet to be commercialized (Kareem and Adebowale, 2002). The pectinase enzyme system consists of a group of enzymes which act together to degrade the pectin molecule (Mirjana et al, 2004). There are three main groups of the pectic Enzymes ; the polyglacturonase (PG) which catalyze the Hydrolytic cleavage of the polygalacturonic acid chain with the

introduction of water across the oxygen bridge (by hydrolysis), the Iyases or polygalacturonate transeleminases (PTE) Which perform non – hydrolytic breakdown of pectates or pectinates and the Pectinesterases, referred to as pectinmethylesterases (PME) which catalyze the desertification of methyl ester linkages of the backbone of the pectic substances to release acidic pectins and methanol (Voragen, 1996; Ranveer et al, 2005 and Castalo et al., 1989), The mode of action of PME varies according to its origin; fungal PMEs act by a multi – chain mechanism, removing the methyl groups at random. In contrast, plant PMEs tend to act either al the non reducing end or next to a free carboxyl group, and proceed along the molecule by a single chain mechanism (Forester, 1988). The increase in the rate of specific enzyme synthesis from basal to maximum levels is caused – by the presence of a substrate analog that act as an inducer. The inducer may be a substrate that inactivates a repressor or a chemical in the cell (Gupta and Ayyachamy, 2012).

SIATERIALS AND METHODS

Trichoderma viride, was isolated from wood, Asperigillus niger, was isolated from onion and Penicillum digitatum was obtained from sudanese orange fruits. The three isolates were cultured and sub cultured on Potato Dextrose Agar (PDA), as was described by Toole (1995), then purified and identified in the Center of Biosciences and Biotechnology, Faculty of Engineering and Technology, University of Gezira.

Medium for enzyme production:

The isolates were grown in a liquid medium containing the following ingredients (g/L) 1.4 g (NH4)2 SO4 : 0.3 g urea 2.0 g

KH2PO4: 0.3 g MgSO4. 7H2O and 0.3 g CaC12. Trace elements were added as FeSO4, 1.0 ppm; ZnC12, 0.8 ppm. MnSO4, 0. 5 ppm: CoC12. 5H2O, 0.5ppm. and CuSO4, 5H2O, 0.5 ppm. Yeast extracts was also added at 0.l g/ liter and 10 g of the carbon substrate source were added per liter distilled water, The final PH of the liquid medium was adjusted to 5.3 with KOH. Each flask of the liquid salt medium was inoculated, using sterile cork borer, with 5 disk (5mm in diameter) of agar on which the fungal mycelia were previously grown.

Measurement of the enzyme activity:

After incubation for the required time, the culture filtrates were centrifuged at 16,000 r.p.m for 15 minutes. The sterile filtrates were then aseptically collected in sterile McCartney bottles and stored in 10 ml portions at 200C. The reaction mixture contained: 8.0 ml w/v substrate in 0.1 M buffer, 1.0 ml 0.01 $CaCl_2$ (0.001 M) and 1.0 ml enzyme. Both autoclaved enzyme and zero time reaction mixtures containing the active enzyme were employed as controls. Pectinase activity was measured in the crude pectinase extract with pectin, using the following methods:

Viscometric method:

Pectinase was assayed by the viscosity reducing method. Using U – tube viscometers (BS \ U, size G') containing 5 ml enzyme solution and 5 ml of a substrate (1%) in citrate buffer (0.1 ml) at. 300C. The reaction mixtures were as described above. The flow time through the tube was recorded using a stopwatch after 4 minutes period, up to 24 minute from the start of the reaction.. The efflux time of substrates alone, without enzyme (zero time), and the efflux time for substrate - plus - enzyme

treatment were measured. Enzyme activity is expressed as 100/t where t is the time\ minutes.

Thiobarbituric acid (TBA) test:

The method of Ayers et al, (1966) was used to characterize the pectic enzyme. The reaction mixtures were as described for reducing group with polygalacturonic acid or pectin as substrate. Following incubation of mixtures at 300C for I hour, enzyme action was stopped. The enzyme protein and excess substrate were precipitated by adding 0.6 ml of 9% ZnSO4 , 9H20 and 0.6 ml of 0.5N NaOH, successively. The mixture was shaken and centrifuged at 6,000 r.p,,m for 15 minutes. Five ml of the clarified reaction mixture were added to tubes containing 3 ml of 0.04M TBA, 1.5 ml of IN HCI, and 0.5 ml of distilled water. The tubes ware placed in a boiling water bath for 30 min and cooled, and then the red colour produced was determined by a spectrophotometer at 515 nm to detect polygalacturonase activity and at 550 nm for the transeleminases activity. The polygalacturonate transeleminase activity in the clarified reaction mixtures was also confirmed by the detection of increased absorbance at 230 nm.

Release of reducing groups;

Reducing groups of the reaction mixtures were estimated by the Nelson- Somogi method (Nelson, 1944). Copper reagents were mixed on the day of use 25 parts A to I part B, the test sample (0.2 ml) was add to 0.8 ml water and 1.0 ml of the mixed copper reagent and heated for 30 min on a boiling water bath.. After cooling 1.0 fill of' arsennomolybdale reagent was

added, the blue colour produced was measured at 660 nm against the control treatments.

Cup-plate assay:

This method was used to test for pectinmethylesterase (PME) and polygalacturonate trans - eliminase (PTE) activities (Abdel-Rahim, 1981)). For pectinmethylesterase [PME), washed pectin (2.5 g) was added to 245 ml water and the PH was adjusted to 6.0. then methyl red solution (5 ml), salicylanilide (0.025 g) and agar (5 g) were added. The mixture was heated to dissolve the pectin and the agar and autoclaved at 121 OC for .15 minutes, 25 ml aliquots were then pipette into 9.0 cm diameter plastic Petri dishes and when set, 7 mm diameter wells were cut into the medium and the bottoms of the wells were sealed with a film of agar to prevent leakage, 0.1 m culture filtrates were pipette into the wells, The plates were incubated at 250C and observed at 24 hours intervals. PME activity was detected as a red zone surrounding the wells,

For polygalacturonate trans - eliminase (P T E). 2.5 g of sodium Polypectate (NaPP) was dissolved in 125 ml water and the pH was adjusted to 9. An equal volume or 0.2M tris – HCI buffer (pH 9) was added to give a solution of 1% NoPP in 0. I M buffer at PH 9. Then 2.5 g agar (1 96 final cone.) was added and the mixture was autoclaved 15 minutes at 121^0C, 25 ml aliquots were pipette into 9.0 plastic petri - dishes and when set, 7 mm diam. wells were cut in the agar with a No, 4 cork borer, 0104 ml CaC12 (0 002M) and 0.04 ml of the enzyme sample were pipetted into each well. The plates were incubated at 28^0C. At intervals of 24 hours the plates were flooded with 5N HCI. White haloes developing around the wells after this treatment indicated PTE activity which was expressed as orcas of the while zone.

Effect of different substrates on enzyme production:

pectin, sodium polypectate (Napp), orange rind and dextrose were used individually as substrates for enzyme production, The substrates were added to the liquid medium at 0.1%. The filtrate of each culture was assayed for enzyme activity.

Effect of incubation time on enzyme production:

Flasks Of the liquid salt medium inoculated with the three fungi were incubated at different incubation times ranging from one week to four weeks and samples were drawn weekly, The filtrates of each culture were tested for the enzyme activity.

Partial purification by ammonium sulphate:

The filtrates of the three fungi (A. niger, P, digitatum and T. viride) were fractionally precipitated with ammonium sulphate saturation, then the reaction mixtures were assayed for enzyme activity by the reducing sugar method.

Determination of the molecular weight of the pectic enzymes

The molecular weight of the pectic enzymes produced by the fungus digitatum was determined by gel electrophoresis as described by Almustafa and Abdel-Rahim (2013).

RESULTS

Production of pectinase enzymes on different substrates:

The three fungi (A niger, P. digitatum and T, viride) were grown individually on a salt medium to which pectin, sodium polypectate (Napp), orange rind and dextrose were added to induce pectic enzymes. The cultures were prepared in 250 ml

conical flasks and were incubated at 30^0C for 7 days. The filtrate of each culture was used as a pectic enzyme source. Pectin was used as a substrate for the polygalacturonate transeleminase (PTE) enzyme assay which was performed at pH 9 by using two methods (TBA test and the cup - plate method). Polygalacturonic acid was used as a substrate for the polygalacturonase **(PG)** at pH 5 also by using TBA test only, while the pectinmethylesterase (PME), was detected at pH 6 and pectin was used as a substrate by using the cup- plate method. The maximum yields of the (PTE) for the three fungi were found when pectin was used as an inducer substrate (Table, 1). The effect of the dextrose and orange rind as inducers resulted in the lowest enzyme induction, while the Napp gave an intermediate effect.

Table. (1). Production of PTE enzyme by the three fungi (mg/ml), cultured on different substances and at pH 9

Substances	T. viride	P. digitatum	A. niger
Pectin	94	91	105
Napp	92	29.3	85
Orange rind	50	86.8	78.5
Dextrose	3.3	8.3	25.0

The polygalacturonase enzyme (PG) activity of the culture filtrate of T. viride was higher than that of P. digitatum. While a very low activity was detected for the same enzyme by A. niger in comparison with the two other fungi studied (Table, 2). Concerning to the pectinmethylesterase enzyme (**PME**), no activity was detected for A. niger. Both T. viride and P. digitatum were able to produce the PME enzyme. However, T. viride gave more production of this enzyme compared with P. digitatum (Plate l).

Table (2). Production of PG enzyme by the three fungi (mg/ml), culturedon different substances and at pH 5

Substances	T. viride	P. digitatum	A. niger
Pectin	69.3	80.6	0.0
Napp	58.8	48.8	0.0
Orange rind	47	25	0.0
Dextrose	3.3	8.3	0.0

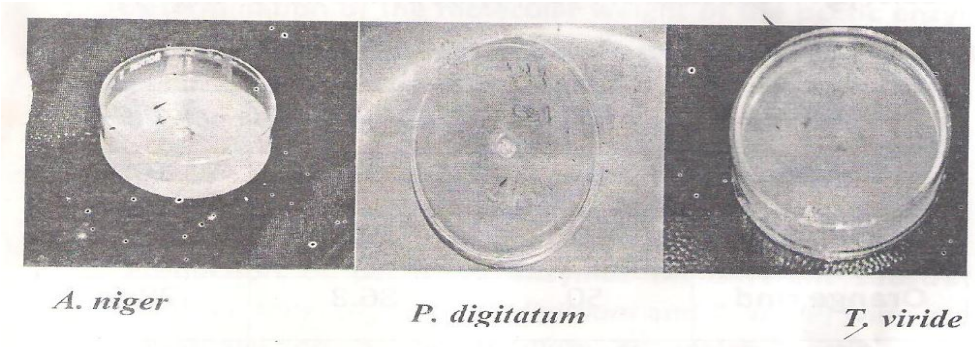
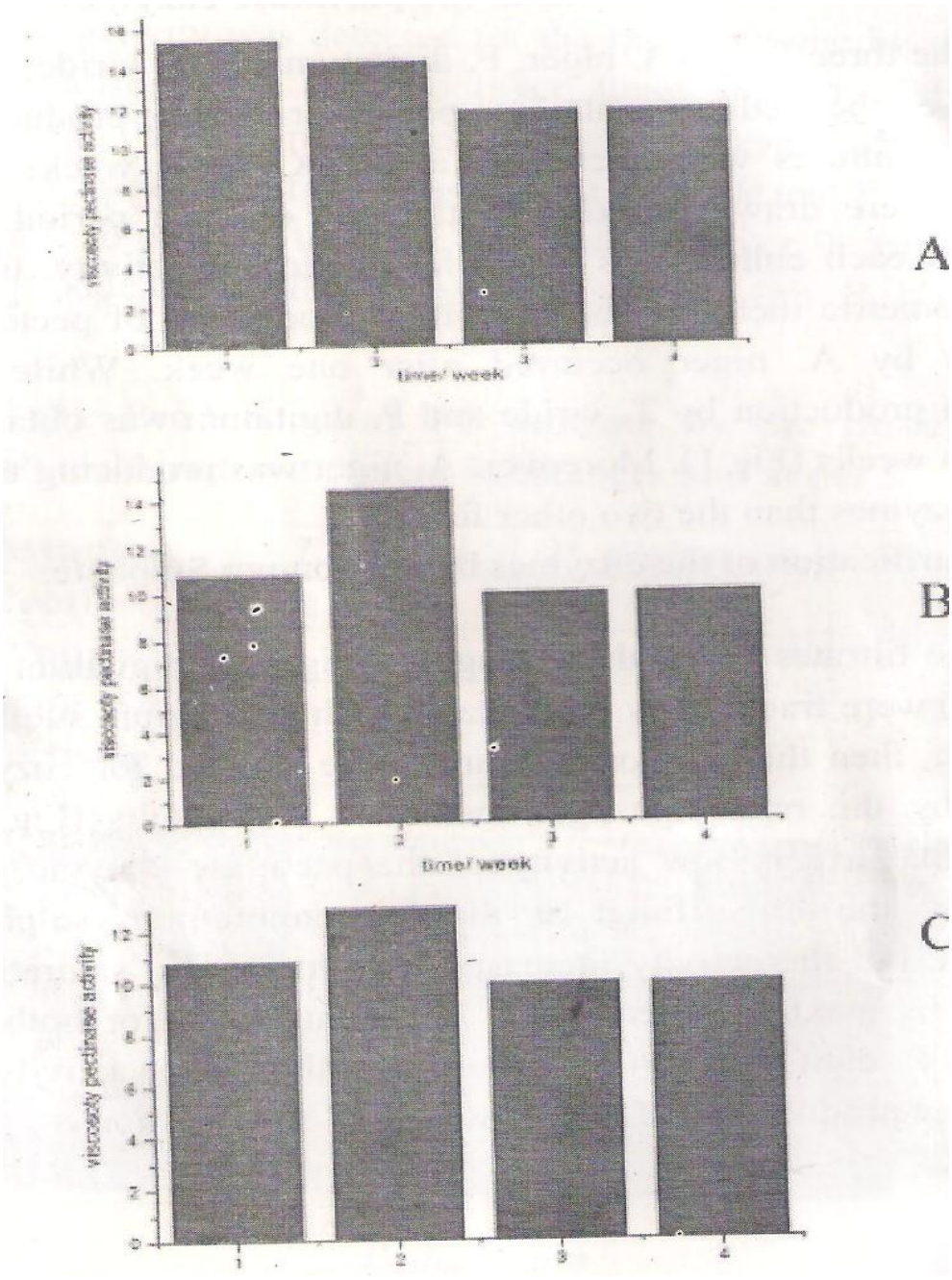


Plate (1) Activity of the pectinmethyksterase enzyme produced by the three fungi at pH 6, using the cup-plate method, after 72 hours,

The effect of incubation time on the pectinase enzymes:

The three fungi (A. niger P, digitatum and T. viride) were cultured on the medium containing pectin for enzyme production. Prepared cultures were incubated at 30 ^{0}C for 4 weeks, and sample were drawn weekly. At the end of each period, the filtrate of each culture was tested for pectinases activity, using the viscometric method. The maximum production or pectinase enzymes by A. niger occurred after one week, While the optimum production by T.viride and P- digitatum was obtained after two weeks (Fig.1). Moreover A. niger was producing more pectic purification of the enzymes by ammonium Sulphate:

The filtrates of the three fungi (A. niger, P. digitatum and T. viride) were fractionally precipitated with ammonium sulphate saturation, then the reaction mixtures were assayed for enzyme activity by the reducing sugar method. The results (Fig. 2) showed that a very low activity of' the pectinase enzyme was saturation, but the activity increased with increasing saturation, reaching its maximum activity at 100% saturation for both A. niger and P. digitatum. On the other hand, the higher activity of the enzyme produced by T, viride was at 60 % saturation.



time/ week

Fig (1). Effect of incubation time on pectinase enzymes activity (units) produced by the three fungi; (A) A. niger, (B) P. digitatum, (C) T.Viride

The molecular weight of the pectic enzymes:

The molecular weight of the pectic enzymes produced by the fungus P. digitatum was determined by gel electrophoresis. The results of the analyses of the enzyme showed that eleven bands have been detected on the gel. Some of them have high molecular Weights more than that known for the pectinases (above 110,000). But five of them have molecular weights in the range of the pectinase molecular weight. These bands were estimated to be about 97.500 for band (1) ; 80.000 for band (2) 55.800 for band (3) ; 35.000 for band (4) and 18.300 for band five.

DISCUSSION

To induce pectinase enzymes production, the three fungi (T. viride, A. niger and P. digitatum) were grown, individually on a salt medium to which either pectin, sodium polypectate, orange rind or dextrose were added. The maximum yields of pectinase enzymes (PG and PTE) were found when pectin was used as an inducer substrate. This result was in agreement with that presented by Maria et al (2000; 2006), who reported that the best values of pectinolytic enzymes were found in the presence of pectin as a substrate, Danial and Immanuel (2011) also found that T. viride isolate is a good source of polygalacturonase when pectin was used as a carbon source, However, Reddy and Sreeramulu (2012) reported that the.

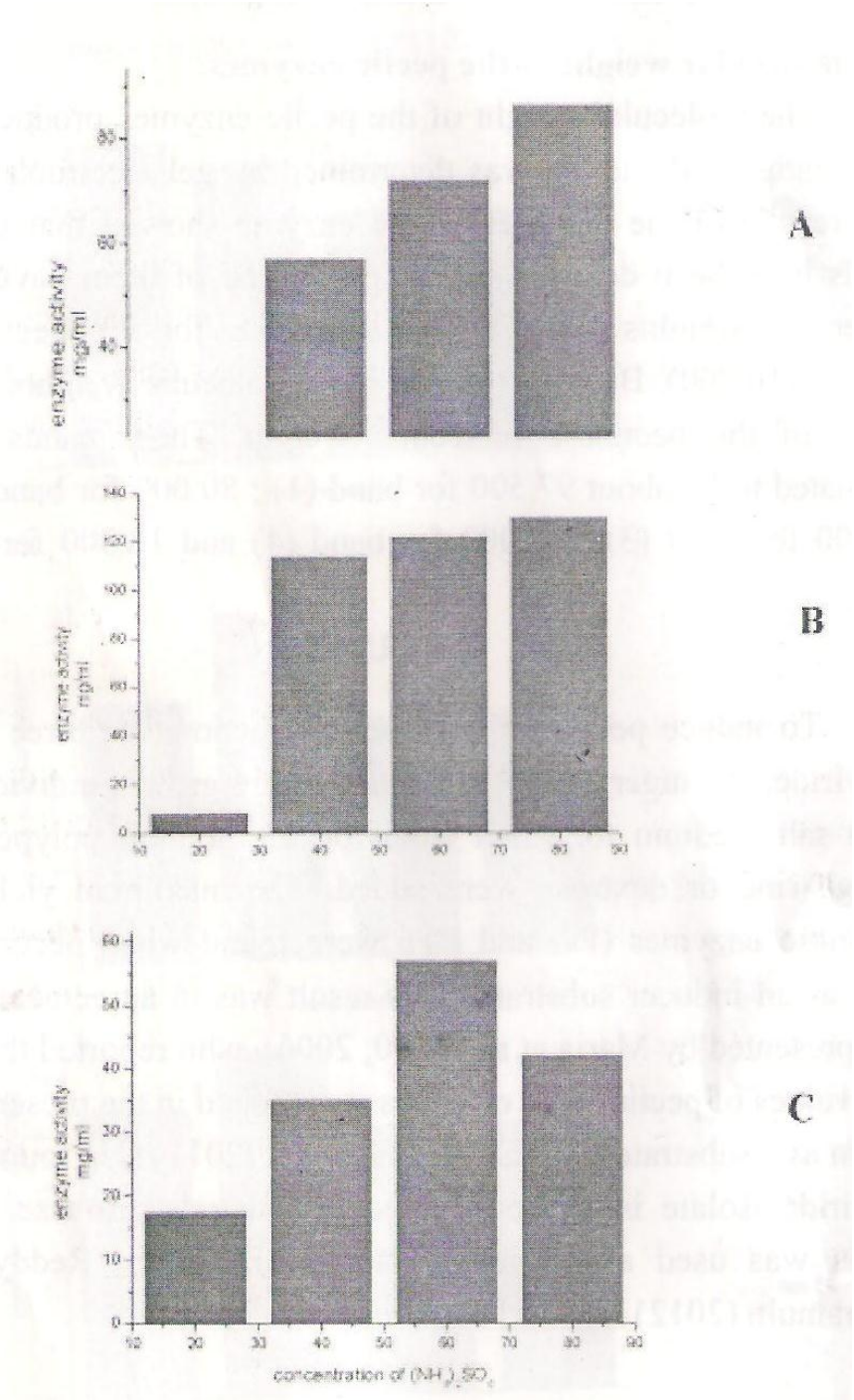


Fig. (2). Partial purification of the pectinase enzyme produced by the three fungi (A) A, niger. (B) P, digitatum, (C} viride using ammonium sulphate.

Maximum pectinase production by four fungi (A. niger, A. flavous, A. jabonicus and Chaetomum globosum) was obtained the medium containing citrus pectin, In this study the results pectinase enzyme, after one week. However, P. digitatum and T. viride showed maximum activity after two weeks. Desouky (2007) found that maximum enzyme productivity attained after 10 and 15 days incubation periods for pectinase enzymes production by Asperigillus ustus and Penicillum hordei, respectively

The filtrates of the three fungi (A.niger, P . digitatum and T. viride) were fractionally precipitated with ammonium sulphate. The results showed that a very low activity of the pectic enzyme was produced by the three fungi at the low ammonium sulphate saturation. but the activity increased with increasing saturation, reaching its maximum at 80% saturation for both A. niger and P. digitatum. On other hand, the higher activity of of 60% Kabli (2007) reported that the active fraction of crude pectinase obtained by kluyveromyces marxianus cultures was at 65% ammonium sulphate. Yogesh et ale (2009) also reported that the highest degree of precipitations was achieved by 65% concentration of ammonium sulphate for pectinase production by A. niger. But Joshi et al. (2011) reported that the pectinmethylesterase activity increased from 8.25 to 21.5 u/g (160% increase) as the ammonium sulphate.

concentration was raised from zero to 80%. While Sangeeta and Shastri (2005) reported that the stepwise precipitation of the pectinlyase enzyme of Penicillum oxalicum at 0-30, 30-60 and

60-90% ammonium sulphate saturation, gave highest purification at 30-60% saturation.

To determine the molecular weight of the pectinase enzyme, P. digitatum was grown in liquid — salt medium containing 1 % pectin as the sole carbon source, The culture was filtrated, and the crude enzyme preparation was subjected to SDS – PAGE. During the electrophoresis of the enzyme, four Bands showing pectinolytic activity were detected. The molecular weights of these bands were calculated to be 97.000 for band (l); 80.000 for band (2); 55.800 for band (3) and 35.000 for band (4). These values fall within the range of molecular weight reported in literature by Rasheedha et aL (2010) for pectinase of Penicillum chrysogenum. While, pectinases of A. japonics showed five bands with molecular weights 38.000; 65,000; 50.000; 46.000 and 47.000 (Semenova et al 2003). Vasanthi and meenakshisundarm (2012) also reported that the molecular weights of purified pectinolytic enzymes of A. niger were determined to be in the range of 35.000 - 60.000 by gel electrophoresis.

REFERANCES

Abdel- Rahim, A. M. (1980), Studies on Sesame Leaf Spot caused by Xanthomonas sesame (Sabet and Dowson), Ph.D. thesis, Department of Botany and Plant Technology, Imperial Collage of Science and Technology. London.

Almustafa, A. A. and Abdel- Rahim, A. M. (2013). Production and characterization of cellulolytic enzymes from three fungi. Gez. J. of Eng. and Appl Sci. 10 (1): 1 — 19.

Ayers, W. Papavizas, GA C, and Diem, A. F. (1966), Polygalacturonate Trans - Eleminase and Polygalacturonase Production b Rhizoctonia solani Journal of Phytopathology. 56: 925 930.

Bateman, D and Miller, R. L. (1966}, Pectic Enzymes in Tissue Degradation. Annual Review of Plant Pathology, 4: 119 124.

Birch, G.G, Blakebrough, N. and Parker, J. (1981). Enzyme and Food Processing. Applied science publisher LTD,, England. Pp 5-13.

Braverman, J. B. F. (1974) Introduction to Biochemistry of Food. 3rd. ed, , Elsevier publishing Company. London. Pp 31.

Castaldo, D.; qualiquuolo, L; Servillo, L; Balestrieri, C. and Giouane, A. (1989). Isolation and Characterization of Pectinmethylesterase from Apple Fruit. Journal of Food Science, 54(3): 653 – 673.

coral, G.; B. M. Nisa, O. and Hatice, G (2001). Some properties of Crude Carboxymethyl Cellulase of Asperigillus niger. Turkish Journol of Biology, 26: 209 213,

Danial, J. and Emmanuel, E. (2011) , Effect of Carbon and Nitrogen sources on Polygalacturanase Production by viride Isolate Tar Sand in Ondo State (Nigeria). 7 (3) : 153 – 158.

Desouky, E. M, (2007), Production of cellulase by Penicillum hordei and by Asperigillus ustus under solid state fermentation condition, Egyptian Journal of Microbiology, 17 : 6 - 64.

Forester, H. (1988). Pectinase from Phytophthora infestants. Journal of Enzymology. 161:307-355.

Gupta, V. K. and Ayyachamy, M. (2012), Biotechnology of Fungal Genesc Science publisher, England. Pp 53-71.

Joshi, V. K. Mukesh, P. and Neerja, R. (2011) Purification and Characterization of Pectinase produced from Apple Pomace and Evaluation of its Efficacy in Fruit Juice Extraction and Clarification. Indian Journal of Natural Products and Resources, 2(2): 189-197.

Kabli, S. A. (2007). Purification and Characterization of Protopectinase produced by Kluyveromyces marxianus, JKAU: Sci, vol. 19: 139 - 153.

Kareem, O. S. and Adebowalej A. A. (2002). Clarification of Orange Juice by Crude Fungi Pectinase from Citrus Peel. Nigerian Food Journal. 25 (1): 130 – 137.