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Research Article

Optimization of Production and Molecular Characterization of Pectinase Enzyme Produced From *Penicillium chrysogenum*

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Abstract: Four moulds isolated from garden soil samples of Andhrahalli area, Bangalore, Karnataka, India and were screened for pectinolytic enzyme production when grown on pectin containing (YPSS) solid media. *Penicillium chrysogenum* was selected based on clearance zones and pectinase enzyme production was carried out in shake flask (submerged) fermentation. Enzyme production by *Penicillium chrysogenum* was higher at pH 6.0 and a temperature of 30°C using sucrose and ammonium sulphate as carbon source and nitrogen source, respectively. The maximal activity of *P. chrysogenum* pectinase was at 60°C, pH 5.0 and was thermostable up to 70°C. Manganese sulphate had hige effect on pectinase enzyme activity. K_m and V_{max} values were 1.0 mg/mL and 78 U/mg protein, respectively and an apparent molecular weight of 32 kDa on SDS-PAGE.
Keywords: *Penicillium chrysogenum*; shakeflask fermentation, pectinase

INTRODUCTION

The utilization of microbial enzymes has found broad technological application in different industrial processes. Among the various enzymes commercialized many are products of fermentation of filamentous fungi [1]. The genus Penicillium is world wide known for production of secondary metabolites and extracellular enzymes of commercial value, including pectinases.

Pectinase are now an integral part of juice and textile industries [2], such as maceration of tea leaves [3]; processing of cotton fabric [4] as well as in various biotechnological applications [5-6]. The filamentous fungi are most often used in the commercial production of pectinases. Microbial production of pectinases has been extensively studied [2, 7]; actinomycetes [8]; Aspergillus flavus[9]; Aspergillus sp [3]; Penicilluim italicum [10]; Penicillium viridicatum RFC3 [11]; Penicillium roqueforti [12]; Penicillium expansum [13] and Pectolytic moulds [14].

New enzymes for use in commercial applications with desirable biochemical and physico-chemical characteristics and a low cost production have been the focus of much research[11,15-16]. Enzyme breakdown of the biomolecules depends up on the type of enzyme, application, temperature, incubation time, agitation, concentration, pH and use of different enzyme preparations[17, 18].

In this context, the objective of the present study was to produce pectinolytic enzymes by a newly isolated strain of Penicillium chrysogenum by submerged fermentation and process evaluation. Furthermore, the physico-chemical characteristics of the purified enzymes are also presented.

MATERIALS AND METHODS

Isolation of fungal species:

The 5g of soil sample was collected from Acharya Bangalore B-School girl's hostel garden. A quantity of 1.0 mg of soil from each of the collected samples was dissolved in 9.0 ml of sterile distilled water and serial dilution was plated on MRBA plate with antibiotics to restrict bacterial growth and incubated at 37^oc. After the growth of each colony, on the basis of its morphological characteristics was picked up and further purified by repeated streaking on YPSS (Yeast soluble starch agar) agar media. Each fungal culture was then stored in refrigerator for further experiment [10, 5].

Screening of soil isolates for pectinolytic activity:

A total of 4 isolates from soil were assayed for polygalacturonase (PG) activity using pectin–containing agar medium. Culture plates with pectin-containing agarose were inoculated with each isolate and incubated for 3-5 days at 27^0C. After incubation, plates were stained with aqueous 0.05% ruthenium red solution for 30 min and rinsed with distilled water. Culture expressing pectinase activity exhibited a clear zone around

the margines of the colony. Macroscopic study a final extension was done by studying growth rate, colour, texture and topography of colony using two standard media namely PDA and Czapek Dox. Microscopic study of *Penicillium* species was done by preparing slide mount with lacto phenol cotton blue stain and observed under light microscope [10,5].

Enzyme production by submerged fermentation:

The fungal culture was used to produce pectinase enzyme using liquid medium (g/L) containing citrus pectin – 10g/L ; $(NH_4)_2SO_4$ -1.4g/L ; K_2HPO_4 - 6g/L ; $MgSO_4.7H_2O$ – 0.1g/L ; pH - 6 . Fermentation was carried out in 500mL conical flask containing 250mL of growth medium with 10% inoculum and incubated at 30ºC under shaking conditions (175 rpm) for 4 days. The biomass was separated by centrifugation at 10,000 *g for 15min at 4^0C. The supernatant was used to evaluate the pectinase enzyme activity.

Culture conditions:

The culture conditions [pH between 6 to 8, temperature from 30ºC to 50ºC, different carbon sources like dextrose, mannitol, sucrose and starch and nitrogen sources like ammonium sulphate, ammonium nitrate, ammonium chloride & peptone] on selected pectinase enzyme production was studied.

Pectinase enzyme assay:

The polygalacturonase activity was determined by measuring the amount of reducing substances liberated from citrus pectin. The reaction mixture consisted of substrate buffer (0.125 g citrus pectin dissolved in 25 mL of 0.2 M sodium citrate buffer, pH 6.5) and enzyme solution (0.5 mL). This mixture was incubated at 37°C for 30 min., heated in a oiling water bath for 5 min and the reaction was stopped by using 3 mL of DNS reagent. The absorbance was read at 570 nm. One unit of enzyme activity (U) was defined as 1 µmol of galacturonic acid released per min[11].

Protein content :

Protein content was determined by the method of Lowry et al., [20], using Bovine Serum Albumin (BSA) as standard.

Enzyme purification:

Enzyme purification was carried out by the following steps:i.) The crude enzyme filtrate was dialysed and clear supernatant (200mL) was mixed with three volumes of n-butanol and kept undisturbed for overnight in the separating funnel. The crude enzyme filtrate was also separated with diethyl ether, isoamyl alcohol, chloroform and petroleum benzene and kept undisturbed for overnight in separating funnel. The resulting precipitates were collected by centrifugation at 9000 X g for 30 minutes at 4^0C. The precipitate was dissolved in 10mL of sodium citrate buffer (pH 6.5) and dialysed against distilled water. For further purification, the dialyzed sample was lyophilized to 5mL and passed through Sephadex G-100 column (1.5 X 45cm) equilibrated with sodium citrate buffer (pH 6.5) and eluted with the same buffer. About 20 fractions were collected at a rate of 10mL/h. The fraction of the enzyme that showed highest enzyme activity and protein content was then pooled and used for characterization [3].

Enzyme characterization:

The enzyme PG activity was determined at 35°C in different pH using phosphate buffer of pH 6-9. The optimum temperature was assayed by incubating each reaction mixtures at 20-80°C and its thermo stability. The reaction speed V_{max} and K_m were determined for the enzyme by varying the substrate concentration from 2-20 mg/mL and plotting substrate/velocity as function of substrate concentration [20]. For determination of the influence of different metal salts, zinc sulphate, magnesium sulphate, manganese sulphate and ferrous sulphate were studied. These salts were added to the reaction mixture (5 mM), and the enzyme activity was determined as described above. SDS-PAGE of protein samples was performed using 12% gel. After migration protein bands were stained with Coomassive Brilliant Blue (CBB) [21].

RESULTS AND DISCUSSION

Screening of isolates:

Four fungal species isolated from garden soil sample and morphological characteristics were examined. Fungal isolates were further screened by yeast soluble starch agar (YPSS) plate method and the zone of clearance was calculated. *Penicillium chrysogenum* culture had a zone of clearance above 3cm. On the basis of screening method, the isolate *Penicillium chrysogenum* found to be a potential source of pectinolytic enzyme. This culture was used for optimization of pectinase production using shake flask submerged fermentation process.

Fig -1: Pure culture of *Penicillium chrysogenum*

Fig-2. Penicillium chrysogenum showing zone of clearance maximum

Optimization of culture conditions:

The temperature and pH of the cultivation medium are important factors in the pectinase production, which may influence the sort and content of those enzymes produced by filamentous fungus. The strong effect of pH on the production of endo pectinase was clearly observed in flask cultures, where pH value 6-8, and temperature 30°C-50°C were tested for crude enzyme production (Fig 3,4,5) . The maximum pectinase activity was found with an initial pH of 6 at temperature 30°C, activity reaching 0.696 µg/ml/min on fifth day of incubation. When the temperature was maintained at 40°C and 50°C with pH 6, the enzyme activity was found to be 0.516 µg/ml/min and 0.468 µg/ml/min respectively on fifth day of incubation. By keeping the pH constant at pH 7, the enzyme activity was measured as 0.576 µg/ml/min at 30°C, 0.504 at 40°C and 0.396 µg/ml/min at 50°C whereas by keeping pH 8 as constant, it was seen that the enzyme activity was found to be 0.612 µg/ml/min at 30°C, 0.444 µg/ml/min at 40°C and 0.336 µg/ml/min at 50°C. So optimum pH for enzyme production was found to be 6 and optimum temperature was found to be 30°C. Either increase or decrease beyond the optimum value show decline in enzyme production. However, the mechanism by which the pH and temperature both acts on the same time for the production of pectic enzyme is not clearly known. Piccolo-valle et al. [1] observed that a high pectinase and pectin esterase activity was showed by *P.griseoroseum* in more acid pH of 4.5 and 5 and of Pectin Lyase, pH was closed to the neutral 5-7. *P. viridicatum* showed maximum production of polygalacturonase and Pectin Lyase at pH 4.5 and 5 respectively [11]. The temperature optima of 30°C, was obtained from a purified culture fluid of *P. frequentans* by Chellegatti [22]. This can give the support to our obtained result.From the observation it was clear that 30°C was the optimum temperature for the growth of micro organisms, variation was done in different pH and it was seen that pH 6 was optimum (Fig 3,4,5).

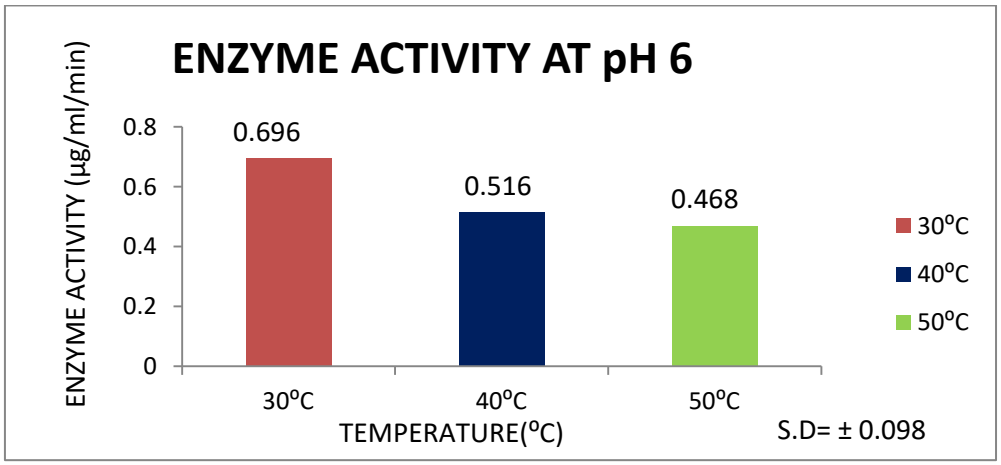

Fig-3: Enzyme Activity measured at different temperatures keeping pH 6

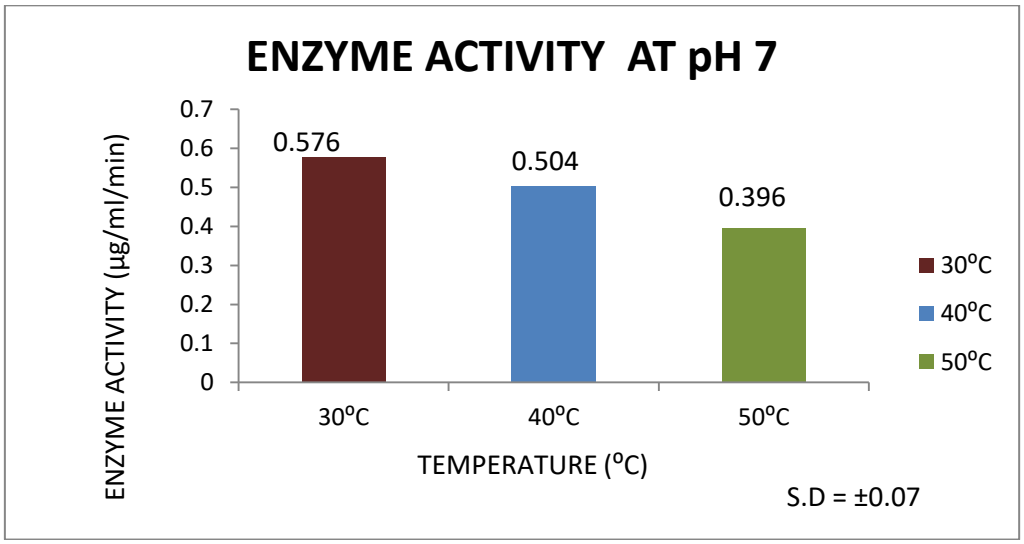

Fig-4: Enzyme Activity measured at different temperatures keeping pH 7

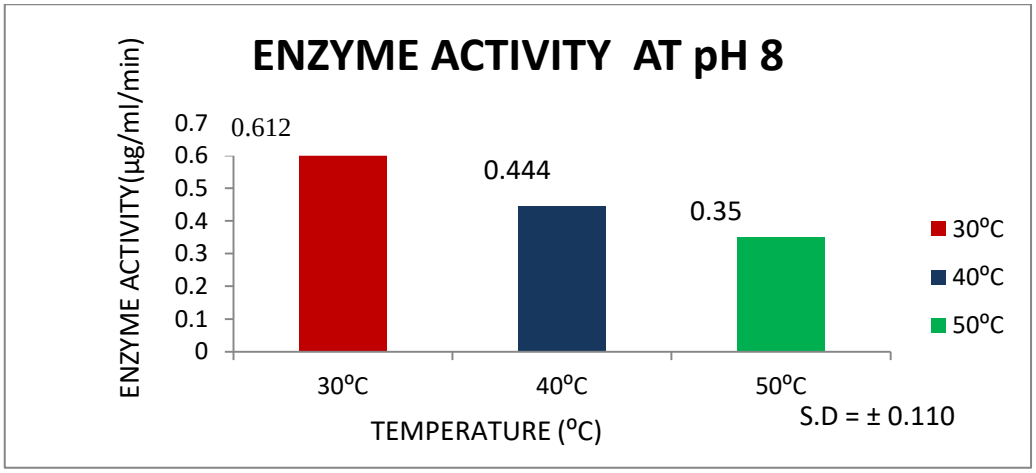

Fig-5: Enzyme Activity measured at different temperatures keeping pH 8

Supplementation of different carbohydrate sources (dextrose, mannitol, sucrose and starch) to the production medium increase the pectinolytic activity of *P.chrysogenum* (Fig 6.7). When pH 6 and temperature 30°C (both optimum for production) were maintained constant in production medium, the different carbohydrate sources showed different effect on pectinase production respectively. In presence of sucrose the crude production was maximum (13.7 µg/ml) and enzyme activity was found 0.876 µg/ml/min. In case of dextrose, mannitol and starch, the production was found 10.2µg/ml, 9.7µg/ml and 5.8µg/ml respectively and enzyme activity was found 0.732µg/ml/min, 0.636µg/ml/min and 0.516µg/ml/min respectively. So from our data it can be concluded that the pectinase production rate was highly repressed in presence of starch and highly induced for enzyme production in presence of sucrose. Piccoli-valle et al. [1] obtained significant Pectin lyase (PL) production by growing *P.griseorosum* in medium containing 60-74 µM sucrose.

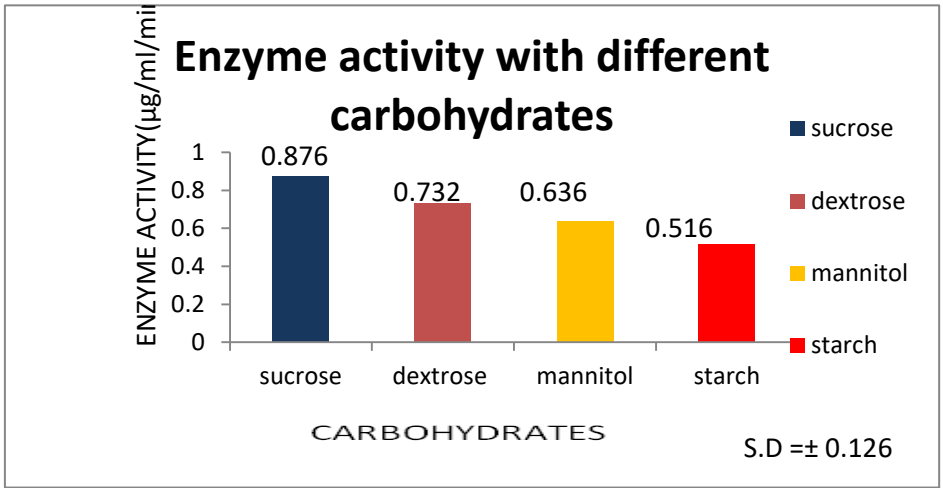

Fig-6: Graph to show the enzyme activity with different carbohydrates

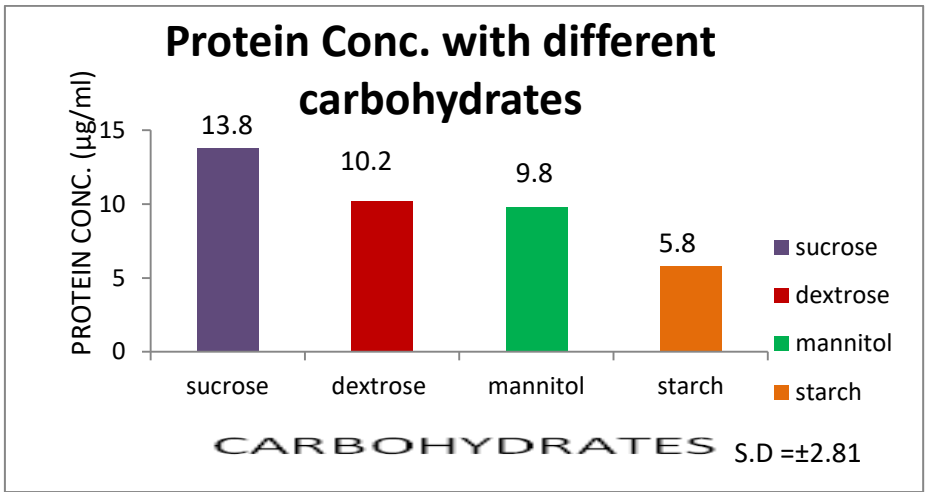

Fig-7: Graph to show the protein concentration with different carbohydrates

Of the different nitrogenous sources (ammonium sulphate, ammonium nitrate, ammonium chloride and peptone) used, peptone has enhanced the production of *P. chrysogenum* pectinase when medium optimum pH and temperature were 6 and 30°C respectively (Fig 8,9). Phutela et al. [16] reported that peptone followed by ammonium sulphate stimulated pectinase production more, as in their absence fungus displayed a slight proteolytic activity and did not produce extracellular pectinases.

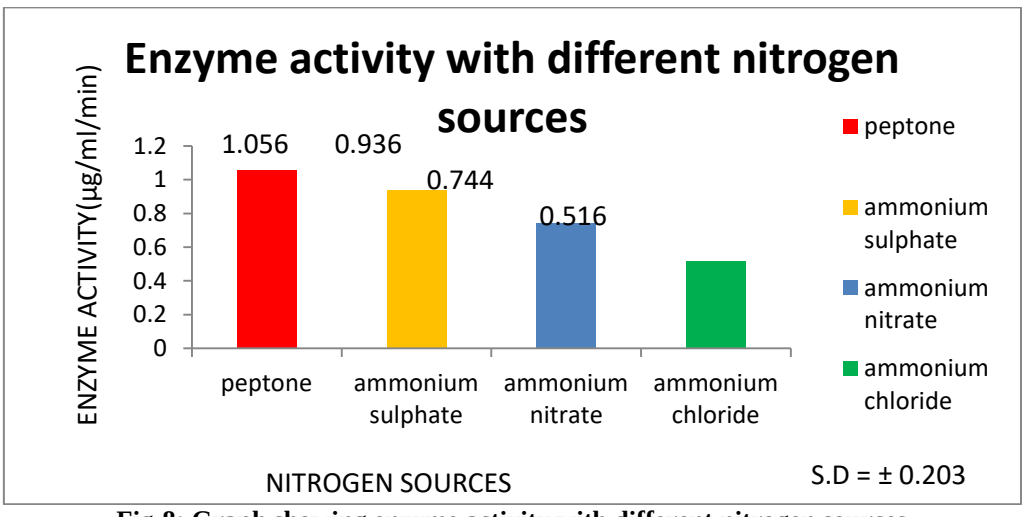

Fig-8: Graph showing enzyme activity with different nitrogen sources

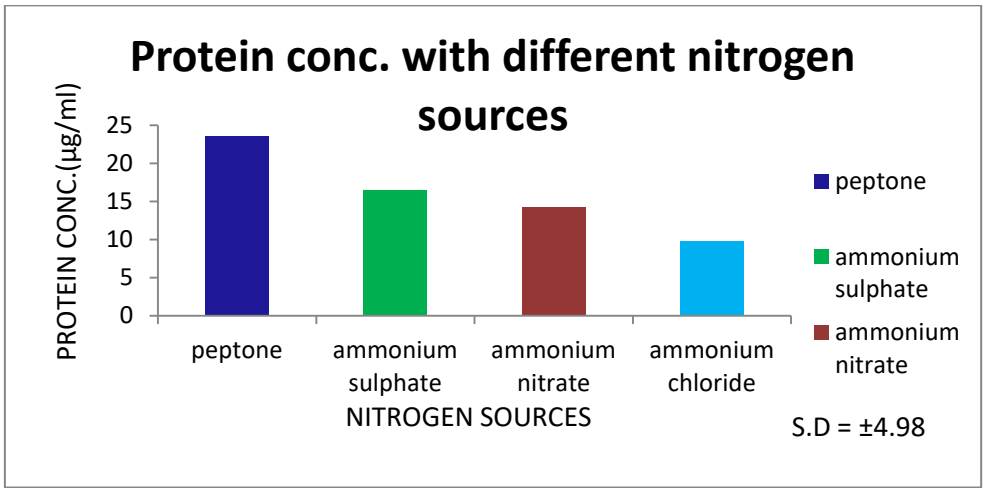

Fig-9: Graph showing Protein concentration with different nitrogen sources

Enzyme purification: Pectinase enzyme was partially purified by Sephadex G-100 column chromatography. It showed an increased specific activity of *P.chrysogenum* pectinase as 24.8µg/min, enzyme activity 1.116µg/ml/min and enzyme recovery of about 68% (Fig 10,11).

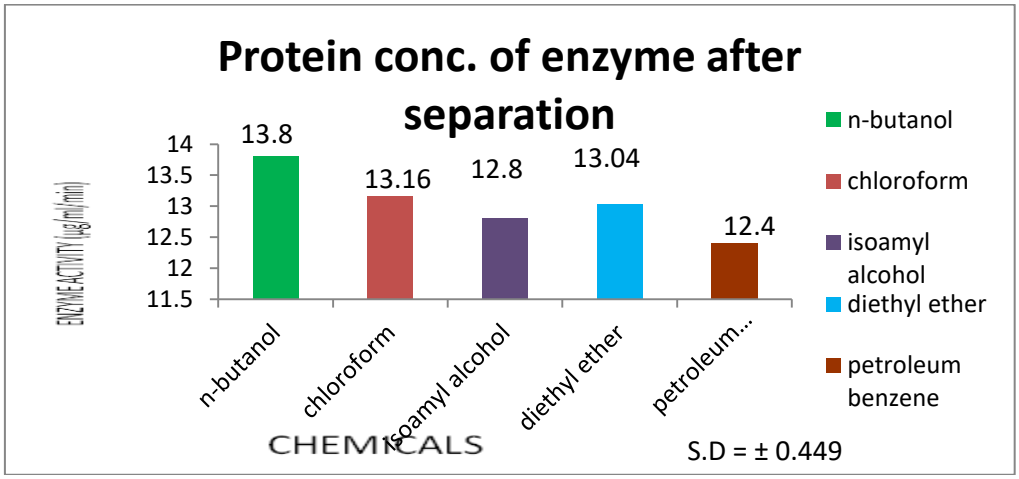

Fig:10. Protein concentration variation after separating with chemicals

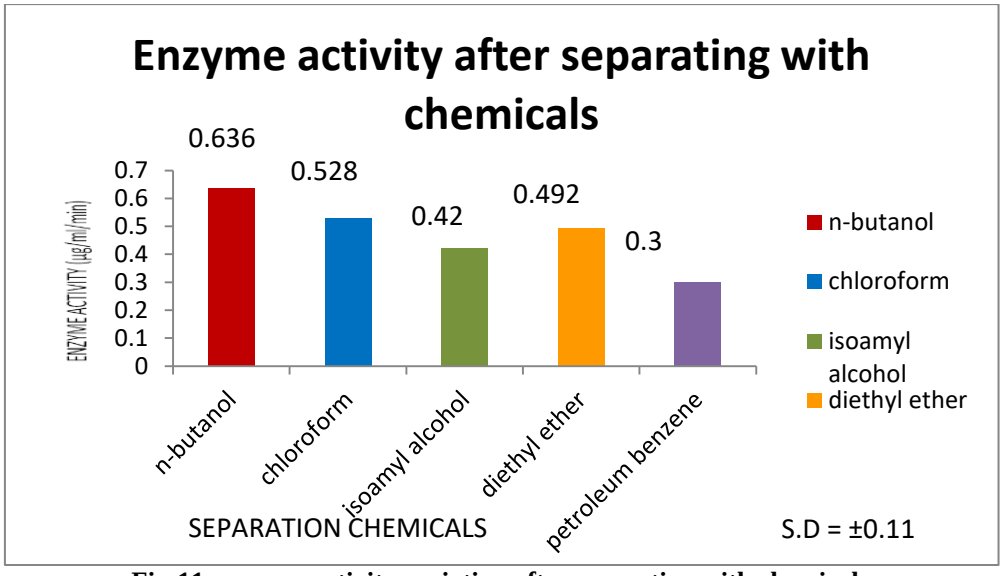

Fig-11: enzyme activity variation after separating with chemicals

Enzyme characterization:

The pectinase activity of *P.chrysogenum* was found to be highest at pH 6.0 using phosphate buffer. Marcia et al.,[23] studied the stability of pectinase against different pH. Their result indicated that the enzyme was stable in a pH 6-8 and showed highest activity at pH 5.0. Martin et al.,[24] reported that pectinase from *Penicillium sp*. was stable at pH range of 3-8 and maintained 70% of initial activity at 70^{0}C. In this study, pectinase produced by *P.chrysogenum* was stable in neutral pH (6-8) and was stable in temperature 60^{0}C (Fig 12,13).

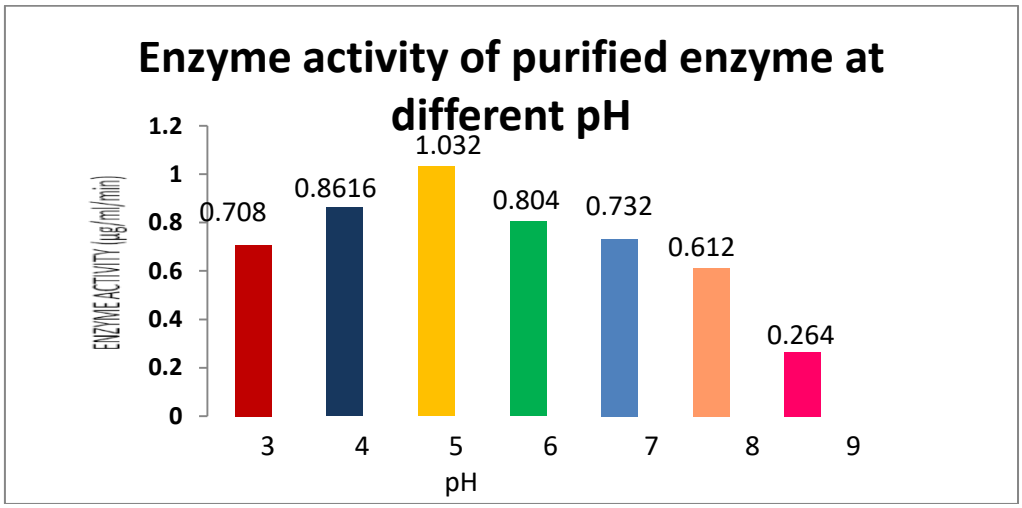

Fig-12: Graph showing enzyme activity with different pH on purified enzyme

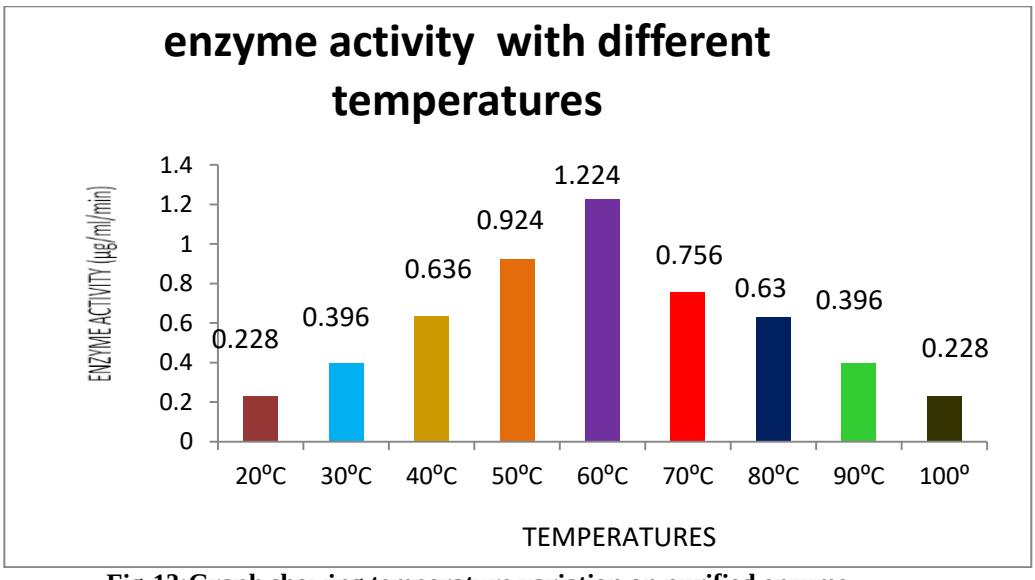

Fig-13:Graph showing temperature variation on purified enzyme

Different metals (zinc sulphate, magnesium sulphate, manganese sulphate and ferrous sulphate) influenced on enzyme activity was also studied (Fig 14). Optimal activity was observed at 1mg/ml of manganese sulphate (0.888µg/mL/min) and least activity was found in presence of 1mg/ml of zinc sulphate (0.348µg/mL/min). It may be suggested that the active sight of pectinase was influenced by magnesium sulphate to catalyse the hydrolytic reactions and negative cooperativity was shown by zinc sulphate [25].

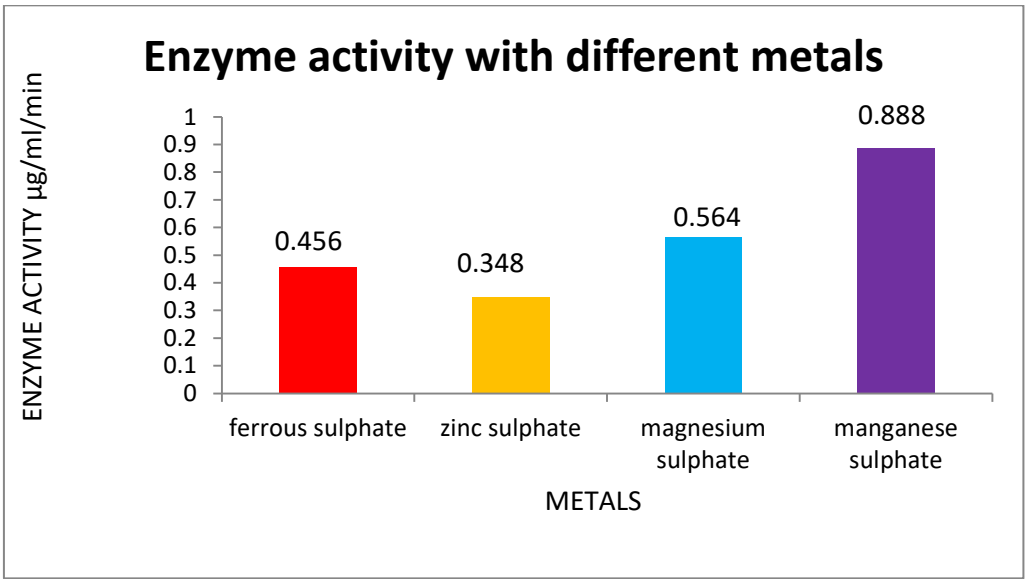

Fig-14: Enzyme activity of purified enzyme by the addition of various metals

The kinetic parameter of purified pectinase enzyme fromm *P.chrysogenum* was also determined. The pectinase enzyme from *P.chrysogenum* showed a V_{max} of 1.2 mg of enzyme protein and K_m of 0.018 mg/ml. It also reported that the V_{max} and K_m values of pectinase enzyme were increased from 11 to 14 which is supporting to our result [26].

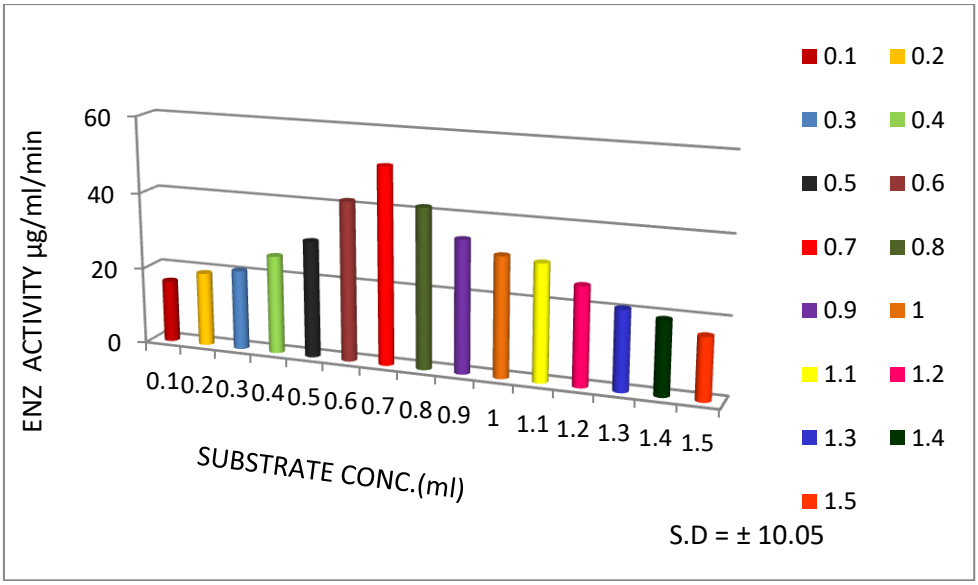

Fig-15: Graph to show enzyme activity vs. substrate concentration.

The molecular weight determination of purified pectinase was done by SDS-PAGE. The molecular weight was found to be 32 KD. Similar result was obtained by Martin et al [24]. This pectinase was found to have low molecular weight (15, 16).

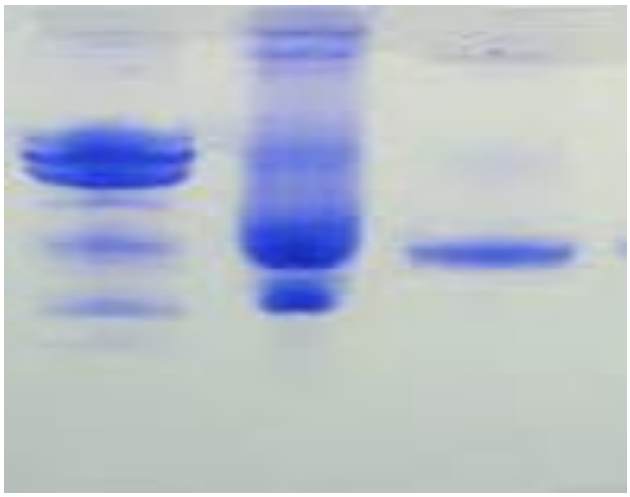

Fig-16. SDS-PAGE of purified pectinase enzyme from *Penicillum chrysogenum.* Lane1: Molecular wt marker, Lane2:Crude pectinase enzyme, Lane 3:single band of highly purified pectinese enzyme (Mol wt appx 32 KD).

>Polygalacturonase SLDS (PGA) (Pectinase)[Penicillum crysogenum]
MRNMINPLKKQFITISACLVLGACGGGSSNDAGTGSASTGTGSTSTGTGSTSTGTGSTSTGTGSTSTGT
GSTSTGTGSTSTGTGSTSTGTGSTSTGTGTTSTSVDATLPAEPQLPTTVCATLTANLKHTAGLLPDSVDANLVDS
NPDSVRIQKQITNCPAGQAVKLIAGSSGQNAFLSGPLTLASGVTLWVDQGVTLFASRNPADFDTGAGNCGTAA
GDGKSCNALITASKTQNSAVVGDGIIDGRGGSVLTSGANAGIMTWWDVAMLNKSTGKNQNNPRLIQVFGGSNF
TLYRITVQNAPAFHIVPNTVSGFTAWGVKILTPTLAYSKPGYSCPTGSSPDPTTPATSPSTCFTPETTKNTDGIDPG
QSSNVLIAYSYFSGGDDNIAIKASGSAPALSHRIVHSHFYYGHGMSIGSETNSGVDGIEMRDLSFDGHDSANGVG
IRIKSDDGRGGEVKNVSYRQICMRNVKEPMVFDTYYSAGNHSSAPNFHDITVSGFHYLGSAKYAGGTLTFNGY
ALNGTVNPLKIALDNVIFDSAPKISNSPHNGGPTPPSNTQFTMGPGPVNFTVASSAANNVTIAELQKNAQAPLDC
SQAFVSFPSSASPF

Fig-17: Sequences of purified enzyme from *Penicillum chrysogenum.*

Table-1:Overall summary of character of pectise enzyme from Penicillum chrysogenum

PROPERTIES	*P.chrysogenum*
Optimum pH	5.0
Optimum temperature	60°C
Metal ions effect	$MnSO_4$
V_{max}(U/mg protein)	72
K_m(mg/ml)	1.2
Molecular weight(kD)	32

CONCLUSION

Pectinase enzyme was isolated from the native strain, P. chrysogenum that can be used for various industrial applications including extraction and clarification of fruit juices, processing of cotton fabric in textile industries, bleaching of paper, removal of pectic waste waters and maceration of tea leaves.

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