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Isolation and Characterization of Pectinolytic enzyme from Soil *Aspergillus* sp.

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ABSTRACT

Pectinases are an important class of enzymes; mostly used in various industries like food, wine and paper to enhance speed of processing for economic increase. This study involved the isolation, partial purification and kinetics of pectinolytic protein from soil samples of decomposing sites of fruits and vegetables. Fungal isolate showing maximum zone of clearance and enzyme activity was identified as *Aspergillus* sp. The enzyme was semi-purified using ammonium sulphate fractionation and anion exchanger, DEAE. The optimum pH and temperature for the fungal isolate was observed as 5.0 and 40˚C while K_m and V_{max} was determined as 1.93 mg/ml and 0.138 U/ml respectively. Therefore, this enzyme can be used as a promising contender for the industrial uses.

INTRODUCTION

Microorganism is cosmopolitan in nature and occupies an important place in human view of life. They serve humans since hundreds of years for the purpose of food, drugs, enzymes and other high-value chemical substances. Enzymes play an essential role in cellular anabolism, catabolism and biochemical reactions by accelerating the biological or biochemical reactions in cytoplasm as well as in outer environment the cells (Gurung *et al.*, 2013). The microbial enzymes was

invented in the 20th century and since then the studies on their purification, characterization and their application in bio-industries has been regularly studied and updated. There are certain microbial enzymes which are of special interest and are categorized to be superior biocatalysts predominantly for application in productions on commercial scale. Some selected microorganisms have been evaluated for the production of various enzymes for commercial application and many such enzymes are being already used in numerous commercial procedures. Microorganisms including prokaryotes, fungi and yeast have been successfully studies and have been universally used for the synthesis of economically useful enzymes (Nigam, 2013).

Pectins are assembly of different polysaccharides present in the outer wall of plants. All the fruits peels are made up of pectin layer and pectinase enzymes are used for dissolving the pectin into smaller units. Microbial pectinase can easily digest the pectin layer to isolate the liquids from various fruitlets. Hence, these are useful for decompose the plant parts and also in rapid extraction of the fruit juices. Naturally pectinolytic enzymes are produced by fungi, bacteria, yeast and actinomycetes. Out of which fungus is mostly used in industries namely *Fusariumoxysporum, Aspergillus sp., Neurosporacrassa, Rhizopusstolonifer, Alternaria mali, Penicillium italicum*, etc.[3]

The initial profitable use of pectinase enzyme was first reported by Kertesz in the year 1930 for the clearing up of apple squash.[4] Pectinases have great importance in industries like in fruits and vegetable processing for reducing viscosity and clarification juice and maceration of vegetable to produce products like paste, in wine processing for intensifying the flavor and color of wine and also helps in its extraction process, in saccharification of agriculture waste, extraction of vegetable oils, processing of textile material, processing of textile material, processing of animal feeds, recycling of waste paper, etc.[5-11]

Pectinase enzymes, functional at extreme conditions, are required to study which can upturn its sturdiness regarding the temperature, pH and reaction rate and important in excluding the usage of harmful diluters in manufacturing methods. In this context, the present study was intended for the isolation of low pH optima pectinase producing fungi and enzyme characterization.

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MATERIAL AND METHODS

Sample collection

From different decomposing sites of fruits and vegetables in village Chunni Kalan of District Fatehgarh Sahib, Punjab, soil samples rich in pectin waste viz. orange peel, decomposing vegetables were procured in autoclaved plastic bags and carried to the laboratory.

Isolation and Screening of pectinolytic fungi

1 gram of soil sample was first consecutively (10^{-2} to 10^{-9}) diluted from using 9 ml of saline water in each test tubes blanks. 20 mg/ml of ampicillin containing potato dextrose agar (PDA) with pH adjusted to 5.0 was spread plated using 0.1 ml sample of each dilution. The plates were kept at 27^{O}C for 5 days. The fungal isolates were re-cultured on Czapek-Dox agar medium (pH 5.0) containing 1.5 percent pectin as the only carbon source. The petriplates were kept for about 72 hours at 27°C and when the colonies grow about 3 mm to 4 mm diameter, potassium iodide-iodine solution (2.5 g potassium iodide and 0.5 g iodine in 165 ml of distilled sterile water) was poured to visualize the zone of clearance. The diameter of the zone of clearance was counted as the extent of pectinolytic activity of the fugal isolate.

Pectinase activity and selection of fungal strain

To produce enzyme from the selected 6 isolates of fungi showing zone of clearance, Czapek-Dox liquid media was used to culture at low pH (5.0) at 27°C for 7 days and enzyme activity was checked in liquid media with substrate, 1% pectin and sodium-acetate buffer (pH 5.0).

Morphological identification of the fungi

The isolate showing the maximum enzyme activity in the previous step was selected and further processed for identification. Identification was carried out by slide culture technique [12] and lacto phenol cotton blue staining [13].

Purification of pectinase enzyme

200 ml of Czapek-Dox liquid media was prepared in 500 ml of conical flasks and was autoclaved. After cooling filter sterilized 0.1% of ampicillin was mixed and its final pH was set to 5.0. The isolated fungal strain was inoculated in the medium and incubated in a shaker at 27°C for seven days. The mycelium was passed through Whatmann filter paper no. 1 and then centrifuged for 15 minutes at 10,000 rpm. The liquid portion was accounted as a source of extracellular enzyme.

Partial purification of the enzyme

Fractional precipitation

Ammonium sulphate fractionation was performed by altering the solubility of protein by adding different concentrations (20%, 40%, 60% & 80%) of ammonium sulphate using magnetic stirrer at 4°C.[14] At a sufficiently high concentration of ions, the protein portion of the supernatant entirely precipitates from the solution. Each fraction mixture was centrifuged at 4°C for 15 min at 10,000 rpm and protein pellets were dissolved in re-suspension buffer (0.05 M sodium-acetate buffer, pH 5.0) and desalting was performed against large volume of 0.05 M sodium-acetate buffer (pH 5.0) by dialyzing using with a membrane tube of pore size of 50 A°.

Ion exchange chromatography – DEAE cellulose column chromatography

DEAE cellulose packed column was equilibrated with equilibrium buffer (0.05 M sodium-acetate buffer, pH 5.0) by passing 10 column volumes at flow speed of 60 ml/hr. Then, the sample was loaded and kept for 10 minutes allowing enzyme to bind to the stationary phase. Washing was carried out by same buffer with 5 column volumes. After that, it was subjected to elution buffer with stepwise increase in salt concentration and the elutes were collected in the fractional collectors**.** The fractions were examined for the enzyme activity.

Characterization of partially purified enzyme

The pectinase activity was calculated by determining the quantity of reducing carbohydrates formed after enzyme action using 3,5-Dinitrosalicylic acid.[15] 100 µl of 1% pectin substrate, 300

µl of sodium-acetate buffer (pH 5.0), 100 µl of applicably diluted enzyme was incubated at 40°C for 15 min. The enzyme catalysis was then terminated with 0.5 ml of DNS reagent and the absorbance was observed at 540 nm. 1 unit of pectinolytic activity was stated as the concentration of enzyme that can release 1 µmol of galacturonic acid in one min under standardized conditions, using galacturonic acid as reference.

By reaction the enzyme-substrate at different pH ranging from 3.0 to 9.0 at 40°C for 30 mins, pH effect on enzyme activity was demonstrated. Various pH buffers used in this study were citrate phosphate buffer (pH 4.0 to 7.0), Tris-Cl buffer (pH 8.0) and glycine-sodium hydroxide buffer (pH 9.0). Temperature effect on pectinase activity was observed by keeping the mixture at temperatures from 20°C to 80°C.

Kinetic characteristics of pectinase enzyme

The K_m and V_{max} value were calculated by the reaction velocity at various substrate concentrations (0.25 to 1.75 mg/ml) and plotting the inverse graph of them. The appropriate concentration pectin substrate (900µl) was incubated with 100µl of suitable diluted enzyme at 40°C for 30 minute and the pectinase enzyme activity was examined.

RESULTS AND DISCUSSION

Isolation and screening of pectinolytic fungal isolates

To isolate pectin producing fungi from the collected soil samples; serial dilution, spread plate and streak plate methods were used. In this study, 20 colonies were isolated on the PDA plates (Figure 1A). The isolated strains were sub-cultured into Czapek-Dox agar medium (pH 5.0) enriched by 1% pectin. The isolates; 1, 3, 9, 10, 13 & 17 which showed highest zone of clearance were considered as high pectinase producers (Table 1 & Figure 1B) and were selected enzyme production and assay.

Table 1. Zone of clearance on the plates

Isolate number	Zone size (mm)
1	1

3	3
9	2
10	2
13	0.5
17	1

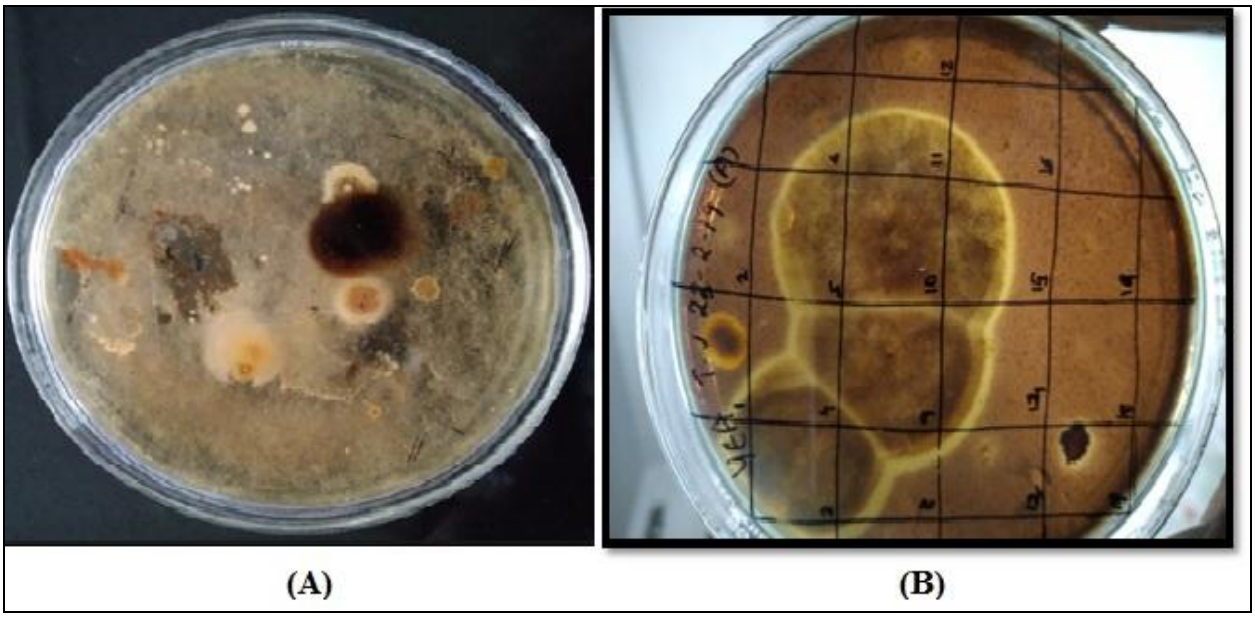

Figure 1. (A) Isolation of pectinolytic fungi on PDA, (B) Screening of pectinolytic fungi by plate assay method.

Pectinase enzyme assay

The selected fungal stains were observed for the pectinolytic activity (Table 2) by using the DNS method suggested by the method of Miller, 1959.[15] Fungal isolate no. 9 showed the most enzyme activity at acidic pH, 5.0 and 40°C temperature with optical density of 0.038 at 540 nm and it was further processed for identification, protein purification and characterization (Table 2).

Table 2. Enzyme activity of different colonies

Isolate number	**Absorbance at 540 nm**
1	0.009

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3	0.036
9	0.038
10	0.029
13	0.005
17	0.008

Morphological identification of the fungi

Whitish colony with the fussy appearance was visible on Czapek-Dox agar medium (Figure 2) and the diameter after 5 days of incubation was observed as 6 cm.

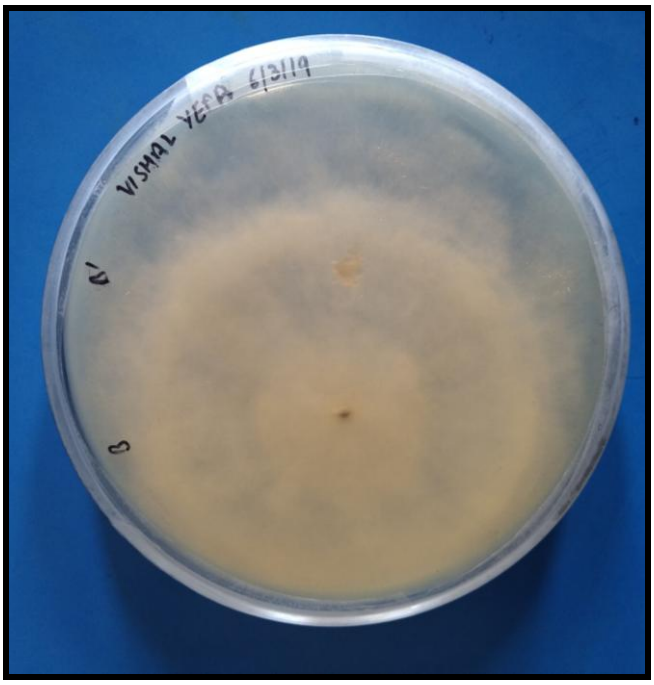

Figure 2. Fungal isolate no. 9 on Czapek-dox agar plate.

On the basics of the structure and morphology observed by slide culture method (Figure 3A) & lactophenol cotton blue staining (Figure 3B), the fungal strain was identified as a species of *Aspergillus.* The present result is in consent with a previous study in which *Aspergillus flavus* showed a maximum pectinolytic activity.[16]

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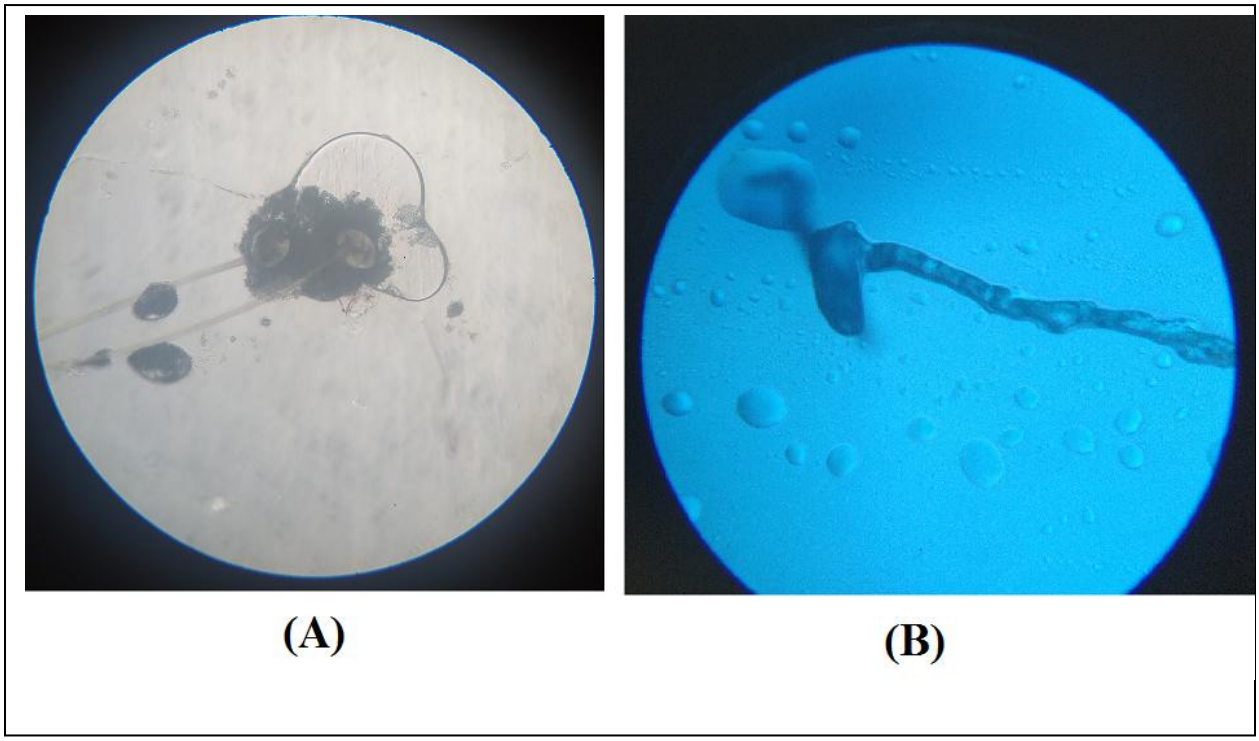

Figure 3. Identification of fungal isolate 9, (A) Slide culture technique (B) Lactophenol cotton blue staining.

Partial purification of enzyme

Production phase

The selected fungal stains was inoculated in 200 ml of Czapek-Dox liquid medium (pH 5.0) and incubated at 27°C 5 days. After incubation, the culture was centrifuged and absorbance was observed at 540 nm which was found to be 0.153.

Ammonium sulphate fractionation

Crude extract was added with different amounts of ammonium sulphate to achieve different saturations. After each saturation, the pectinolytic activity was measured (Table 3). At 80% ammonium sulphate saturation, maximum pectinolytic activity was observed at 80% saturation

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with absorbance 0.485 at 540 nm. The present findings coincide with the results of an earlier study reporting 80% ammonium sulphate saturation with maximum pectinase activities.[17]

Table 3. Pectinase activity at different ammonium sulphate concentration

Ammonium sulphate saturation (%)	**Absorbance at 540nm**
20	0.102
40	0.284
60	0.241
80	0.485
100	0.203

80% ammonium sulphate saturated enzyme was dissolved in 0.5 molar sodium-acetate buffer (pH 5.0) and was dialyzed against the 0.5 M sodium-acetate buffer for 24 hours. The enzyme activity of petinase was observed and absorbance was found to be 0.624 at 540 nm.

Ion exchange chromatography

The dialyzed pectinase enzyme was more purified via ion exchange chromatography using DEAE and from the elution profile, it was seen that the proteins were fractionated into three major peaks (Figure 4) and the column with highest pectinolytic activities were pooled and used for further characterization.

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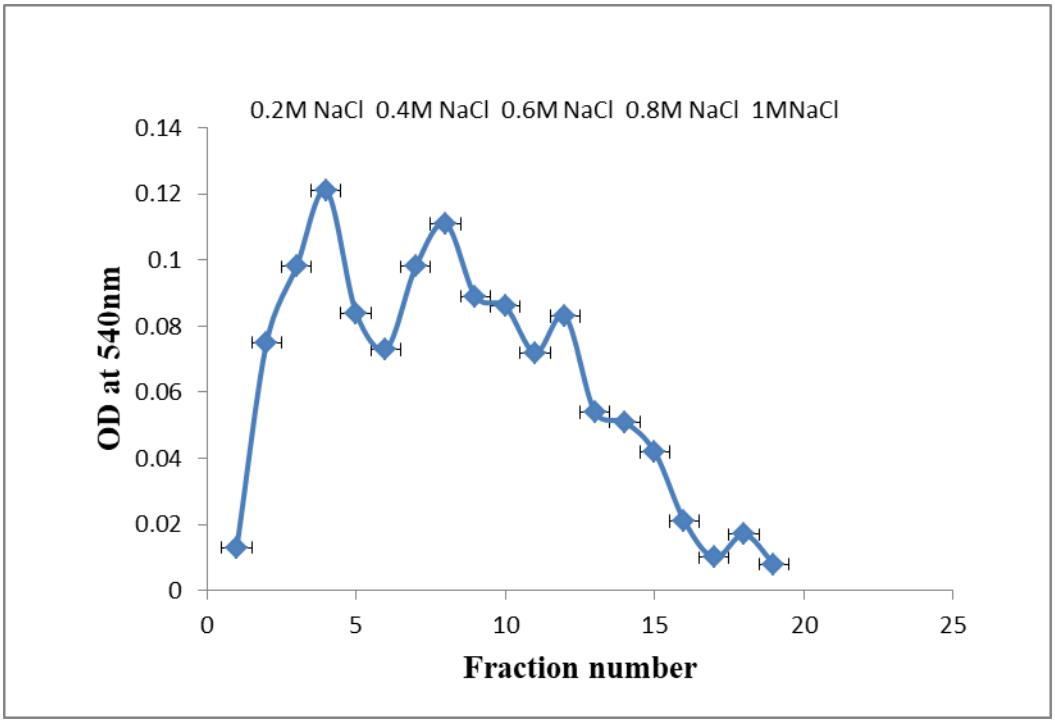

Figure 4. Elution profile of DEAE-Cellulose Ion exchange chromatography

Characterization of pectinase enzyme

Partial purification was confirmed by SDS-PAGE. The number of proteins visualized in sample after ion exchange chromatography was much lower than that of 80% ammonium sulphate fractionation (Figure 5).

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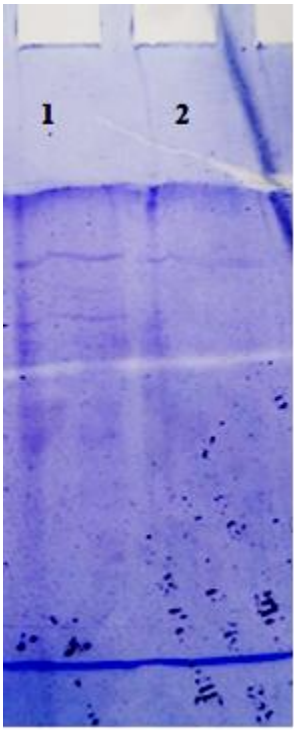

Figure 5. Partial purification of enzyme; Lane 1, protein after 80% ammonium sulphate fractionation, Lane 2, protein after ion exchange chromatography

Effect of pH on enzyme activity

The effect of pH on pectinase activity was studied by keeping reaction mix at different pH (3-9) and the activity was measured at 540 nm (Figure 6). It was observed that the enzyme activity was highest at acidic pH of 5.0 and the same parameter was obtained by some previous studies who observed the maximum pectinase activity at pH 5.0 from *Sclerotinia sclerotiorum*[18] and from *Aspergillus niger*[19] and *Aspergillus awamori*[20].

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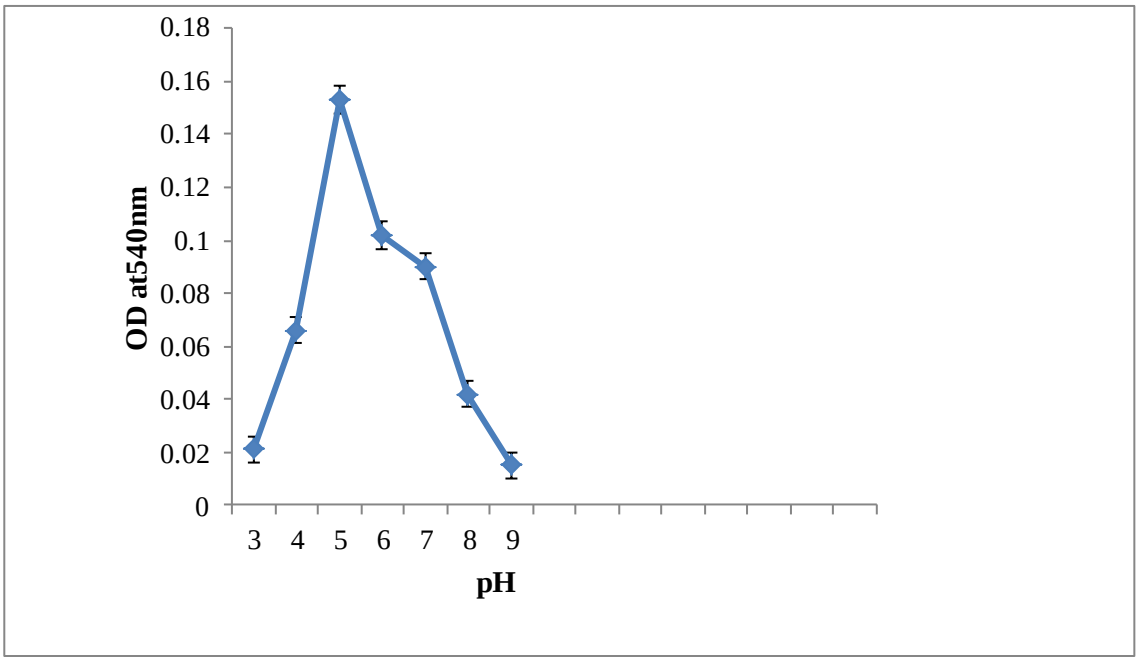

Figure 6. Effect of pH on enzyme activity

Effect of temperature on enzyme activity

Enzymes exhibit an optimal temperature at which it shows maximum activity. Temperature higher than this, denature it 3D structure, therefore losses its activity. Below the optimum temperature, the enzyme becomes less active because of intra-molecular hydrogen bonding.[21] The temperature factor effect on pectinase was estimated by keeping the enzyme-substrate mix at various temperatures of 20-80°C and the activity was measured at 540 nm (Figure 7). Arotupin et al. (2007) It has been observed that pectinases secreted by *Aspergillus flavus*, *A. repens*, and *A. fumigatus* exhibited highest activity at 35°C, 45°C and 40°C respectively.[22,23]

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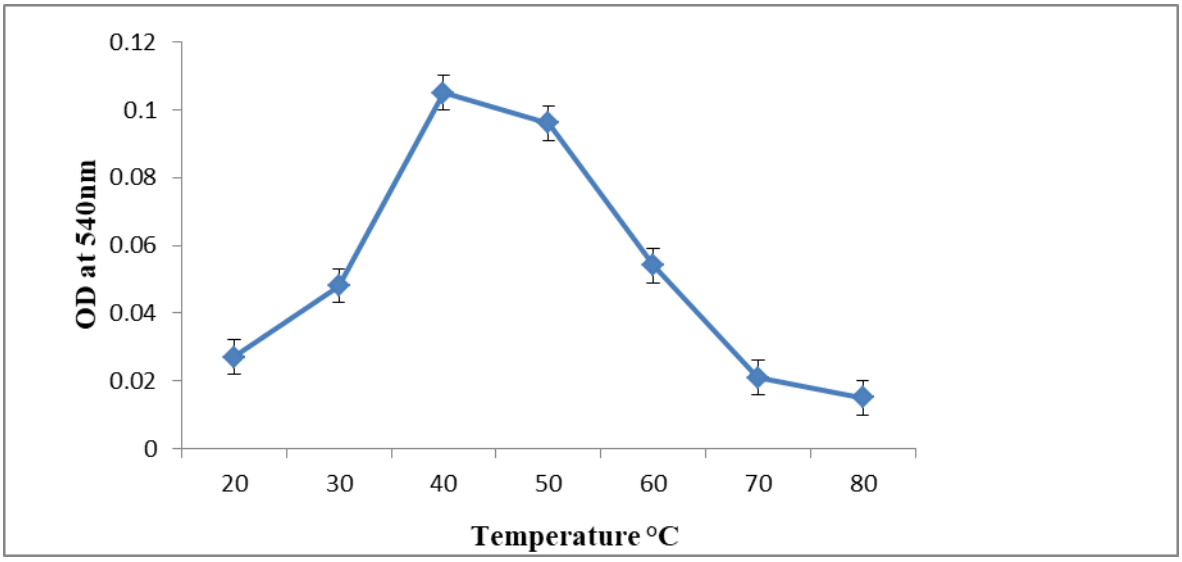

Figure 7. Effect of temperature on pectinolytic activity.

Kinetics characteristics of pectinase enzyme

The substrate concentration effect on pectinase activity was at substrate concentration ranging from 0.25-1.75 mg/ml (Figure 8). The maximum pectinase activity was seen at substrate concentration of 1 mg/ml.

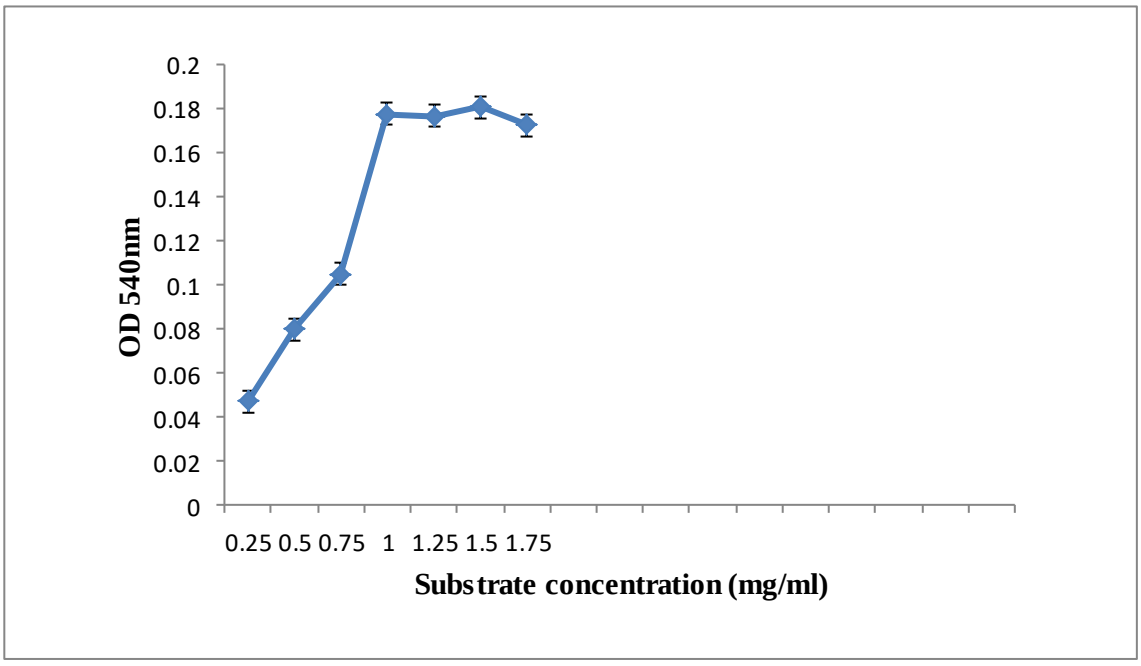

Figure 8. Effect of substrate concentration on pectinolytic activity

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The K_m and V_{max} value of the enzyme were determined by drawing lineweaver burk plot of the velocity inverse and the inverse of different substrate concentration. Michaelis-Menton constant (K_m) & maximum velocity (V_{max}) were evaluated as 1.93 mg/ml and 0.138 U/ml respectively (Figure 9).

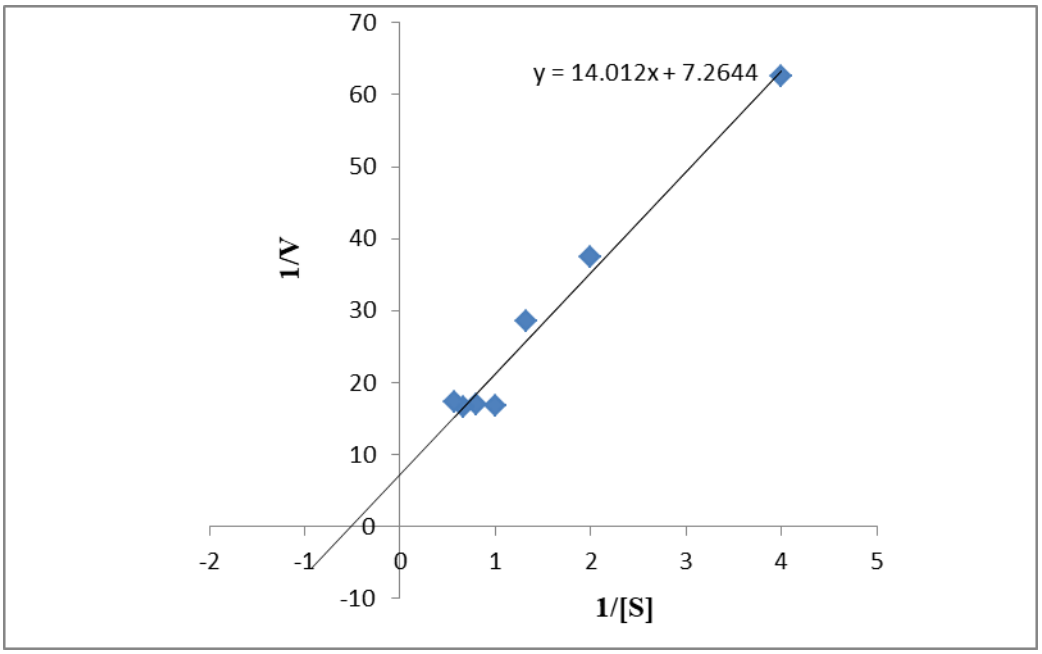

Figure 9. Double reciprocal (Lineweaver-Burk) plot of the substrate, pectin

CONCLUSION

The pectinase enzyme was isolated and purified from soil *Aspergillus* sp. that showed optimum activity at pH 5.0. Acidic pectinases are broadly used in fruit product industries as well as wine industries. It can be studied further for its structure elucidation and detailed biochemical characterization.

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