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RESEARCH ARTICLE

Effect of Various Parameters on Activity of Pectinase Enzyme

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Abstract

Pectinase enzymes are today one of the upcoming enzyme of commercial sector. It has been reported that microbial pectinase account for 25% of global food sales. Primarily, these enzymes are heterogeneous group of enzymes that hydrolyse the pectin substance, present mostly in plants. The present study was under taken for pectinolytic enzyme production and selection of more efficient isolates for industrial applications. The objective of this work consists of determining the optimum parameters for maximum activity of pectinase enzyme. The characterization of crude enzyme was done for temperature, pH, time course, substrate concentration, enzyme concentration and metal ions. It was found that for isolate P1 and P57 the optimum temperature was 37°C and for isolate P13 and P58 the optimum temperature was noted at 30°C. The isolate P1 and P58 showed maximum activity at pH 6 which is slightly acidic pH while the crude enzyme from isolates P13, P57 showed highest activity at pH 8 which is slightly alkaline pH. It was observed that Zinc enhanced the activity of isolate P1, P57 and P58 while it showed inhibitory effect on isolate P13. Magnesium, Calcium, Barium and Manganese acted as good activators for all the isolates. Iron showed inhibitory effect on isolate P1, P13 and P57 while it enhanced the activity of enzyme from isolate P58. Potassium enhanced activity of enzyme from isolates P1, P13 and P57 while it showed strong inhibition on the enzyme isolated from isolate P58.

INTRODUCTION

Enzymes are biocatalysts which are synthesized by living cells. It is also defined as the catalyst that increases the velocity or rate of a chemical reaction. Microbial enzymes are routinely used in many environment friendly and economic industrial sectors (Hoondal *et al.,* 2000). Microbes are the best source of enzymes as they allow an economical technology with low resource consumption and low emission involving no social and political issues, as in the case of animal and plant sources (Dalvi *et al*., 2007). Such enzymes may be discovered by screening microorganisms sampled from diverse environments or developed by modification of known enzymes using modern methods of protein engineering or molecular evolution. As a result, several important food-processing enzymes such as amylases and lipases with properties tailored to particular food applications have become available (Beer *et al*., 2006). New enzymes for commercial applications with desirable biochemical and physico-chemical characteristics and a low cost production have become a focus of research (Silva *et al*., 2002).

Pectinases were some of the first enzymes to be used in homes. Their commercial application was first observed in 1930 for the preparation of wines and fruit juices. Only in the 1960s did the chemical nature of plant tissues become apparent and with this knowledge, scientists began to use a greater range of enzymes more efficiently. As a result, pectinases are today one of the upcoming enzymes of the commercial sector. Primarily, these

enzymes are responsible for the degradation of the long and complex molecules called pectin that occur as structural polysaccharides in the middle lamella and the primary cell walls of young plant cells (Kashyap *et al.*, 2000).

Study of reaction rates is an important tool to investigate the chemical mechanism of catalysis. Kinetic studies provide information on substrate and product affinity to the enzyme. Knowledge of the dynamic properties of enzyme catalysis is a prerequisite for the optimization.

The present study was under taken for pectinolytic enzyme production and selection of more efficient isolates for industrial applications. The objective of this work consist of determining the optimum parameters for maximum activity of pectinase enzyme.

MATERIAL AND METHODS:

Isolation of pectinolytic bacteria was done at P. G. Department of Microbiology, Shri Shivaji College of Arts, Commerce and Science, Akola by stepwise technique. Soil samples were collected from different places of Akola region. Four previously isolated pectinase producers namely isolate P1 (*B. firmus*), P13 (*B. coagulans*), P57 (*B. endophyticus*) and P58 (*B. vietnamensis*) were subjected for further optimization studies to get higher yield of pectinase.

- **Extracellular enzyme production :**

The production of crude enzyme was carried out by taking 200 ml production medium in 250 ml flask by shake flask fermentation. After incubation media was centrifuged at 3000 rpm for 30 min to obtain cell free supernatant. The supernatant was used as crude enzyme for further studies.

- **Total protein estimation of crude enzyme :**

The protein concentration was determined by the Lowry's method, as described by Lowry's (1951). Standard graph was plotted between concentration of BSA and optical density. The protein content of crude enzyme sample was calculated from standard graph by comparing the O.D with standard graph.

- **Enzyme assay:**

Standard graph for enzyme assay:

The activity was determined by measuring the release of reducing groups using dinitrosalicylic acid reagent DNS assay (Miller., 1959).

Formula for enzyme activity:

$$Enzyme\ activity\ (IU/mL/min) = \frac{Absorbance\ of\ enzyme\ solution \times Standard\ factor}{Time\ of\ incubation\ (min)}$$

Where,

$$Standard\ factor = \frac{Conc.\ (\mu Mol/mL)\ of\ Standard}{Absrobance\ of\ 540\ nm}$$

- **Characterization of crude enzyme:**

The characterization of crude enzyme was done for the effect of pH, temperature, time course, metal ions, enzyme concentration and substrate concentration.

- **Effect of temperature on pectinase activity:**

For determination of temperature, 1 ml pectin, 1 ml buffer solution was taken in different test tubes. It was kept for activation at 37°C for 10 minutes. 1 ml enzyme solution was added and mixed. Tubes were kept at different temperature ranging from 5°C to 80°C for 30 minutes. After 30 minutes 3, 5- dinitrosalysilic acid was added in all tubes. All tubes were mixed and kept in boiling water bath for 10 minutes. 8 ml distilled water was added. Absorbance was read at 540 nm.

- **Effect of pH on pectinase activity:**

Citrate buffer and phosphate buffer of different pH were prepared. 1 ml pectin and 1 ml buffer solution was added in test tubes of pH 3.6 to pH 8 and kept at 37°C for 10 minutes. After that 1 ml enzyme solution was added and kept for incubation at 37°C for 30 minutes. After 30 minutes 1 ml of 3, 5- dinitrosalysilic acid was added in all

tubes. All tubes were mixed and kept in boiling water bath for 10 minutes. 8 ml distilled water was added. Absorbance was read at 540 nm.

- **Effect of time course on pectinase activity:**

For determination of time course, 1 ml pectin, 1 ml buffer solution was taken in different test tubes. It was kept for activation at 37°C for 10 minutes. 1 ml enzyme solution was added and mixed. Tubes were kept for incubation at different time period ranging from 5 to 50 min. After incubation 3, 5- dinitrosalysilic acid was added in all tubes. All tubes were mixed and kept in boiling water bath for 10 minutes. 8 ml distilled water was added. Absorbance was read at 540 nm.

- **Effect of substrate concentration on pectinase activity:**

Different concentrations of pectin from 0.1 to 1.0 ml was taken, 1 ml buffer solution was added in different test tubes. It was kept for activation at 37°C for 10 minutes. 1 ml enzyme solution was added and mixed. Tubes were kept at 37°C for 30 minutes. After 30 minutes 1 ml of 3, 5- dinitrosalysilic acid was added in all tubes. All tubes were mixed and kept in boiling water bath for 10 minutes. 8 ml distilled water was added. Absorbance was read at 540 nm.

- **Effect of enzyme concentration on pectinase activity:**

1 ml pectin, 0.9 to 0.1 ml buffer solution was taken in different test tubes. It was kept for activation at 37°C for 10 minutes. 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 ml enzyme solution was added and mixed. Tubes were kept at 37°C for 30 minutes. After 30 minutes 1 ml of 3, 5- dinitrosalysilic acid was added in all tubes. All tubes were mixed and kept in boiling water bath for 10 minutes. 8 ml distilled water was added. Absorbance was read at 540 nm.

- **Effect of metal ions on pectinase activity:**

0.2 ml pectin, 0.1 ml buffer solution was taken in different test tubes. It was kept for activation at 37°C for 10 minutes. 1 ml enzyme solution was added and mixed. Different metal ion solutions were added. Tubes were kept at 37°C for 30 minutes. After 30 minutes 3, 5- dinitrosalysilic acid was added in all tubes. All tubes were mixed and kept in boiling water bath for 10 minutes. 8 ml distilled water was added. Absorbance was read at 540 nm.

- **Application of pectinase in fruit juice clarification:**

Label two tubes one as test and other as blank. Add 10 ml of crude pectinase enzyme into the first test tube. Finally, add 10 ml of distilled water to the remaining blank tube. Stir the Apple juice well to distribute any suspended particles evenly through it. To each test tube add 20 ml of Apple juice. Agitate or stir the contents of the tubes to mix the enzymes throughout the juice. Put the tubes into the water bath. Observe the tubes and record the appearance of their contents at 5 minute intervals over a half hour period. After incubation filter the solution (Mehta *et al.,* 2013).

RESULTS AND DISCUSSION :

The crude enzyme produced by shake flask fermentation from isolate namely isolate P1 (*B. firmus*), P13 (*B. coagulans*), P57 (*B. endophyticus*) and P58 (*B. vietnamensis*) was subjected for further characterization.

- **Protein estimation:**

- **Standard graph:**

For the determination of concentration of protein in the crude enzyme sample, a standard graph was plotted (Fig. 1) with the known concentration of a standard protein (BSA).

- **Protein estimation for crude enzyme:**

Protein concentration from the crude enzyme sample was determined by Folin-Lowry method. The highest protein content was found in the cell free supernatant of isolate P1 that is 1.98 µg/ml followed by P58 which is 1.97 µg/ml while protein content of P13 was found to be 1.93 µg/ml and lowest protein content was found in crude enzyme of P57 which is 1.90 µg/ml (Fig. 2).

- **Assay of crude enzyme:**

The activity was determined by measuring the release of reducing sugar using dinitrosalicylic acid reagent DNS assay (Miller., 1959). The standard graph of glucose was plotted (Fig. 4). From the standard graph the activity of crude enzyme was calculated and isolate P1 showed highest activity which is 0.0053 IU/ml/min followed by P57 which is 0.005 IU/ml/min and P13 and P58 showed 0.0044 IU/ml/min.

- **Characterization of crude enzyme:**

The characterization of crude enzyme sample was done. The effect of temperature, pH, time course, substrate concentration, enzyme concentration and metal ions was studied and results were noted as follows.

- **Effect of temperature on pectinase activity:**

The effect of temperature on the activity of crude enzyme was studied by DNS method. It was found that for isolate P1 and P57 the optimum temperature was 37°C and for isolate P13 and P58 the optimum temperature was noted at 30°C. At low temperature the enzyme showed less activity and at high temperature also the enzyme activity was lowered down. The isolate P1 exhibited highest activity among all isolates at 37°C which is 0.0053 IU/ml/min (Fig. 5).

- **Effect of pH on pectinase activity:**

The effect of pH on the activity of crude enzyme was studied by DNS method. The isolate P1 and P58 showed maximum activity at pH 6 which is slightly acidic pH while the crude enzyme from isolates P13, P57 showed highest activity at pH 8 which is slightly alkaline pH. The highest activity was shown by isolate P1 at acidic pH which is 0.010 IU/ml/min (Fig. 6).

- **Effect of time course on pectinase activity:**

The DNS method was employed for studying effect of time course on pectinase activity. The optimum time for the activity of pectinase was found to be between 30-40 minutes. The isolate P1 and P58 showed highest activity after incubation of 35 minutes while isolate P13 showed maximum activity after 30 minutes incubation and isolate P57 showed highest activity after 40 minutes incubation. Before 30 minutes and after 40 minutes the enzyme activity was found to be less (Fig. 7).

- **Effect of substrate and enzyme concentration on pectinase:**

The effect of substrate concentration on the activity of crude enzyme was studied by DNS method. It was found that for all isolate the increase in the concentration of substrate increases the activity up to certain limit and it levels off (Fig. 8). While same results were noted for enzyme concentration as well (Fig 9).

- **Effect of metal ions on pectinase activity:**

The effect of different metal ions was studied on pectinase activity. It was observed that Zinc enhanced the activity of isolate P1, P57 and P58 while it showed inhibitory effect on isolate P13. Magnesium, Calcium, Barium and Manganese acted as good activators for all the isolates. Iron showed inhibitory effect on isolate P1, P13 and P57 while it enhanced the activity of enzyme from isolate P58. Potassium enhanced activity of enzyme from isolates P1, P13 and P57 while it showed strong inhibition on the enzyme isolated from isolate P58 (Fig. 10).

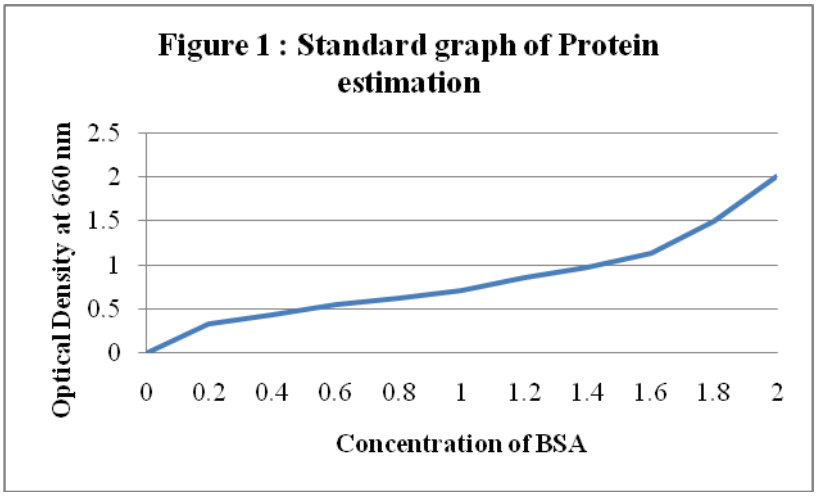
Figure 1 : Standard graph of Protein estimation
Optical Density at 660 nm
2.5
2
1.5
1
0.5
0
0 0.2 0.4 0.6 0.8 1 1.2 1.4 1.6 1.8 2
Concentration of BSA

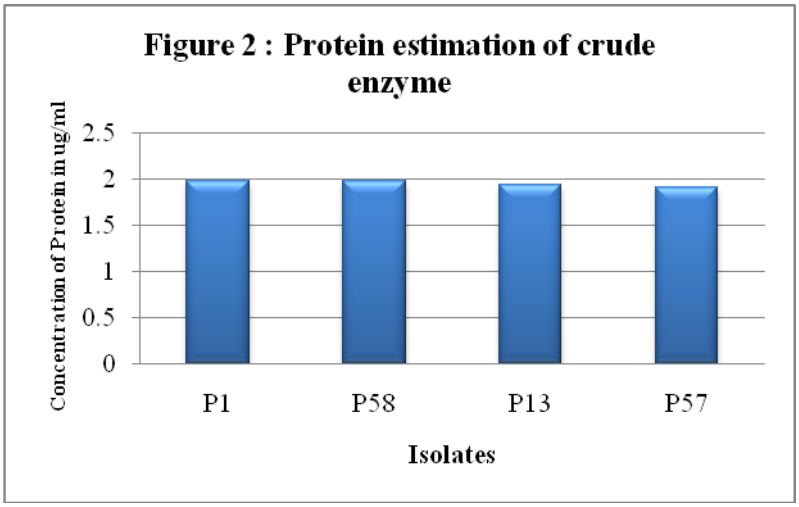
Figure 2 : Protein estimation of crude enzyme
Concentration of Protein in ug/ml
2.5
2
1.5
1
0.5
0
P1 P58 P13 P57
Isolates

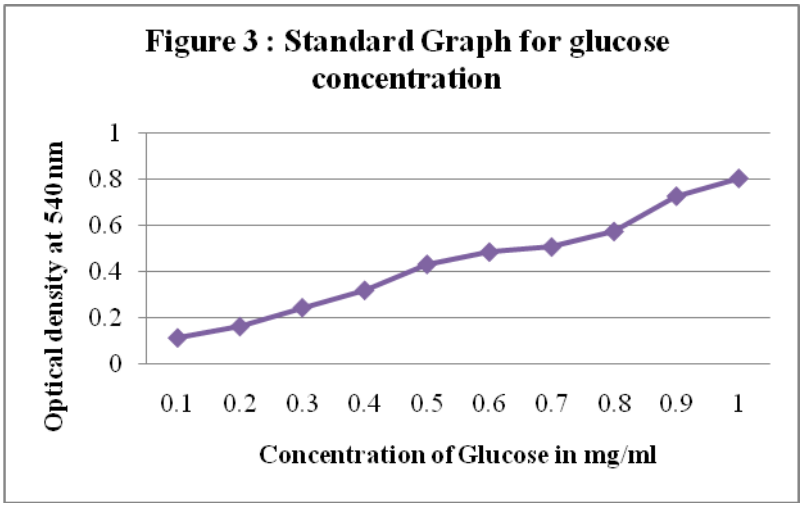
Figure 3 : Standard Graph for glucose concentration
Optical density at 540 nm
1
0.8
0.6
0.4
0.2
0
0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1
Concentration of Glucose in mg/ml

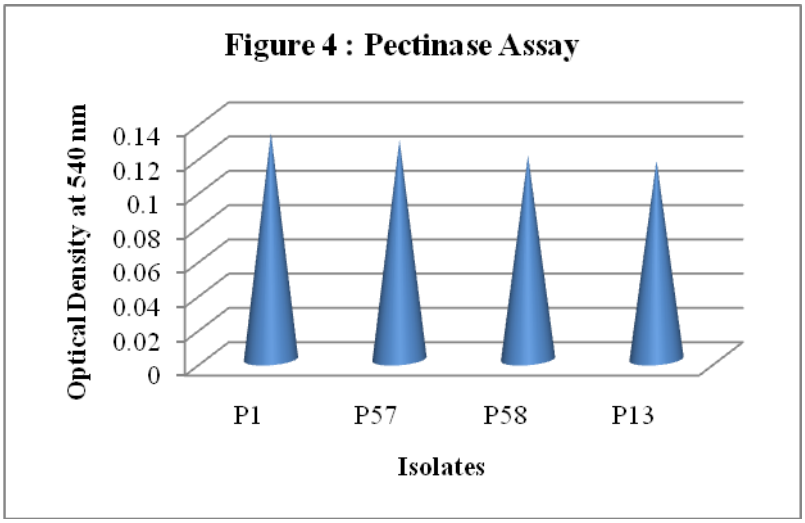
Figure 4 : Pectinase Assay
Optical Density at 540 nm
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
P1
P57
P58
P13
Isolates

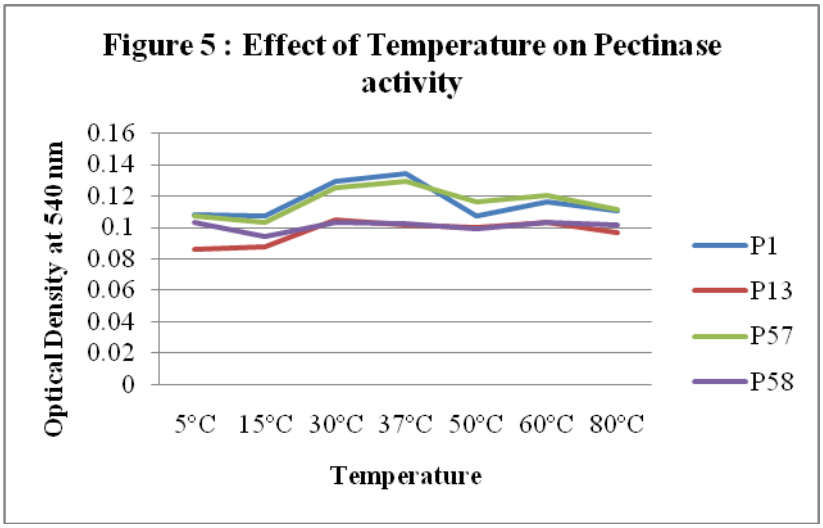
Figure 5 : Effect of Temperature on Pectinase activity
Optical Density at 540 nm
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
5°C
15°C
30°C
37°C
50°C
60°C
80°C
Temperature
P1
P13
P57
P58

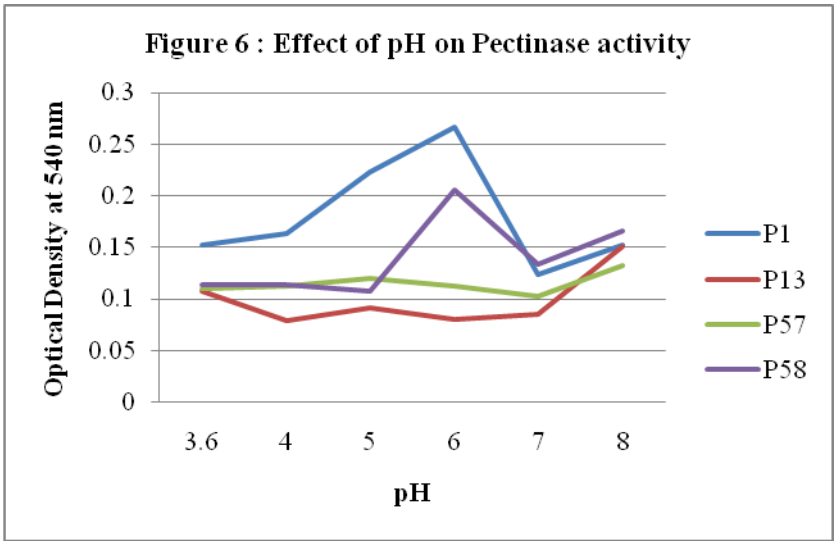
Figure 6 : Effect of pH on Pectinase activity
Optical Density at 540 nm
0.3
0.25
0.2
0.15
0.1
0.05
0
3.6
4
5
6
7
8
pH
P1
P13
P57
P58

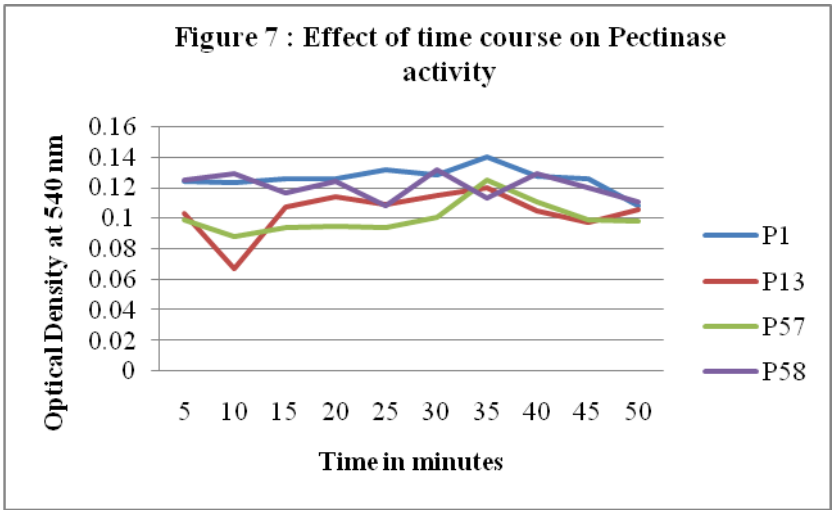
Figure 7 : Effect of time course on Pectinase activity
Optical Density at 540 nm
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
5 10 15 20 25 30 35 40 45 50
Time in minutes
P1
P13
P57
P58

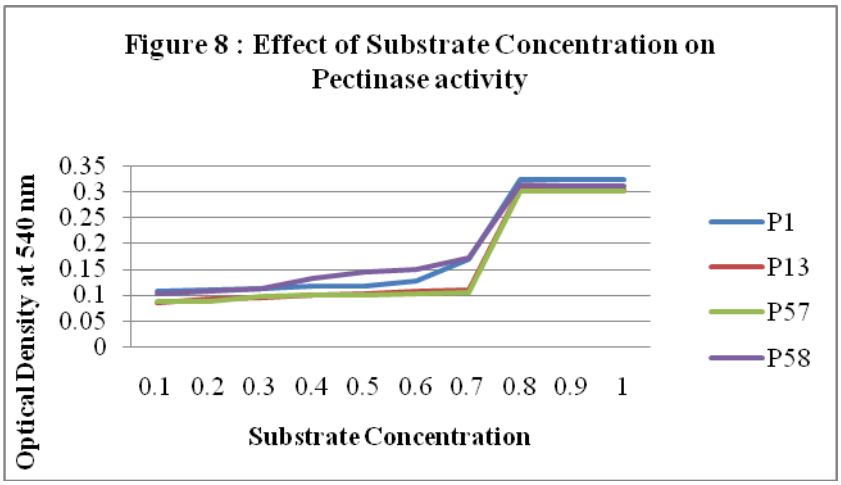
Figure 8 : Effect of Substrate Concentration on Pectinase activity
Optical Density at 540 nm
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1
Substrate Concentration
P1
P13
P57
P58

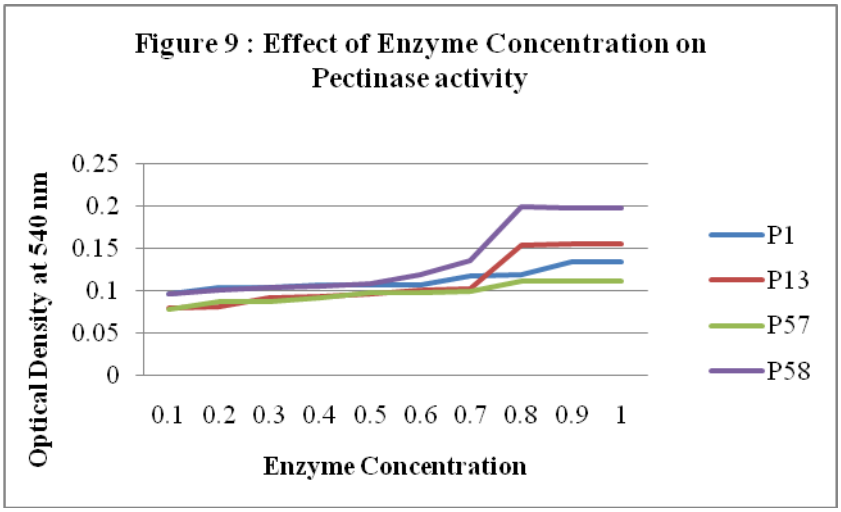
Figure 9 : Effect of Enzyme Concentration on Pectinase activity
Optical Density at 540 nm
0.25
0.2
0.15
0.1
0.05
0
0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1
Enzyme Concentration
P1
P13
P57
P58

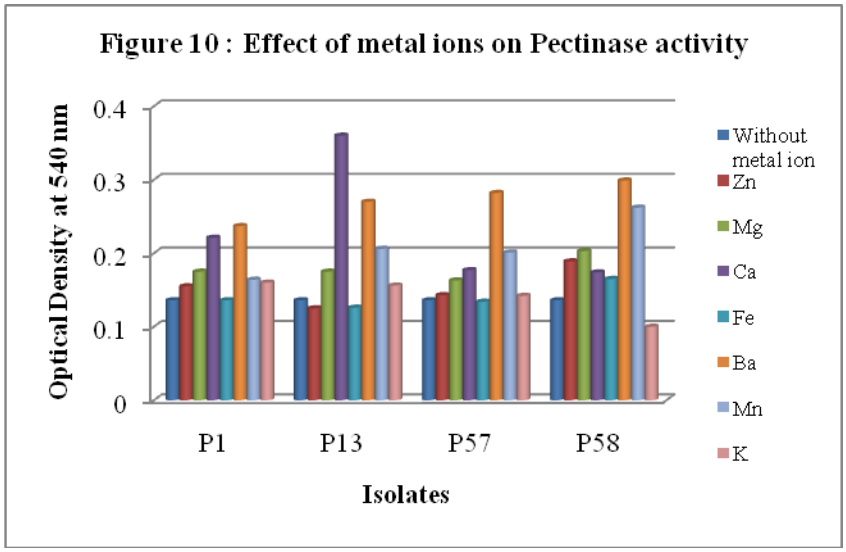

Photo no. 1 : Application of pectinase in fruit juice clarification:

Control **Test**

DISCUSSION:

Enzyme production is one of the broad areas of biotechnology which accounts for about 1.5 billion of the world market. The enzymes of microbial origin were found to be more advantageous than others. Strains belonging to genus *Bacillus* have played an important role in biodegradation and bioconversion of various macro-molecules particularly pectin. In present investigation the optimization studies of pectinase was done. The extracellular pectinase was isolated from bacterial source.

The enzyme production was done by shake flask fermentation method (Mehta *et al*., 2013). The crude enzyme was utilized for protein estimation by Folin-Lowry method. The highest protein content was found in crude extract of P1 (*Bacillus firmus*) that was 1.98 µg/ml and lowest protein content was found for P57 (*Bacillus endophyticus*) 1.525 µg/ml. Enzyme assay of crude enzyme was done and highest activity was found by P1 (*Bacillus firmus*) and lowest activity was found by P13 (*Bacillus coagulans*). This is in accordance with Roosdiana *et al.*, in 2013 worked on pectinase from *Bacillus firmus*.

In the study, it was found that rise in temperature increases the activity upto certain limit and after that it decreases. The influence of temperature on pectinase activity is shown in Fig.5. The maximum activity was recorded at 30°C for *B. coagulans* and *B. vietnamensis* and 37°C for *B. firmus* and *B. endophyticus.* Below or above these temperature values the activity was less. These temperature values are in concordance with some other studies like Roosdiana *et al*., (2013) who reported *B. firmus* pectinase showed maximum activity at 50°C. Torimiro *et al*., (2013)

also reported pectinases from various *Bacillus species* were most active at 50°C & 60°C . Some findings support our study that they reported maximum pectinase activity can be obtained at 37°C and 35°C too (Namasivayam *et al*., 2011; Banu *et al*., 2010)

The effect of pH on enzyme activity was also studied (Fig. 6). It was found that optimum pectinase activity was recorded at alkaline pH 8 for *B. coagulans*, and *B. endophyticus*. Other reports also have shown pectinase activity to be highest at alkaline pH (Torimiro and Okanji, 2013). While *Bacillus firmus* and *Bacillus vietnamensis* showed maximum activity at acidic pH 6. This is supported by reports of Banu *et al*., (2011); Sores *et al*., (1999) & Ghani *et al*., (2013) that maximum pectinase activity can be obtained at slightly acidic pH. This is in concordance with the study of Roosdiana *et al*., (2013) who found *B. firmus* pectinase was most active at neutral pH 7. This may happen because at this pH range, ionization of active site of pectinase (-COOH) can gradually increase following the increase of pH condition.

In addition to pH and temperature effect of time course on pectinase activity was also studied. It was observed that for *B. firmus* (P1) & *B . vietnamensis* (P58) the reaction time was 35 minutes while for *B. coagulans* (P13) it was 30 minutes and for *B . endophyticus* (P57) it was 40 minutes. At this reaction times pectinase activity reached maximum product and enzyme was saturated with substrates for these isolates. This fact is also supported by other studies (Torimiro and Okanji., 2013).

In the study metal ions Zn^{++}, Mg^{++}, Ca^{++}, Ba^{++}, Mn^{++} and K^{+} was found to enhance the activity of pectinase from the isolates while Fe^{++} showed slight inhibitory effect on the enzymes. Similarly, Ca^{+}, Mg^{++}, Mn^{++} and Zn^{++} were reported to activate pectinase from *Penicillin italicum* but was inhibited by Cu^{++} and Fe^{++} (Alana *et al*., 1990). Banu *et al*., (2010) observed little effect of Mg^{++} and Ca^{++} on pectinase from *P. chrysogenum* while Ca^{++} enhanced the production of pectinase by *B. cereus* obtained from market solid waste (Namasivayam *et al*., 2011). The application of pectinase from the isolates was studied for apple fruit juice clarification. The isolates showed good activities by clarifying it as compared to control

CONCLUSION :

The demand of industrially important enzymes in food industries is rising enormously. So, research should be done to alleviate the demand of these enzymes. Pectin degrading enzyme like pectinase has received a great attention because of their wide application and economic benefits. The *Bacillus species* was found to be potent producers of pectinase. The isolate *Bacillus firmus* showed highest activity than others. The pectinase isolated from two isolates *B. coagulans* and *B. endophyticus* showed maximum activity at pH 8 which is slightly alkaline pH, possibly making it a better source for industrial purpose.These enzymes may be scaled up for juice production after careful investigations.

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