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Partial Purification and Characterization of Cellulase, Pectinase and Xylanase from *Penicillium chrysogenum*

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Abstract : *Today's growing industrial world is in great need of the enzymes that can effectively process the plant fibers. Especially in the textile industries, the processing of these fibers is of great need wherein the process mainly consists of the degradation of compound such as cellulose, xylan and pectin. Since textile processing industries have become one of the major causes for environmental pollution. Cellulases, pectinase and xylanase enzymes that can effectively hydrolyze polysaccharides are of great interest in industrial point of view. These enzymes are optimally produced from the fungus Penicillium chrysogenum using single factorial design. Results of the single factorial design can be scaled-up for the future large-scale and an eco-friendly processing in textile and many other industries. These enzymes were also partially purified, and characterized.*
Keywords: *cellulose, pectin, xylan, cellulase, pectinase, xylanase, Penicillium chrysogenum*

I. Introduction

Enzymes are biocatalysts, which speed up the rates of reactions without undergoing any permanent change. Enzymes differ from chemical catalysts at least in two ways: i) they work at room temperature, and ii) 1 atmospheric pressure. Enzymes are classified into 6 major groups and hydrolytic enzymes find various applications in the industries such as food, agriculture and textile. Plant cell wall mainly consists of cellulose, hemicelluloses, and pectin. A wide variety of enzyme are used to breakdown the aforesaid carbohydrates. Break down of these polysaccharides is achieved by cellulases, pectinases and xylanases, respectively. These enzymes are widely used in the production and clarification of fruit juices, processing of plant-based animal feeds, extraction oils from oil seeds and other textile applications.

1.1 Cellulase

Cellulases or beta-1,4-glucanases (EC 3.2.1.4) are a class of hydrolytic enzymes that catalyze the hydrolysis of cellulose. Cellulase is a group enzymes comprising of endo-ß-glucanase (EC 3.2.1.4), exo-ß-glucanase (EC 3.2.1.91) and ß-glucosidase (EC 3.2.1.21). These enzymes are majorly extracellular and are produced by a wide range of bacteria and fungi. Their main substrate is the cellulose which can be abundantly found in the plant cell walls and hence their processing needs the degradation of these carbohydrates. Hence the successful hydrolysis of the cellulosic materials majorly depends on its nature, source and the optimal conditions of the enzyme acting on it.

1.2 Structure of cellulose

Cellulose is a polymer of β-1,4 linked glucose units. The structure and function of the enzymes that degrade cellulose can be well studied by recombinant DNA techniques. It has been found that the cellulases which are capable of breaking down cellulose have two or more structural and functional domains. The hydrolysis of the cellulose happens through its binding with the cellulose binding domain which is joined to the catalytic domain of the cellulases [1]. Lignocellulosic biomass has numerous structural features that make it very difficult to deconstruct enzymatically. The majority of biopolymers, including cellulose, hemicellulose and lignin, are not just individual units in a plant cell wall but are intimately interconnected. The main commercial purpose of enzymatic hydrolysis of cellulose is to deconstruct cellulose and other carbohydrate polymers into fermentable sugars, including glucose and oligomers that can be further converted into valuable products through biological or chemical approaches. Its crystalline structure and insoluble nature represents a big challenge for enzymatic hydrolysis [2]. It has been reported that the hydrolysis of cellulose by microbial cellulases considerably increased with the decrease in the substrate's crystalline nature [3].

1.3 Pectinases

Pectinases are a group of enzymes that can hydrolyse or transform pectins, polysaccharide substrates that are found abundantly in plant cell walls. Pectinases are commonly called as polygalactouronases. It has been reported that the pectinases are abundantly found in many bacteria like *Bacillus subtilis* [4] and also in many fungi such as *Aspergillus niger* [5]. These pectinases have been increasingly used in industries such as

food, textile and paper. In textile industries, pectinases have been greatly used in the processes such as scouring of cotton fabrics, the removal of pectin and wax compounds from them [6].

1.4 Structure of pectin

Pectins are a family of complex polysaccharides that contain 1,4-linked α-D-galactosyluronic acid residues. They have a jelly-like texture which help greatly in holding the plant cells together and are present in the middle lamella. Hence pectinase enzymes are often used in the degradation of plant materials and their processing. The primary wall of pectin exists as a continuous three dimensional cross-linked network. The functional characteristics of this network depend on the chemical structures, the conformations, and the interactions of the individual macromolecules [7]. It has been reported that the hydrolysis of pectin increased with the decrease in the methylation of pectin [8].

1.5 Xylanases

Xylanases are a class of enzymes that are majorly involved in the degradation of plant matter into usable nutrients. This is done by xylanases by specifically hydrolysing the 1,4-β-D-xylosidic linkages in xylans that are a structural component of plant cell walls. Arabinoxylans are the highly branched xylans that occur in the rice and wheat flour. Hence, the breakings down of these highly complex polysaccharides have gained a lot of importance lately. These industrially important xylanases have been reportedly found in many bacteria and fungi.

1.6 Xylan

Xylans are those complex polysaccharides that are present majorly in plants along with the above cellulose and pectin. They contain β-D-xylose units as monomers and hence yield xylose on hydrolysis. The main chain of xylan in composed of β-xylopyranose residues. Xylans also posses side chains where the softwood xylans have fewer acidic side chains than the hardwood xylans [9].

II. Materials and Methods

2.1 Chemicals:

Birchwood xylan and carboxymoethyl cellulose (CM cellulose) were purchased from Sigma-Aldrich chemicals, United States.

2.2 Growth of *Penicillium chrysogenum:*

The *Penicillium chrysogenum* culture was grown on the commercially available Czepak-dox broth. Initially 100 ml broth was prepared in each of 3 conical flasks at the specified substrate concentrations and autoclaved. The sterilized flasks were inoculated and flasks were incubated on an orbital shaker for 3 days at 28±2°C. At the end of the 3rd day were filtered through Whatman filter paper No.1 and the filtrate thus obtained was used as sources of enzyme for the further studies.

2.3 Enzyme Assay:

2.3.1 Assay of Cellulase:

The reaction mixture contained 0.025 ml of the enzyme, 1.5 ml acetate buffer (pH 5.0, 0.2 M), and 1ml cellulose (1%, w/v) as substrate. The mixture was incubated at 50°C for 15 minutes and then the reaction was arrested by adding 3 ml of DNS reagent followed by its boiling the tubes exactly for 5 minutes. The absorbance of the solution was measured at 540 nm.

2.3.2 Assay of Pectinase: The reaction mixture contained 0.5 ml of the enzyme, 1.5 ml acetate buffer (pH 5.0, 0.2 M), and 1ml pectin (1%, w/v) as substrate. The mixture was incubated at 50°C for 15 minutes and then the reaction was arrested by adding 1ml of DNS reagent followed by its boiling for 5 minutes. The absorbance of the solution was measured at 540 nm.

2.3.3 Assay of Xylanase: The reaction mixture contained 0.025 ml of the enzyme, 1.5 ml acetate buffer (pH 5.0, 0.2 M), and 1ml birchwood xylan (1%, w/v) as substrate. The mixture was incubated at 50°C for 15 minutes and then the reaction was arrested by the addition of 3 ml DNS reagent followed by its boiling for 5 minutes. The absorbance of the solution was measured at 540 nm.

2.4 Characterization of enzymes

2.4.1 Cellulase

2.4.1.1 Determination of optimum temperature:

The 3 days grown fungal broth was filtered through Whatman filter paper No.1 and the filtrate obtained was assayed for the cellulase activity. The reaction mixture in duplicate were incubated separately at 30°C, 40°C, 45°C, 50°C, 55°C, 60°C, 65°C, and 70°C for 15 min. The reaction was then arrested by adding DNS and the tubes kept in a water bath at 100°C. The absorbance of the solution was measured at 540 nm.

2.4.1.2 Determination of optimum pH:

The 3 days grown fungal broth was filtered through Whatman filter paper no.1 and the filtrate obtained was assayed for the cellulase activity. The reaction mixtures were prepared with buffers of different pH 3.4, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and were incubated for 15 minutes at the optimum temperature. The reaction was then arrested by adding DNS and boiled at 100°C. The colour obtained was then read spectrophotometrically at 540 nm.

2.4.1.3 Optimization of substrate concentration for cellulase production

The Czapek-Dox broth was prepared at different concentrations (1%, 2.5%, 5%, 7.5%, and 10%, w/v) in five different conical flasks. The flasks were autoclaved and inoculated with three days grown *Penicillium chrysogenum* mycelia. Inoculated flasks were placed on an orbital shaker for 5 days at 28±2°C and then filtered through Whatman filter paper no.1. The filtrate thus obtained was assayed for enzyme activity.

2.4.1.4 Optimization of inoculum size for cellulase production

The Czapek Dox broth was prepared at five different conical flasks with the optimum substrate concentration. The flasks were autoclaved and inoculated with *Penicillium chrysogenum* by varying the inoculum size 5%, 7.5%, 10%, and 12.5% (v/v). Inoculated flasks were placed on an orbital shaker for 5 days at 28±2°C and 120 rpm. At the end of 5th day, fermented broth was filtered through Whatman filter paper no.1. The filtrate was assayed for enzyme activity.

2.4.1.5 Optimization of pH for cellulase production

The Czapek-Dox broth was prepared at six different conical flasks with the optimum substrate concentration. The pH of the medium in six conical flasks was adjusted to pH 3, 4, 5, 6, 7, and 8. The flasks at different pH were autoclaved and were inoculated with the optimum inoculum size. Inoculated flasks were placed on an orbital shaker for 5 days at 28±2°C and 120 rpm. At the end of 5th day, fermented broth was filtered through Whatman filter paper no.1. The filtrate was used as source of cellulose enzyme and assayed for enzyme activity.

2.4.2 Pectinase

2.4.2.1 Determination of optimum temperature:

The 5 days grown fungal broth was filtered through Whatman filter paper no.1 and the filtrate obtained was assayed for the pectinase activity. The reaction mixture in duplicate were incubated separately at 30°C, 40°C, 45°C, 50°C, 55°C, 60°C, 65°C, and 70°C for 15 min. The reaction was then arrested by adding DNS and the tubes kept in a water bath at 100°C. The absorbance of the solution was measured at 540 nm.

2.4.2.3 Determination of optimum pH:

The 3 days grown fungal broth was filtered through Whatman filter paper no.1 and the filtrate obtained was assayed for the pectinase activity. The reaction mixtures were prepared with buffers of different pH 3.4, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and were incubated for 15 minutes at the optimum temperature. The reaction was then arrested by adding DNS and boiled at 95°C. The colour obtained was then read spectrophotometrically at 540 nm.

2.4.2.4 Optimization of substrate concentration for pectinase production

The Czapek-Dox broth was prepared at different concentrations 1%, 2.5%, 5%, 7.5%, and 10% (v/v) in five different conical flasks. The flasks were autoclaved and inoculated with *P. chrysogenum.* The cultures were incubated on an orbital shaker for 5 days and then the contents were filtered. The filtrate thus obtained was source of enzyme and was assayed at the optimum temperature and pH for the pectinase production at these different substrate concentrations.

2.4.2.5 Optimization of inoculum size for pectinase production

The Czapek-Dox broth was prepared at five different conical flasks with the optimum substrate concentration. The flasks were autoclaved and inoculated with *Penicillium chrysogenum* by varying the inoculum size (5%, 7.5%, 10%, and 12.5%, v/v). The cultures were incubated on an orbital shaker for 5 days and then filtered and assayed at the optimum temperature and pH for the pectinase production at these different inoculum sizes.

2.4.2.6 Optimization of pH for pectinase production

The Czapek-Dox broth was prepared at six different conical flasks with the optimum substrate concentration. The pH of the medium in six conical flasks were adjusted to pH 3, 4, 5, 6, 7, and 8 and then autoclaved. The flasks at different pH were inoculated individually with 7% inoculum. Inoculated flasks were placed on an orbital shaker for 5 days at 28±2°C and 120 rpm. At the end of 5th day, fermented broth was then filtered through Whatman filter paper no.1. The filtrate was assayed for enzyme activity.

2.4.3 Xylanase

2.4.3.1 Determination of optimum temperature:

The 5 days grown fungal broth was filtered through Whatman filter paper no.1 and the filtrate obtained was assayed for the cellulase activity. The reaction mixture in duplicate were incubated separately at 30°C, 40°C, 45°C, 50°C, 55°C, 60°C, 65°C, and 70°C for 15 min. The reaction was then arrested by adding DNS and the tubes kept in a water bath at 100°C. The absorbance of the solution was measured at 540 nm.

2.4.3.3 Determination of optimum pH:

The 5 days grown fungal broth was filtered through Whatman filter paper no.1 and the filtrate obtained was assayed for the cellulase activity. The reaction mixtures were prepared with buffers of different pH 3.4, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0. The tubes were then incubated for 15 minutes at the optimum temperature. The reaction was then arrested by adding DNS and boiled at 100°C. The orange-red color obtained was then measured spectrophotometrically at 540 nm.

2.4.3.4 Optimization of substrate concentration for xylanase production

The Czapek-Dox broth was prepared at different concentrations (1%, 2.5%, 5%, 7.5%, and 10%, w/v) in five different conical flasks. The flasks were autoclaved and inoculated with three days grown *P.chrysogenum* mycelia. Inoculated flasks were placed on an orbital shaker for 5 days at 28±2°C and 120 rpm. At the end of 5th day, fermented broth was filtered through Whatman filter paper no.1. The filtrate thus obtained was assayed for enzyme activity.

2.4.3.5 Optimization of inoculum size for xylanase production

The Czapek-Dox broth was prepared at five different conical flasks with the optimum substrate concentration. The flasks were autoclaved and inoculated with *Penicillium chrysogenum* by varying the inoculum size 5%, 7.5%, 10%, and 12.5% (v/v). Inoculated flasks were placed on an orbital shaker for 5 days at 28±2°C and 120 rpm. Then the contents were filtered through Whatman filter paper no.1. The filtrate thus obtained was assayed for enzyme activity.

2.4.3.6 Optimization of pH for xylanase production

The Czapek-Dox broth was prepared at six different conical flasks with the optimum substrate concentration. The pH of the medium in six conical flasks was adjusted to pH 3, 4, 5, 6, 7, and 8. The flasks at different pH were autoclaved and inoculated with the optimum inoculum size. Inoculated flasks were placed on an orbital shaker for 5 days at 28±2°C and 120 rpm. At the end of fifth day, contents were filtered through Whatman filter paper no.1. The filtrate was assayed for enzyme activity.

2.5 Partial Purification

2.5.1 Ammonium sulphate fractional precipitation

All the purification steps were carried out at 0 to 4°C to prevent the denaturation of enzymes. The culture filtrate was used as an enzyme source. Solid ammonium sulphate (75%, w/v) was added to the cell-free culture filtrate with gentle stirring and the resultant solution was kept at 0 to 4°C at least for 6 h and the resultant precipitate was collected by centrifugation at 10,000 rpm for 20 minutes at 4°C. Then the precipitate was dissolved in a small volume of 0.2 M acetate buffer, pH 5.5 and centrifuged. Then the supernatant was dialyzed against 25 mM acetate buffer (pH 5.5) at 4°C to remove the salts.

2.5.2 Acetone Precipitation

Acetone precipitation was carried out by using the chilled acetone (-20°C) as a solvent. Chilled acetone was added to cell-free fermented broth in the ratio 1:2 slowly with continuous stirring and the mixture was stored at 4°C for 4 h. After 4 h, the protein precipitate in the bottom layer containing the protein was recovered by its centrifugation at 10,000 rpm and 4°C for 20 min. The precipitate in the centrifuge tubes were kept open for complete evaporation of acetone, because even trace amount of acetone in the precipitate prevents the solubility of enzymes. Precipitate in the centrifuge tubes were dissolved in minimum amount of buffer, vortexed, centrifuged 10,000 rpm and 4°C for 20 min. The clear supernatant thus obtained dialyzed and was stored at 4°C until further use.

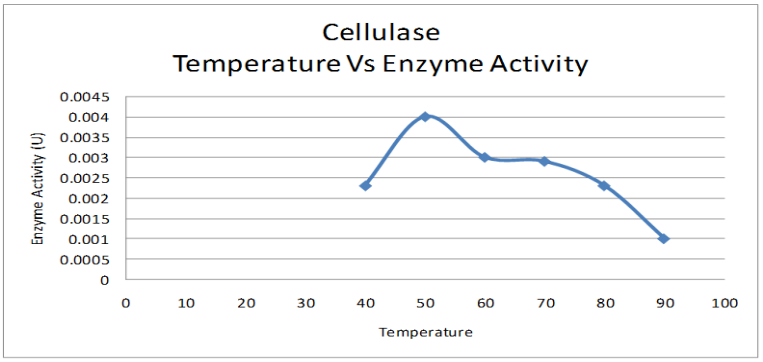
III. Results and Discussions

3.1 Characterization of Enzymes

3.1.1 Cellulase

3.1.1.1 Optimization of Temperature for Cellulase Activity

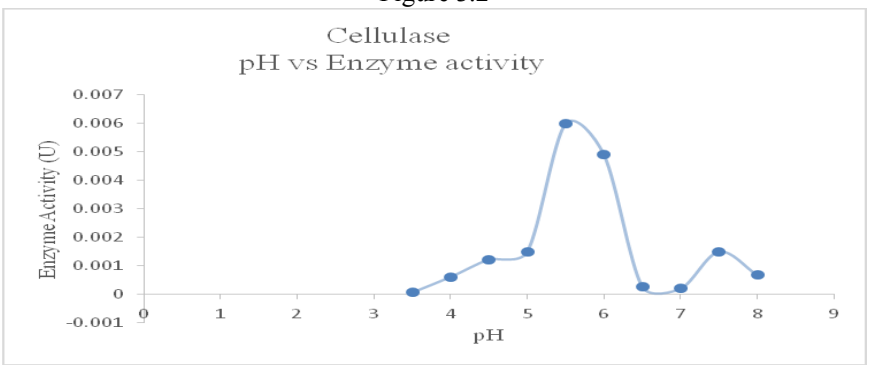
Figure 3.1



It is evident from Fig. 3.1 that cellulase enzyme exhibited its optimal activity at 50°C.

3.1.1.2 Optimization of pH for Cellulase Activity

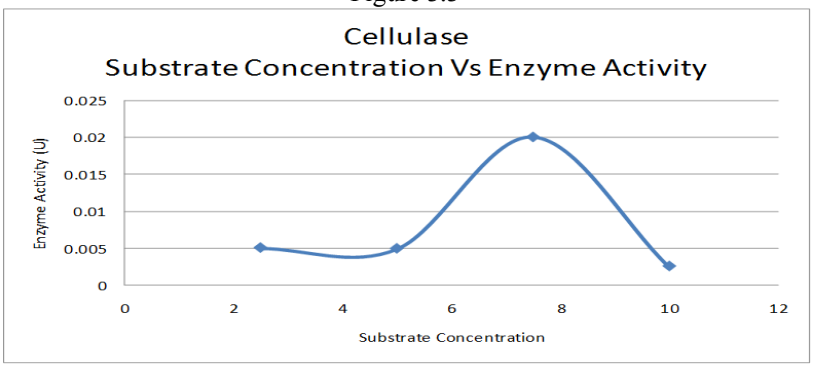
Figure 3.2



It is evident from Fig. 3.2 that the maximum cellulase activity was observed with the buffer of pH 5.5

3.1.1.3 Optimization of Substrate Concentration for Cellulase Production

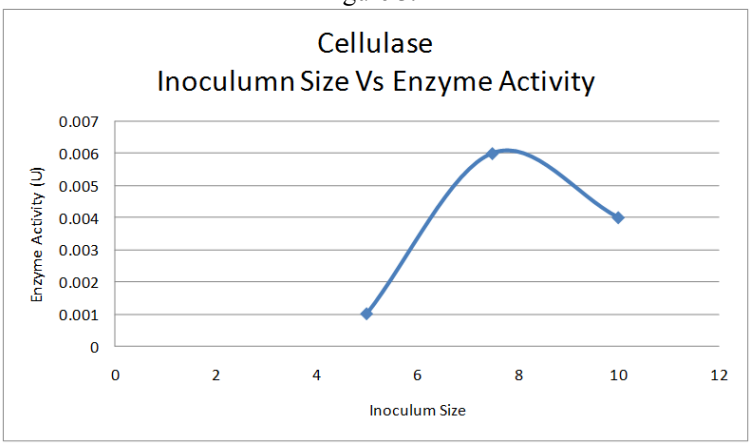
Figure 3.3



It is evident from Fig. 3.3 that that the maximum cellulase production from *P.chrrysogenum* was observed at 7.5% (w/v) Czapek-Dox broth.

3.1.1.4 Optimization of Inoculum Size for Cellulase Production

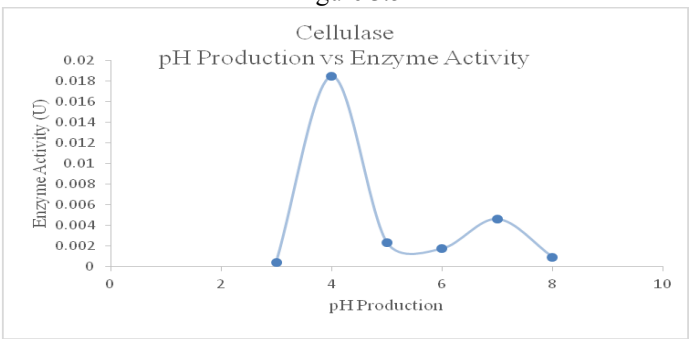
Figure 3.4



It is evident from Fig. 3.4 that the maximum cellulase production from *P.chrrysogenum* was observed at 7.5% (v/v) inoculum.

3.1.1.5 Optimization of pH for Cellulase Production

Figure 3.5

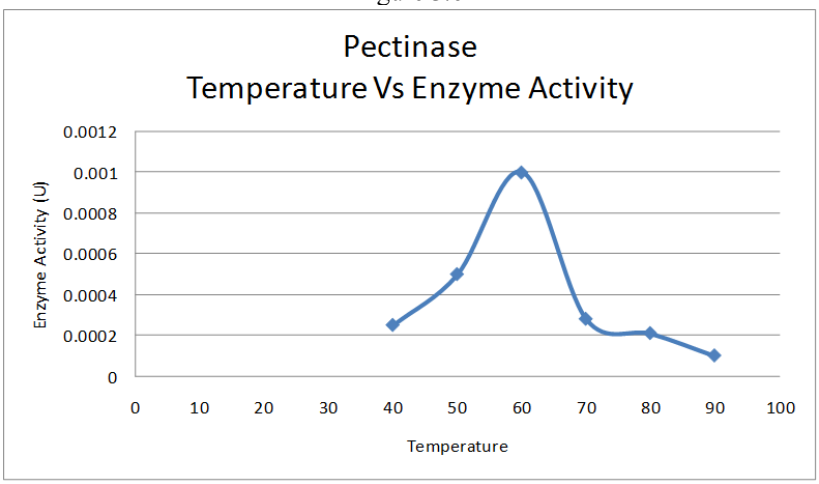


It is evident from the Fig. 3.5 that the maximum cellulase production from *P.chrrysogenum* was observed at pH 4.

3.1.2 Pectinase

3.1.2.1 Optimization of Temperature for pectinase Activity

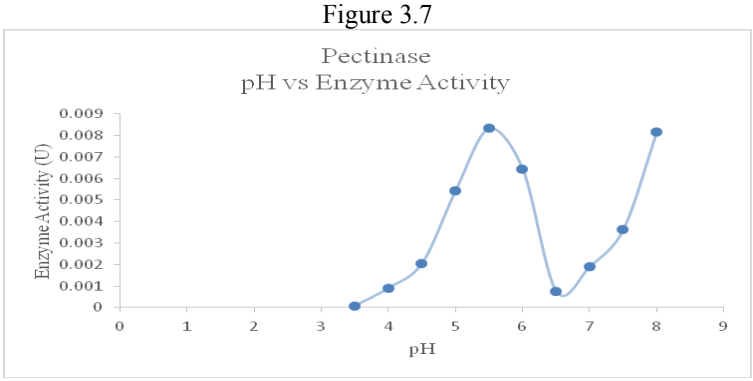
Figure 3.6



It is evident from Fig. 3.6 that the pectinase enzyme from *P.chrysogenum* exhibited its maximum activity at 60°C.

3.1.2.2 Optimization of pH for Pectinase Activity

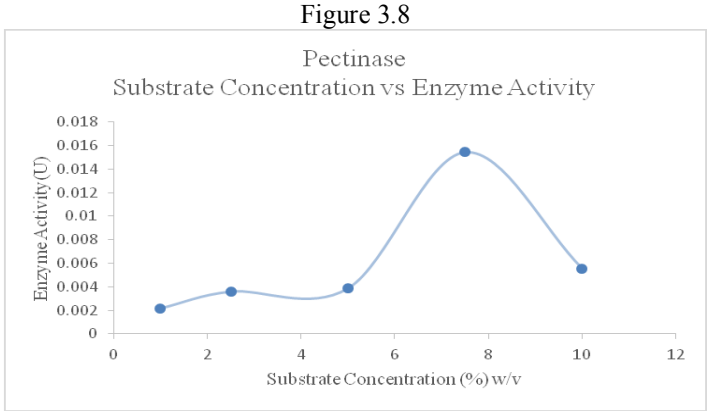
Figure 3.7



It is evident from Fig. 3.7 that pectinase enzyme from *P.chrysogenum* exhibited its optimal activity at pH 5.5.

3.1.2.3 Optimization of Substrate Concentration for Pectinase Production

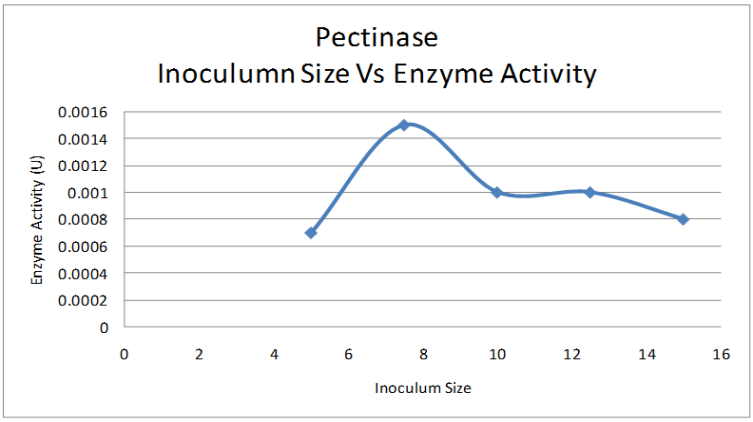
Figure 3.8



It is evident from Fig. 3.8 that the maximal pectinase production from *P.chrrysogenum* was observed at 7.5% (w/v) Czapek -Dox broth concentration.

3.1.2.4 Optimization of inoculum size for pectinase production

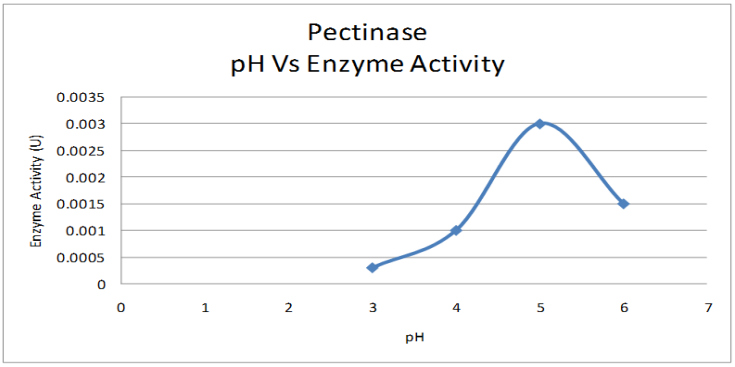
Figure 3.9



It is evident from Fig. 3.9 that the maximal pectinase production from *P. chrrysogenum* was observed at 7.5% (v/v) inoculum.

3.1.2.5 Optimization of pH for Pectinase Production

Figure 3.10

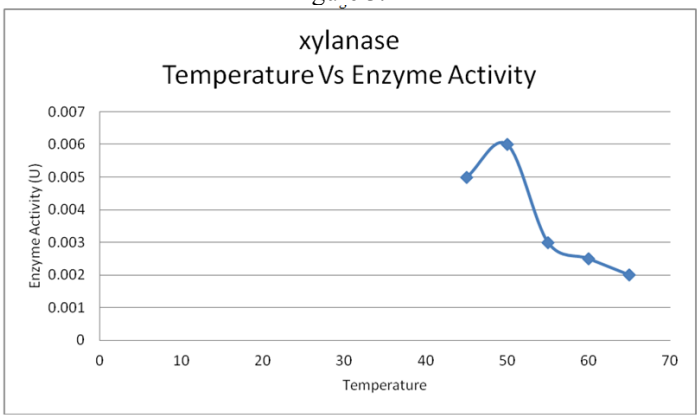


It is evident from Fig. 3.10 that optimal pectinase production from *P. chrrysogenum* was observed at pH 5.

3.1.3 Xylanase

3.1.3.1 Optimization of Temperature for Xylanase Activity

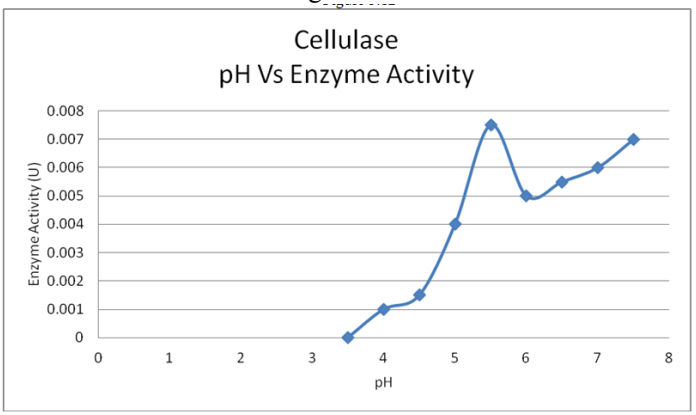
Figure 3.11



It is evident from Fig. 3.11 that xylanase enzyme from *P.chrysogenum* was exhibited its optimal activity at 50°C.

3.1.3.2 Optimization of pH for Xylanase Activity

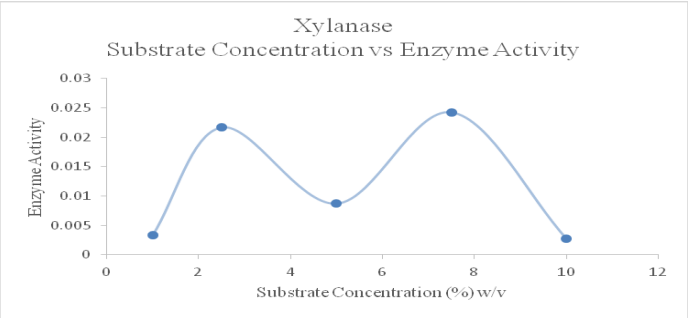
Figure 3.12



It is evident from Fig. 3.12 that the xylanase enzyme from *P.chrysogenum* was exhibited its optimal activity at pH 5.5.

3.1.3.3 Optimization of Substrate Concentration for Xylanase Production

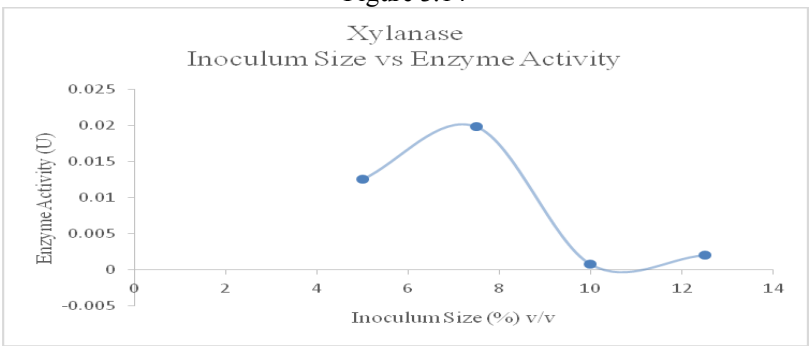
Figure 3.13



It is evident from Fig. 3.13 that the maximal pectinase production from *Penicillium chrrysogenum* was observed at two substrate level (Czapaek-dox broth) concentrations (2.5% and 7.5, v/v).

3.1.3.4 Optimization of Inoculum Size for Xylanase Production

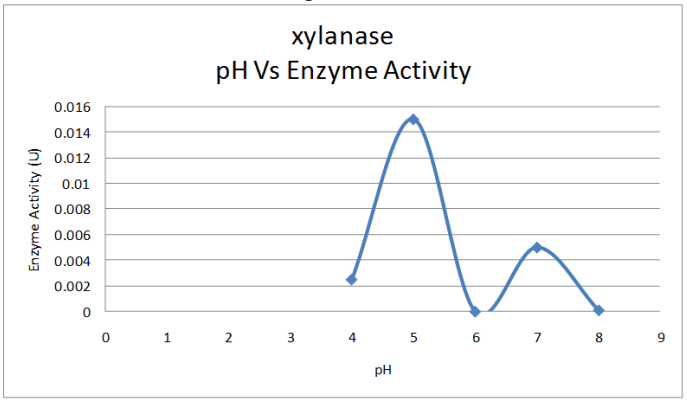
Figure 3.14



It is evident from Fig. 3.14 that the maximal xylanase production from *P. chrrysogenum* was observed at 7.5% (v/v) inoculum.

3.1.3.5 Optimization of pH for Xylanase Production

Figure 3.15



It is evident from Fig. 3.15 that the optimal xylanase production from *P.chrysogenum* was observed at pH 5

4.2 Partial Purification of Enzymes

4.2.1 Ammonium Sulphate Fractional Precipitation

Table 4.1 Cellulase

Purification step	Enzyme activity (U/ml)	Total activity (U)	Protein (mg/ml)	Specific Activity (U/mg)	Fold Purification	Yield (%)
Crude Enzyme	0.030	24	0.710	0.042	1	100
Ammonium Sulphate precipitation	0.015	12	0.510	0.029	0.693	69.3

Table 4.2 Pectinase

Purification step	Enzyme activity (U/ml)	Total activity (U)	Protein (mg/ml)	Specific Activity (U/mg)	Fold Purification	Yield (%)
Crude Enzyme	0.026	20.8	0.710	0.014	1	100
Ammonium Sulphate precipitation	0.016	12.8	0.510	0.032	0.88	88

Table 4.3 Xylanase

Purification step	Enzyme activity (U/ml)	Total activity (U)	Protein (mg/ml)	Specific Activity (U/mg)	Fold Purification	Yield (%)
Crude Enzyme	0.010	8	0.710	0.014	1	100
Ammonium Sulphate precipitation	0.002	1.6	0.510	0.0028	0.21	21

It has been found from table 4.1, 4.2 and 4.3 that cellulose, pectinase and xylanase enzymes from *P. chrysogenum* were precipitated using ammonium sulphate (at 75% saturation) to yield an active pellet. It is also evident from the above results that protein contaminants in the crude enzyme solution were removed.

4.2.2 Acetone Precipitation

Table 4.4 Cellulase

Purification step	Enzyme activity (U/ml)	Total activity (U)	Protein (mg/ml)	Specific Activity (U/mg)	Fold Purification	Yield (%)
Crude Enzyme	0.032	25.6	1.01	0.030	1	100
Acetone precipitation	0.014	11.2	0.96	0.014	0.467	46.7

Table 4.5 Pectinase

Purification step	Enzyme activity (U/ml)	Total activity (U)	Protein (mg/ml)	Specific Activity (U/mg)	Fold Purification	Yield (%)
Crude Enzyme	0.041	32.8	1.01	0.040	1	100
Acetone precipitation	0.026	20.8	0.96	0.027	0.677	67.7

Table 4.6 Xylanase

Purification step	Enzyme activity (U/ml)	Total activity (U)	Protein (mg/ml)	Specific Activity (U/mg)	Fold Purification	Yield (%)
Crude Enzyme	0.058	46.4	1.01	0.057	1	100
Acetone precipitation	0.037	29.6	0.96	0.03	0.676	67.6

Results from Table 4.4, 4.5, and 4.6 indicated that addition of ice chilled acetone to the crude enzyme precipitated out cellulose, pectinase and xylanase enzymes. Among the two methods tested, chilled acetone precipitated more enzymes from crude enzyme solution than using ammonium sulfate. Of the above purification techniques performed,

IV. Conclusion

The enzymes produced by *P.chrysogenum* were optimized by the single factorial design for their production. It was thus concluded that highest enzyme titres were obtained when the fungus was grown under the optimized conditions. Since these enzymes are a great deal of importance in the industries such as textile, food, leather for the processing of raw materials, it can be done by extraction of these enzymes by their mass production from *P.chrysogenum* under the optimized growth conditions. Use of chilled acetone for the precipitation of enzymes was preferred to ammonium sulfate because of two reasons: a) higher enzyme activity, and b) acetone can be recovered from the crude enzyme solution by distillation for further use. Plackette Burman and response surface methodology (RSM) methods should be followed during scale-up studies for the production of these enzymes.

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