PRODUCTION AND OPTIMIZATION OF EXTRACELLULAR FUNGAL PECTINOLYTIC ENZYMES FROM ASPERGILLUS ORYZAE USING APPLE PECTIN AS A SUBSTRATE

BY

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CERTIFICATE

This is to certify that the work entitled "production and optimization of extracellular fungal pectinolytic enzymes from Aspergillus Oryzae using apple pectin as a substrate", submitted by Pooja Raina and Jasmeen Kaur in partial fulfilment of for the award of degree of Bachelor of Technology in Bioinformatics of Jaypee University of Information Technology has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institution for the award of this or any other degree or diploma.

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ABSTRACT

Crude pectinolytic enzyme was produced by *Aspergillus Oryzae* in solid-state fermentation (SSF) using Apple pectin substrate. This substrate was chosen for its easy availability and low cost.Effect of different parameters such as temperature, pH, incubation time and pectin concentration on this enzyme preparation was studied.

The maximum yields obtained were – 11.56 U/mg at 4% pectin concentration, 11.53 U/mg at pH 5.0, 13.21 U/mg at 30° C, 15.31 U/mg in 96 hrs. Furthermore, increase in pectin concentration decreased the pH of media. Flasks gave more favorable results then petri plates.

CHAPTER 1

Introduction

1.1 PECTINOLYTIC ENZYMES

Pectinolytic enzymes or pectinases are a heterogeneous group of related enzymes that hydrolyze the pectic substances, present mostly in plants. They are of prime importance for plants as they help in cell wall extension and softening of some plant tissues during maturation and storage. They also aid in maintaining ecological balance by causing decomposition and recycling of waste plant materials. Pectinase enzymes include pectin methylesterase (pectin esterase) and depolymerising enzyme (polygalacturonase and lyases). Pectin esterase hydrolyses the pectin to methanol and polygalacturonic acid and the enzyme polygalacturonase further hydrolyses the polygalacturonic acid into monogalacturonic acid by breaking the glycosidic linkage.

Pectinases find extensive applications in fruit processing industries including clarification of fruit juices, wines, extraction of fruit juice, in the manufacturing of pectin free starch, or curing of coffee. (Evans et al, 2002)

Pectinolytic enzymes are used mainly in the food industry, particularly for fruit juice clarification and for the isolation of essential oils and pigments from citrus. They are also used in the textile industry to release cellulose fibers from fibrous stalks. (Rombouts et al, 1980)

These processes usually employ crude enzyme preparations containing more than one pectic enzyme, often requiring even cellulases and hemicellulases. These enzymes are mostly formed as a mixture or complex made up of hydrolases (depolymerases), lyases and esterase.

In addition to their industrial importance, pectinases are of interest because they participate in the infection process together with other plant cell wall-degrading enzymes, thus facilitating the installation of pathogens in plants. (Riou et al, 2000)

Pectinase is produced by several fungi including *Aspergillus* sp., *Botrytis cinerea, Fusarium moniliforme, Rhizoctonia solani, Rhizopus stolonifer, Trichoderma* sp., *Neurospora crassa, etc.*, but *Aspergillus* is the major source. Among pectinases, pectin esterase (E.C. 3.131.11) is the first enzyme of pectin hydrolysis that catalyses the hydrolysis of methylated carboxylic ester group in pectin into pectic acid and methanol.

TRIVIAL NAME	CLASSIFICATION	SOURCE	SYSTEMATIC NAMES (IUB)[1]
Pectinase[2]	Carbohydrase	(1) *Aspergillus niger* var.	(1) poly(1,4-α-D-galacturonide) glycanohydrolase
		(2) *Rhizopus oryzae* var.	(2) pectin pectylhydrolase
		(3) *Aspergillus aculeatus*	
		(4) *Aspergillus oryzae*[4] d- *Aspergillus aculeatus*	
		(5) *Aspergillus niger*[4] d-*Aspergillus niger*	
			(3) (1→4)-6-*O*-methyl-α-D-galacturonan lyase
			(4) (1→4)-6-O-methyl-α-D-galacturonan lyase
			(5) α-L-arabinofuranoside arabinofuranohydrolase
			(6) 1,5-α-L-arabinan 1,5-α-L-arabinanohydrolase
			(7) poly(1,4-α-D-galacturonide) galacturonohydrolase
			(8) arabinogalactan 4-β-D-galactanohydrolase
			(9) acetic-ester acetylhydrolase
			(10) (1→4)-α-D-galacturonan reducing-end-disaccharide-lyase

Table 1.1 Sources of pectin

1.2 SOLID STATE FERMENTATION (SSF)

The term solid state fermentation (SSF) is applied for the processes in which insoluble materials in water are used for the microbial growth (Moo-Young et al, 1983). In the fermentative processes of this type, the quantity of water should not exceed the capacity of saturation of the solid bed in which the microorganisms grow (Laukevics et al, 1984). Water is essential for the microbial growth and in SSF and it is present in thin layers and in occasions, absorbed inside the substrates (Mudgett R., 1986).

Solid state cultures have more advantages than liquid cultures: that is higher product yield; better product quality; cheaper product recovery and cheaper technology.

Solid state cultures (SSC) are becoming a viable alternative technique for large-scale industrial processes for enzyme production. Filamentous fungi are the most important group of microorganisms used in SSC processes owing to their physiological, enzymological and biochemical properties, the hyphal mode of fungal growth and their good tolerance to low water activity (Aw) and high osmotic pressure conditions. All these characteristics make fungi efficient and competitive for bioconversion of solid substrates (Gervais et al., 1988; Oriol et al., 1988a, 1988b). Also the hydrolytic enzymes which are excreted in this case are more concentrated than with liquid substrate culture (LSC) (Moo-Young et al., 1983).

This cultivation technique is acquiring a special relevancy in the field of the biotechnological processes, as an alternative to the traditional submerged fermentation (SmF), because has lower energy requirements, produces less wastewater, gives high product concentrations, avoids the foaming and has lower risks of contamination .In addition, SSF allows the use of different agricultural and agroindustrial residues as substrates such as wheat bran (Castilho et al., 1999; Soares et al., 1999; Singh et al., 1999), soy bran (Castilho et al., 2000), sugar cane bagasse (Acuña- Agüelles et al., 1995), lemon and orange peels (Garzón and Hours, 1992; Ismail, 1996, Martins et al., 2002, Silva et al., 2002 and 2005), etc
Of these characteristics some advantages and disadvantages of the SSF in comparison to the SmF are derived and presented (Hesseltine, C.W., 1977).

Advantages:

· The culture media are simple. Some substrates can be used direclty as a solid media or enriched with nutrients

· The product of interest is concentrated, that which facilitates its purification.

· The used inoculum is the natural flora of the substrates, spores or cells.

· The low humidity content and the great inoculums used in a SSF reduce vastly the possibility of a microbial contamination

· The quantity of waste generated is smaller than the SmF.

· The enzymes are low sensitive to catabolic repression or induction.

Disadvantages:

· The used microorganisms are limited those that grow in reduced levels of humidity.

· The determination of parameters such as humidity, pH, free oxygen and dioxide of carbon, constitute a problem due to the lack of monitoring devices. The scale up of SSF processes has been little studied and it presents several problems.

1.3 PECTIN

Pectin (Polymethyl galacturonate) is the polymeric material in which, at least, 75% of the carboxyl groups of the galacturonate units are esterified with methanol. It confers rigidity on cell wall when it is bound to cellulose in the cell wall.

Pectin has a very complicated and heterogeneous structure. The biosynthesis of such a complex structure probably requires 50-100 different enzymes.

Pectic substance is a generic name used for the compounds that are acted upon by the pectinolytic enzymes. They are high molecular weight, negatively charged, acidic, complex glycosidic macromolecules (polysaccharides) that are present in the plant kingdom. They are present as the major components of middle lamella between the cells in the form of calcium pectate and magnesium pectate.

Pectic substances mainly consist of galacturonans and rhamnogalacturonans in which the C-6 carbon of galactate is oxidized to a carboxyl group, the arabinans and the arabinogalactans. These substances are a group of complex colloidal polymeric materials, composed largely of a backbone of anhydrogalacturonic acid units.

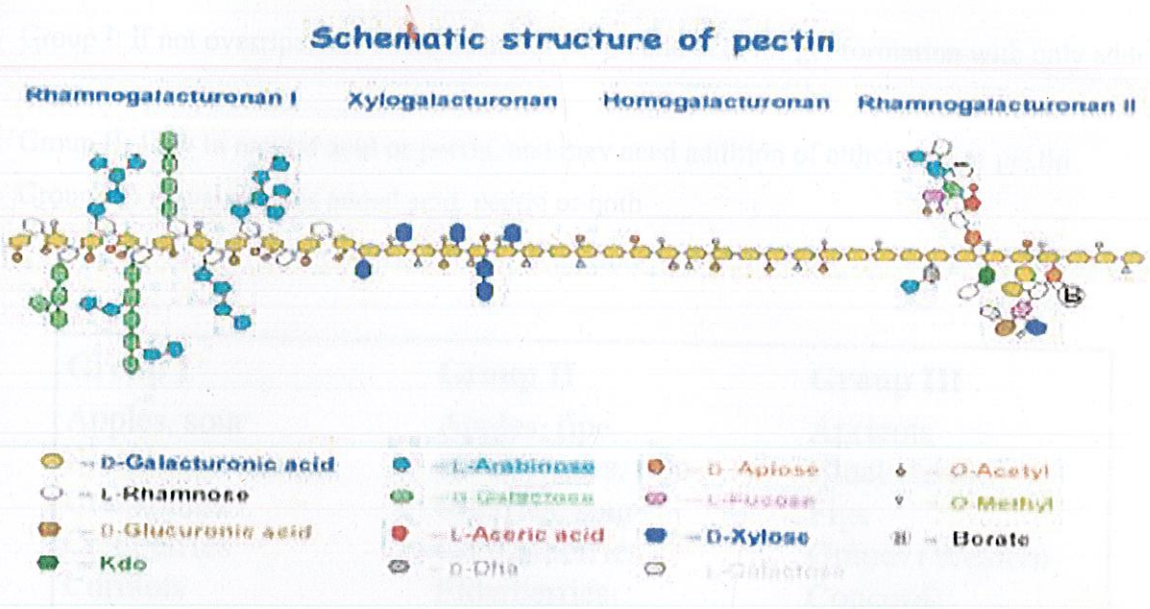

Figure 1.1: Schematic structure of pectin

Apple pulp is a rich source of pectin besides other nutrients; hence it can serve as an important natural substrate for pectinase production. The production of pectinase from apple mash is promising. It has several advantages like easy availability of cheaper raw material and easier processing of the substrate. Being economical, it may lead to reduction in cost of the enzyme and may prove as an efficient method of pomace utilization.

An important factor influencing the synthesis of pectic enzymes in the mixture is the medium composition, particularly the carbon and nitrogen sources. Studies have been conducted by different workers using both synthetic and complex media for the production of this enzyme. The synthesis of pectic enzymes is reported to be induced by either pectin or its derivatives or by complex forms such as beet-pulp, wheat bran, etc. On the Other hand, pectin has also been found to completely suppress the synthesis of the enzyme, while polygalacturonic acid is found to stimulate the enzyme synthesis.

Fruits have varying amounts of pectin. The chart below groups fruits according to the amount of natural pectin and acid found inside the fruit.

Group I: If not overripe, has enough natural pectin and acid for gel formation with only added sugar

Group II: Low in natural acid or pectin, and may need addition of either acid or pectin

Group III: Always needs added acid, pectin or both

Group I	Group II	Group III
Apples, sour	Apples, ripe	Apricots
Blackberries, sour	Blackberries, ripe	Blueberries
Crabapples	Cherries, sour	Figs
Cranberries	Chokecherries	Grapes (Western
Currants	Elderberries	Concord)
Gooseberries	Grapefruit	Guavas
Grapes (Eastern	Grape Juice, bottled	Peaches
Concord)	(Eastern Concord)	Pears
Loganberries	Grapes (California)	Plums (Italian)
Plums (not Italian)	Loquats	Raspberries
Quinces	Oranges	Strawberries

Table 1.2 Pectin and Acid Content of Common Fruits Used to Make Jelly

1.4 MECHANISM OF ACTION

Pectinase enzymes include pectin methylesterase (pectin esterase) and depolymerising enzyme (polygalacturonase and lyases). Pectin esterase hydrolyses the pectin to methanol and polygalacturonic acid and the enzyme polygalacturonase further hydrolyses the polygalacturonic acid into monogalacturonic acid by breaking the glycosidic linkage.

Pectin esterase

Pectin + H_2O → Pectate + CH3OH

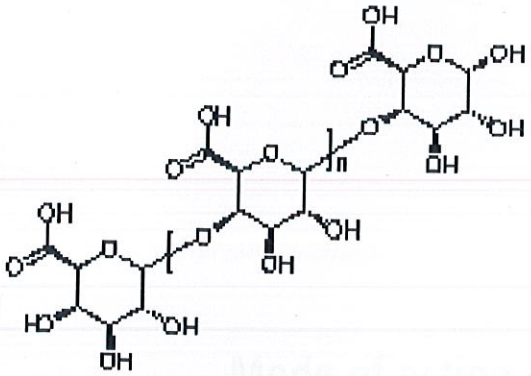

Figure 1.2 Pectate structure

The degradation of pectic substances involves the combined action of different pectinases, viz., esterases and depolymerises (hydrolases and lyases). Pectic transeliminases or pectic lyases are one among the pectinases, which degrade pectic substances by 3- elimination mechanism yielding 4:5 unsaturated oligogalacturonates. Pectin Lyase acting on pectin and polygalacturonate lyase or pectate lyase acting on polygalacturonic acid is two important transeliminases acting on pectic substances .Fungal strains are mainly found to produce pectate lyase and bacterial strains were used for the production of polygalacturonase .Pectin lyase and polygalacturonase can be differentiated by their substrate requirement and the absolute requirement of calcium for polygalacturonase activity (Henrissat et al., 1995).

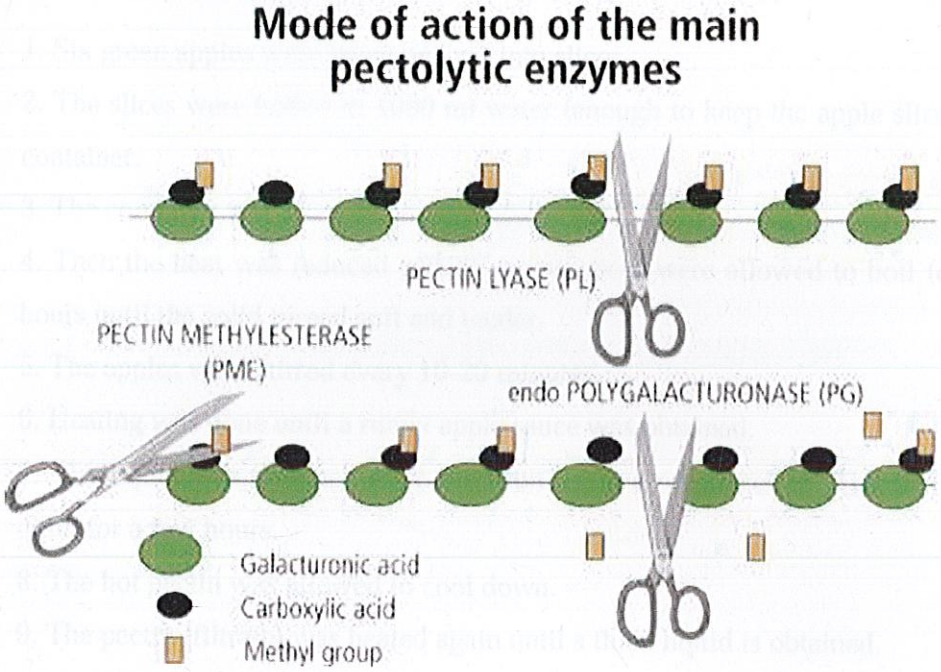

Figure 1.3 Mode of action of the main pectolytic enzymes

CHAPTER 2

MATERIAL AND METHODS

2.1 PECTIN PRODUCTION

Home made apple pectin was made using locally available apples.

1. Six green apples were taken and cut into slices.
2. The slices were boiled in 1000 ml water (enough to keep the apple slices afloat) in a steel container.
3. The container was covered and brought to boil.
4. Then the heat was reduced and the apple slices were allowed to boil (covered) for a few hours until the solid turned soft and tender.
5. The apples were stirred every 10-20 minutes.
6. Heating was done until a runny apple sauce was obtained.
7. The liquid was obtained after filtration using a muslin cloth. The liquid was allowed to drain for a few hours.
8. The hot pectin was allowed to cool down.
9. The pectin (filtrate) was heated again until a thick liquid is obtained.
10. Pectin was cooled and stored 4 C.

2.2 TEST FOR PECTIN

1. The test can only be performed once the pectin is cooled.
2. A few drops of ethanol were added in a beaker and followed by a spoonful of pectin.
3. The pectin coagulated forming a jelly-like mass.

2.3 INOCULA PREPARATION

1. *Aspergillus oryzae* were kept on potato dextrose agar (PDA) on PDA slants.
2. The inocula were made after the growth of these microorganisms on PDA during 7 days at 30°C, until the complete fungus sporulation.

2.4 CULTURE CONDITIONS

1. Lyophilised stock of *Aspergillus Oryzae* was revived in PDA broth.
2. Flask was kept at 25 C in a water bath shaker at 110 rpm for 3 days.
3. The Aspergillus spores were grown on PDA slants.
4. In all cases, 45 mL of mineral salts solution (containing (g/L):

   KH_2PO4 -2

   Urea -1.8

   MgSO4 -1

   was added to each 100 g of substrate. Fermentation carried out at 30°C for 96 hrs.

2.5 PARAMETERS CONTROLLING THE PECTINASE PRODUCTIVITY

1. Different pectin Concentrations

Different concentrations of pectin (%) (1, 2, 3, 4, 5, 6) were applied. At the end of incubation period, enzyme productivity was assayed.

2. Different incubation Periods:

Fermentation was carried out for different incubation periods of 24 to 144 hrs.

3. Different pH Values:

The pH was adjusted at different pH values (4.0, 4.5, 5.0, 5.5, and 6.0).

4. Different Temperatures:

Fermentation carried out at various temperatures (25°C, 30°C, 35°C, 37°C, 40°C).

2.6 PREPARATION OF CRUDE ENZYME

- 2 g of culture material was placed in a flask.
- 8 ml of distilled water was added.
- Culture material was centrifuged at 100 rpm for 30 minutes at 30°C

- The debris was removed by filtering through blotting paper followed by Whatman #41 filter-paper.

2.7 ACETONE PRECIPITATION

1. The required volume of acetone was cooled to -20°C.

2. Protein sample placed in acetone-compatible tube.

3. Four times the sample volume of cold (-20°C) acetone was added to the tube.

4. The tube was vortexed and incubated for 60 minutes at -20°C.

5. The tube was centrifuged for 10 minutes.

6. The supernatant was properly disposed off, without dislodging the protein pellet.

7. The acetone was allowed to evaporate from the uncapped tube at room temperature for 30 minutes.

CHAPTER 3

ENZYME ASSAYS

3.1 PECTINASE ASSAY

Pectinase activity principle

This assay method is based on the enzymatic hydrolysis of pectin, the resulting galacturonic acid being determined spectrophotometrically at 235 nm.

Apparatus

Spectrophotometer set at 235 nm

Water bath set at 30.0 ± 0.1°

Reagents and solutions

Citrate buffer pH 5.8

35.6 g Disodium hydrogen phosphate ($Na_2HP0_4.2H_20$) are dissolved in 1000 ml of water (A). 21 g of Citric acid monohydrate are dissolved in 1000 ml of water (B). 57 ml of solution A and 43 ml of solution B are mix to obtain working buffer solution. pH can be adjusted to 5.8 by adding one of the aforesaid solutions.

Pectin substrate solution

0.5 g of pectin substrate are mashed with 2 ml ethanol in a beaker and stirred to homogeneity with a magnetic stirrer. Slowly (allow 2 minutes for this step) add 80 ml of 0.1 M citrate buffer and continue stirring, avoiding foam formation. Measure pH and if necessary adjust to pH= 5.8 with solutions A or B respectively. Transfer solution to a volumetric flask and add 0.1 M citrate buffer up to 100 ml. Keep substrate solution cool over night (refrigerator). Next day centrifuge solution 10 minutes.

Samples preparation

Weigh 2 g of sample and dissolve with buffer solution into a volumetric flask. Fill up to 100 ml and transfer 3 ml of this solution to a next volumetric flask and fill up with buffer to 25 ml. Sample solutions have to be prepared immediately before analysis

Standard enzyme solution

Dilute standard enzyme with known activity in buffer solution.

Procedure

Measurement of enzyme activity

Add 0.1 ml of sample or standard solution to 3.0 ml of substrate solution pre-warmed to 30 ± 0.1° for 5 min. After short mixing, the absorbance at 235 nm is registered each minute over 8 minutes.

Calculation

Pectinase activity units is calculated as:

$$\textit{PECTINASE ACTIVITY, U/mg} = \frac{A235/t}{0.01 \times C \times V}$$

Where:

V = final reaction volume, ml (0.1 ml of sample or standard solution plus 3.0 ml of substrate solution)

C = final sample or standard concentration, g/ml

3.2 TOTAL CARBOHYDRATE CONTENT

1. For quantification of the total carbohydrate (TC) content in the different media the samples were hydrolyzed with concentrated HCI in an autoclave at 121 ∞C for 15 min.
2. Then after cooling they were alkalinized to pH 14 with 40% NaOH

3. Then it was treated with potassium ferricyanide and cupric sulfate solutions to remove proteins.

4. It was kept for 5 mins.

5. After which the concentration of soluble carbohydrate was estimated by the 3,5 dinitrosalicylic acid (DNS) method (Miller, 1959) as total reducing sugars.

3.3 SOLUBLE CARBOHYDRATE CONTENT

1. It was measured without prior hydrolysis of sample.

2. 0.3-mL sample was added to a solution containing 1 mL of substrate and 0.7 mL of 0.1 M acetate buffer (pH=4.5).

3. Reducing sugars were determined from the samples incubated at 45 °C for 30 min by dinitrosalicylic acid (DNS) method using Pectin as reference

3.4 TOTAL PROTEIN CONTENT

1. Firstly standard curve with different concentrations of bovine albumin serum (BSA) namely-0.1mg/ml, 0.2mg/ml, 0.3mg/ml, 0.4mg/ml , 0.5mg/ml was made and final vol was raised to 1 ml each with distilled water.
2. Its absorbance was noted.
3. Standard curve was plotted by taking Absorbance on Y-axis and BSA concentration on X-axis.
4. For analysis of total protein, 3 ml of Biuret reagent was added to 1ml of test protein solution in a sterile test tube and the mixture was properly mixed.
5. The tubes were then warmed at 37^0C for 10 min with shaking and finally the tubes were cooled and absorbance was noted at 540nm.
6. From Standard Graph find the Protein concentration of unknown samples.

CHAPTER 4

RESULTS AND DISCUSSION

Time (min)	Absorbance
1	0.012
2	0.016
3	0.020
4	0.023
5	0.027
6	0.030
7	0.033
8	0.041

Table 1.3 Observed pectinase activity

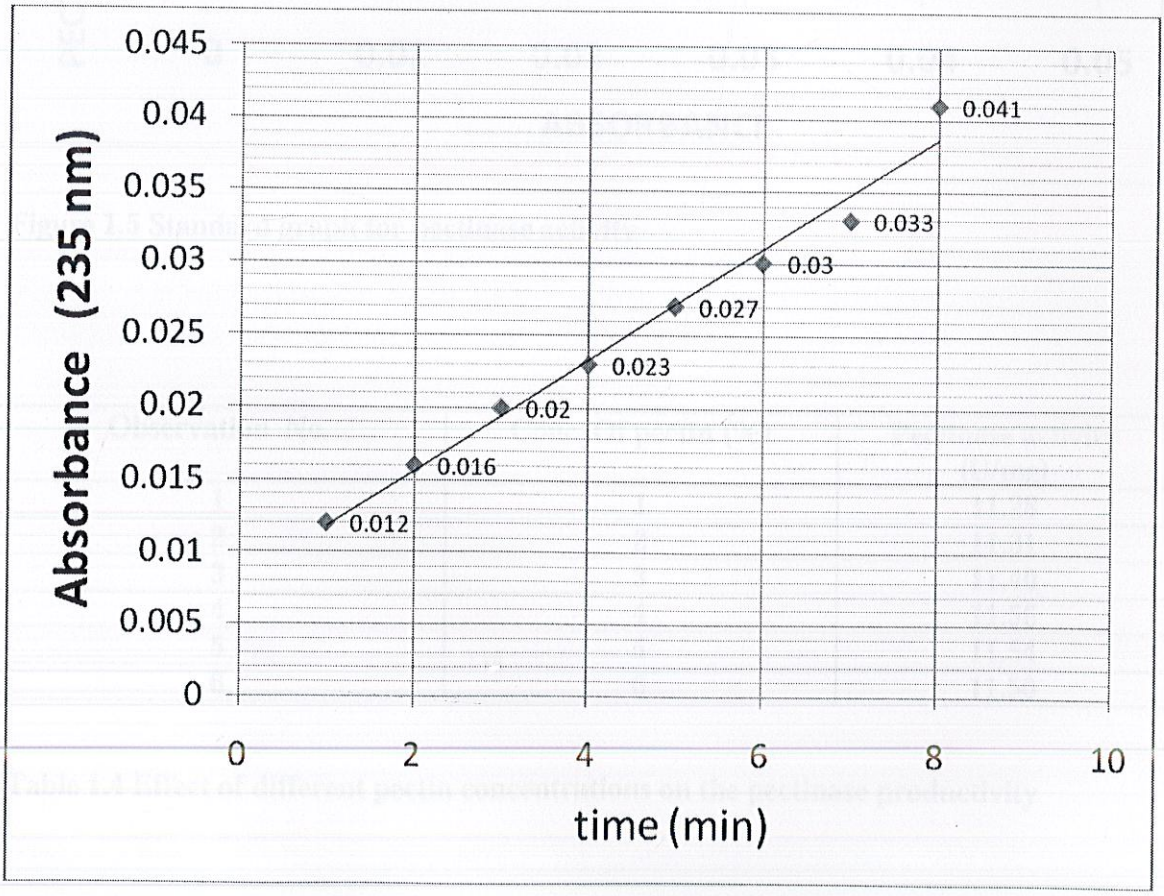

Figure 1.4 Observed pectinase activity

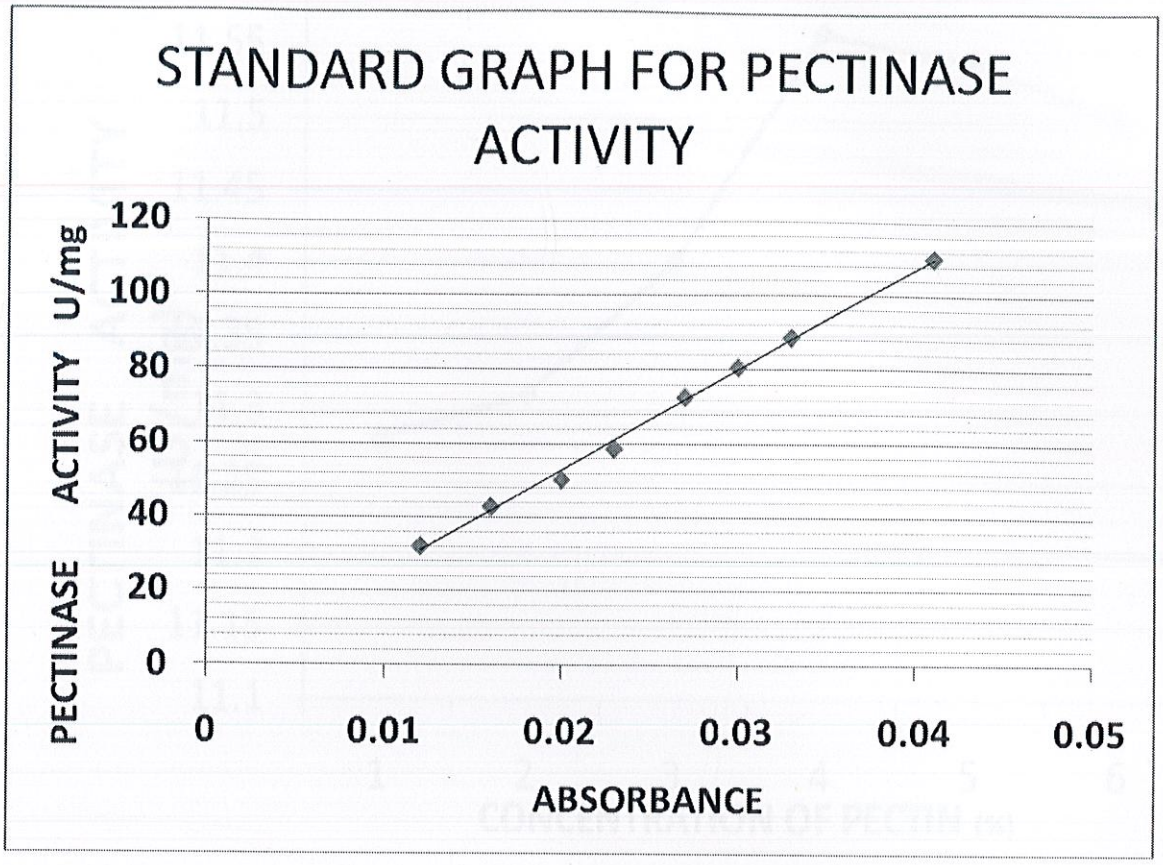

Figure 1.5 Standard graph for pectinase activity

Observation No.	Conc. Of pectin (%)	Pectinase activity (U/mg)
1	1	11.28
2	2	11.31
3	3	11.40
4	4	11.56
5	5	11.54
6	6	11.50

Table 1.4 Effect of different pectin concentrations on the pectinase productivity

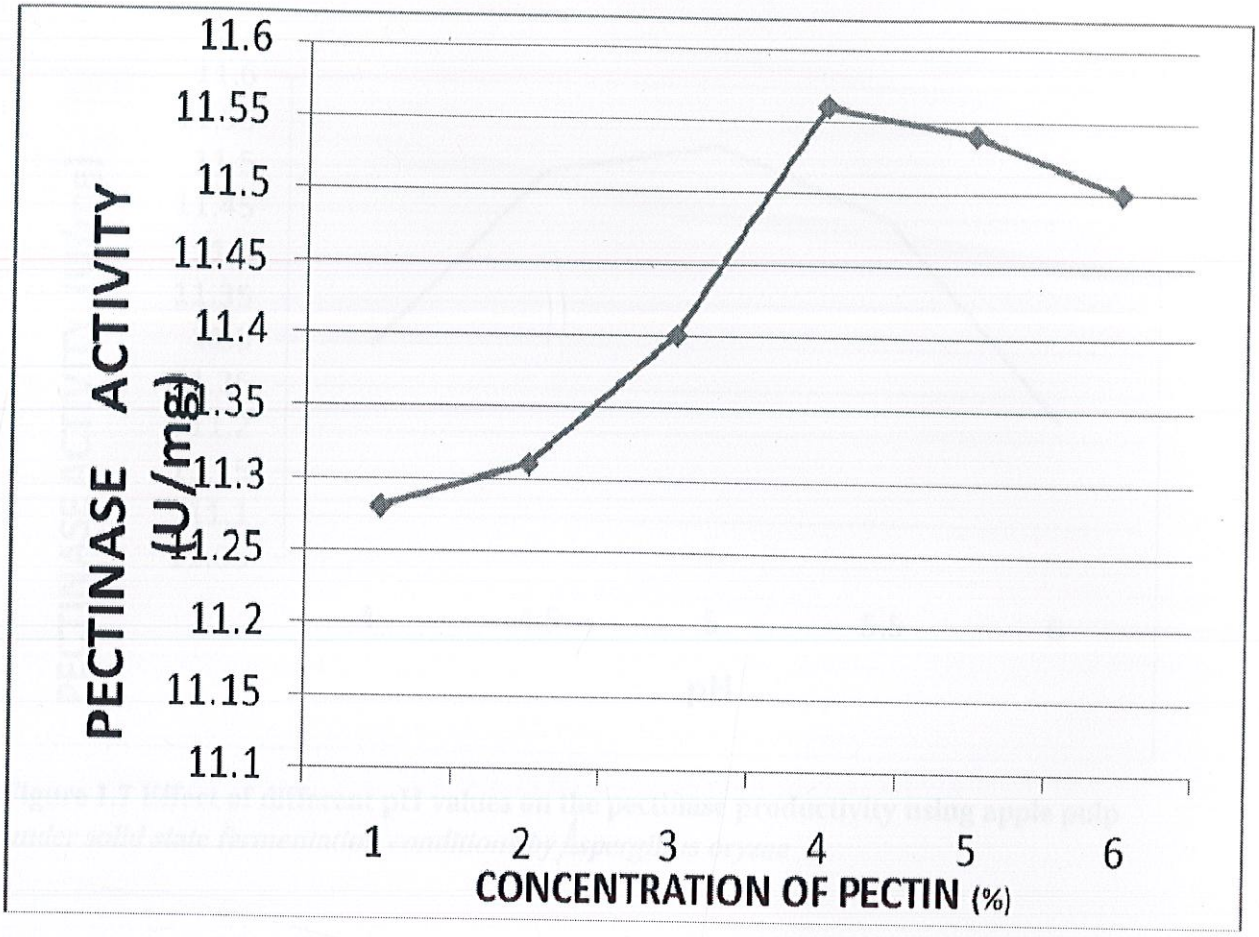

Figure 1.6 Effect of different pectin concentrations on the pectinase productivity using apple pulp *under solid state fermentation conditions by aspergillus oryzae*

Observation No.	pH	Pectinase activity (U/mg)
1	4.0	11.30
2	4.5	11.50
3	5.0	11.53
4	5.5	11.45
5	6.0	11.22

Table 1.5 Effect of different pH values on the pectinase productivity

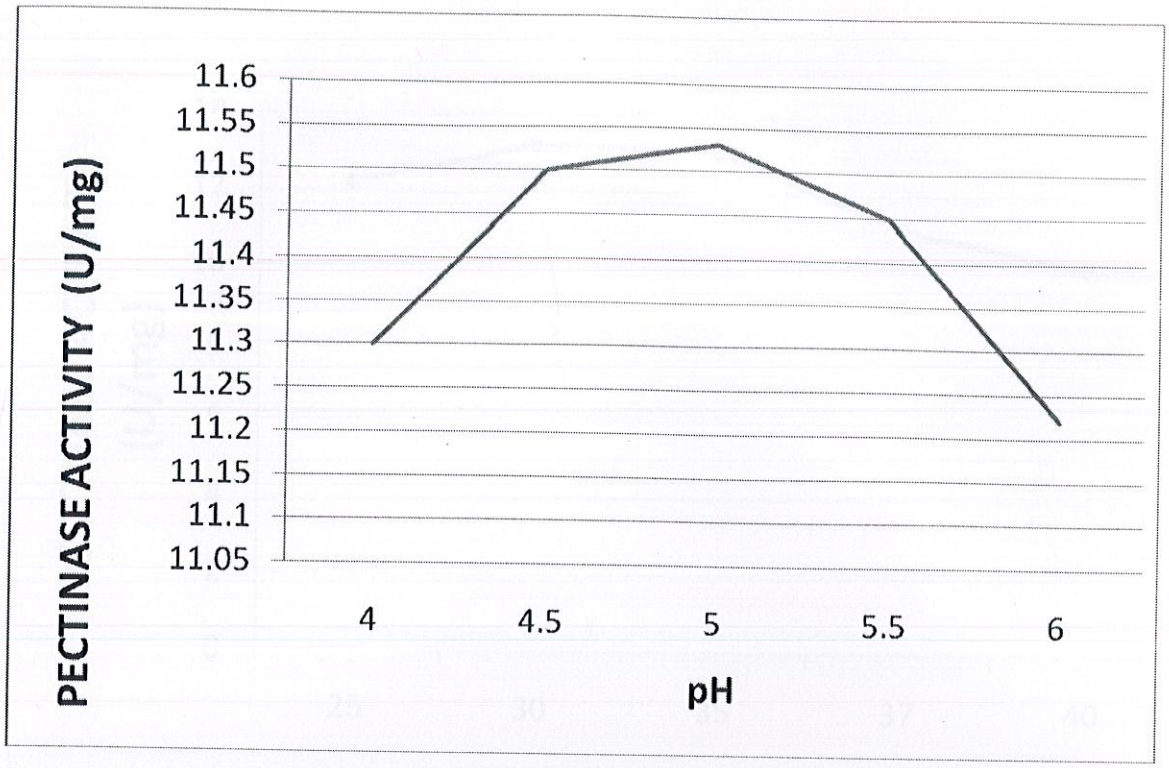

Figure 1.7 Effect of different pH values on the pectinase productivity using apple pulp *under solid state fermentation conditions by Aspergillus oryzae*

Observation No.	Temperature (°C)	Pectinase activity (U/mg)
1	25	12.23
2	30	13.21
3	35	13.01
4	37	11.10
5	40	10.09

Table 1.6 Effect of different temperature values on the pectinase productivity

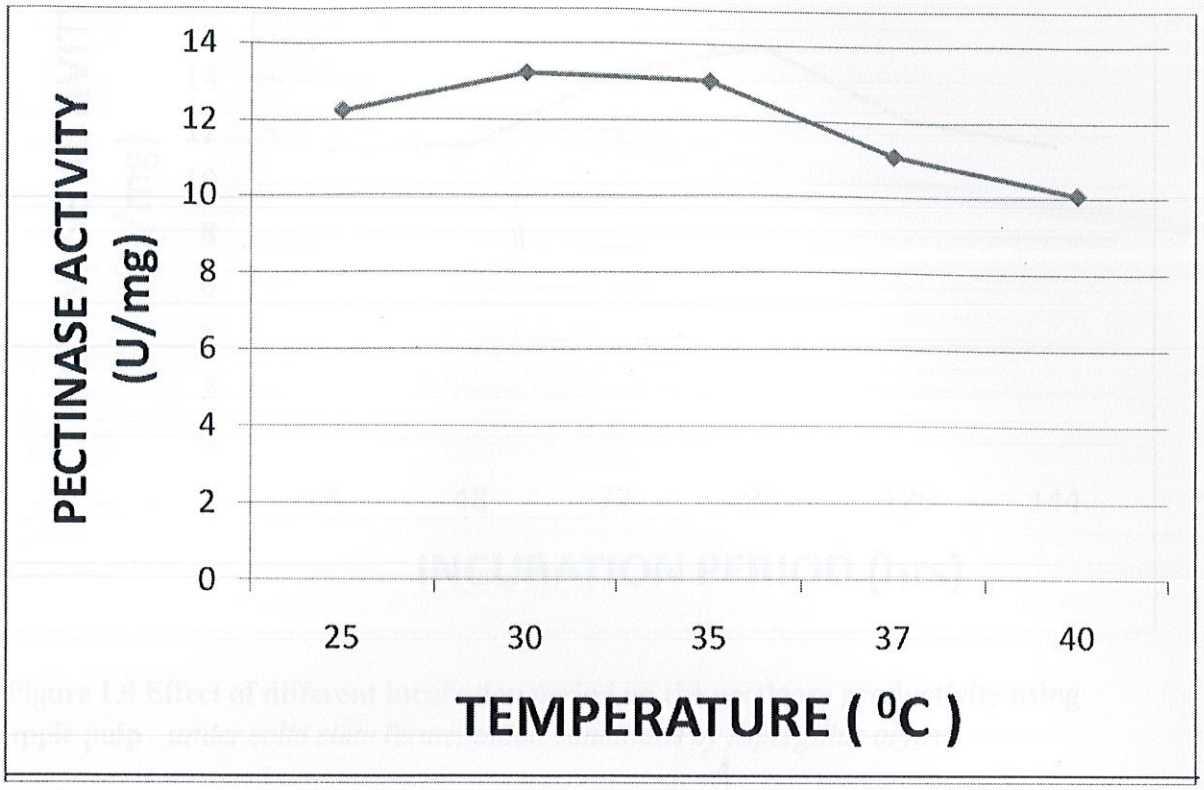

Figure 1.8 Effect of different temperature values on the pectinase productivity using apple pulp ***under solid state fermentation conditions by Aspergillus oryzae.***

Observation No.	Incubation period (hrs)	Pectinase activity (U/mg)
1	24	11.31
2	48	11.43
3	72	14.28
4	96	15.31
5	120	12.52
6	144	11.52

Table 1.7 Effect of different incubation period on the pectinase productivity

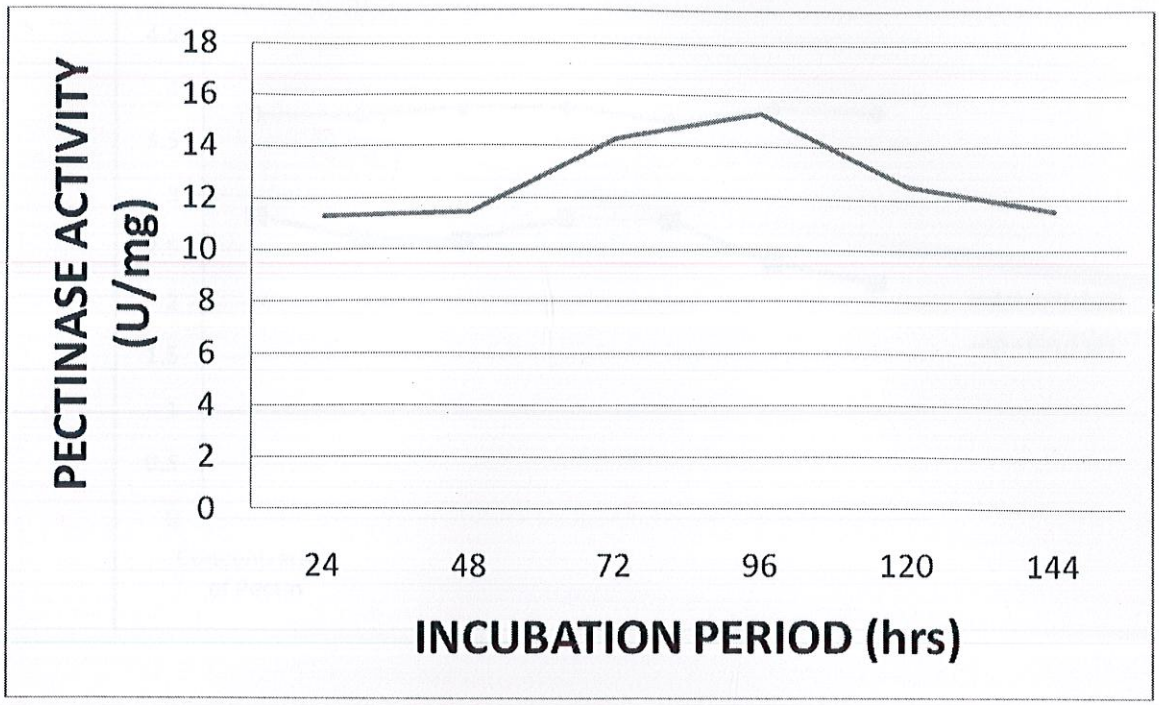

Figure 1.9 Effect of different incubation period on the pectinase productivity using apple pulp ***under solid state fermentation conditions by aspergillus oryzae***

A

Sample	Conc. Of Pectin (g/10 ml)	Initial pH	Final pH
1	15(p)	3.77	2.82
2	15(f)	3.76	2.6
3	20(p)	3.82	2.58
4	20(f)	3.83	2.8
5	25(p)	3.7	2.77
6	25(f)	3.83	2.37
7	30(f)	3.8	2.2

Table 1.8 Initial and final pH of media in SSF

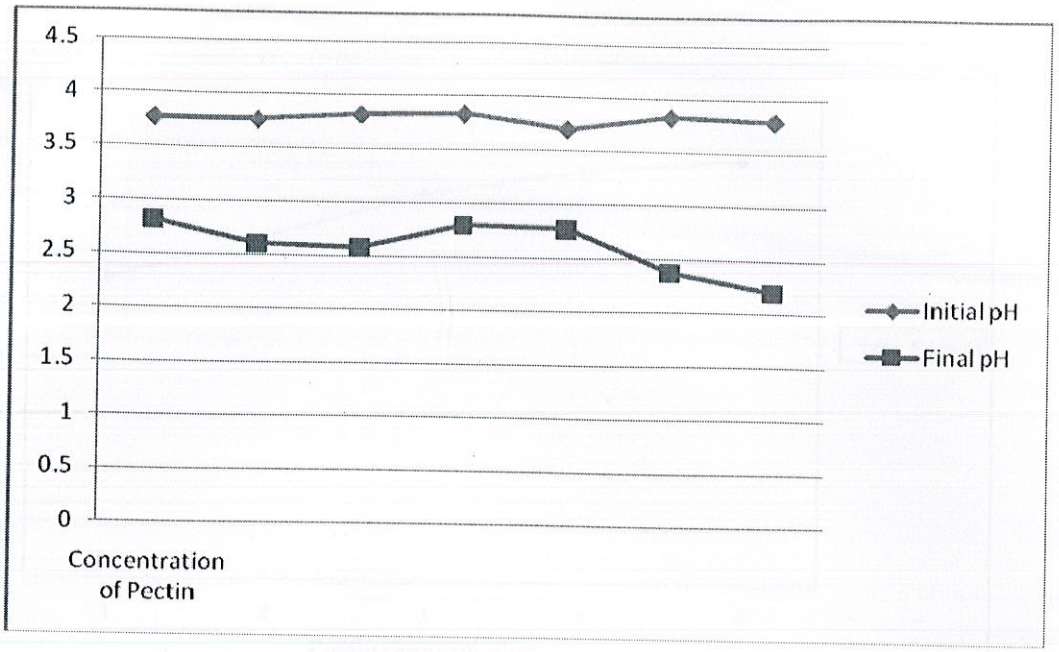

Figure 2.0 Change in Initial and final pH of media ***under solid state fermentation conditions by Aspergillus Øryzae***

Sample	Crude enzyme solution(mL)	Carbohydrate Conc (mg)
1	1	3.6
2	1	3.2
3	1	5.4
4	1	7.6
5	1	6.4
6	1	4.15

Table 1.9 Total carbohydrate concentration

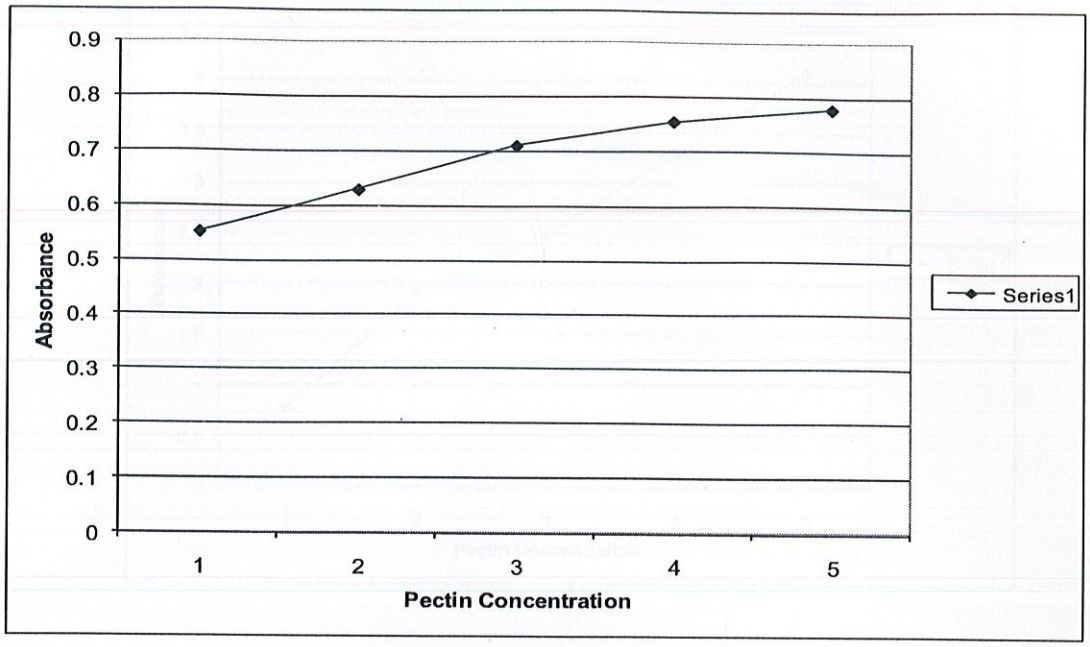

Figure 2.1 Total carbohydrate content of media ***under solid state fermentation conditions by Aspergillus Oryzae***

Sample	Crude enzyme solution(mL)	Sol.Carbohydrate Conc (mg)
1	1	0.85
2	1	0.86
3	1	1.2
4	1	1.56
5	1	2.1
6	1	3.05

Table 2.0 Soluble carbohydrate concentration

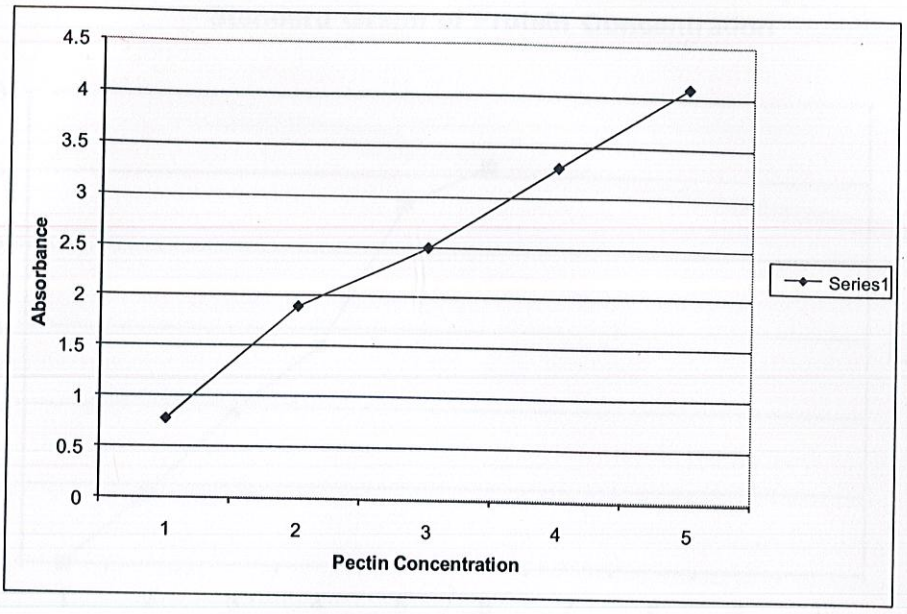

Figure 2.2 Soluble carbohydrate content of media ***under solid state fermentation conditions by Aspergillus Oryzae***

Sample	Crude enzyme solution(mL)	Protein Conc (mg)
1	1	8.14
2	1	14.2
3	1	8.55
4	1	11.6
5	1	11.18
6	1	10.14

Table 2.1 Protein content concentration

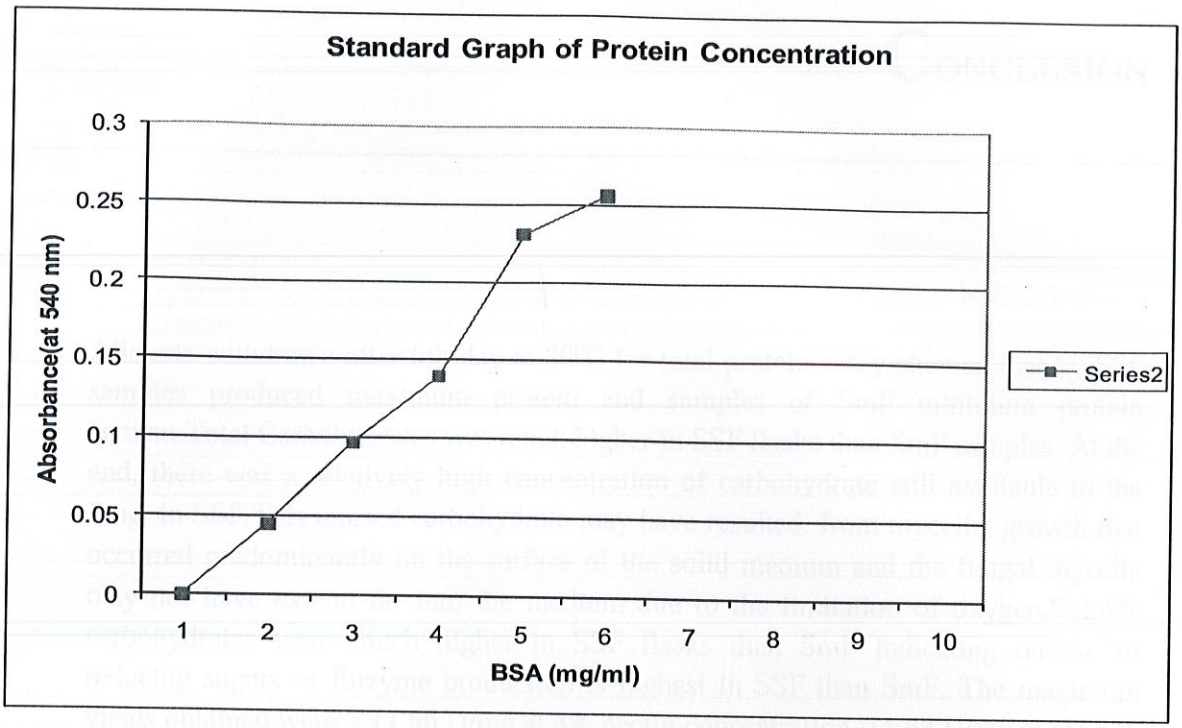

Figure 2.3 Protein content of media ***under solid state fermentation conditions by Aspergillus Oryzae***

CONCLUSION

Aliquots withdrawn after 5th day at 30^0C for total protein assay showed that by SSF samples produced maximum protein and samples of SmF minimum protein content.Total Carbohydrates was much higher in SSF flasks than SmF samples. At the end, there was a relatively high concentration of carbohydrate still available to the fungi in SSF.This unused carbohydrate may have resulted, from mycelial growth that occurred predominantly on the surface of the solid medium and the fungal mycelia may not have extend far into the medium due to the limitation of oxygen.Soluble carbohydrates were much higher in SSF flasks then SmF indicating release of reducing sugars or Enzyme production is highest in SSF than SmF. The maximum yields obtained were – 11.56 U/mg at 4% pectin concentration, 11.53 U/mg at pH 5.0, 13.21 U/mg at 30° C, 15.31 U/mg in 96 hrs. pectinase activity varied with variations in the different parameters.

Bibliography

Agrios, G.N., (1988). How pathogens attack plants. Ch.3 In: Plant pathology 3rd ed., (pp. 63-86).*Academic Press, Inc., San Diego, C.A.*

Ahlawat, S., B. Battan, S.S. Dhiman, J. Sharma and R.P. Mandhan, (2007). Production of thermostable pectinase and xylanase for their potential application in bleaching of kraft pulp.*J.Ind.Microbiol Biotechnol., 34: 763-770.*

A. Pandey, C.R. Soccol, P. Nigam, V.T. Soccol, (2000) Biotechnological potential of agro-industrial residues. I: Sugar cane bagasse, *Bioresour. Technol. 74 69–80.*

Bateman, D.F. (1968). *Neth.J.Plant Path. 25*: 161.

B.K. Lonsane, N.P. Ghildyal(1992): Exoenzymes. In: Solid Substrate Cultivation, H.W. Doelle, D.A. Mitchell, C.E. Rolz (Eds.), *Elsevier Applied Science, London, UK.*

Debing, J., L. Peizun, F. Stagnitti, X. Xianzhe and L. Li, 2005. Pectinase production by solid fermentation from Aspergillus niger by a new prescription experiment. *Ectoxicology and Environ. safety, 64: 244-250.*

Dingle, J., Reid, W.W. and Soloman, G. (1950). *J.Sc. Ed. Agric.***1**:234.

D.R. Kashyap, S.K. Soni, R. Tewari, (2003)Enhanced production of pectinase by Bacillus sp. DT7 using solid state fermentation,*Bioresour. Technol. 88 251–254.*

E. Favela-Torres, C. Aguilar, J.C. Contreras-Esqivel, G. Viniegra- González(2005): Pectinase. In: Enzyme Technology, A. Pandey, C. Webb, C.R. Soccol, C. Larroche (Eds.), *Springer Verlag, Berlin, Germany pp. 273–296.*

E. Oriol, B. Schettino, G. Viniegra-González, (1988) Solid–state culture of Aspergillus niger on support, *J. Ferment. Technol. 66 57–62.*

Gewali, M.B., Thapa, S., Baral, A., Bhattarai, S. and Shrestha, J.K.(1994), Proceeding of Second National Conference on Science and Technology, *Royal Nepal Academy of Science and Technology, Kathmandu, Nepal* , June 8-11.

Gewali, M.B. (1995), Proceedings of the 4th Symposium on Traditional Medicines in Toyama. Toyama, Japan. 217

Gewali, M.B., Manandhar, S., Thapa, S., Banskota, A. and Shrestha, J.K. (1997). *Journal of Nepal Chemical Society.* **16**: 23-26.

Gewali M.B., Maharjan J., Thapa S. and Shrestha J.K. *(* 2007) STUDIES ON POLYGALACTURONASE FROM ASPERGLLUS FLAVUS. (*Scientific World, Vol. 5, No. 5,*

Gummadi S.N., Kumar D.S. and Aneesh C.N.A. (2007): Effect of Salts on Growth and Pectinase Production by Halotolerant Yeast, Debaryomyces nepalensis NCYC 3413. *Current Microbiology, 54, 472-476.*

Gummadi S.N. and Kumar D.S. (2005): Microbial pectic transeliminases. *Biotechnol Lett., 27, 451-458.*

Gummadi S.N. and Kumar D.S. (2006): Pectin lyase and pectate lyase from Deharyornyces nepalensis isolated from apple. *Res J Microbiol., 1, 152-159.*

G. Reed: Enzymes in Food Processing, Academic Press, New York, USA (1966).

G. Sakellaris, A.S. Nikolaropoulos, A.E. Evangelopoulos, (1988) Exolygalacturonase biosynthesis by Lactobacillus plantarum effect of cultural conditions on enzyme production, *J. Appl. Bacteriol. 65 397–404.*

J.D. Evans, D.E. Akin, J.A. Foulk, (2002) Flax retting by polygalacturonase containing enzyme mixtures and effects on fibre properties, *J. Biotechnol. 97 223–231.*

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Makky E.A. *(2009)* Comparison of Osmotic Stress on Growth and Pectinase Production by Aspergillus flavus in Liquid and Solid-State Cultures *Asian J. Exp. Sci., Vol. 23, No. 1,; 19-26*

Miller, G.L. (1959). *Anal.Biochem.* **31**: 426.

M.E. Acuña-Argüelles, M. Gutiérrez-Rojas, G. Viniegra- -González, E. Favela-Torres, (1995)Production and properties of three pectinolytic activities produced by Aspergillus niger in submerged and solid state fermentation, *Appl. Microbiol. Biotechnol. 43 808–814.*