

Full Length Research Paper

# The influence of carbon and nitrogen sources on pectinase productivity of *Penicillium chrysogenum* in solid state fermentation

Tariq, A.L.<sup>1</sup> and Reyaz, A.L.<sup>2</sup>

<sup>1</sup>Department of Microbiology Sree Amman and Science College Erode-638003

<sup>2</sup>Department of Biotechnology Periyar University Salem-636011

## Abstract

*Penicillium chrysogenum* strain MTCC \*160 was used for the production of pectinase in a solid state fermentation and enzyme activity was found 15U/mL. The pectinase concentration was found 2.7mg/mL. The pectinase activity was greater when carbon sources were added it showed sucrose 82.5 U/ml, glucose 67.5U/mL and lactose 79.1U/mL. The pectinase activity were greater in the presence of nitrogen sources such as ammonium sulphate 65U/mL, peptone shows 37.5U/mL and yeast extract 31.6U/mL. The pectinase activity was maximum at substrate concentration of 7g and activity found 25U/mL. The quick germination took place in the cashew nut seed after treating with pectinase and no germination was found in untreated cashew nut seeds. The good clarified was obtained after treatment with pectinase.

**Keywords:** Pectinase enzyme, solid state fermentation, carbon sources, nitrogen sources, seed germination and juice clarification.

## INTRODUCTION

Pectinase are a group of enzymes that break the glycosidic bonds of the long chains of galacturonic acid residues of pectic substances, which are structural polysaccharides of plant cells, major pectinases are polygalacturonase, pectin lyase, pectate lyase and pectin esterase (Ernesto et al., 2006). The fungi *Penicillium chrysogenum* is used for pectinase production, is a filamentous organism which consists of branched and unbranched hyphae (Nazia et al., 2003). Orange bagases contains large amount of soluble carbohydrates, particularly fructose, glucose, sucrose, pectins as well as insoluble cellulose has been used as fermentation feed stock for the production of enzymes (Ellan et al., 2008). The major industrial application of these enzymes include the extraction and clarification of fruit juices, grape musts, macerator vegetables, fruit and production of cloudiness in soft drinks (Alana et al., 1990).

*Penicillium sp* produced polygalacturonase and pectin lyase on mixture of orange bagases as substrate (Natalia et al., 2004). *Penicillium chrysogenum* showed the best performance of production of pectinases was measured by an enzymatic index in solid pectin medium (Silvia et al., 2001). Pectinases are widely used in industrial processes that need solubilization of the cell wall of plants, wood, fruit or paper (James et al., 2004; Sarvamangala and Agasar et al., 2006). The pectinase identified in this study has potential application in processing of fruits and vegetables, agricultural and agro industrial waste, coffee and tea fermentation, and in textile and paper making industry (Reshmi et al., 2008). The Polygalacturonases were produced in more amounts from cheap raw material in solid state fermentation under all optimal condition for application in the clarification of juice (Reeda et al., 2008).

Pectic substances and their hydrolysis products are used as carbon sources by a limited number of yeasts and hence these enzymes must be involved in the colonization of different part of plants, including fruits (Pilar et al., 1999). Low amount of water available in solid

---

\*Corresponding Author E-mail: tariqtasin@gmail.com

state bioprocess of the class of microorganism that is most commonly used in fungi (Zheng and Shetty, 2000). The production of exo-pectinase was more influenced by both sugars than endo-pectinase in submerged as well as solid state conditions (Aguilar and Huitron, 1990).

## MATERIALS AND METHODS

### Culture Maintenance

*Penicillium chrysogenum* strain number MTCC\*160 obtained from CEPC culture collection was activated by subculturing on malt extract agar.

### Substrate Preparation

Orange bagasse was collected from Bismmulah fruit stall Erode and substrate was dried by sunlight then made powdered. The powdered substrate was weighed 30gm in three Erlenmeyer flask for fermentation and sterilized at 121 °C for 15 minutes for 3 successive days.

### Mineral Media Preparation

The fermentation basal mineral media for *Penicillium chrysogenum* was used having the composition of Potassium Phosphate -3g/L, Di-sodium Sulphate -0.5g/L, Magnesium Sulphate -0.25g/L, Zinc Sulphate -0.02g/L, Manganese Sulphate -0.02g/L, Ferrous Ammonium Sulphate -0.10g/L, Copper Sulphate -0.005g/L, Calcium Chloride -0.05g/L, Lactose -30g/L, Glucose -10g/L, Ammonium Lactate -6.0g/L, B-phenyl ethylamine-1g/L, pH 6.2. and media was sterilized at 121 °C for 15 minutes.

### Solid State Fermentation

Spore inoculum was prepared following Sarvamangala and Agasar, 2006 method by adding 2.5mL of sterile distilled water containing 0.1% Tween 80 to a fully sporelated culture. The spores were dislodged using a sterile inoculation loop under aseptic conditions. Solid state fermentation was carried out by inoculated 10ml of spore suspension into Erlenmeyer flask contained 30grams of sterilized orange bagases as substrate, to this 50mL of mineral media was added and mixed uniformly by using sterilized spatula then incubated at 30<sup>0</sup> C far four days.

### Submerged Fermentation

Submerged fermentation was carried out (Sarvamangala and Agasar, 2006) in Erlenmeyer flask, containing 30g of

3 successive days' sterilized orange bagases as substrate. This substrate was inoculated with 10mL aliquots of spore suspension under aseptic conditions followed by inoculation of 110mL of mineral media. The contents of the flask were mixed by using sterile spatula and incubated on shaker at 30 °C for 4days

### Liquid State Fermentation

Liquid state fermentation was carried out (Sarvamangala and Agasar, 2006) in Erlenmeyer flask, containing 30g of successive day's sterilized orange bagases as substrate. This substrate was inoculated with 10 ml aliquots of spore suspension under aseptic conditions and followed by the inoculation of 170mL of mineral media. The contents of the flask were mixed by using sterile spatula and incubated on shaker at 30 °C for 4 days.

### Extraction and Partial Purification of Enzyme

After fermentation (Sangeeta and Shastri, 2007) fermented mass from solid state fermentation was mixed with 20ml of sodium acetate buffer then grinded by using motor and pestle. Similarly submerged and liquid fermentation were grinded with mortar and pestle. The pH of the Sodium acetate buffer was adjusted to pH 5 by mixing the 14.8ml of 0.2 M of acetic acid solution and 35.2mL of 0.2 M sodium acetate solution and total volume made up to 100 ml by adding 50 ml of distilled water. The fermentation product was filtered by using muslin cloth and then filtered product was centrifuged at 9000g for 10 minutes. After centrifugation the filtrate was collected and Ammonium sulphate was added slowly and mixed till the 90% saturation took place then kept at 4 °C for 24 hrs. After 24 hrs, the mixture was again centrifuged and the pellet was dissolved in 3mL Acetate buffer at pH 5 and stored at 4 °C for further studies.

### Pectinase Assay

Pectinase activity was determined by measuring the release of reducing groups from pectin solution using the 3, 5- dinitrosalicylic acid (DNS) reagent assay galacturonic acid was used as standard. The reaction mixture containing 1mL of 1% pectin and 1mL of partially purified enzyme solution was incubated at room temperature for 1 hour. Then the reaction was stopped by adding ethanol, from this mixture 0.5 ml was taken then added to 3ml of DNS reagent. This mixture was incubated for 5 minutes at 100 °C in water bath. After incubation 1 ml of 40% sodium potassium tartrate was added, cooled and OD value was taken at 510 nm. Standard Galactouronic was prepared by taking 100 mg galecturonic acid in 100 ml standard flask and made up

the volume to 100 ml. From this 0.2, 0.4, 0.6, 0.8, and 1.0 ml was taken and made up to 2 ml by adding distilled water. Then 3 ml of DNS reagent was added into all the test tubes and incubated at 100°C for 5 minutes in water bath. Then 1 ml of 40% Sodium potassium tartrate was added, cooled and taken the optical density value at 510 nm (Lizu et al., 2008).

### **Determination of Protein Concentration**

Total protein of enzyme was determined by Lowry's, et al. (1951) by using standard Bovine Serum Albumin and OD value was taken at 650 nm.

### **Effect of Additional Carbon Sources on Pectinase Activity**

The carbon source was detected (Reda et al., 2008) by taking the dried, weight of 20grams of sterilized substrate orange bagases in three conical flask for solid state fermentation. The substrate of each Erlenmeyer flask was inoculated with 10mL aliquots of spore suspension then 1% of Carbon sources: sucrose, glucose, and lactose were added to the substrate and mixed well. The 30mL of basal mineral media were added to the substrate and contents of the flask were mixed by using sterile spatula then incubated at 30°C for 5 days

### **Effect of Additional Nitrogen Sources on Pectinase Activity**

Inorganic Nitrogen source and organic Nitrogen source were used to determine the pectinase activity by following the Reda et al., 2008. The substrate of orange bagases was taken, dried, weighed 20g and sterilized in 3 Erlenmeyer flask for solid state fermentation. The substrate of each flask was inoculated with 10 ml aliquots of spore suspension, then 1% of Nitrogen sources such as peptone, yeast extract and ammonium nitrite were added into the substrate. After inoculation and mixing of Nitrogen sources 30 ml of Basel mineral media were added to the substrate. Contents of the flask were mixed by using sterile spatula and incubated at 30°C for 5 days.

### **Effect of Different Substrate Concentration on Pectinase Activity**

Different concentration of substrate (3g, 5g, 7g and 15g) was taken by following method of Reda et al., (2008) it was dried and sterilized in Erlenmeyer flask for solid state fermentation. The substrates of each flask were inoculated with 10mL aliquots of spore suspension. After inoculation of spores 30 ml of basal mineral media were

added to the substrate and contents of the flask were mixed by using sterile spatula then incubated at 30°C for 5 days.

### **Clarification of Orange Juice by Crude Pectinase**

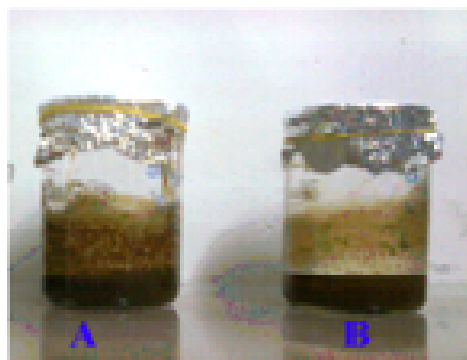
Clarification of orange juice by crude pectinase was carried by following Kareem and Adebawale (2002). The mature ripened orange fruits (*Citrus sinensis*) were obtained from market in Erode, Tamil Nadu. The orange bagases were sorted, washed and peeled. The juice was extracted using a domestic juice extractor. The extracted juice was pasteurized at 85°C for 3minutes to inactivate the natural fruit enzymes or microbes present and then cooled down to 40°C before the addition of extracted pectinase enzyme. Varying concentration of enzyme (0, 0.25, 0.5, and 1%) was added. The samples were incubated at 40°C for 1 hour. After incubation, the samples were treated at 85°C for 3 minutes to inactivate enzyme.

### **Pectinase Treatment for Seed Germination**

Few cashew seeds were taken and immersed in partially purified enzyme in a beaker and kept for 15 days. After incubation place the seed into soil for germination. Few cashew seeds were taken and immersed in distilled water in a beaker as control and kept for room temperature for 15 days, after incubation the seeds were placed into soil for germination (Draginga et al., 2004).

## **RESULT**

Solid state fermentation, submerged and liquid state fermentation were carried out at room temperature by *Penicillium chrysogenum* for 4 days using the substrate orange bagases. The fungal growth was observed after 2 days of incubation and maximum growth obtained on the fourth day of fermentation. After incubation the crude enzyme saturated completely with ammonium sulphate (Figure 1). *Penicillium chrysogenum* produce pectinase more amount from solid state fermentation than submerged and liquid state fermentation having the enzyme activity of 15U/ml, 14.16U/mL and 11.66 U/ml respectively presented in Table 1. The Pectinase concentration of solid state fermentation was found to be 2.78mg/mL, in submerged fermentation and liquid state fermentation was calculated as 2.5mg/mL and 2.08mg/mL respectively. Pectinase activities were greater when supplied the carbon source sucrose, the activity was found to be 82.5U/mL. The pectinase activity from other carbon glucose and lactose were calculated as 67.5 U/ml and 79.1U/mL respectively (Table 2). Pectinase activities were greater when supplemented the Nitrogen



**Figure 1.** Ammonium sulphate precipitation of crude pectinase enzyme after 24 hours of an incubation at 4°C where A is before centrifugation and B is after centrifugation and filtration.

**Table 1.** Pectinase activity obtained from different Fermentation concentration in which amount of galacturonic acid released in  $\mu\text{g}$  and the enzyme activity in U/mL

Fermentation	Amount of tannin Degraded ( $\mu\text{g}$ )	Pectinase activity (U/mL)
SSF	180	15.0
SMF	170	14.16
LSF	140	11.6

Where SSF- solid state fermentation, SMF-Submerged fermentation, LSF-Liquid state fermentation

**Table 2.** Pectinase activity at different Carbon sources concentration in which amount of galacturonic acid released in  $\mu\text{g}$  and the enzyme activity in U/mL

Carbon sources	Amount of tannin Degraded ( $\mu\text{g}$ )	Pectinase activity (U/mL)
Sucrose	990	82.2
Glucose	810	67.5
Lactose	950	79.1

**Table 3.** Pectinase activity at different Nitrogen sources concentration in which amount of galacturonic acid released in  $\mu\text{g}$  and the enzyme activity in U/mL

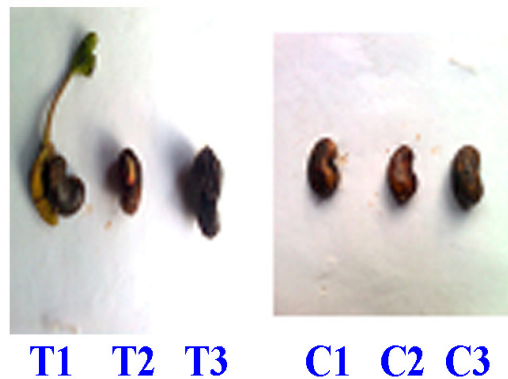
Nitrogen sources	Amount of tannin Degraded ( $\mu\text{g}$ )	Pectinase activity (U/mL)
Ammonium sulphate	780	65.0
Peptone	410	37.5
Yeast Extract	380	31.6

source Ammonium sulphate, the activity was found to be 65U/mL. The pectinase activity from other Nitrogen sources; Peptone and Yeast extract were calculated as

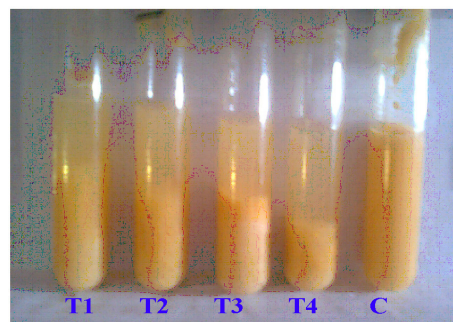
37.5 U/mL and 31.6 U/mL (table 3). A Pectinase activity was maximum at a substrate concentration of 7g, and the activity was found to be 25U/mL (table 4). In the cashew

**Table 4.** Pectinase activity at different Substrate concentration in which amount of galacturonic acid released in  $\mu\text{g}$  and the enzyme activity in U/mL

Substrate concentration of Orange bagases in grams	Amount of tannin Degraded ( $\mu\text{g}$ )	Pectinase activity (U/mL)
3g of Orange bagases	115	9.58
5g of Orange bagases	275	22.9
7g of Orange bagases	300	25.0



**Figure 2.** Germinated Cashew seeds when treated with pectinase where C1, C2, C3 are the control seeds and T3 (0.50%), T2 (0.75%), T1 (1%) are the pectinase treated seeds which showed the germination at different percentages.



**Figure 3.** Pectinase treated fruit juice where C is the control and T1 (0.25%), T2 (0.50%), T3 (0.75%) and T4 (1%) pectinase treated juices. After incubating the orange juice with different amount of pectinase enzyme for 1 hour, it was found that the juice was clarified at the top and the remaining are settled at the bottom

seed germination, the germination was observed in enzyme treated cashew seeds. Whereas no germination occurred in the control cashew seed (figure 2). After incubating the orange juice with different amount of

pectinase enzyme for 1 hour, it was found that the juice was clarified at the top and the remaining is settled at the bottom (Figure 3).

## DISCUSSION

Solid state fermentation holds tremendous potential for the production of enzyme. This system offers numerous advantages over submerged and liquid state fermentation (Aguilar and Huitron, 1990). In addition to high level stability of enzymes results from solid state fermentation cultures. Production of pectinase by solid state fermentation is a attractive proportions because of low water activity so prevention of bacterial contamination. Agricultural wastes containing pectin can be considered as an alternate substrate for the production of pectinase. In this study solid state fermentation, submerged and liquid state fermentation were used for pectinase production. The enzyme extracted from different fermentation was purified and the activity was compared (Zhang, 2009). It has been observed that the pectinase activity was higher in solid state fermentation compared to submerge and liquid state fermentation (Denis et al., 2002). Purification of three alkaline endo polygalacturonases (endo PGs) an exopolygalacturonase (exo pg) and exopolymethyl galacturonase (exo pmg) from the crude pectinases extract was carried out by ammonium sulphate fractionation and their enzyme activities were characterized (Lizu et al., 2008).

Protein estimation was done to estimate the concentration of total protein in enzyme extract produced from Solid state fermentation, submerged fermentation and liquid state fermentation. It reveals that all enzymes are proteins (Lowry et al., 1951). Sucrose supplied more carbon content in to the systems, which are utilized by organisms. So the pectinase activity was also increased (Reda et al., 2008; Solis et al., 1993). Ammonium sulphate supplied more nitrogen in to the fermentation systems, which are utilized by organisms. So the pectinase activity was also increased (Reda et al., 2008). The pectinase activity of substrate concentration 3g, 5g, and 7g found to be 9.58U/mL, 22.9 U/mL, and 25.0U/mL respectively (Manpreet et al., 2005). It reveals that the pectinase degraded the pectinase content in shell of cashew; it helps in easy germination. The pectinase can able to act on pectin and degrade completely. Pectinase attack de-polymerize pectin by hydrolysis and trans-elimination as well as by de-esterification reaction which hydrolysis the ester bond between the carboxyl and methyl of pectin (Reshmi, 2008). The treating of fruit juice with pectinase, the pectin content was degraded. Pectinase was produced by Fungi and Bacteria. It was capable of hydrolysing the pectin in to galacturonic acid. Pectinase is used in the fermentation of tea and coffee, soft drinks, juice clarification (Ellan, 2008).

## REFERENCE

- Aguilar G, Huitron C (1990). Constitutive exo pectinase produced by *Aspergillus niger* sp. CH- y- 1043 different carbon source Biotechnol. Lett. 12(9): 655- 660.
- Alana A, Alkorta I, Dominguez JB, Llama MJ, Serra J.I. (1990). Pectin lyase activity in a *Penicillium italicum* strain. Applied And Environmental Microbiology. 56(12):3755-3759.
- Denis S, Eduardo DSM, Roberto DS, Eleni G (2002). Pectinase Production by *Penicillium Viridactum* RFC3 by solid state fermentation using agricultural wastes and Agro-industrial by-products. Brazilian Journal of Microbiology. 33:318-324.
- Draginja MP, Ljiljana MR, Senka S (2004). Extraction of Endo-Pectinase Activity filtrate of *Polyporus squamosus* by aqueous two-phase systems composed of low Molecular mass polyethylene glycol and Phosphate salt. Acta Periodica Technologica. 35: 215-224.
- Ellen CG, Robert FHD, Aneli MB (2008). Orange Bagasse as substrate for the production of Pectinase and Laccase by *Biotryosphaeria rhodina* MAMB-05 in Submerged and Solid state Fermentation. Bio Resources. 3(2): 335-345.
- Ernesto FT, Tania VS, Gustavo VG (2006). Production of Hydrolytic Depolymerising Pectinases. Food Technol. Biotechnol. 44(2): 221-227.
- James S, Wendy AU, Bess W (2004). Genotypic variation in *Penicillium chrysogenum* from indoor environments. Mycology. 96(5): 1095-1105.
- Kareem SO, Adebowale AA (2002). Clarification of Orange juice by crude fungal pectinase from citrus peel. Nigerian Food Journal. 25(1):0189-7241.
- Lizu M, Jin B, Zhang HX (2008). Purification and Characterization of three alkaline endopolygalacturonase from a Newly isolated *Bacillus gibsonii*. The Chinese Journal of Process Engineering. 8 (4): 768-773
- Lowry OH, Rusen BPJ, Fass AL, Randall RJ (1951). Protein measurement with folin- phenol reagent. J. Biol. Chem, 193:265-275.
- Manpreet S, Sawraj S, Sachin D, Pankaj S, Banerjee UC (2005). Influence of Process Parameters on the production of Metabolites in solid- state Fermentation. Malaysian Journal of Microbiology. 1(2):21-9.
- Natalia M, Simone RDS, Roberto DS, Aleni G (2004). Pectinase production by fungal strains in solid state fermentation using Agro-industrial bioproduct. Brazilian Archives of biology and Technology. 47(5): 813-819,
- Nazia GN, Asgher M, Shah AH (2003). Production of pectinase by *Trichoderma harzianum* in solid state fermentation of citrus peel. Pak J. Agri. Sci. 40 (3-4): 193-201.
- Pilar B, Carmen S, Tomas GV (1999). Production of pectic enzymes in yeasts. Fems Microbiology Letters 175:1-9.
- Reshmi R, Siddalinga MK, Sneha R (2008). Partial purification and biochemical characterization of extra cellular pectinase from *Aspergillus niger* isolated from groundnut seeds. Journal of Applied Bioscience. 9(1): 378-384.
- Reda AB, Hesham M, Yassin M (2008). Production of Bacterial Pectinase (s) from Agro-industrial Wastes Under solid state fermentation conditions. Journal of Applied Science research. 4(12): 1708-1721.
- Sangeeta Y, Shastri N (2007). Purification and properties of an extracellular pectin lyase produced by the strain of *Penicillium oxalicum* in solid state fermentation. Indian Journal of Biochemistry and Biophysics. 44:247-251.
- Sarvamangala RP, Agasar D (2006). Exploration of regional Agro wastes for the production of Pectinase by *Aspergillus niger*. Food technol. Biotechnol. 44 (2): 289-292 .
- Silvia MGM, Francisco AP, Claudia BMV (2001). Screening and Genetic Improvement of Pectinolytic Fungi for Degumming of textile fibers. Brazilian Journal of Microbiology. 32:320- 326.
- Solis PS, Favela TE, Viniestra G, Gutierrez RM (1993). Effect of different carbon sources on the synthesis of pectinase by *Aspergillus niger* in submerged and solid state fermentation. Appl Microbiol. Biotechnol. 39:36-41.
- Zhang C, Li Z, Peng X, Jia Y, Zhang H, Bai Z (2009). Separation, Purification and Characterization of Three Endo-Polygalacturonase from a newly isolated *Penicillium oxalicum*. The Chinese Journal of Process Engineering. 9(2):243-249.
- Zhen Z, Shetty K (2000). Solid state production of polygalacturonase by *Lentinus edodes* I using fruit process in wastes. Process Biochemica. 35: 825-830.

