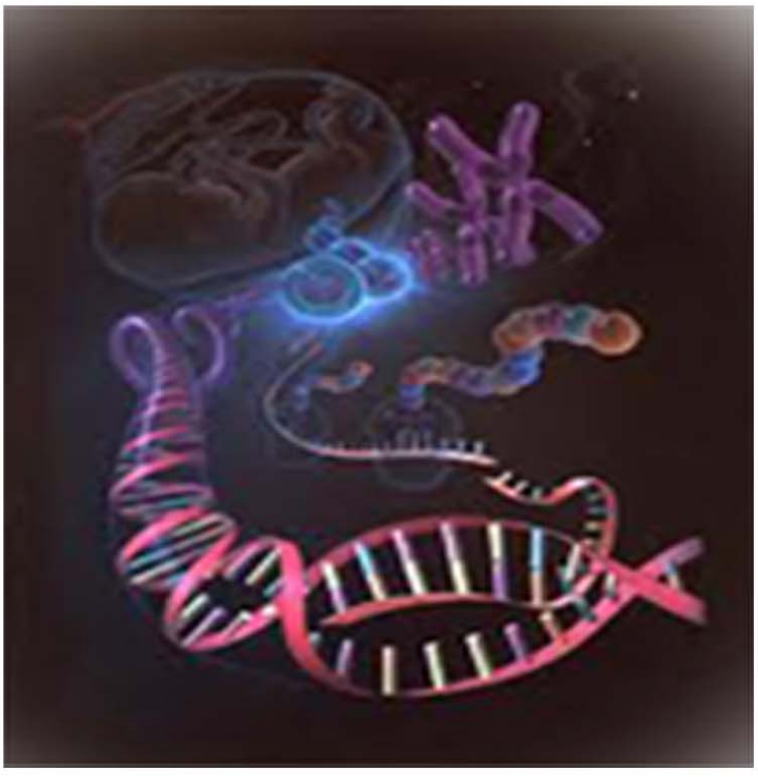
IJLBPR

ISSN 2250 - 3137
Vo1. I, No. 3, July 2012

# International Journal of

## Life Sciences Biotechnology and Pharma Research



International Journal of Life Sciences Biotechnology and Pharma Research
Hyderabad, INDIA

www.ijlbpr.com

Email : editorijlbpr@gmail.com or editor@ijlbpr.com

International Journal of Life Sciences Biotechnology and Pharma Research

*ISSN 2250-3137 www.ijlbpr.com*
*Vol. 1, No. 3, July 2012*


*Research Paper*

# ISOLATION, IDENTIFICATION AND SCREENING OF PECTINOLYTIC FUNGI FROM DIFFERENT SOIL SAMPLES OF CHITTOOR DISTRICT

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The aim of this present study was isolation and screening of pectinase producing fungi from different agricultural and non agricultural soils in chittoor district, Andhra Pradesh, South India. Total 44 fungal strains were isolated from these soils and only 4 strains were positive in pectinolytic activity. The extracellular pectinase was partially purified by ammonium sulphate precipitation and dialysis. These 4 strains were identified as *Aspergillus niger, A. flavus, A. japonicus, and Chaetomium globosum* and they produced very high levels pectinase by submerged fermentation. Maximum enzyme production was obtained in the medium containing citrus pectin. The effect of different temperature shows the optimum temperature for enzyme production was 30°C.

***Keywords:*** *Aspergillus* sp. Pectinase, Submerged fermentation

## INTRODUCTION

Fungi can produced diverse extracellular enzymes those are used to break down complex polysaccharides into simple sugars to be assimilated and used for growth and reproduction. These enzymes are useful for environmental and industrial applications such as food processing, brewery, biofuels, bioremediation etc. For those applications, exploration of new sources of useful fungal extracellular enzymes is demanded.

Pectin is a polymeric material having carbohydrate group esterifies with methanol. It is an important component of plant cell wall. It is present in highest concentration in the middle lamella, where it acts as a cementing substance between adjacent cells. Plant pathogens attack target cells by producing number of cell degrading enzyme which facilitates the entry and expansion of pathogen in the host tissue (Jayani *et al.*, 2005). The history of pectinases began with an understanding the structure of pectins substances and the mechanism by which pectolytic enzymes degrade pectic substances. Later the microbial production of pectinases

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became prominent for many decades. Many microorganisms viz.., bacteria, yeast, fungi could produce pectinases (Yadav *et al*., 2009). Evidence showed that pectinases are inducible and they can produce from different carbon sources. In the course of time, numerous reports have appeared on the optimization of fermentation and microbiological parameters and different fermentation strategies for the production of pectinases (Bayoumi *et al*., 2008). With the advent of molecular biology, vigorous research has been carried out on cloning and expression of pectinase genes in various hosts. *Apergillus niger* pectinases are most widely used in industries because this strain posses GRAS (Generally Regarded As Safe) status so that metabolites produced by this strain can be safely used. This fungal strain produces various pectinases including polymethyl-galacturonase (PMG), polygalacturonase (PG), and pectin esterase (PE). Apple and citrus fruits are the main source of commercial pectin at present. Pectin, a major constituent of cereals, vegetables, fruits; fibers are complex, high molecular weight heterogeneous and acidic structural polysaccharide (Doco *et al*., 1997). D-galacturonic acid is one of the major components of pectin (Gummadi and Panda, 2003). The present study was designed to isolate and purify the pectinase from fungi and also identify the potential pectinase producing strains through conventional methods.

## PECTIC SUBSTANCES

Pectic substance is the 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 (Rastogi, 1998) That the middle lamella is largely composed of pectic substances, has been conûrmed by the comparable uptake of ruthenium red by known pectic substances (Sterling, 1970) and from the estimation of pectin by the use of alkaline hydroxylamine (Gee *et al*., 1959; McCready and Reeve, 1955; and Albersheim and Killias, 1963). Pectic substances account for 0.5-4.0% of the fresh weight of plant material (Sakai *et al*., 1993; Kashyap *et al*., 2001; Whitaker, 1990) (Table 1).

**Table 1: Composition of Pectin in Different Fruits and Vegetables**

| Fruit/Vegetable | Tissue | Pectic Substance (%) |
|---|---|---|
| Apple | Fresh | 0.5-1.6 |
| Banana | Fresh | 0.7-1.2 |
| Peaches | Fresh | 0.1-0.9 |
| Strawberries | Fresh | 0.6-0.7 |
| Cherries | Fresh | 0.2-0.5 |
| Peas | Fresh | 0.9-1.4 |
| Carrots | Dry matter | 6.9-18.6 |
| Orange pulp | Dry matter | 12.4-28.0 |
| Potatoes | Dry matter | 1.8-3.3 |
| Tomatoes | Dry matter | 2.4-4.6 |
| Sugar beet pulp | Dry matter | 10.0-30.0 |

**Table 2: Molecular Weights of Some Pectic Substances**

| Source | Molecular Weight (kDa) |
|---|---|
| Apple and lemon | 200-360 |
| Pear and prune | 25-35 |
| Orange | 40-50 |
| Sugar beet pulp | 40-50 |

*Source: Sakai et al. (1993)*

Contrary to the proteins, lipids and nucleic acids, being polysaccharides, pectic substances do not have a defined molecular weight. The relative molecular masses of pectic substances range from 25 to 360 kDa (Table 2).

## STRUCTURE, CLASSIFICATION AND NOMENCLATURE OF PECTIC SUBSTANCES

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 (Whitaker, 1990). These substances are a group of complex colloidal polymeric materials, composed largely of a backbone of anhydrogalacturonic acid units (Cho *et al*., 2001; and Codner, 2001).The carboxyl groups of galacturonic acid are partially esteriûed by methyl groups and partially or completely neutralized by sodium, potassium or ammonium ions (Kashyap *et al*., 2001). Some of the hydroxyl groups on $C_2$ and $C_3$ may be acetylated (Alkorta *et al*., 1998).The primary chain consists of aD-galacturonate units linked $\beta$(1-4), with 2-4% of L-rhamnose units linked $\beta$-(1-2) and $\beta$-(1-4) to the galacturonate units (Whitaker, 1990) (Figure 1). The rhamnogala-cturonans are negatively charged at pH 5. The side chains of arabinan, galactan, arabinogalactan, xylose or fucose are connected to the main chain through their $C_1$ and $C_2$ atoms (Blanco *et al*., 1999; Sathyanarayana and Panda, 2003; and van der Vlugt-Bergmans *et al*., 2000). The above description indicates that the pectic substances are present in various forms in plant cells and this is the probable reason for the existence of various forms of pectinolytic enzymes.

The American Chemical Society classiûed pectic substances into four main types as follows (Alkorta *et al*., 1998):

(I) Protopectin: is the water insoluble pectic substance present in intact tissue. Protopectin on restricted hydrolysis yields pectin or pectic acids.

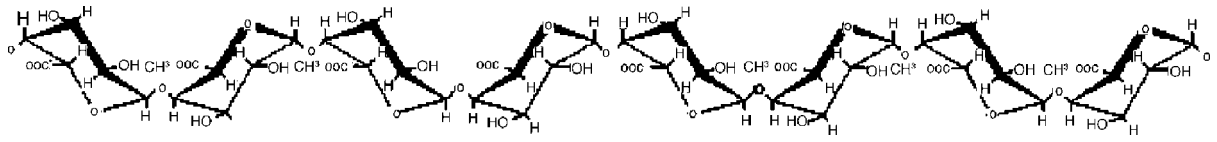
(II) Pectic acid: is the soluble polymer of galacturonans that contains negligible amount of methoxyl groups. Normal or acid salts of pectic acid are called pectates.

(III) Pectinic acids: is the polygalacturonan chain that contains >0 and <75% methylated galacturonate units. Normal or acid salts of pectinic acid are referred to as pectinates. (IV) Pectin (Polymethyl galacturonate): is the polymeric material in which, at least, 75% of the carboxyl groups of the galacturonate units are esteriûed with methanol. It confers rigidity on cell wall when it is bound to cellulose in the cell wall.

### The Pectinolytic Enzymes

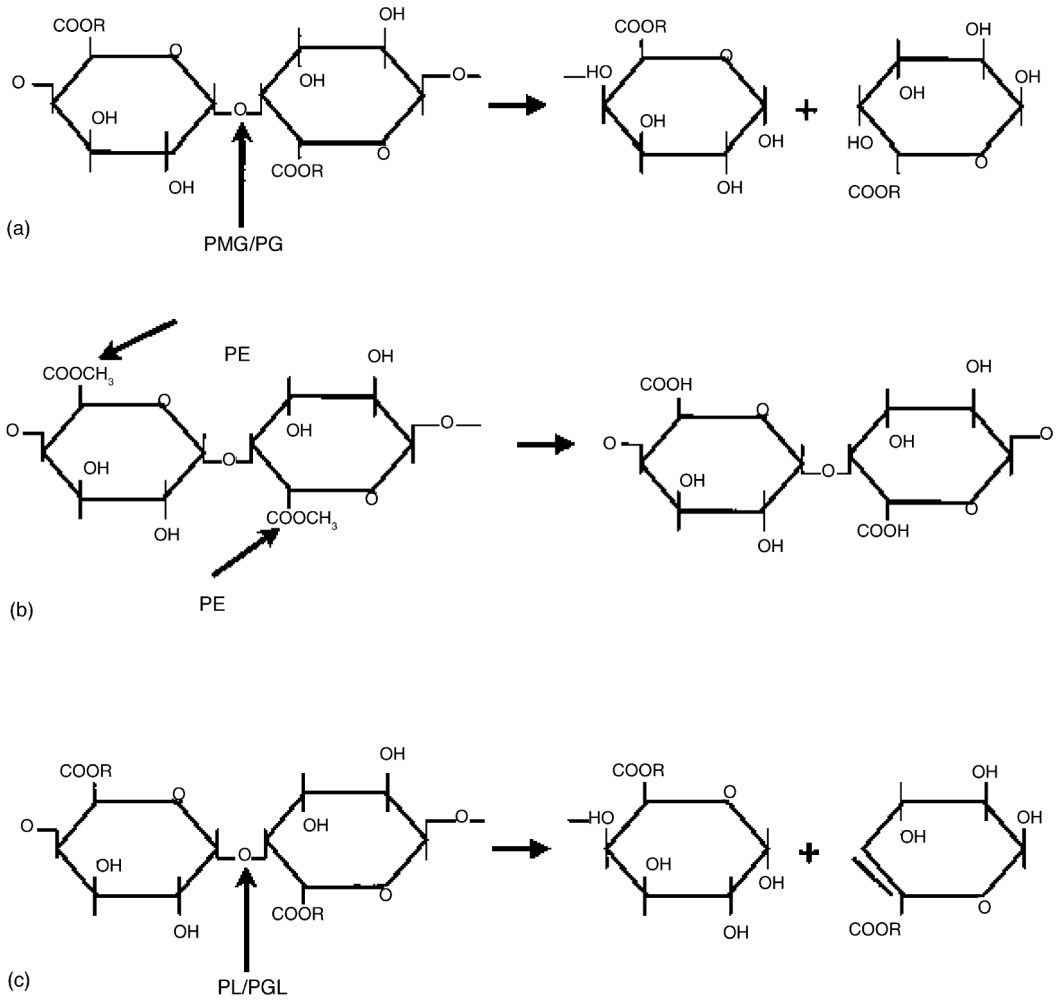
The pectinolytic enzymes may be divided in three broader groups as follows (Sakai, 1992; and Paloma ki and Saarilahti, 1997):

**Figure 1: Primary Structure of Pectic Substances**



*Source: Rexova Benkova and Markovic (1976)*

**Figure 2: Mode of Action of Pectinases: (a) R = H for PG and $CH_3$ for PMG; (b) PE; and (c) R = Hfor PGL and CH for PL**




**Note:** The arrow indicates the place where the pectinase reacts with the pectic substances. PMG, polymethylgalacturonases; PG, polygalacturonases (EC 3.2.1.15); PE, pectinesterase (EC 3.1.1.11); PL, pectin lyase (EC-4.2.2.10).

*Source: Sathyanarayana and Panda (2003)*

1. Protopectinases: degrade the insoluble protopectin and give rise to highly polymerized soluble pectin.
2. Esterases: catalyze the de-esteriûcation of pectin by the removal of methoxy esters.
3. Depolymerases: catalyze the hydrolytic cleavage of the $\alpha$-(1-4)-glycosidic bonds in the D-galacturonic acid moieties of the pectic substances.

## BIOTECHNOLOGICAL APPLICATIONS OF MICROBIAL PECTINASES

Over the years, pectinases have been used in several conventional industrial processes, such as textile, plant fiber processing, tea, coffee, oil extraction, treatment of industrial wastewater, containing pectinacious material, etc. They have also been reported to work on purification of

viruses (Salazar and Jayasinghe, 1999) and in making of paper (Reid and Richard, 2004; and Viikari *et al*., 2001). They are yet to be commercialized.

# MATERIALS AND METHODS

## Sample Collection

### *Isolation of Microorganisms*

One gram of soil samples from each collection site were pooled and homogenized in sterile distilled water and 10-fold serial dilutions were prepared. 1 ml aliquot's from each dilution was inoculated by spread plate method on to the sterile petriplates containing mineral salt agar medium with pH 7.0 containing pectin at 37°C. Pure cultures were sub cultured onto slant media and maintained for identification and enzyme studies.

Pectinolytic activity:

Pectinase activity was detected by growing fungi in a petriplate on mineral salt agar medium ( $NaNO_3$-2.0 g, KCl-0.5 g, $MgSO_4.7H_2O$-0.5 g, $K_2HPO_4$-1.0 g, $FeSO_4.7H_2O$-0.01 g, Citrus pectin -10.0 g, Agar-20.0 g, pH-6.8/7.0, Distilled water-1000 ml) containing 1% (Mandel and Weber) citrus pectin as sole carbon source. After incubation (at 30° C for 5 days) the plates were stained with ruthenium red and purple halos around the colonies were observed for pectinase.

### *Enzyme Extraction*

22 ml of 0.1 M phosphate buffer (pH 6.5) was added to the cultures, the mixtures were shaken for 30 min at 19 ºC and 140 rpm on a rotary shaker. The mixture was filtered through cheese cloth and centrifuged at 8000 rpm at 4 ºC for 15 min.

**Table 3: Pectinolytic Fungi**

| S. No. | Name of the Fungi | Clear Zone (mm) |
|---|---|---|
| 1. | *Aspergillus niger* | 10 |
| 2. | *Aspergillus flavus* | 15 |
| 3. | *Chaetomium globosum* | 12 |
| 4. | *Aspergillus japonicus* | 10 |

**Figure 3: A. (*Aspergillus niger*), B. (*Aspergillus japonicus*), C. (*Aspergillus flavus*), D. (*Chaetomium globosum*) Purple Halos Around the Colonies were Observed for Pectinase Activity; E. Pectinase Assay by DNSA Method**

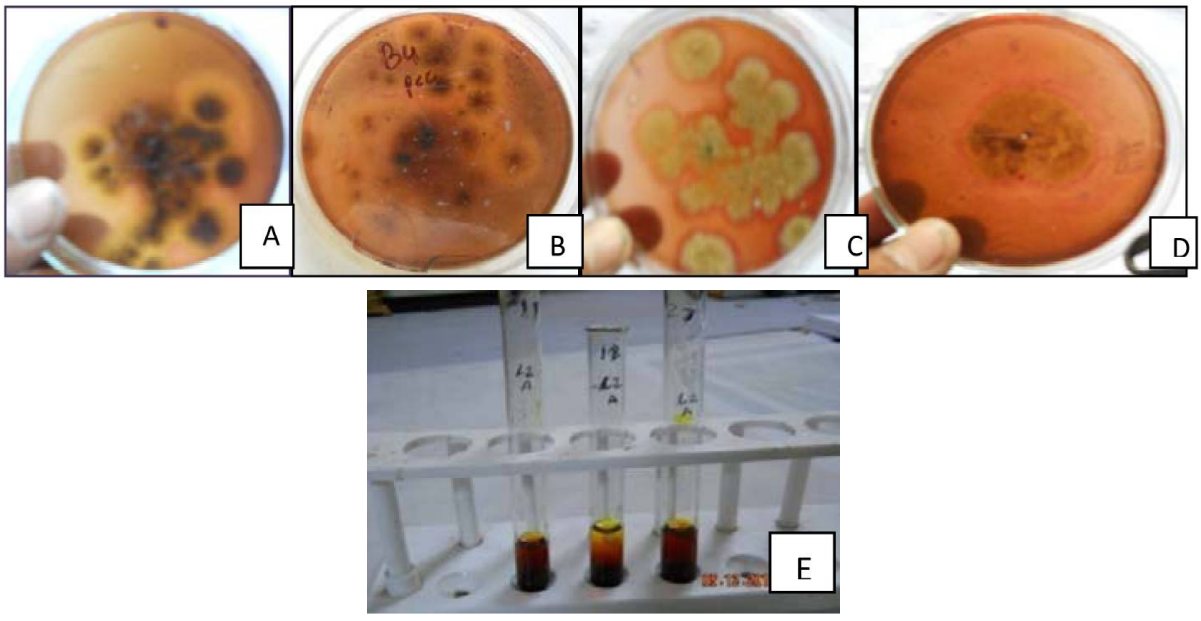
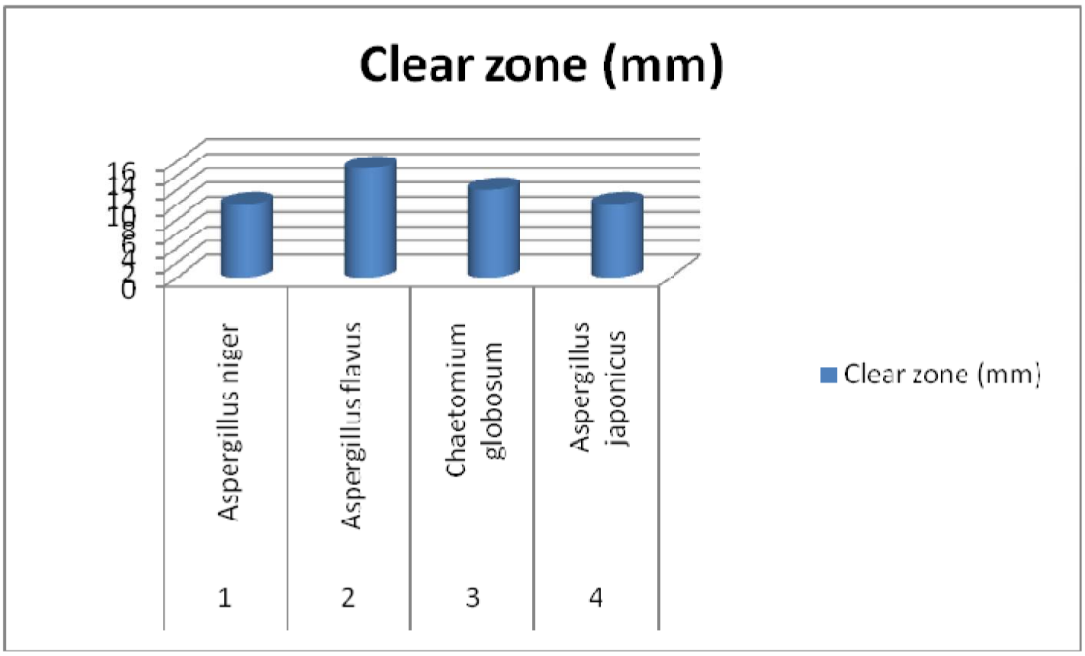


**Figure 4: A Graph Showing a Clear Zone of Pectinolytic Activity**




The supernatant was filtered through What- mann number 1 filter paper and the filtrate was used as the crude enzyme preparation.

***Enzyme Assay***

Cell-free culture filtrate was used as crude enzyme preparation to assay (Dinitrosalicylic acid (DNSA) method ) pectinase activity. Crude enzyme preparation was incubated in the presence of 500 µl (1% w/v) substrate citrus pectin for pectinase.

## DISCUSSION

Microbial Pectinase can be stated as the most important enzyme for the juice industry. Although pectinase production is an inherent property of most all organisms, only those microbes that produce a substantial amount of extracellular pectinase are of industrial importance and have been exploited commercially. Of these, strains of *Aspergillus* species dominate the industrial sector. Aspergillus Niger showed maximum growth at pH 6.8 as compared its growth at other various respective pH range varying from pH 6.8 to pH 7.0. During growth studies, the maximum biomass or cell mass was obtained in between 24 hrs to 48 hrs. The production of Pectinase from *Aspergillus niger* is greatly influenced by initial culture pH. The optimum pH for production of pectinase by *A. niger* was 6.8. In the present study, however, the most significant level of growth and production of pectinase were supported by pH ranging between 6.8 to 7.0. The entire fermentation process was carried out at room temperature (30° C). Earlier studies have stated that the optimum temperature for the production of Pectinase from A.niger was found out to be 28° C (Rombouts *et al*., 1988) and 30° C (Sayem *et al*., 2006).

## CONCLUSION

Most of the studies performed so far have been concentrated with the isolation, screening, production, purification, characterization and applications of pectinolytic enzymes in increasing the fruit juice yield and its clarification. Some reports are available on the applications of pectinases in other industries. Study of the molecular aspects of pectinases and engineering of enzymes that are more stout with respect to their pH and temperature kinetics by the techniques of protein engineering and site directed mutagenesis should receive increased attention in the Future times. Future studies on pectic enzymes should be devoted to the understanding of the regulatory mechanism of the enzyme secretion at the molecular level and the mechanism of action of different pectinolytic enzymes on pectic substances.

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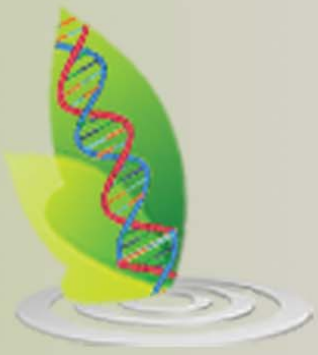

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