Journal of Applied Biosciences (2008), Vol. 9(1): 378 - 384.
ISSN 1997 – 5902: www.biosciences.elewa.org

Partial purification and biochemical characterization of extracellular pectinase from *Aspergillus niger* isolated from groundnut seeds

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ABSTRACT

Objectives: To produce and partially purify pectinases from *Aspergillus niger* isolate JGIm2, characterize the enzymes for potential industrial applications in clarifying fruit juices.

Methodology and results: Isolates of *Aspergillus niger* were screened for ability to produce pectinases by the enzymatic index method. All the 34 isolates screened were producers with isolates JGIm2, JGIm3 and JGIm5 being the best. Optimum enzyme production was found with medium containing 1.5% pectin after 48 h of fermentation. Partial purification of the enzyme was carried out by ethanol precipitation to give two fold purification and 56% yield. The enzyme had pH & temperature optima of 4.0 and 45°C, Km and Vmax values of 0.178 g/dl & 11.62 IU/mg protein, respectively. Clarification of banana and pineapple juice using the partially purified enzyme resulted in 38 and 41% reduction in viscosity as determined spectroscopically.

Conclusions and potential application of findings: The results illustrate that a naturally secreted pectinase, produced by *A. niger* which is a 'generally regarded as a safe' organism has good potential for industrial application in that it's enzyme activity is in the acidic range which is suitable for fruit juices. Low Km, appreciable Vmax and good yield values also enhance its capacity to clarify fruit juices. Pectinases are widely used in industrial processes that need solubilization of the cell wall of plants, wood, fruit or paper. The pectinase identified in this study has potential applications in processing of fruits and vegetables, agricultural and agro-industrial waste, coffee and tea fermentations, and in textile and paper making industry.

Key words: pectinase, partial purification, *Aspergillus niger,* juice clarification

Citation: Rashmi R, Siddalingamurthy KR, Sneha G, Shabana S, Syama A. and Radhika VS, 2008. Partial purification and biochemical characterization of extracellular pectinase from A*spergillus niger* isolated from groundnut seeds. *Journal of Applied Biosciences* 9 (1): 378 - 384.

INTRODUCTION

Recent years have seen a great increase in industrial applications of enzymes owing to their significant biotechnological potential. Pectinases have attracted attention globally as biological catalysts in numerous industrial processes. The enzymes are used in processing agricultural and agro-industrial waste (Jin *et. al.*, 2001; Kashyap *et al.,* 2001; Bai *et al.,* 2004; Sarvamangala & Dayanand, 2006); in clarification of fruit juices and wines, in extraction of vegetable oils and decreasing the viscosity of concentrates (Demir, 2001; Chen *et al.*, 2001; Mantovani *et al.*, 2003), coffee and tea fermentations (Yarkanni *et al*., 2002; Hoondal *et. al.,* 2004), paper making

(Reid *et al.*, 2000; Hoondal *et al.,* 2004), in textile industry for treatment of natural fibers such as linen and ramie fibers, and degumming of plant bast fibers (Baracat *et al.*,1991; Jin *et al.*, 2001; Molina *et al.*, 2001).

Fungi, with their well characterized biology, have been widely exploited as sources of industrially important enzymes. *Aspergillus* and *Penicillium* species are among the principal fungi used in this regard. Members of the genus *Aspergillus* exhibit a high degree of variability in their morphological and physiological characteristics, often caused by the external growth conditions (Samson, 1994) and two species, *A. niger* and *A. oryzae,* are well known enzyme producers (Oxenbull, 1994). *A. niger* has mainly been exploited in the food industry (bakery and starch industry) and beverages (wine and juice, brewery and distilling industry). Many of the products obtained through *A. niger* activity have earned the GRAS (Generally Regarded As Safe) status (Aidoo *et al*., 1994). In the pectin industry, fungal pectinases have major applications and preparations derived from *A. niger* are the most frequently used (Parenicova, 2000).

Microbial pectinases are produced mostly by submerged fermentation in a process that is influenced by the type and concentration of the carbon source, the culture pH, the incubation temperature and the oxygen concentration depending on whether the process is aerobic or anaerobic. Recent work on microbial pectinase production has been oriented in three main directions, i.e. comparison between submerged and solid state fermentation systems, optimization of culture conditions for obtaining better yields and genetic improvement of strains. Studies on media composition and culture conditions have demonstrated that pectinase production can be considerably improved (Jayani *et al*., 2005; Favela-Torres *et al.,* 2006). Fungal pectinases are mainly extracellular enzymes, prominent among them being polygalacturonase, which is also the most commonly assayed to determine pectinase activity. The aim of this work was to identify a potent strain of *Aspergillus* species capable of producing extracellular pectinase by submerged fermentation and to partially purify and characterize the enzyme produced.

MATERIALS AND METHODS

Organism: Thirty-four *A. niger* isolates from different seed sources were obtained from the Department of Microbiology and Department of Biotechnology, Center for Post-graduate Studies, Sri Bhagawan Mahaveer Jain College, Bangalore. All the 34 fungal isolates were maintained by subculturing on to Sabauraud's Dextrose Agar slants at 27°C and stored at 4°C.

Plate assay to screen for pectinolytic activity: Czapek-Dox agar (Atlas, 2004) with 1.5% pectin as the sole source of carbon was centrally inoculated with 2% (v/v) fungal spore suspension (so as to maintain constancy in the amount of inoculum for semi quantitative comparison of clearance zones) and incubated for 3 days at room temperature (28±2°C). Agar medium was amended with ampicillin (200µg/ml) to restrict bacterial growth. Pectin utilization was detected by flooding the culture plates with freshly prepared Iodine-Potassium iodide solution (Iodine-1.0g, Potassium iodide-5.0g in 330ml distilled water) (Hankin *et al*., 1971). This solution gives color to the medium containing pectin resulting in a translucent halo in the region where pectin is degraded.

Enzymatic Index (EI) for the positive isolates was calculated as per Phutela *et al.* (2005). An average of three readings per colony was considered. The isolates JGIm2, JGIm3 & JGIm5 which showed maximum EI were chosen for further studies.

Optimization of culture conditions and enzyme production: The enzymatic activity of the three isolates was compared by biochemical assays in order to confirm the results obtained by the enzymatic index method. The results were further used to optimize culture conditions. The tested culture media were: Medium 1: 2.0g $NaNO_3$; 1.0g K_2HPO_4; 5.0g $MgSO_4$; 5.0g KCl; 0.001g $FeSO_4$; 15.0g Pectin; H_2O up to a liter, pH 3.8 (Sadashivam & Manickam, 1997); Medium 2: 2.9g $(NH_4)_2SO_4$; 1.0g K_2HPO_4; 1.0g Urea; 0.066g $MgSO_4$; 0.096g $FeSO_4$; 0.001g $MnCl_2$; 0.001g $CuSO_4$; 0.001g $ZnSO_4$; 15.0g Pectin in 1000ml of distilled water, pH 4.5 (Diaz-Godinez *et al.*, 2001). The media (250 ml) were inoculated with 0.2 ml of 2% (v/v) fungal spore suspension. The flasks were incubated on shaker at 160 rpm at 28±2°C for one, two and three days. Inclusion of pectin as the substrate was tested at 1.0,

1.5 and 2.0% to determine suitable rate for optimum growth and enzyme production. After incubation, the biomass was separated by filtration and the culture filtrate used as the source of crude enzyme.

Enzyme assay: Polygalacturonase activity was assayed by quantifying the liberated reducing sugars using the DNS method (Miller, 1959). One international unit (IU) of pectinase activity was defined as micromoles of reducing sugars liberated per milliliter of the enzyme per minute. Protein was estimated using the modified method of Lowry (Peterson, 1977).

Partial purification of enzyme: The culture filtrate produced by *A. niger* isolate JGIm2 was used for all further investigations. The culture filtrate was cooled to 4^{o}C for 30min, treated with three volumes of chilled absolute alcohol/acetone and allowed to stand for 15min. The precipitate obtained by centrifugation (5000 rpm, 10 min) was dissolved in distilled water and used for further studies (Yarkanni *et al.,* 2002).

Determination of kinetic parameters: The optimum pH was determined in reactions carried out at pH values ranging from 3.0 - 10 using 0.1M buffers-Citrate (pH 3.0 and 4.0); Acetate (pH 4.5 - 5.5); Phosphate (pH 6.0 - 7.0) and Tris (pH 8.0 to 10.0). Optimum temperature was determined for reaction mixtures with temperature ranging from 27 - 80°C. Optimal incubation period was determined by measuring enzyme activity between 10 - 80 min. Michaelis-Menten constants were determined with substrate concentration between 0.025 to 0.7 % using Lineweaver-Burk plots (Benen *et al.,* 1999).

Juice clarification: The efficacy of the enzyme to clarify juices was tested using pineapple and banana juice. Fruit samples (1.0 g) were blended in 3.0 ml distilled water and the solution filtered through a layer of muslin cloth to remove the solid matter. Two milliliter of the juice extract was incubated with 0.2 ml of the partially purified enzyme and percent clarification was determined spectroscopically at 660 nm (Chen *et al.*, 2001).

RESULTS

Thirty-four *A. niger* isolates obtained from different seeds were screened by the Enzymatic Index method. EI ranged between 1.0 and 1.88 (Fig. 1) and isolates with values above 1.5 were considered to be potential candidates. Nine isolates showed significant EI of which three isolates from groundnut seeds, i.e. JGIm2, JGIm3 and JGIm5 were the best.

The three strains JGIm2, JGIm3 & JGIm5 were chosen for optimization of enzyme production by submerged fermentation. Of the two media investigated, medium 2 showed better activity (Fig. 2) and hence was chosen as the growth medium. Specifically, strain JGIm2 showed highest enzyme production activity in media 2 and hence it was used for purification.

The optimal concentration of pectin for fungal growth was 2.0% but this interfered with purification due to gelling, which was identified to be due to the interaction of alcohol with the unutilized pectin present in the culture filtrate. However 1.5% substrate was found to be optimal for growth as well as purification and hence chosen as the ideal substrate concentration. Optimum fermentation time was 48 h after which the activity declined (Fig. 3).

The enzyme from *A. niger* isolate JGIm2 was subjected to partial purification. Precipitation with absolute alcohol was found to be ideal resulting into two fold purification and 56% yield. Precipitation with acetone resulted in approximately 40% less enzyme activity. The partially purified enzyme had pH optima of 4.0 (Fig. 4A), temperature optima of 45°C (Fig. 4B), Km value of 0.178 g/dl and Vmax of 11.62 IU/mg protein. Juice clarification determines the efficacy of pectinases for fruit pulp defibrillation. By measuring the increase in transmittance, the reduction in viscosity is quantified in comparison with untreated juice as the control. Partially purified pectinase from isolate JGIm2 clarified pineapple juice to an extent of 41 and banana juice up to 38% (Table 1).

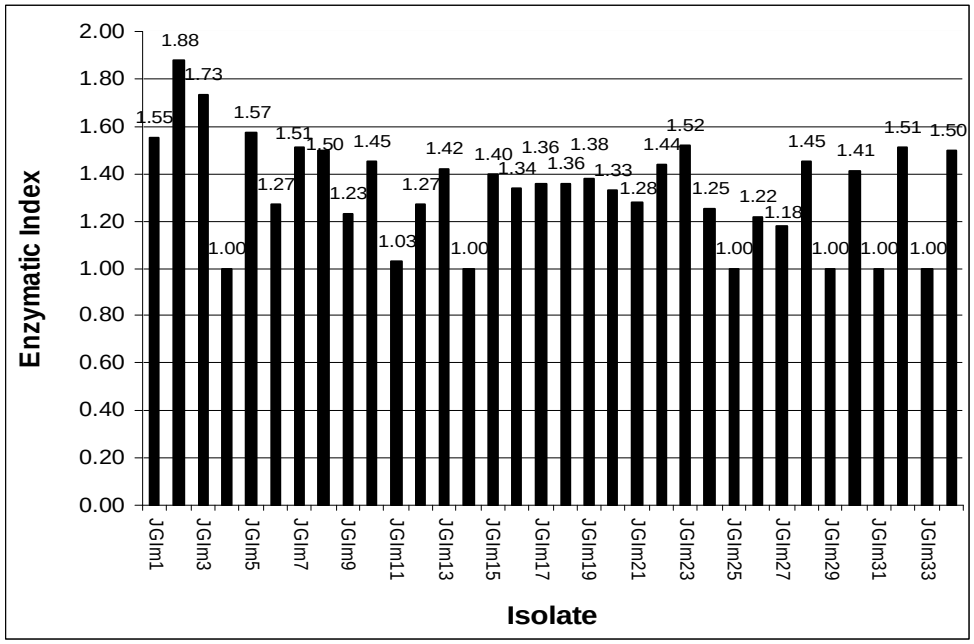

Fig. 1: Enzymatic indices of 34 *Aspergillus niger* isolates.

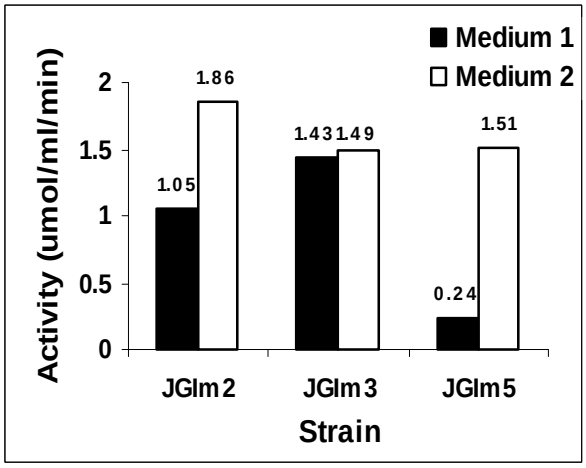

Figure 2: Comparison of pectinase activity (IU) in two different media.

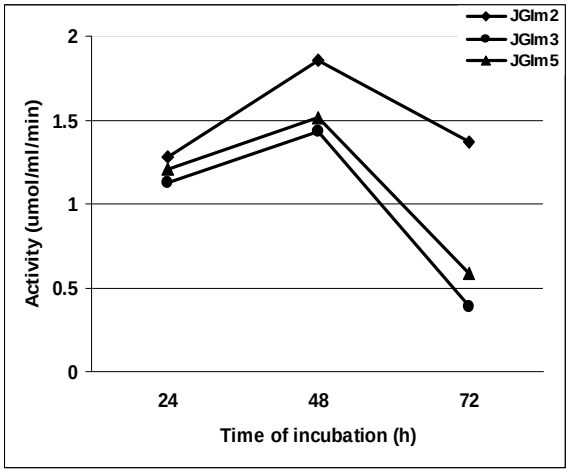

Figure 3: Optimization of time of incubation for pectinase production.

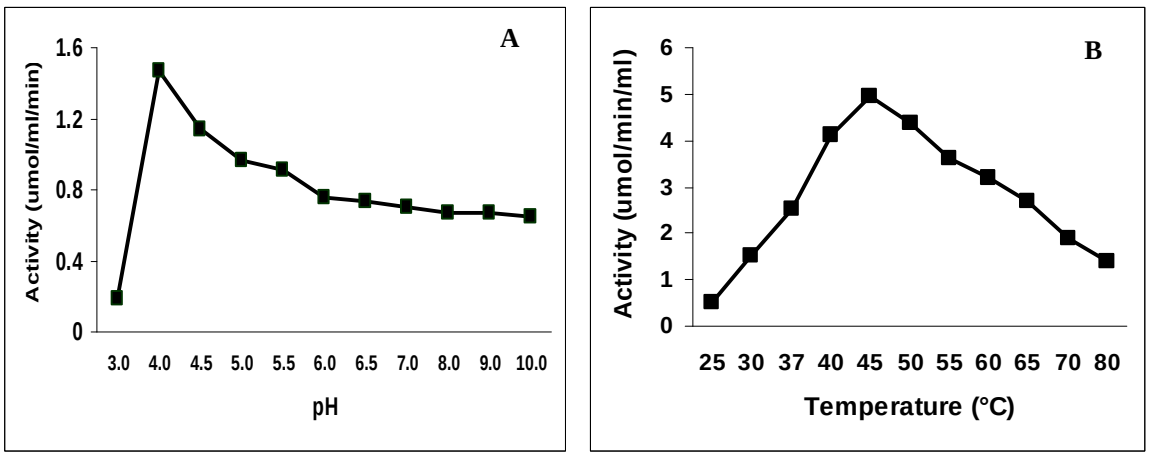

Figure 4: pH (A) and temperature optima (B) of pectinase from *Aspergillus niger* isolate JGIm2.

Table 1: Properties of the partially purified pectinase from *Aspergillus niger* isolate JGIm2.

Parameter	Values
pH optima	4.0
Temperature optima	45 °C
Km	0.178 g/dl
Vmax	11.62 IU/mg
JC* (Banana)	38
JC* (Pineapple)	41

*JC-Percentage of juice clarification

DISCUSSION

Pectinases from *A. niger* have been studied widely due to their ease of adaptability to various uses. In this study, 34 mesophilic fungal strains possessing pectinolytic activity were identified. Further biochemical assays identified isolate JGIm2 from groundnut seeds as the best producer of pectinase. The plate assay approach that was used in this study has been widely employed for isolating and screening many enzyme producers (Hankin *et al.,* 1971; Soares *et al.,* 1999).

Under conditions of submerged cultivation, higher activities were found in media supplemented with urea and trace elements. The highest pectinase activity was observed after two days of incubation. This shorter fermentation period can be advantageous for industrial production. Said *et al*. (1991) have reported maximum pectinase activity for *Penicillium frequentans* after culturing for 48h in media containing urea and trace elements.

A. niger JGIm2 ably produced pectinase when grown on pectin as the carbon source. Activities of pectinase have been found to be highest when grown on pectin rather than on other sources of carbon (Teixeira *et al.,* 2000). Purification with ethanol is a common method employed for many enzymes including pectinases and cellulases (Dalal *et al., 2002*). In this study pectinase that was precipitated using ethanol retained more activity than that precipitated with acetone. Comparing the kinetic parameters and biochemical properties of the purified enzyme, it seems to be equivalent to the endoPG I reported by Benen *et al*. (1999).

The pH optimum of 4.0 is in agreement with that reported by Martin *et al.* (2004) with *Penicillium* sp. and also by Jyothi *et al.* (2005) and Diaz-Godinez *et al*. (2001) with *A. niger*. Alana *et al.* (1989) have also reported low pH values as being favorable for high pectinase production in *Penicillium italicum*. The optimum pH of mesophilic pectinases has been established to range between 4.0-5.5 and temperature optimum between 36-45°C (Favela-Torres *et al*., 2006).

Pectinases increase juice yields and accelerate juice clarification producing clear and stable single-strength juices, juice concentrates and wines. The use of pectinase not only increases juice yields, but also increases the color and health-promoting antioxidants in fruit and vegetable juices. By reducing fruit mash viscosity and improving solid/liquid separation, they increase color extraction and juice volume by as much as 15 percent. The juice clarification capacity of pectinase is determined by monitoring reduction in viscosity.

The method of evaluating reduction in viscosity i.e., increase in transmittance at 660nm, which signifies clarification, has been reported with a variety of fruit juices viz. mosambi, orange, tomato, grape etc, using pectinases from *A. niger* (Rai *et al.*, 2004) as well as those from other fungi like *Penicillium expansum* (Mantovani *et. al.,* 2003) for clarification of apple, grape and passion fruit juices, *Saccharomyces cerevisiae* for clarification of grape, orange and apple juices (Gainvors *et.al.,*1994) etc. The partially purified enzyme clarified pineapple and banana juice to an extent of 41 and 38% respectively, which is good from industrial point of view.

Acknowledgement: The authors would like to acknowledge the financial support given by Sri Bhagawan Mahaveer Jain College, Bangalore.

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