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Full Length Research Paper

Screening of natural substrates and optimization of operating variables on the production of pectinase by submerged fermentation using *Aspergillus niger* MTCC 281

M. Palaniyappan[1]*, V. Vijayagopal[2], Renuka Viswanathan[1], T. Viruthagiri[3]

[1]Department of Chemical Engineering and Biotechnology, St. Joseph's College of Engineering, Chennai - 600 119, Tamil Nadu. India.
[2]Department of Chemical Engineering, Eritrea Institute of Technology, Eritrea, North-East Africa.
[3]Department of Chemical Engineering, Annamalai University, Annamalai Nagar - 608 002. Tamil Nadu, India.

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Pectinases are a group of hydrolytic enzymes that play an important role in food processing industry and alcoholic beverage industry. The present work aims at studying different natural substrates such as wheat flour and corn flour in comparison with synthetic pectin for the production of pectinase using *Aspergillus niger* (MTCC: 281). The work involves optimizing various parameters like substrate concentration, pH, temperature, rpm, time of fermentation for the production of pectinase and the effect of carbon sources on the synthesis of the pectinase enzyme to suggest a plausible, commercially suitable substrate than the standard. The experimental studies indicate that maximum synthesis of pectinase (6.1 U/mL) was obtained with *A. niger* (MTCC: 281) by using wheat as substrate under the influence of the additional carbon source, starch. The optimal conditions are found to be substrate concentration - wheat (1%), pH 5.5, temperature 30°C, time 72 h, rpm 170, and carbon source starch (0.025%).

Key words: Pectinase, polygalacturonase, *Aspergillus niger*, catabolite repression, pectin, pectolytic enzymes.

INTRODUCTION

The pectic substances, located primarily in the middle lamella between cells in higher plant tissues, are complex polysaccharides (Whitaker, 1984). The term "pectins" encompasses a group of acidic heteropolysaccharides with distinct structural domains. They are subjected to both biosynthetic and cell wall-based modifications. The chemical structure of pectins has been the subject of many scientific investigations for decades (Perez et al., 2003). On the other hand, pectinases are a group of related enzymes involved in the breakdown of pectin from a variety of plants. These enzymes are classified based on their preferred substrate (pectin, pectic acid or oligo-D-galacturonate), the degradation mechanism (transelimination or hydrolysis) and the type of cleavage (random [endo-] or terminal [exo-]) (Kashyap et al., 2001).

Pectinases are extensively used in fruit juice processing (extraction and clarification), vegetable oil extraction, processing of alcoholic beverages and a variety of application in food industries (Brawman, 1981; Phutela et al., 2005). The synthesis of pectolytic enzymes by microorganisms has been reported to be highly influenced by the components of the growth medium. Most extracellularly induced enzymes are known to be synthesized in higher quantities when inducers are present in the cultivation medium (Alkorta et al., 1998; Lang and Dornen-burg, 2000).

The production of pectolytic enzymes using different sources and the effect of physical parameters such as temperature, aeration rate and type of fermentation were investigated and reported in literature (Nair et al., 1995; Naidu and Panda, 1998). Pectolytic enzymes have been reported to be induced by several substances. In many cases pectin itself has been used.

*Corresponding author. E-mail: tmpalaniyappan@gmail.com. Tel: +91 9443678571. Fax: +91 4424500861.

Other investigators had used complex media such as beet sugar, wheat bran, ground nut meal, citrus fruit peels etc (Kilara, 1982; Hoondal et al., 2002). In the industrial market pectolytic enzymes contribute to at most 25% of the global enzyme sales, where its contribution is estimated to increase further by the year 2009 (Tari et al., 2007). As compared to solid state fermentation, submerged fermentation has been extensively employed for the production of enzymes and to understand physio-ogical aspects of the synthesis of enzymes (Pereira et al., 1993; Patil and Dayanand, 2006).

Due to the potential and wide applications of pectinases, it is necessary to study on several aspects related to pectinase production. The idea of using cheaper raw materials for pectinase production is an important para-meter in useful technological development (Panda et al., 2004). The aim of this experimental paper is to present an overview of the pectinase activity values obtained by fungal strain *A. niger* from different substrates in submerged fermentation (Friedrich et al., 1989; Bailey, 1990). The addition of carbon sources like glucose (monosaccharides), sucrose (disaccharides) and starch (polysaccharides) with various concentrations in fermentation medium were studied.

MATERIALS AND METHODS

Selection of microorganisms

More than 30 different genera of bacteria yeasts and moulds have been used for the production of pectinases. However, *Erwinia*, *Bacillus*, *Saccharomyces, Kluyveromyces*, *Aspergillus*, *Penicillum*, *Fusarium* and *Rhizopus* have been the genera most frequently studied in the last 15 years, with strains of *Aspergillus, Penicillum* and *Erwinia* mainly used for enzyme production studies. Selection of the microbial source for pectinase production depends on several features, such as the type of culture (solid-state or submerged fermentation), number and type of the produced pectinases (esterases, hydrolytic depolymerases and eliminative depolymerases), pH and thermal stability of the enzymes (Panda et al., 2004; Patil and Dayanand, 2006; Tari et al., 2007).

Large-scale production of pectinases industrially and comercially has been carried out with fungal organisms especially of the genus *Aspergillus.* Therefore for the present study *A. niger* (MTCC 281) has been chosen for the production of pectinases with different substrates such as wheat flour, corn flour and pectin.

Subculture and maintenance of microorganism

The strains were subcultured on Potato Dextrose Agar slants and incubated for 72 h at 30°C. The subcultured strains were maintain-ned in a refrigerator at 4°C and subcultured at regular intervals.

Inoculum preparation

The inoculums were prepared by growing the microorganism in Potato Dextrose Agar slants for 72 h at 30°C. The spores were harvested by addition of 5 ml of isotonic NaCl solution. After inoculation of the different media with the inoculums, the flasks were placed on a rotary shaker at room temperature, kept at 160 rpm. After 4 to 5 days, clear growth of species in all media was observed.

Fermentation medium and culture conditions

The medium which was optimized for pectinase synthesis contained (g/L), $KHPO_4 3H_2O$ 1; yeast extract 5; sucrose 30 and Czapek concentrate 10 ml. Czapek concentrate contained (g/100 ml), $NaNO_3$ 30; KCl 5; $MgSO_4.7H_2O$ 5; $FeSO_4.7H_2O$ 0.1. Submerged cultivation was carried out in 500 ml Erlenmeyer flasks containing 100 ml of the sterile cultivation medium. Slants of *A. niger* (MTCC: 281) was used as inoculum (1 ml). Cultures were incubated in a rotary shaker maintained for 5 days and samples were withdrawn every 24 h for analyzing the extracellular enzyme activity.

Pectinase assay

Exopectinolytic activity in cell free filtrates was assayed by quantification of reducing sugars using the DNS method (Miller, 1959), that were liberated by 0.1 ml filtrate mixed with 0.5 ml 1.0% pectin and 0.4 ml acetate buffer pH 5.0 incubated for 20 min at 45ºC. Results were expressed as galacturonic acid equivalents. One unit (U) of exopectinolytic activity was defined as the amount of enzyme that catalyzes the formation of 1 µmol galacturonic acid under the assay conditions (Minjares Carranco et al., 1997).

RESULTS AND DISCUSSION

Effect of substrate concentration on enzyme activity

The effect of substrate concentration is depicted in Figure 1. The enzyme activity was maximum (5.17 U/ml) at 84 h of fermentation for 1% wheat flour as substrate when compared with corn flour for *A. niger*. But the activity of wheat flour and corn flour is almost comparable with that of pectin (5.84 U/ml) as substrate. There is a decrease in the activity of wheat flour, corn flour and pectin when there is an increase in the substrate concentration beyond 1%; this may be due to the fermentation of other metabolites during fermentation and also because of increase in viscosity of the broth. The temperature, pH and rpm were maintained at 28°C, 5 and 160 respectively. The substrate concentration was maintained at 1% w/v for optimization of operating variables in the further investigation.

Effect of temperature on enzyme activity

Figure 2 shows the effect of temperature on the activity of the enzyme. It can be seen that the optimum temperature for the production of pectinases is 30°C (ambient temperature) for all the substrate at 1% (w/v), after which there is a decrease in the activity of the enzyme. From this we can infer that higher temperature affect the production and the activity of the enzyme. Thus a maximum of 5.05 U/ml for wheat flour, 4.59 U/ml for corn flour and 5.15 U/ml for pectin was obtained with *A. niger*. From the data it can be observed that *A. niger* using wheat as a substrate gives better result (5.15 U/ml) when compared with synthetic substrate pectin.

The temperature was identified as 30°C for maximum production and maintained the same in further investiga-

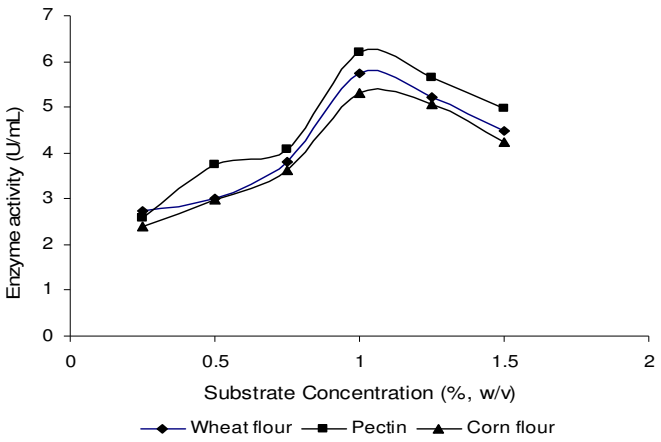

Figure 1. Effect of substrate concentration on pectinase production by *A. niger*.

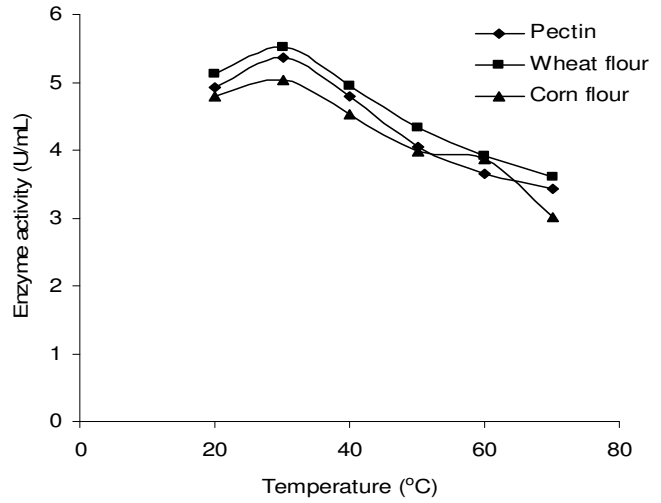

Figure 2. Effect of temperature on pectinase production by *A. niger*.

tion of pH, time and agitation speed. The operating variables such as agitation speed, time and pH were maintain-ed at 160 rpm, 84 h and 5, respectively.

Effect of pH on enzyme activity

Figure 3 shows the effect of pH on enzyme activity. The pH for the maximum production of pectinase was found to be 5.5 for the wheat flour (4.74 U/ml) compared to pectin (4.97 U/ml) substrate using *A. niger*. The same was found to be optimum pH for corn and pectin. It can be inferred that pH below or above 5.5 results in decrease in the activity of the enzyme, which could be due to the fact that as the acidity of fermentation medium increases the activity or production of the enzyme decreases. The optimum pH of 5.5 was used for further

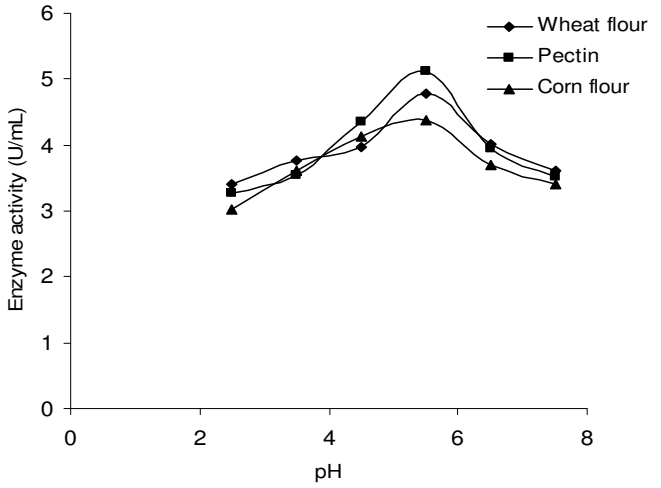

Figure 3. Effect of pH on pectinase production by *A. niger*.

studies. The operating variables such as agitation speed and time were maintained at 160 rpm and 84 h respectively.

Effect of time on enzyme activity

Figure 4 shows the effect of time of fermentation on the production of the enzyme. It can be noticed that the optimum time of fermentation was found to be 72 h after which there is decrease in the production of the enzyme. 5.74 U/ml for wheat as substrate was obtained compared to pectin (5.54 U/ml) using *A. niger*. The decrease in the activity can be due to the depletion of nutrients in the medium. The time was identified as 72 h for maximum production which was maintained to study the effect of other parameters. The operating conditions such as agitation speed, pH were maintained at 160 rpm, 5.5 respectively which was identified as optimum in the previous section.

Effect of agitation speed on enzyme activity

The effect of rpm for the production of the enzyme is depicted in Figure 5. It can be noticed that optimum level of rotation is needed for the maximum production of the enzyme is 170 rpm. With an increase in the rpm level from 100 rpm with a gradation of 20 there has been an increase in the production of pectinase and the maximum activity was 5.04 U/ml after 72 h of fermentation for wheat as substrate compared to pectin (5.21 U/ml) using *A. niger*. With further increase in the rpm level, there is a decrease in the activity; this could be due to the fact that the increase in the rpm level has resulted in the coa-gulation of the organism to form as lumps and decrease in rate of mass transfer.

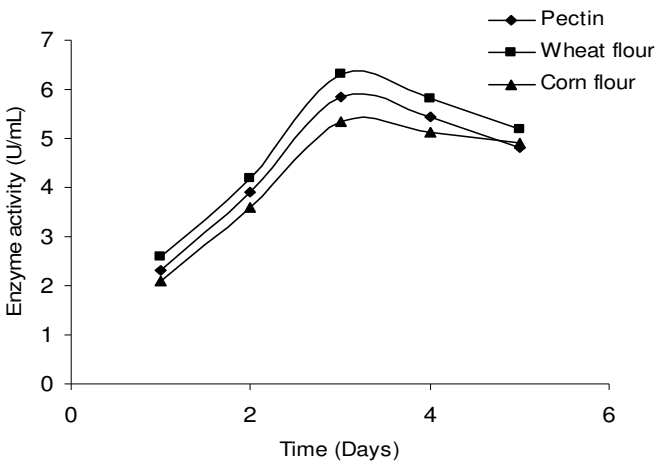

Figure 4. Effect of time on pectinase production by *A. niger*.

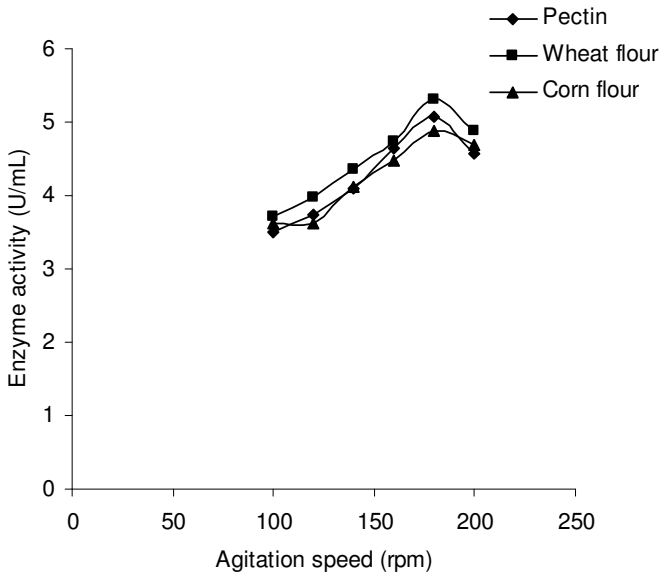

Figure 5. Effect of rpm on pectinase production by *A. niger*.

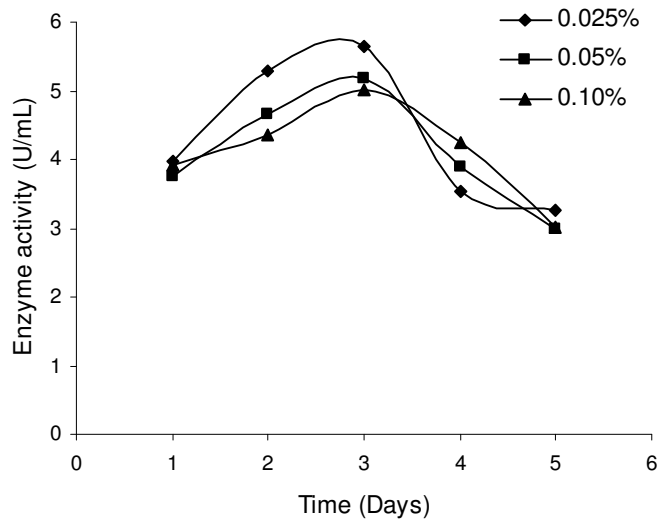

Figure 6. Influence of glucose on pectinase production by *A. niger*.

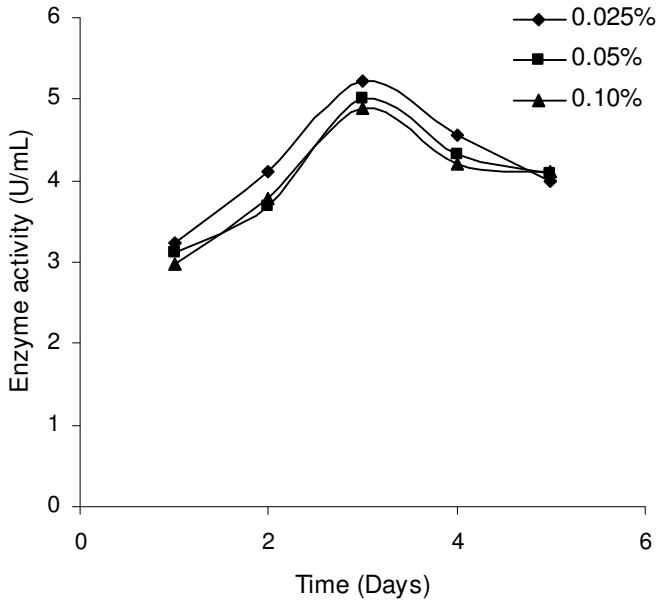

Figure 7. Influence of sucrose on pectinase production by *A. niger*.

Influence of carbon sources

The addition of carbon sources like glucose (monosaccharides), sucrose (disaccharides), starch (polysaccharides) with various concentrations; 0.025, 0.05, 0.1% to the fermentation medium and their effect were studied. The substrate was chosen as wheat with 1% (w/v) concentration based on its maximum productivity when compared to corn. The operating variables such as time, temperature, pH and rpm were maintained at its optimal values 72 h, 30ºC, 5.5 and 170 rpm, respectively, which was identified from previous work represented in Figures 1 to 5.

From Figures 5 - 8 it can be inferred that starch with concentration of 0.025% produced maximum enzyme activity using wheat as a substrate. It shows that the addition of carbon sources acted as a catabolite repressor at higher concentration.

Conclusion

It can be summarized that of the three sources (wheat, corn and pectin), wheat gave better results when compared with the other two and the results were comparable with that of synthetic substrate pectin. The effect of carbon sources such as glucose, sucrose and starch on the production of pectinase was investigated and starch

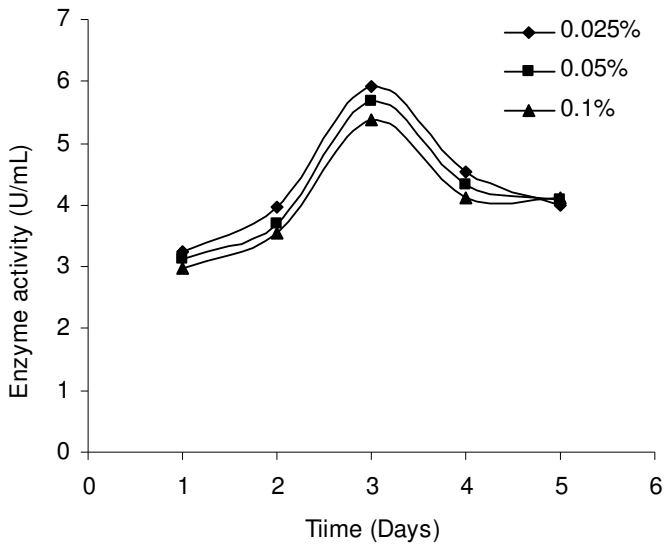

Figure 8. Influence of starch on pectinase production by *A. niger*.

was identified to induce maximum pectinase at 0.025% (w/v). The optimal conditions are found to be substrate concentration wheat (1%, w/v), pH 5.5, temperature 30°C, time 72 h, rpm 170, and carbon source starch (0.025%, w/v).

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