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Full Length Research Paper

Production and partial purification of pectin lyase by *Aspergillus niger* grown on orange peels

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The peel of citrus fruits contains a large percentage of pectin which can be a good substrate for pectinolytic microorganisms. These microbes secrete large amount of extracellular enzymes to degrade the cell wall of substrates. The current study was conducted for the production and characterization of pectin lyase from *Aspergillus niger* using citrus fruits peel as a substrate. The optimum pectin lyase production was analyzed from pectin lyase activity assay. *A. niger* showed maximum production after 96 h at 30°C, 8.0 pH, 4 mL inoculums and 0.1% peptone. Tween-80 was used as a surfactant and showed negative effect on pectin lyase production. Pectin lyase was purified by the addition of 60% of ammonium sulfate and showed maximum activity at 30°C and 8.0 pH.

Key words: Citrus fruits, pectin lyase, *A. niger,* peptone.

INTRODUCTION

Citrus fruits are one of the important fruits, produce all over the world. These include orange, kinnow, Malta, Mausami and sweet orange (Dhillon et al., 2004). Punjab is one of the major producer of citrus fruits in Pakistan (Kareem and Adebowale, 2007) Pectin is the major component of primary cell wall of all citrus fruits. Pectin is a polysaccharide which have important nutritional and gelleting properties in foods (Mohnen, 2008).

Pectinolytic enzymes can be produced in large amount by microorganisms, using citrus peel as a substrate because it contains considerable quantity of pectin. It works as inducer for the synthesis of pectinolytic enzymes by microbial systems (Dhillon et al., 2004). These enzymes have the ability to degrade and chemically modify pectin (Zhang, 2006).

Pectinases are commonly employed in juice, textile, paper and pulp industries. These enzymes catalyzed the conversion of complex polysaccharides into simpler molecules like galacturonic acids (Kashyap et al., 2000; Giese et al., 2008). These have wide industrial applications like oil extraction, tea extraction, juice clarification and waste water treatment (Hoondal et al., 2002; Botella et al., 2007; Mohnen, 2008).

Microorganisms have various advantages and can be used for enzymes production at higher level. Pectinolytic enzymes have great biotechnological potential and can be employed in many important industrial processes (Tewari et al., 2005; Zhong and Cen, 2005). *Aspergillus niger* belongs to ascomycota group of fungi, genus *Aspergillus*. It is an opportunistically infectious microbe to human being and well adapted to environmental changes (Samson et al., 2001; Baker, 2006). The current study was designed for the optimization of production of pectin lyases by *A. niger* and then its characterization after partial purification.

MATERIALS AND METHODS

Substrate preparation

Orange (*Citrus sinensis*) peel was used as substrate, which was obtained from local fruit market of Rawalpindi, Pakistan. It was

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Table 1. Composition of PDA media for *A. niger.*

Number	Chemical	Concentration (g/100 mL)
1	Starch from potato	2
2	D- glucose	2
3	Agar	2
4	Urea	0.3
5	$MgSO_4.7H_2O$	0.05
6	Potassium chloride	0.015
7	KH_2PO_4	0.008
8	$ZnSO_4.7H_2O$	0.001
9	D. H_2O	to100 mL mark

Table 2. Composition of mineral salts solution for moistening substrate.

Number	Chemical	Concentration (g/L)
1	Urea	3
2	$MgSO_4.7H_2O$	0.5
3	KCl	0.15
4	KH_2PO_4	0.08
5	$ZnSO_4.7H_2O$	0.01
1	Distilled water	Upto 1L mark

sliced, air dried and meshed with 40 mm mesh.

Fermentative organism and sporulation medium

Pure culture of *A. niger* was obtained from Plant Pathology Department, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi. (PMAS AAUR). It was maintained on potato dextrose agar (PDA) medium (Table 1) and preserved in inoculum medium (pH 4), lacking agar, for pectin lyase production (Asghar et al., 2000). Numbers of spores were adjusted at 10^7-10^8 spores/mL microscope (Kolmer et al., 1951).

Solid state fermentation

All experimental treatments were performed in triplicate flasks containing 5 g substrate moistened with mineral salts solution (Table 2). Flasks were plugged with cotton and growth medium was autoclaved at standard conditions. 2 mL of *A. niger* inoculum was added under aseptic conditions in autoclaved flasks. These flasks were then placed at 28°C temperature for specific time period.

Enzyme harvesting

Enzyme was harvested from growth media by sample contact method as described by Krishna and Chandrasekaran (1996). Harvested crude enzyme was stored at 4°C before performing enzyme assay.

Optimization of enzyme production

Pectin lyase production was increased by optimizing various fermentation parameters. These parameters effect the growth and production of fungus. Classical method was adopted for optimization of fermentation parameters by varying one parameter in an experiment and to incorporate it at a standardized level before optimizing the next parameter. Parameters optimized during the current study are as follows.

Growth conditions

Suitable growth conditions like time period, temperature, pH and moisture level are necessary for the growth of fungus. All these conditions were optimized in order to produce maximum pectin lyase.

Nutritional conditions

Presence of additional nutrients in the vicinity of fungus enhances its ability of enzyme production. During the current study, the effect of Tween-20, yeast extract and peptone was checked as additional nutrients.

Enzyme characterization

Pectin lyase was characterized for pH and temperature to increase its activity. Tris HCl buffer (50 mM) was used to adjust the pH (pH 4 to 9) of the assay mixture. Enzyme assay was performed at different temperatures for temperature optimization.

Partial purification

Ammonium sulphate precipitation

Ammonium sulphate is water soluble ionic compound, maintain high ionic strength and precipitate out proteins by salting out. At high ionic strength, salt may remove water of hydration from proteins and reduce solubility, hence proteins were coagulated. Various concentrations of ammonium sulfate were used to obtained maximum precipitation and purification.

Analytical methods

Pectin lyase assay

Assay of pectin lyase was performed by the method described by Preiss and Ashwell (1963). 0.5 mL of enzyme was incubated for 1 h with 0.5 mL of 0.5% pectin and 1 mL of 50 mM Tris HCl buffer (pH 8) and 1 mL of 0.2 mM $CaCl_2$. After 1 h, absorbance was measured at 548 nm against blank. One unit of Pectinlyase activity was defined as “the amount of enzyme present in 1 mL of original enzyme solution which released 1 µM of galcturonic acid in 1 min.”

$$\text{Enzyme activity (U/ml/min)} = \frac{\text{Absorbance of enzyme solution x standard factor}}{\text{Time of incubation}}$$

Whereas,

$$\text{Standard factor} = \frac{\text{Concentration (µM/ml) of standard}}{\text{Absorbance}}$$

Protein estimation

The sample protein was estimated by Biuret method (Gornall et al.,

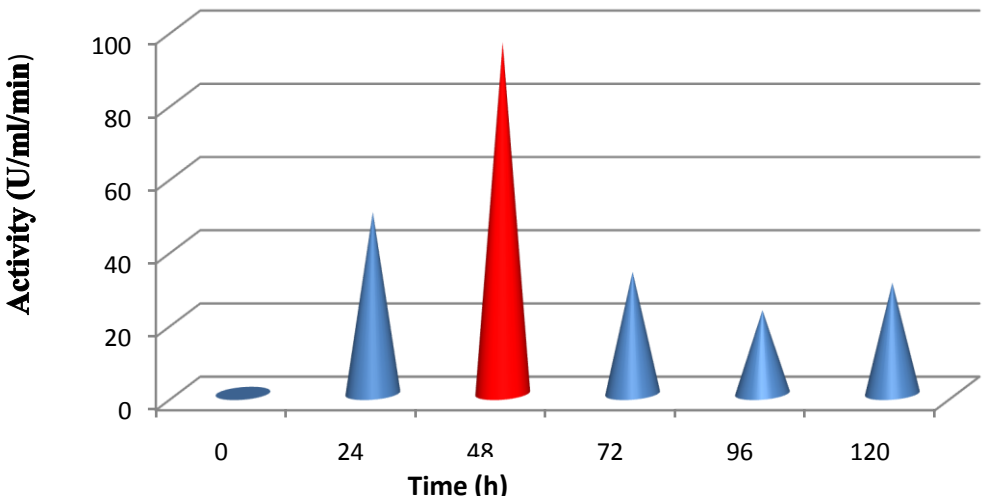

Figure 1. Pectinlyase activity with varying time period.

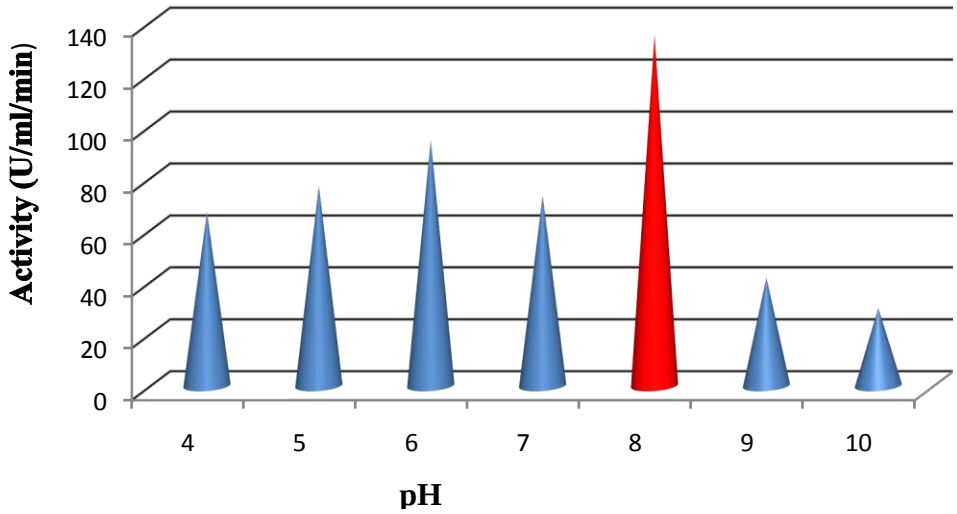

Figure 2. Pectin lyase activity with varying pH.

1949) using bovine serum albumin (BSA) as a standard.

Specific activity

It is defined as number of units of enzyme activity per mg of protein.

RESULTS AND DISCUSSION

Microbes are the best source to obtain the important enzymes for human needs (Shafique et al., 2009). Enzymes synthesis by microorganisms is affected mainly by substrate, size of substrate particles, surface area of substrate, oxygen utilization, water %, humidity, fermentation temperature, period of incubation and carbon dioxide removal (Jacob and Prema, 2008; Palaniyappan et al., 2009).

In the current investigation, maximum pectin lyase activity was observed after 48 h of incubation (Figure 1). With the increase in incubation period, production of enzyme decrease due to accumulation of waste material and unavailability of nutrients. Pectin lyase has maximum activity at pH 8 of the growth media, indicating that pectin lyase produced by *A. niger* is alkaline in nature (Figure 2). Presence of 60% water contents other than inoculums is the most suitable for both, fungal growth as well as pectin lyase secretion (Figure 3). Similarly, 30°C is the most suitable temperature for the growth and production of pectin lyase by *A. niger* (Figure 4). *A. niger* is a mesophilic fungi, growing well in moderate conditions and

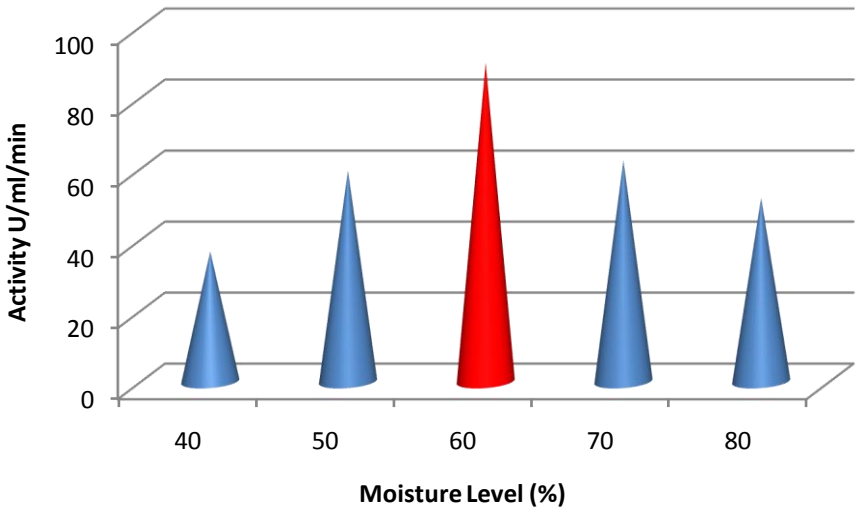

Figure 3. Pectin lyase activity with varying moisture level.

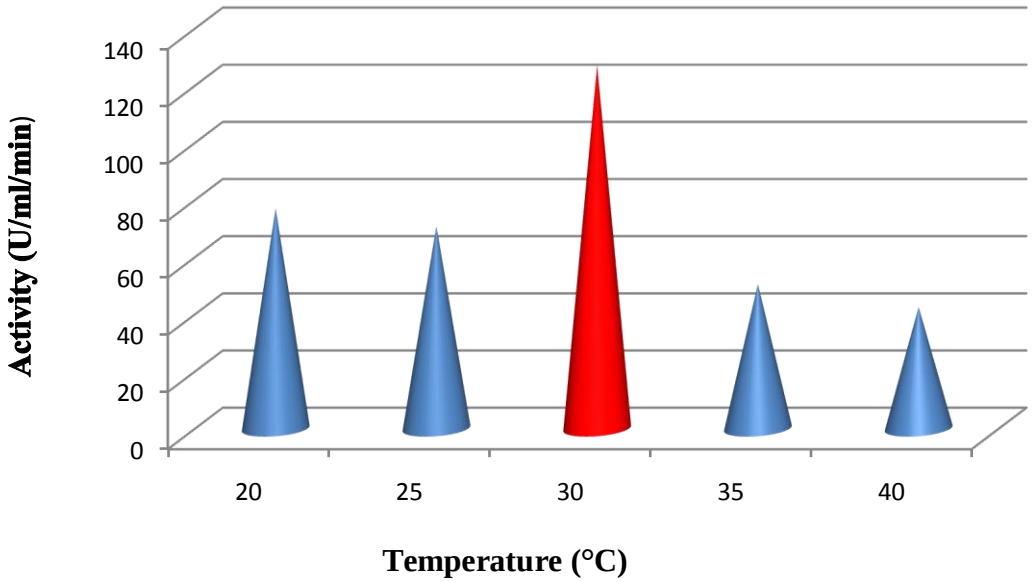

Figure 4. Pectinlyase activity with varying incubation temperature.

temperature.

Additional nutrients (other than substrate) in the growing media enhance the growth of fungi and growth related enzymes secretion. Addition of 0.1% of peptone (% of total dry substrate) as a nitrogen source further enhanced the production of pectin lyase (Figure 5). Yeast extract was added as additional carbon source and it was found that 0.4 % (% of total dry substrate) was the most suitable quantity for the growth and production of pectin lyase (Figure 6). There was negative effect of Tween-80 on the production of pectin lyase as indicated by decreased in its activity (Figure 7).

Various optimum time periods (24 to 120 h) were reported for the production of pectin lyase and other extracellular enzymes by fungi. This difference in time is due to variation in substrate and fungal species (Kashyap et al., 2000; Jacob et al., 2008; Gummadi and Kumar, 2006). Pectin lyase is an alkaline enzyme with range of pH values, varying from organisms to organisms (Yadav and Shastri, 2007). Yadav et al. (2008) reported pH 8 as optimum pH for pectin lyase production by fungi. Depending upon fungi species, various suitable temperatures for the growth and production of pectin lyase were reported (25°C to 35°C) by mesophilic fungal

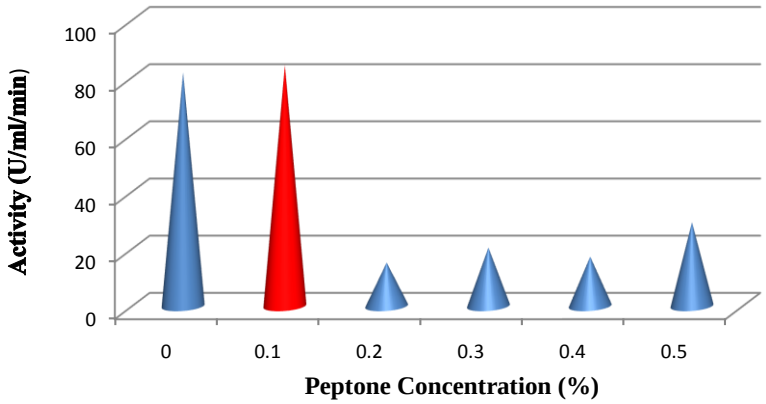

Figure 5. Effect of peptone on pectin lyase production by *A. niger.*

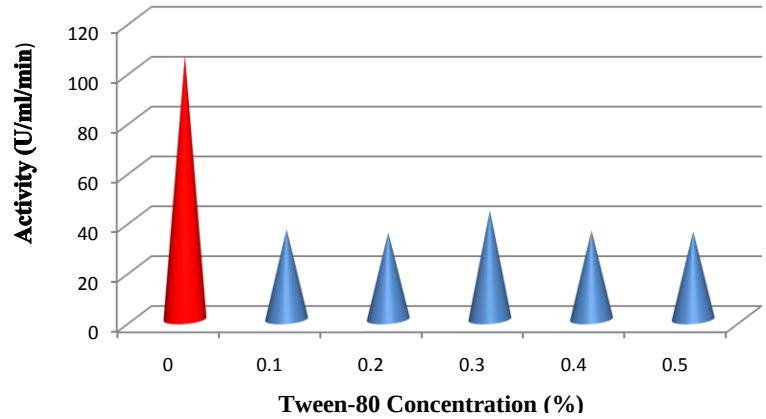

Figure 6. Effect of yeast extract on pectin lyase production by *A. niger.*

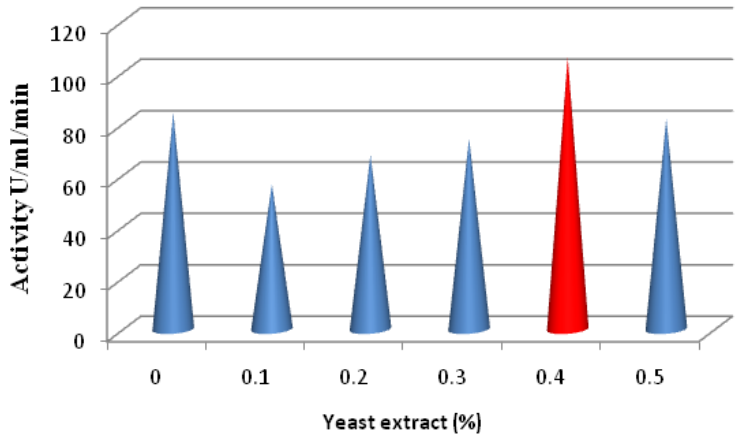

Figure 7. Effect of Tween-80 on pectin lyase production by *A. niger.*

species (Bai et al., 2004; Palaniyappan et al., 2009).

Enhanced pectin lyase production might be related with the growth of fungi at suitable nutritional conditions. Presences of yeast extract as a carbon source and additional nitrogen sources in the growing media increase pectic lyase production (Phutela et al., 2005). Tween-80

interacted with pectin lyase and disrupts its 3-dimensional functional structure and made its non-functional. Peptone contains various amino acids that release nitrogen for the growth of fungi in the media, in the presence of easily available nitrogen source growth of fungi increase (Martin et al., 2004; Margesin et al., 2005).

Effect of pH and temperature on pectin lyase activity

Pectin lyase had maximum enzymatic activity at pH 8 of the mixture and at 30°C. These findings indicate that *A. niger* pectin lyase is alkaline in nature and effective if those processes takes place at moderate temperature level.

Pectin lyase purification

Ammonium sulfate was used for the partial purification of crude pectin lyase; it precipitate protein by salting out process. Maximum protein was purified at 60% of ammonium sulfate, observed from enzyme activity (382.45 U/ml/min.).

Conclusion

From the current study, it can be concluded that *A. niger* can be a good source of pectin lyase. Supplementation of additional carbon and nitrogen are necessary for good enzymatic yield. In order to achieve further active pectin lyase further sophisticated purification techniques should be followed.

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