

Optimization of Cultural Conditions for the Production of Pectinase from Selected Fungal Strain

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*Pectinase are the group of enzymes that catalyze the degradation of pectin substances through de-polymerisation and de-esterification. This study is concerned on the isolation, screening and selection of suitable strain of pectinolytic organism and optimization of cultural conditions for the biosynthesis of pectinase. From the soil samples collected from Lalitpur, Kathmandu, Gulmi, Manang and Dang, 18 fungal colonies were isolated on the basis of halozone formation on Potato dextrose agar and identified. Enzyme production was carried out by submerged state fermentation. The partially purified enzymes showing higher pectinolytic activity were characterized on the basis of temperature of incubation, substrate concentration and pH of the substrate by Dinitro salicylic acid assay (DNSA) method. The fungal isolate showing highest enzyme activity was subjected to optimization of culture medium for the production of enzymes. On optimization, it was found that MG₁ (*Aspergillus niger*) was the most potent strain at 1.5% substrate (pectin) concentration, pH 4.5 and temperature of 30°C. On the enzyme application, the yield of the orange juice, Total Soluble Solid and absorbance increased as the concentration of the enzyme increased and hence increasing the possibility of being used commercially for maximum pectinase production.*

Keywords: Pectinase, optimization, substrate concentration, pH, temperature, MG₁

Introduction

Pectin is an important component of middle lamella and primary cell wall of higher plants. These are high molecular weight acid polysaccharide primary made up of α (1-4) linkage of D-galacturonic acid residues (Patil *et al.*, 2010). Pectic substances are glycosidic macromolecules with high molecular weight. These substances consist of protopectins, pectinic acids, pectins and pectic acids. The main chain of pectin is partially methyl esterified 1,4-D-galacturonan. Demethylated pectin is known as pectic acid (pectate) or polygalacturonic acid. Three major pectic polysaccharide group are recognized, all containing D-galacturonic acid to a greater or a lesser extent. They are homogalacturonan (HG), rhamnogalacturonan I (RGI), and rhamnogalacturonan II (RGII). Similarly, American Chemical Society has classified pectic substances into four main types as protopectin, pectic acid, pectinic acid and pectin (Polymethylgalacturonate) (Singh *et al.*, 2005).

Pectinases are commercially important enzymes belonging to group hydrolase. Pectinase enzyme hydrolyzes the pectin present in plant cell wall and the middle lamella and play a vital role in physiological process that take place in plant like cell wall extension, fruit ripening, softening of plant tissues during maturation

etc. (Sakai, 1993). Pectinases have wide applications in food industries. The applications include clarification of wine and juice, scouring of cotton, degumming of plant fibers, waste water treatment, vegetable oil extraction, tea and coffee fermentations, bleaching of paper, and in poultry feed additives (Rangarajan *et al.*, 2010).

Pectinolytic enzymes have been reported from different genera of bacteria, including actinomycetes, yeast, and fungi. Pectinases from *Erwinia*, *Bacillus*, *Saccharomyces*, *Kluyveromyces*, *Aspergillus*, *Penicillium*, *Fusarium* and *Rhizopus* have been isolated and characterized. Most of the commercial preparations of pectinases are produced from fungal sources *viz* *Aspergillus*, *Erwinia* and *Penicillium* (Khairnar *et al.*, 2009), however, *Aspergillus niger* is the major source of commercially important pectinase (Rangarajan *et al.*, 2010).

Pectic substance is a generic name used for the compounds that are acted upon by the pectinolytic enzymes. These are negatively charged acidic glycosidic macromolecules and have high molecular weight (Patil and Dayanand, 2006). Microbial pectinases account for 25% of the global industrial enzymes among overall pectinase production (Jayani *et al.*, 2005).

There are multitudes of food and beverage industries in Nepal which require pectinase enzyme during various processing steps. Keeping in view the importance of enzyme pectinases in the food processing industry and the problems associated with the disposal of waste from food processing industry, the present study was undertaken with the purpose of isolation and screening of pectinase producing micro-organism. Simultaneously, optimization of different conditions for the production, extraction and purification of this enzyme will overcome the acute demand of this enzyme.

Materials and Methods

Sample

From different areas of Lalitpur, Kathmandu, Gulmi, Manang and Dang, soil sample was collected from the areas where soil was rich in composts, agro-industrial wastes and organic fertilizers.

Isolation, screening and identification of pectinolytic fungi

Potato Dextrose Agar (PDA) enriched with 1% pectin was used. Isolation of fungi was done by soil dilution plate method and incubated at 25°C for 7 days. Different colonies were selected and duplicated. Then 1% Cetyl Trimethyl Ammonium Bromide (CTAB) was flooded on one of the plate and incubated at 26°C for an hour. Pectinolytic fungi were indicated by the zone of hydrolysis around the colonies and the zone of hydrolysis was measured. Similarly, secondary screening was done by measuring the diameter of halozone formed by the fungal isolates in PDA enriched with 1% pectin. Identification was done on the basis of cultural and morphological characteristics by Lactophenol Cotton Blue staining method (Aneja, 2008). The stock cultures of isolated colonies were maintained on PDA slant and preserved at 4°C.

Enzyme production

Submerged state fermentation was carried out using 50ml Hankin's broth was prepared and autoclaved. After sterilization, 1ml of each pre-fermenter inoculum was inoculated. The flasks were shaken at 150 rpm and incubated at 30°C for 7 days and fermentation was monitored for PG activity using Dinitro Salicylic Acid Assay (DNSA) and cup plate method.

Extraction and partial purification of enzyme produced:

After 7 days of fermentation, the fungal cultures were separated from crude enzyme by filtration using Whatmann No.1 filter paper. The filtered enzymes were then were partially purified by acetone precipitation.

Characterizations of partially purified pectinases

The partially purified enzymes obtained after the precipitation with acetone showing highest PG activities were subjected to different enzymatic parameters for identifying their optimal working ranges. The effects of different parameters like pH, temperature and substrate concentration were studied.

Optimization of cultural conditions for pectinase production

The fungal strains showing high pectinase production and high pectinolytic activity were selected. The optimum growth characteristics of the fungal strains for the yield of pectinase were studied. The effects of pH, substrate concentration and temperature of incubation on production of pectinase were studied.

Effect of pH on pectinase production

To study the effect of pH on pectinase production, Yeast Glucose Pectin (YGP) media (1000ml) with varying pH of 2.5, 3.5, 4.5, and 5.5 were made, autoclaved and cooled. Then 0.5ml of spore inoculum was inoculated in each media. These media were then incubated at 30°C for 2 days. After incubation the enzyme assay was carried out by DNS assay method (Miller, 1959).

Effect of temperature on pectinase production

To study the effect of temperature on pectinase production, 100 ml of Yeast Glucose Pectin (YGP) media was prepared, autoclaved and cooled. Then the 0.5ml of spore inoculums was added and the flasks were incubated separately at 20°C, 30°C, 40°C, 50°C and 60°C for 2 days. After incubation, the enzyme assay was done by usual DNS assay method (Miller, 1959).

Effect of substrate (pectin) concentration on pectinase production

For the study of effect substrate concentration on pectinase production, YGP (100ml) media with pectin concentrations of 0.1%, 0.5%, 1.0%, 1.5%, and 2.0% were prepared, autoclaved and cooled. Then about 0.5ml of spore inoculums was added to each substrate containing YGP media and incubated at 30°C for 2 days. After incubation enzymatic assay was performed by usual DNS method (Miller, 1959).

Purification of enzyme

The partially purified enzyme produced from the fungal sample was subjected to dialysis for further purification. The enzyme sample suspended in acetate buffer was placed in a sterile dialysis bag and placed in a beaker containing distilled water. The beaker containing the dialysis bag was kept in a magnetic stirrer for 24 hours. Finally, the purified enzyme in the dialysis bag was

poured into a sterile test tube and suspended in acetate buffer of pH 4.2.

Application of enzyme

Extraction of orange juice and enzyme treatment

Mature ripened orange fruits (*Citrus sinensis*) were sorted, washed and peeled. The juice was extracted using a domestic juice extractor. The extracted juice was pasteurized at 85°C for 3 minutes to inactivate the natural fruit enzymes and cooled down to 4°C. Varying concentration of enzyme (0, 0.25, 0.5, 0.75 and 1%) was added. The samples were incubated at 40°C for 1hr. After incubation, the samples were heated at 85°C for 3 minutes to inactivate the enzyme.

Determination of Yield, Total Soluble Sugar and Clarity of juice

The clarified juice was filtered using Whatman No. 1 filter paper. The volume of fruit juice obtained was measured using 100ml measuring cylinder. The TSS was determined using refractometer. For clarity, the juice was shaken and 10 ml portion of juice was centrifuged at 5000rpm for 10 min to remove pulp and coarse cloud particles. Percent transmittance and absorbance was determined at 560 nm by spectrophotometer.

Data Analysis

The data were analysed using SPSS computer program (version 17.0). Analysis of Variance (ANOVA) in one way classification system was used in the study for testing the statistical significance of variation of mean at 5% level. The Least Significant Difference (LSD) was used to compare the significance difference between the different parameters at 5% level.

Results and Discussion

The study was carried out to isolate and screen pectinolytic organisms from environmental soil sample from different parts of Nepal for the production of valuable product pectinase. A total of 9 different soil samples [Lalitpur (3), Kathmandu (2), Gulmi (2), Manang (1) and Dang (1)] were collected and analyzed for the growth of fungal colonies. From those, 40 different colonies were screened primarily on the basis of hydrolysis around the colonies. Among them only 18 colonies were selected for enzyme production by secondary screening using PDA enriched with pectin. From microscopy and cultural characteristics, 14 strains were identified as genus *Aspergillus* whereas remaining four were found to be *Fusarium*, *Penicillium*, *Rhizopus* and *Syncephalastium*. Submerged state fermentation was carried out for 7 days and analysis of crude enzymes produced by these 18 fungal isolates was done routinely at

24 hours interval for 5 days. The enzymes were partially purified by filtration followed by acetone precipitation. Out of 18 enzyme sample produced from 18 different fungal isolates, 3 enzyme samples produced from three isolates namely MG₁, MM₁ and TC₅(B₁) were selected for enzyme characterization. The activities of these three enzymes were characterized on the basis of three factors namely; temperature of incubation, substrate (pectin) concentration and pH of the substrate.

Table 1. Assay of enzymes at different temperatures

Enzyme Samples	OD at 540nm at different temperatures			
	25°C	37°C	45°C	55°C
MM ₁	0.04	0.06	0.10	0.13
MG ₁	0.04	0.07	0.20	0.16
TC ₅ (B ₁)	0.06	0.06	0.16	0.14

Table 2. Assay of enzymes at various substrate concentrations

Enzyme Samples	OD at 540nm at various substrate concentrations					
	0.3%	0.5%	1%	1.5%	2.0%	2.5%
MM ₁	0.10	0.01	0.02	0.02	0.04	0.04
MG ₁	0.14	0.01	0.01	0.02	0.06	0.06
TC ₅ (B ₁)	0.11	0.02	0.02	0.01	0.06	0.06

Table 3. Assay of enzymes at various pH of substrates

Enzyme Samples	OD at 540nm at various pH of substrate		
	pH 3.6	pH 4.2	pH 5.2
MM ₁	0.09	0.09	0.10
MG ₁	0.08	0.11	0.12
TC ₅ (B ₁)	0.11	0.11	0.11

From the three enzyme samples, enzyme sample MG₁ exhibited higher pectinolytic activity as compared to other two samples. It showed whitish margin with brownish black with conidial production and reverse was white to pale yellow on PDA. The growth characteristic of the fungi on PDA after 7 days of incubation revealed that colonies on PDA at 28°C were initially white and became greenish black with conidial production. Reverse was white to pale yellow and with growth produced in the agar. The microscopic result showed large, globular spore bearing heads and were tightly packed. Chains of conidia were also seen. The conidia were greenish in color. The feature resembled that of *Aspergillus niger*.

For the production of pectinase in optimum cultural condition the fungus was grown on Yeast Glucose media. Factors such as substrate concentration, pH of the substrate media and temperature were varied to analyze the optimum enzyme production by the fungus. The

maximum enzyme production was observed on the YGP media enriched with pectin at the concentration of 1.5% which was 21 U/ml (Figure 1). Similarly, maximum pectinase production i.e. 7U/ml was observed at pH 4.5 of YGP media (Figure 2). The optimum temperature for maximum enzyme production was found to be at 30°C. At this temperature, 32 U/ml enzyme was produced (Figure 3). The enzyme activity was found to be significantly related with substrate concentration, pH and temperature of incubation ($p < 0.05$).

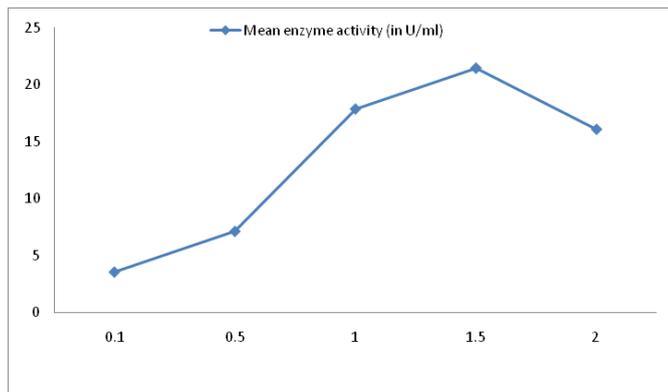


Figure 1. Effect of substrate concentration on pectinase production (Strain MG₁)

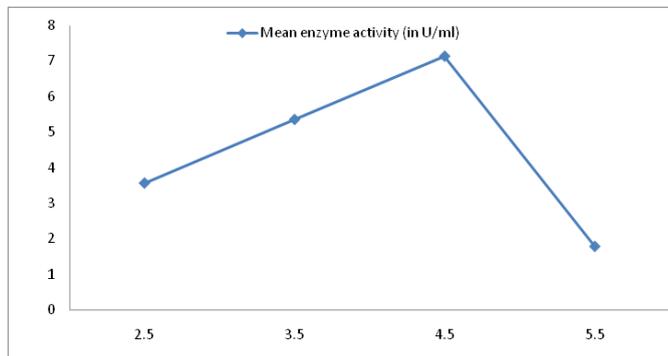


Figure 2. Effect of pH on pectinase production (Strain MG₁)

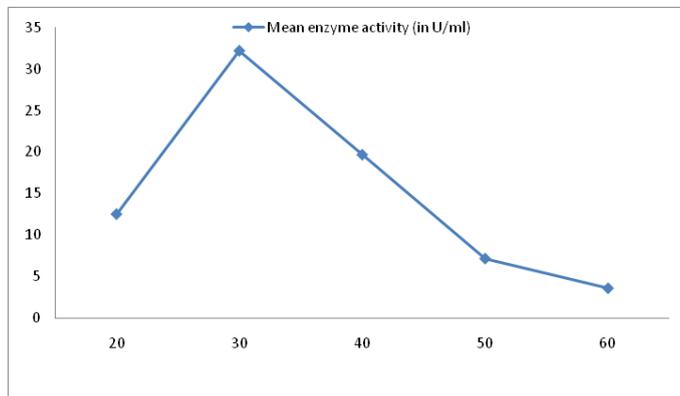


Figure 3. Effect of temperature on pectinase production (Strain MG₁)

The yield of the orange juice and TSS increased as the concentration of the enzyme increased. Highest activity was observed in the enzyme concentration 1%, absorbance 0.75 at 540nm and enzyme activity of 20.9407 U/ml (Table 4).

Table 4. Effect of different pectinase concentrations on yield and total soluble solids

Enzyme concentration (%)	Yield		Total Soluble Solids (TSS)		Absorbance at 540nm
	Initial volume(ml)	Final volume(ml)	Initial (°Brix)	Final (°Brix)	
0	20	20	6	6	0
0.25	20	20.2	5	5.5	0.48
0.5	20	20.5	5	5.5	0.66
0.75	20	21.0	5	6	0.70
1	20	21.7	5	7	0.75

The present study revealed that all the 18 isolates have shown extracellular enzyme activity. Among the 18 fungal colonies assayed for the extracellular enzyme activity, majority of the fungal culture showed that the pectinase activity was decreasing after 48 hours of incubation. It might be due to the depletion of the nutrients, death phase of organisms or due to the production of pectinase in the medium as suggested by the previous report. The *Aspergillus niger* strain showed maximum activity after 24 hours of incubation and later on the activity started depleting when the incubation time was increased (Khairnar *et al.*, 2009). Three fungal strains namely MM₁, MG₁ and TC₅ (B) were selected on the basis of maximum diameter of halozone formation on the media enriched with pectin. These three fungal strains were selected for further study. The isolated fungal strains were characterized on the basis of pectinase production using three parameters i.e. temperature, pH and substrate concentrations. The enzyme produced by these three isolates were assayed varying the temperature of incubation (25-55°C), the substrate concentrations (0.3%-2.5%), and varying the pH of the substrate (3.6-5.2). The enzyme activity was found to increase within temperature range of 45°C-55°C. The enzyme showed highest activity at temperature of 55°C which indicates the pectinolytic enzymes are active at high temperatures. But, the activity of enzymes decreases when temperature is further increased. In a similar research on pectinase, the activity of enzyme activity remained stable up to 70°C. The assay of the three enzymes using various concentrations of substrate showed that the enzyme activity increased up to 2% substrate concentration and then remained stationary on increasing the substrate concentration. This may be due to fact that at high substrate concentration

no more enzymes are available free for carrying the reaction (or catalysis) since the enzyme is constant. The partially purified enzymes were assayed for the activity in varying pH condition of the substrate media. Maximum pectinase production was observed at pH 5.2, which indicates that slightly acidic condition is suitable for the production of pectinase. It has been reported that the exopolysaccharidase and endopolysaccharidase obtained from *Aspergillus niger* URM4645 showed its maximum activity at pH 7.0 and 5.0 respectively (De Holanda Cavalcanti Macial *et al.*, 2011). Fahmy *et al.*, (2008) found that the pectinase isolated from *Aspergillus niger* has optimum activity at pH 5.0. Among the enzyme produced from the various fungal strains, MG₁ showed higher pectinolytic activity as compared to other two isolates. Three different parameters namely substrate concentration, pH of the substrate and temperature of incubation were taken for optimization. The media cultures were tested for pectinase production for two days. The fungus inoculated into the YGP media with different concentrations of substrate showed that 21U/ml enzyme was produced at 1.5% substrate concentration. The maximum yield of pectinase was obtained at pectin concentration of 1.5% by *Penicillium spp* (Patil and Chaudhari, 2009). Similarly, by doing the assay of the fungus inoculated into YGP media of different pH showed maximum pectinase production at pH 4.5 shown in figure 4.2. I.e. 7U/ml enzyme was produced. It was also found that maximum production of pectinase from *Aspergillus niger* and *Aspergillus awamori* was observed at pH 5.0 (Suresh *et al.*, 2009; Suresh and Viruthagiri, 2010). These findings are very close to our results which indicate that the result obtained must be true for the pectinase production from *Aspergillus spp*. The optimum temperature for the enzyme production was found to be at 30°C as shown in figure 4.3, i.e. 32U/ml enzyme was produced at 30°C. So, the maximum enzyme production was observed at normal temperature (30°C) at which majority of fungi are found to grow. On the enzyme application, the yield of the orange juice, TSS and absorbance increased as the concentration of the enzyme increased. The result of °Brix analysis on enzyme treated orange juice indicated a slight increase in soluble solids in enzyme concentration of 1%. Earlier studies have also revealed a similar kind of result where they changed from 6.0 to 6.2 and 8.6 to 11.20

Conclusions

The demand of industrially important enzymes in food industries is rising enormously. So, research should be done to alleviate the demand of these enzymes. Pectin degrading enzyme like pectinase has received a great attention because of their wide application and economic

benefits. On the other hand the production of pectinases for industrial application can be achieved by growing the potent organisms in a cheap substrate. The fungal isolate MG₁ could be potential candidate for industrial production of pectinase. It is thought that more efficient strains with enzymes with required quality could be isolated if the search for such organisms is carried out over different ecological regions of Nepal. In addition more specific and quality research work as well as development of gene cloning technique to produce the enzymes in large scale so as to meet industrial demands.

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