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RESEARCH ARTICLE

PRODUCTION AND OPTIMIZATION OF FUNGAL PECTINASE FROM *Fusarium* sp.,

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ABSTRACT

In the current study *Fusarium* sp., capable of producing novel pectinase was isolated from natural environment. The isolate was subjected to varying parameters of incubation conditions as time, pH, temperature and substrate concentration for optimal enzyme production. An optimal production of pectinase was observed when the culture was maintained at 27°C producing upto 40 U/ml of the enzyme. At pH 6 and at an initial substrate concentration (pectinase) of 0.5% the productivity was about 46 U/ml and 40 U/ml of pectinase enzyme respectively. At optimized conditions mass production of the enzyme was done and purified using acetone precipitation. The activity of the crude enzyme was further characterized by subjecting it to varying pH and temperature ranges. The crude enzyme was able to retain its activity well between the pH 4 and 8 respectively and at a temperature range of 48°C.

INTRODUCTION

Pectinases (EC 3.2.1.15) are a group of enzymes that break the glycosidic bonds of the long chain galacturonic acid residues of pectic substances. These enzymes are classified according to the criteria whether pectin, pectic acid or oligo-D-galacturonate is the preferred substrate, whether pectinase act by trans-elimination or hydrolysis, or whether the cleavage is random. They are Pectin Esterases (PE), depolymerizing enzymes and protopectinases. Pectinolytic enzymes are of significant importance in the current biotechnological era with their all embracing applications in fruit juice extraction and its clarification, scouring of cotton, degumming of plant fibers, waste water treatment, vegetable oil extraction, tea and coffee fermentations, bleaching of paper, in poultry feed additives and in the alcoholic beverages and food industries (Rashida Banu *et al*., 2010). Pectinase are now an integral part of juice and textile industries (Kashyap *et al*., 2001) such as maceration of tea leaves (Angayarkanni *et al*., 2002), processing of cotton fabric as well as in various biotechnological applications (Alkorta *et al*., 1998, Jacob *et al.,* 2006). They have a share of 25% in the global sales of food enzymes (Rashida Banu *et al*., 2010).

Among the commercial pectinolytic enzymes, preparations obtained by the industrial cultivations of *Aspergillus niger* are some of the most popular (Godfrey *et al*., 1996). Since Pectinases are widely used enzyme for different industrial applications, it is necessary to use inexpensive and readily available raw materials for its production. Carbon sources especially of agrarian source are more suitable because they are cost effective, renewable and available in larger quantities.

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The filamentous fungi are most often used in the commercial production of pectinases. Microbial production of pectinases has been extensively studied (Kashyap *et al*., 2001). New enzymes for use in commercial applications with desirable biochemical and physico-chemical characteristics and a low cost production have been the focus of much research (Solbak *et al*., 2005, Malyessi *et al*., 2004 and Rao, 1996). Enzyme breakdown of the biomolecules depends up on the type of enzyme, application, temperature, incubation time, agitation, concentration, pH and use of different enzyme preparations (Dominguez *et al*., 1994, Chadha *et al*., 2003). The current study is focused mainly on the isolation of efficient fungal strain capable of producing the enzyme pectinase. The isolated fungi will then be optimized by subjecting it to different conditions of pH, temperature, substrate concentration, retention time etc. The activity of the produced enzyme will also be characterized under different physical conditions as mentioned before. The enzyme was produced on a large scale and this could be made use of in the bioscouring of 100% pure cotton fabric.

MATERIALS AND METHODS

Isolation, screening and identification of pectinase producing strains

Soil samples collected from various places were serially diluted and plated on pectin media comprising pectin-10gm, sucrose-10gm, tryptone-3gm, yeast extract-2gm, potassium chloride-0.5gm, manganese sulphate-0.01gm, magnesium sulphate-0.5gm, ammonium sulphate-2gm, and Mineral salt solution-1ml for 1 liter distilled water. The plates were observed for the formation of clear zone around the colonies after the addition of 1% cetrimide solution. The organisms which were positive pectinase producers were identified

through macroscopic observation on potato dextrose agar to study the colony morphology and color of spores produced and through microscopic observation through lactophenol cotton blue staining.

Optimization of cultural conditions for pectinase production

The isolated fungal strain was inoculated in the pectin media (production media) and was subjected to various pH, temperature, incubation period, substrate concentration and incubation conditions for the optimal production of the enzyme. All sets have been performed in triplicates and the standard error has been reported.

Effect of incubation time and incubation conditions on pectinase production

The fungal strain isolated was inoculated in two conical flasks containing 100ml of the production medium with one incubated under shaking condition at 120rmp in a metabolic shaker and another incubated at static condition for a period of 120hrs of incubation at 27 ºC. Aliquots of samples were retrieved at every 24 hours and the enzyme activity was estimated. All sets have been performed in triplicates and the standard deviation of the enzyme activity has been reported.

Effect of pH on pectinase production

The fungal strain was inoculated in 100 ml of pectin media which was previously adjusted to various pH ranges (3, 4, 5, 6, 7 & 8). At the end of 48 hrs of incubation samples were collected and the enzyme activity was estimated. All sets have been performed in triplicates and the standard deviation of the enzyme activity has been reported.

Effect of temperature on enzyme production

The fungal strain was inoculated in 100ml of production media and incubated at varying temperature ranges (7˚C, 17˚C, 27˚C, 37˚C, 47˚C and 57˚C) under shaking condition in an incubator shaker. At the end of 48 hrs of incubation samples were collected and the enzyme activity was estimated. All sets have been performed in triplicates and the standard deviation of the enzyme activity has been reported.

Effect of substrate concentration on enzyme production

The isolated fungal strain was inoculated in the production media containing varying concentrations of initial pectin concentration (0.5%, 1%, 1.5%, 2%, 2.5% & 3%). Following incubation the pectinase activity of each trail was estimated. All sets have been performed in triplicates and the standard deviation of the enzyme activity has been reported.

Mass production, extraction and partial purification of enzyme produced

Following optimization procedures large-scale production of the enzyme was carried out in bulk volume containing 1000ml of production media under the studied optimal conditions for the isolate. The fungal culture was separated from the crude enzyme by means of filtration using Whatmann No.1 filter paper. The crude enzyme thus obtained through filtration was stored in a sterile container at 4˚C for further use. The culture filtrate stored at 4˚C was mixed with three volumes of ice cold acetone and was allowed to stand for 15 minutes. The entire content was centrifuged at 5000rpm for 10 minutes and dialyzed using a dialyzing tube in phosphate buffer for partial purification.

Dialysis of partially purified crude enzyme

The dialysis tube was cut to the required length and allowed to boil for ten minutes in a volume of 2% sodium bicarbonate and 1 M of EDTA (pH-8). The tubing was rinsed thoroughly in distilled water. The tubing was cooled down and stored at 4˚C, if required. One end of the tube was tied securely with thread and water was added to the dialysis bag and checked was leakage. Then sample was poured in to the bag and sealed. The dialysis bag was then placed in a solution of potassium phosphate buffer (pH-7) at 4˚C for 48 hours. The potassium phosphate buffer was replaced periodically (Alkorta *et al*., 1998).

Characterization of crude enzyme

The crude enzyme obtained after dialysis was subjected to varying pH and temperature ranges for identifying its optimal working ranges.

Effect of pH on pectinase activity

The crude enzyme was subjected to 0.5% of initial substrate concentration whose initial pH was adjusted between 3 and 9. Then enzyme assay was carried out for all the sets. The maximum enzyme activity was determined from the titer value among all the sets and was noted to be optimal pH for pectinase activity.

Effect of temperature on pectinase activity

The influence of temperature on the pectinase activity was determined by measuring the enzyme activity at temperatures range from 7, 17, 27, 37, 47 and 57°C under the standard assay conditions.

Assay of Pectinase

The culture supernatant taken as enzyme extract containing extracellular pectinases was used to assay the enzyme activity. Ten milliliter of 1% pectin in 0.15 M NaCl (pH 7.0) was incubated with 2 mL of enzyme extract in 100 mL flask at 35°C for 1 hr. The reaction was stopped by keeping the flask in boiling water bath for 10 min. The contents were cooled and the free carboxyl groups released were determined by titrating against 0.02 N NaOH using phenolphthalein as indicator (Kertesz, 1955). One unit of Pectinase was defined as the amount of enzymes required to release one micro equivalent of carboxyl group/ml/hr.

Protein Estimation

Difference in the quantity of protein in the partially purified extract and crude extract was determined using bovine serum albumin as standard (Lowry *et al*., 1951).

RESULTS AND DISCUSSION

Isolation, screening and identification of pectinase producing strains

One fungal strain was selected following the formation of zone of clearance around it in the pectin media inoculated with the dilution samples of agricultural soil from in and around Coimbatore city. The selected fungal strain was grown on potato dextrose agar for its colony morphology and spore stains. Colonies on Potato Dextrose agar at 25°C initially looked like white wool later turned to pink color. On microscopic observation (Figure 1) of the isolated fungal strain using lactophenol cotton blue showed oval microconidium with 0-1 septum crescent-shaped macroconidium with 3-5 septa and 1-2 linkage of round chlamydospores on the side branch of the hypha. From the above characteristics the isolate was found to be *Fusarium sp.,* (Mutsui, 1991). The selected isolate was then stored on Potato Dextrose agar plates for further use. The fungal cultural characteristics were similar to the work of Nelson *et al*., 1983 and Rao, 1996.

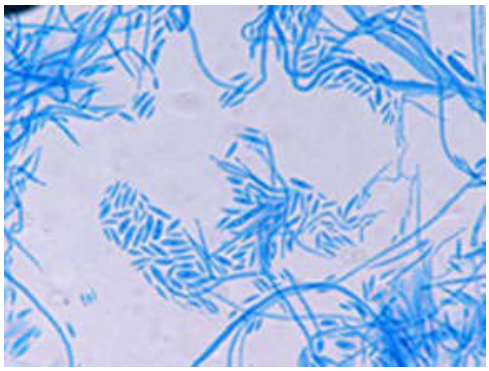

Fig. 1. Microscopic observation of the isolated *Fusarium* sp., using Lactophenol cotton blue staining

Effect of incubation time and incubation conditions on pectinase production

After about 48 hours of incubation there was a maximum production of pectinase in the production media. There was a gradual raise in the production of pectinase during the first 48 hours of incubation after which it showed a reduction in the pattern. A maximum of 40 U/ml of the enzyme was produced after 48 hours of incubation (Figure 2). The standard deviation of the enzyme activity after 24, 48, 72, 96 and 120 hours were calculated and were found to be 0.1, 0.05, 0.2, 0.13 and 0.2 respectively. Also the culture incubated under shaking condition was found to be more efficient in the production of the enzyme than those incubated under static condition. Under shaking condition about 40 U/ml of enzyme was produced after 48 hours on compared to the 19 U/ml of enzyme for the cultures incubated under static conditions. Acuna-Argulles *et al.,* 1995 has already reported the production of pectinase by *Aspergillus flavus* was maximum at 48hrs. The maximum yield of Pectinase production by *Moniliella sp.,*was achieved at sixth day (Solbak *et al*., 2005). According to Saravamangala and Dayanand, 2006, a maximum pectinase activity was

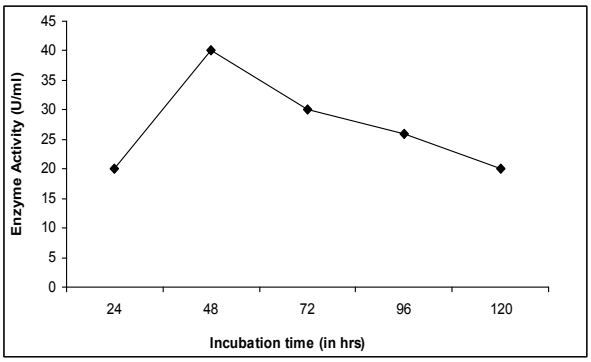

Fig. 2. Effect of incubation time on Pectinase production

observed after 72 hours of incubation in submerged fermentation when deseeded sunflower head was used as the substrate. Palaniyappan *et al.,* 2009 stated that the maximum enzyme production was obtained at 170 rpm.Fungi being aerobic organism require aeration for growth as well as secondary metabolite production. Acuna-Argulles *et al*., 1995 have said that the pectinase production by *Aspergillus* sp., in pectin medium is more in aerated condition of growth.

Effect of pH on pectinase production

An increase in the production of pectinase was observed between the pH range 3 and 6 respectively (Figure 3). There was a continuous increase in the production of pectinase between this range and optimal enzyme units were observed at pH 6. Beyond this range there is a considerable deterioration in the production of pectinase by the isolated fungal strain. This change in activity or reduced Pectinase production at increased pH levels may be due to the fact that the selected isolate was intolerant to alkaline conditions. The maximum enzyme production was at pH 6 (46 U/ml).

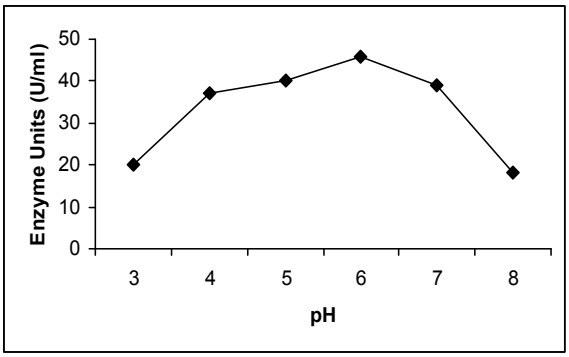

Fig. 3. Effect of pH on Pectinase production

The standard deviation of the enzyme activity at 3, 4, 5, 6, 7 and 8 pH were calculated and were found to be 0.2, 0.05, 0.07, 0.14, 0.1 and 0.1 respectively. The maximum yield of Pectinase was obtained at pH 6 by *Penicillium italicum* (Ranveer *et al*., 2005). Acuna-Argulles *et al.,* 1995 has reported that the production of acidic pectinase by *Aspergillus niger* was optimum at pH 6. In the work of Reda *et al.,* 2008, a maximum pectinase activity was obtained for *Bacillus firmus* at around pH 6 which is similar to the results observed in the current study. Similarly for the work of Rasheedha banu *et al*., 2010, there was an optimal enzyme production at a pH of 6.5 similar to the results observed for the current work.

Effect of temperature on enzyme production

The isolated fungal strains were able to optimally produce the enzyme at a temperature of 27°C of about 40 U/ml of the enzyme. At 37°C there was a deteriorated production of about 35 U/ml of enzyme produced. The production efficiency of the isolated organism at various temperature ranges is depicted in the fig 3. The optimum temperature range for enhanced Pectinase activity was in 27º C to 47º C (Figure 4). The standard deviation of the enzyme activity at 7, 17, 27, 37, 47 and 57°C were calculated and were found to be 0.06, 0.04, 0.12, 0.15, 0.05 and 0.1 respectively. Pectinase from *Penicillium sp.,* showed a maximum activity at 70˚C (Sarvamangala *et al*., 2006). In the work of Reda *et al.,* 2008, about 310 U/ml of pectinase was observed at 37°C for *Bacillus firmus* at 96 hrs of incubation. Similarly for the work of Rasheedha banu *et al.,* 2010, optimal enzyme productivity was observed at a temperature of 37°C for the fungal culture *Penicillium chrysogenum*.

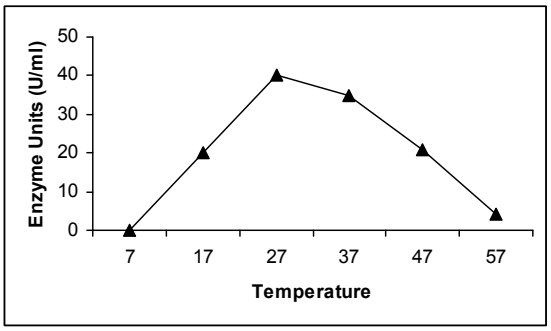

Fig. 4. Effect of temperature on Pectinase production

Effect of substrate concentration on enzyme production

The medium was supplemented with various concentration of pectin as substrate such as from 0.5% to 3%, to find out at which concentration the maximum enzyme yield occurs. Among this the maximum activity was obtained at 0.5% about 44 U/ml (Figure 5).

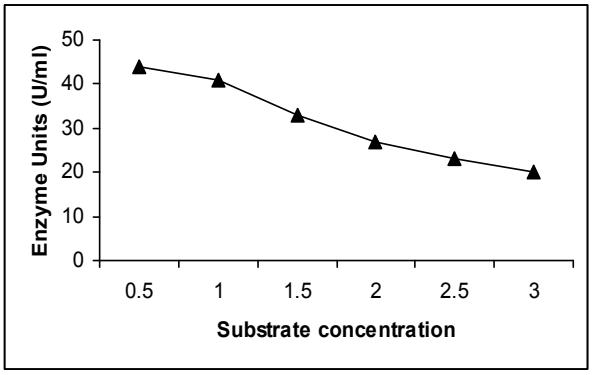

Fig. 5. Effect of initial substrate concentration on Pectinase production

The activity decreased gradually when substrate concentration increased to 3.0% (20 U/ml). The standard deviation of the enzyme activity in 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0% of substrate were calculated and were found to be 0.04, 0.1, 0.08, 0.08, 0.1 and 0.1 respectively.Thus maximum yield of pectinase was obtained at 1% substrate concentration by Acuna-Argulles *et al.,* 1995. Maximum production of pectinase was observed at an initial substrate concentration of 5% for the work of Reda *et al.,* 2008, for the bacterial culture *Bacillus firmus*. Similarly for the work of Palaniappan et al., 2009, at an initial substrate concentration of 1% of pectinase there was an optimal production of about 6 U/ml of enzyme.

Protein estimation

The protein content of the samples was determined using 0.1% Bovine Serum Albumin as standard. The concentration of protein present in 0.01ml of the partially purified culture filtrate was found to be 38 µg of protein and 22 µg in the case of crude extract. This shows that the acetone precipitation and dialysis have increased the concentration of proteins two folds than the crude enzyme extract.

Characterization of crude enzyme

Effect of pH and temperature on crude Pectinase activity

A maximum enzyme activity was determined from the titer value among the different sets and was noted to be optimal pH at 8 and showed a maximum activity of 47 U/ml (Figure 6).

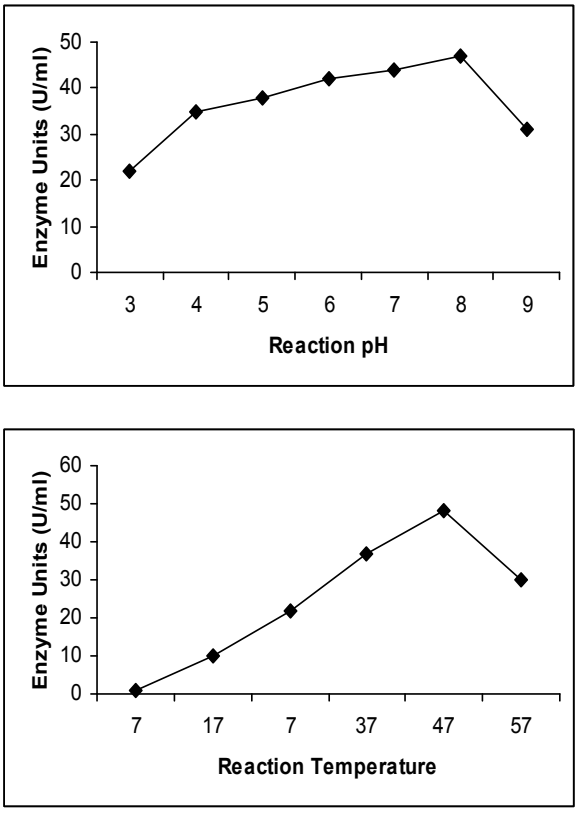

Fig. 6. Characterization of enzyme activity at different reaction pH and temperature

The enzyme activity was found to be most active at a temperature range of 47ºC with a pectinase activity of a maximum of 48 U/ml. The optimum temperature range for enhanced Pectinase activity lies in 27º C to 47º C. Riou *et al.,* 1992 stated that the polygalacturonase and pectate lyase from

Penicillium sp., showed a maximum activity at pH 4.5 and 9.0 respectively. Pectinase from *Penicillium sp.,* showed a maximum activity at 70˚C (Sarvamangala *et al*., 2006). Martin et al., 2004, reported that polygalacturanase from Penicillium sp. was stable at pH range of 3-8 and maintained 70% of initial activity at 70°C. According to the work of Rasheedha Banu et al., 2010, the enzyme produced from *Pencillium chrysogenum* was found to be active at a pH range of 5 and 7 and at a temperature range of 40-60°C. Similarly for the work of Natalia martin *et al*., 2004, the crude enzyme was found to be active at the acidic to neutral pH range between 3.0 and 7.0. and at a temperature of below 40°C.

Conclusion

Pectinase enzyme was isolated from the native strain, *Fusarium* sp., which can be used for various industrial applications including processing of cotton fabric in textile industries, extraction and clarification of fruit juices, bleaching of paper, removal of pectic waste waters and maceration of tea leaves. The mass produced pectinase enzyme using the native fungal strain *Fusarium* sp., could be used in the bioscouring of 100% cotton fabrics.

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