



PRODUCTION OF FUNGAL PECTINASE AND EFFECT OF SILVER NANOPARTICLES ON ITS ACTIVITY

^aKanika Poonia, ^aAbha Verma, ^bGyanika Shukla and ^cPoonam Rani

^aDepartment of Biotechnology, Meerut Institute of Engineering and Technology, Meerut (U.P.) – 250005, India.

^bDepartment of Genetics and Plant Breeding, Chaudhary Charan Singh University, Meerut (U.P.) – 250004, India.

^cDepartment of Soil Science, Jayoti Vidyapeeth Women's University, Jaipur (Rajasthan) – 305001, India.

ABSTRACT

Pectinase is a potential enzyme used for various purposes in food, chemical and paper industries. This enzyme is commercially produced by different bacterial and fungal biomasses. The *Aspergillus* is one of the most significant fungi for the production of various enzymes at commercial scale. In the present study, two species *Aspergillus niger* and *Aspergillus fumigatus* were evaluated for their pectinase producing ability. The biogenic silver nanoparticles were prepared by plant extracts of *Solanum nigrum* and *Sonchus asper* and were characterized with UV-Vis spectroscopy. Further, the silver nanoparticles were applied to the fungal pectinase synthesis bioreaction system to observe its effect on the pectinase synthesis. The results showed that the biogenic silver nanoparticles enhanced the enzyme activity of the fungal pectinase. It was concluded by the study, that the silver nanoparticles can be applied to enhance production of fungal pectinases at commercial level.

KEYWORDS: Fungal pectinase, silver nanoparticle, biogenic nanoparticles, *Aspergillus* etc.

1. INTRODUCTION

Enzymes are biological catalyst that speeds up reaction in the presence or absence of cofactors without any change in their activity. The interaction of enzymes with ligands offered stimulating opportunities for a wide variety of applications in the field of biotechnology and medicine. Some of the extra cellular enzymes produce by fungi include pectinase (Pedrolli *et al.*, 2009). Pectinase are complex of enzymes that hydrolyze the structural pectin found in the cell wall of mainly terrestrial plant. Extra cellular pectinases are easier to harvest and thus their scale up is easier and simpler (Soleimani and Gheibi, 2014). Pectinases find a wide variety of applications in food and paper industries. This enzyme can be used to clarify different juices and is found to be suitable to be used as a clarifying agent. In the present study pectinase producing fungi *Aspergillus niger* and *Aspergillus fumigatus* were used to evaluate their enzyme activity (Garg *et al.*, 2016). One of the aspects that may increase the activity can be association of enzyme with nanoparticles (Dutta *et al.*, 2013). The effect of pectinase enzyme and its activity on silver nanoparticles have many industrial and commercial benefits. Silver nanoparticles are nanoparticles of silver of between 1nm and 100 nm in size. Silver nanoparticles can be produced either intra-or extra-cellular by using organism. Among the various biological methods used for nanoparticles synthesis plant based materials seem to be best candidates and they are suitable for large scale biosynthesis of nanoparticles (Ahmed *et al.*, 2016). Plant parts such as leaf, roots, latex, seed and stem are being used for metal nanoparticles synthesis. Plant have been known to bio-mineralize calcium carbonate, silica and even be induced to hyper-accumulate within different parts

of plants. The internal accumulation of metal in plants can occur via complexation of the metal ion with a suitable bioligand in its native oxidation state after its reduction to a lower oxidation state (Homaee and Ehsanpour, 2016).

Generally, proteins (enzymes) undergo structural changes when interacting with ligands. Nobel nanoparticles (silver and gold) serve as novel entities for the binding of protein due to their large surface to volume ratios, high biocompatibility, non-toxic, chemical stability and ease of preparation (Khan *et al.*, 2017).

In light of the above facts involving the interaction of silver nanoparticles with proteins (enzymes), the present study was designed with following objectives:

1. Screening and optimization of *A. niger* and *A. fumigatus* for pectinase production.
2. Studying the effect of various parameters on pectinase activity.
3. Biological synthesis of silver nanoparticles using leaves and fruit extracts of *Solanum nigrum* (Makoi) and leaves extract of *Sonchus asper* (peeli katili).
4. Characterization of synthesized silver nanoparticles.
5. To study the effect of silver nanoparticles on fungal pectinase activity.

2. MATERIALS AND METHODS

2.1 Preparation and sterilization of medium

All the media were prepared as per standard formulation given in microbiology manual (Aneja, 2007). The dry ingredients were placed in beaker suspended in distilled water and dissolved completely. The prepared medium was dispensed into flask and test tubes which were finally sterilized by autoclaving at 121°C for 15 minutes.

2.2 Enzyme production

2.2.1 Preparation of fungal slants

Procured fungal strains of *Aspergillus niger* and *Aspergillus fumigatus* was reactivated on the sabrouds agar medium slants which were prepared by pouring around 20ml of freshly prepared media in test tubes and then holding them to solidify in a slanting position after sterilization at 121°C for 15 minutes. The transfer of spores was done aseptically and slants were incubated at 27°C for 3-5 days.

2.2.2 Screening of fungal strains for pectinase production

The fungal isolates selected from sabrouds agar plate were streaked over the screening media and the plates were incubated at 27°C and were flooded with iodine to observe and detect the pectinolytic activity of the fungal strain.

2.2.3 Inoculum preparation

The spores obtained from 5 days old sabrouds agar slants culture of *Aspergillus niger* and *Aspergillus fumigatus* were scrapped off from the slant surface by adding sterile distilled water in it. The spores were dispensed in autoclaved distilled water and optical density of the inoculums was set up to 0.12-0.15 at 530nm that correspond 10 CFU/ml.

2.2.4 Crude enzyme production

The prepared media (200 ml) for pectinase was transferred to two separate 250 ml of conical flasks. The flasks were plugged and sterilized in an autoclave and cooled at room temperature, 4ml of inoculum was transferred to each flasks. The flasks were placed in shaking conditions for 5 days at 26°C temperature. The extract of each flask were then filtered through Whatmann filter paper no.1 and the filtrate was used as crude enzyme extract for further study and store at 4°C temperature until further used.

2.2.5 Standard curve for the estimation of reducing sugar by dinitro salicylic acid method

Preparation of a standard curve, glucose (1mg/l) was used as standard solution. Increasing concentration of standard glucose solution was added to the tube (0-10) and amount was equalized up to 3ml by mixing distilled water. The 2ml dinitro salicylic acid was added and the reaction tubes were immersed for 15 minutes in water bath at 100°C. The color change of the reaction mixture was observed visibly and the absorbance was measured at 540nm. The optical density was plotted against standard glucose concentration on a graph paper. This was used as reference standard curve for enzymatic assay.

2.3 Optimization parameters for pectinase production:

To study the effect of incubation time, substrate concentration, inoculum concentration and fermentation rate on pectinase production, 10% of the inoculum was transferred to the production medium contained in different flask, which were kept for incubation at 27°C. After 2-12 days sample were withdrawn. Enzyme activity was determined.

2.4 Synthesis of silver nanoparticles

2.4.1 Plant material collection

Leaves of *Sonchus asper* and *Solanum nigrum* were collected from the fields of M.I.E.T., Meerut. The leaves were washed and the cleaned leaves were air dried for 15 days and then crushed into fine powder using mortar and pestle.

2.4.2 Preparation of the aqueous plant extract

About 200 mg of powdered leaves were dispensed in 100 ml of sterile distilled water and heated for 30 minutes at 80°C. Then the leaves extract was collected in separate conical flask by standard filtration method. The same method was applied for all the samples of plant material.

2.4.3 Plant mediated synthesis of silver nanoparticles

0.001 M silver nitrate (10⁻³M) and aqueous plant leaves extract were mixed in ratio 95:5 ml. The time of addition of leaves extract into the aqueous silver nitrate solution was considered as the start of the reaction. Then the solution was kept at 80°C. At shaking condition for 24 hours and color change was observed. This reaction mixture was used for further study.

2.4.4 Recovery of silver nanoparticles

The reaction mixture was centrifuged at 10,000 rpm for 15 minutes in order to obtain the pellet which was used for further study.

2.4.5 Detection and characterization of phyto silver nanoparticles

2.4.5.1 Visual observation

The reaction mixture containing 95 ml of 0.001 M silver nitrate and 5 ml of aqueous plant leaves extract was examined after every 30 minutes up to 24 hours and the change in color was observed with respect to time for the detection of silver nanoparticles.

2.4.5.2 U.V- Visible spectroscopy analysis

For the U.V- visible spectrum analysis the aliquots of reaction mixture were subjected to the measurement of the absorbance by U.V-visible spectrophotometer at the wavelength 200 to 800 nm for the detection of silver nanoparticles and the baseline was always set with a relevant blank.

2.5 Enzymatic assay

The effect of silver nanoparticles on the enzymatic activity of fungal pectinase was checked. One unit of enzyme activity was defined as the amount of enzyme causing the release of 1 mg of reducing sugar in 1 minute under the assay condition.

2.5.1 Fungal pectinase

Fungal pectinase activity was studied using a reaction mixture that comprises of 0.5 ml crude enzyme, 0.5ml of 0.5% w/v soluble pectin solution in 0.1M of sodium citrate buffer solution (pH-5.0). The reaction mixture was then incubated at 37°C for 30 minutes and the reaction was terminated by adding 2ml of dinitro salicylic acid in the reaction tube. Then the reaction tubes were immersed in water bath at 100°C for 10 minutes and then the absorbance was measured at 540 nm. A positive control containing enzyme and substrate (in absence of silver nanoparticles) and a negative control *i.e.*, enzyme blank was also made.

2.5.2 Effect of silver nanoparticles on fungal pectinase activity

To study the effect of silver nanoparticles on fungal pectinase, 0.5 ml of phytosilver nanoparticles were added to the reaction mixture containing 0.1 ml of crude enzyme, 0.5ml of 0.5 % w/v soluble pectin solution in 0.1 M of sodium citrate buffer solution (pH-5.0). In this reaction mixture 0.5ml of phyto silver nanoparticles were added. The reaction mixtures were then incubated at 37°C for 30 minutes and the reaction was terminated by adding 2ml of

dinitro salicylic acid in the reaction tube. Then the reaction tubes were immersed in water bath at 100°C for 10 minutes and then the absorbance was measured at 540 nm. A positive control containing enzyme and substrate (in absence of silver nanoparticles) and a negative control *i.e.*, enzyme blank was also made.

One unit of pectinase activity was defined as the amount of enzyme causing the release of 1 mg of reducing sugar in 1 minute under the assay condition.

3. RESULTS

3.1 Screening of the fungal strains for pectinase production:

Figure 1 (a) and (b) are showing the results of screening the isolates on czapek's dox media containing pectin for pectinase production. Zone of hydrolysis was observed around the fungal colony on adding iodine, which clearly indicates hydrolysis of pectin by fungus.

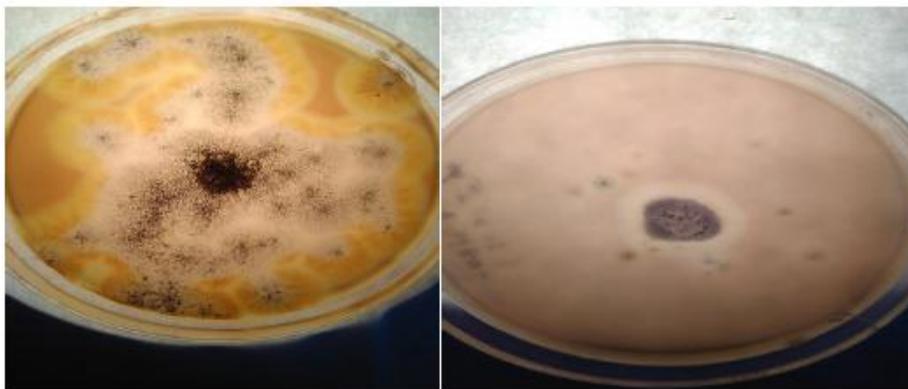


FIGURE 1. Zone of hydrolysis as shown due to pectinolytic activity of
(a) *Aspergillus niger*
(b) *Aspergillus fumigatus*

3.2 Crude enzyme production

On performing pectinase enzyme assay using the crude enzyme extract there was an observable change in color of the reaction mixture containing the crude enzyme extract.

3.3 Optimization of parameters for pectinase production:

The production medium was optimized by varying the types and concentration of different constituents of medium. The results obtained keeping all parameters constant while varying only one at a time is summarized below:

3.3.1. Incubation period

Figure 2 shows the time course for pectinase production was studied by incubating the fungus (*Aspergillus niger* and *Aspergillus fumigatus*) in production medium for varying time period at 27°C for 12 days. As shown in the graph that activity of enzyme increases with increase in time which after 6 days shows a decline clearly indicating the secretion of certain proteolytic enzyme which might have degraded the enzyme of interest. The activity of pectinase was found to be maximum on 6th day in case of *Aspergillus niger* and on 5th day in case of *Aspergillus fumigatus*.

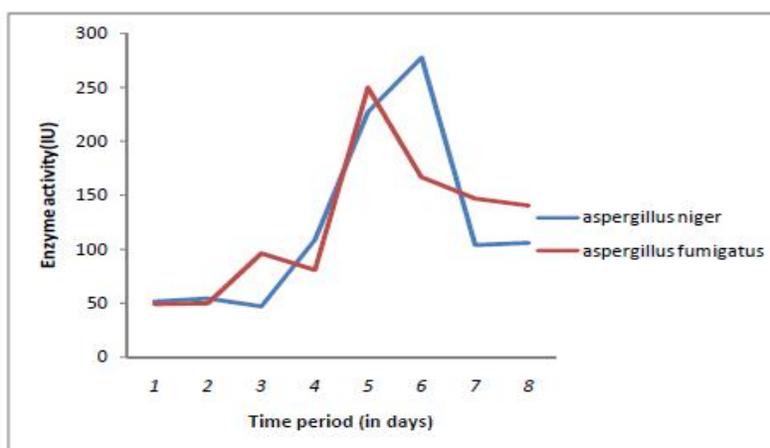


FIGURE 2. Graph showing effect of incubation period on pectinase production

3.3.2. Substrate concentration

Figure 3 shows the effect of substrate concentration on pectinase production. The optimum enzyme activity was found to be at 1% substrate concentration. Above this

substrate concentration the enzyme activity was suppressed. Which may be explained on the basis of the fact that on increasing the substrate concentration while keeping the enzyme concentration constant, all the active

sites of the enzyme are saturated at a point that gives a maximum enzyme activity after which the increase in

substrate concentration will not affect the enzyme activity further.

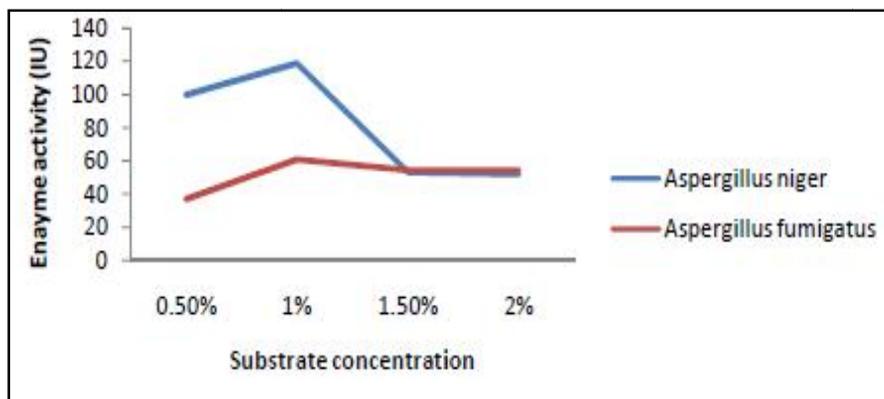


FIGURE 3. Graph showing effect of substrate concentration on pectinase production

3.3.3. Inoculum concentration

Figure 4 shows the effect of inoculum concentration on pectinase production. The optimum enzyme activity was

found to be at 10% inoculum concentration. Above this inoculum concentration the enzyme activity is suppressed.

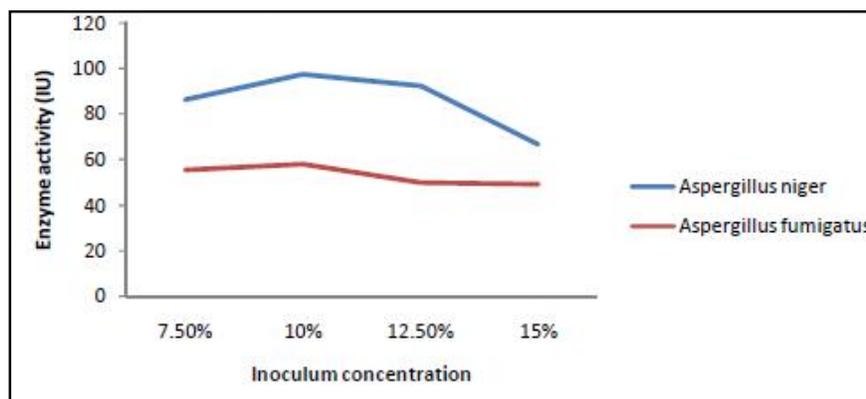


FIGURE 4. Graph showing effect of inoculum concentration on pectinase production

3.3.4 Fermentation process

Figure 5 shows the effect of fermentation process on pectinase production. The maximum pectinase production

was found to be in shake flask method, which may be due to proper agitation, aeration and nutrient availability to the fungi.

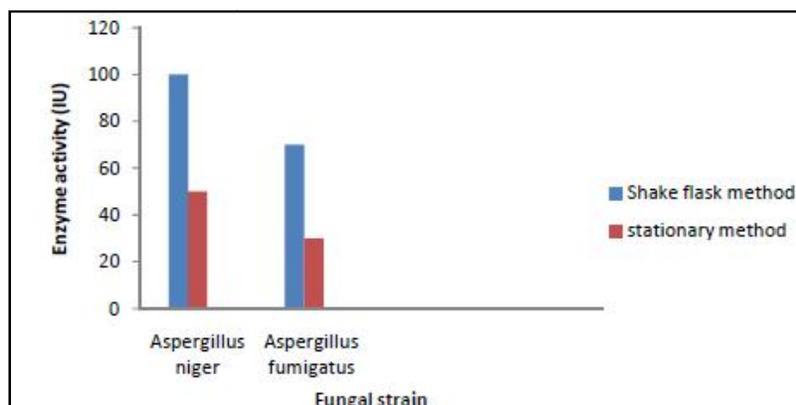


FIGURE 5. Graph showing effect of fermentation process on pectinase production

3.4 Characterization of phyto silver nanoparticles

3.4.1 Color based characterization

The synthesis of silver nanoparticles by leaves extract of *Sonchus asper* and *Solanum nigrum* was performed in the

present study. The color was changed to reddish brown within three hours in case of *Solanum nigrum* and brownish yellow within two hours in case of *Sonchus asper* indicating the synthesis of silver nanoparticles

(figure 6). The color intensity increases within two hours and became stable after four hours in case of *Sonchus asper* and *Solanum nigrum*.

It is an efficient and rapid synthesis which corroborate with the result obtained by other researchers who had

worked with different plant systems (Chandran, 2006; Song and Kim, 2009; Iravani, 2011; Mittal, 2013; Aljabali *et al.*, 2018). Color change was due to excitation of surface plasmon vibrations in the synthesized phyto silver nanoparticles.



FIGURE 6. Indications of color change in phyto silver nanoparticles solution.

3.4.2 U.V- visible spectroscopy analysis

U.V visible absorption spectra had proved to be quite sensitive for the detection of silver nanoparticles because silver nanoparticles exhibit an intensive absorption peak due to surface plasmon resonance.

The change in color from transparent to pale yellow and further to dark brown in cases of *Solanum nigrum* and *Sonchus asper* illustrates the formation of silver nanoparticles and with a peak at 435 nm in *Solanum nigrum* and 445 nm in case of *Sonchus asper* (figure 7).

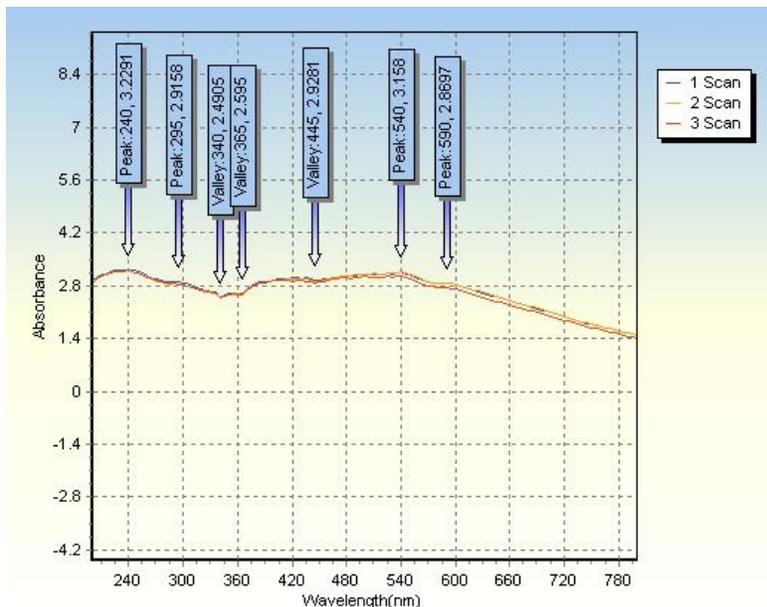


FIGURE 7. UV-Vis spectrum graph of *Sonchus asper*.

3.5 Enzymatic assay

Figure 8 shows that the enzyme activity of fungal pectinase in presence of silver nanoparticles synthesized by *Solanum nigrum* (SNP 1) and silver nanoparticles

synthesized by *Solanum asper* (SNP 2). The enzyme activity enhancement was observed in case of *Sonchus asper* while no enhancement in the activity was observed in case of *Solanum nigrum*.

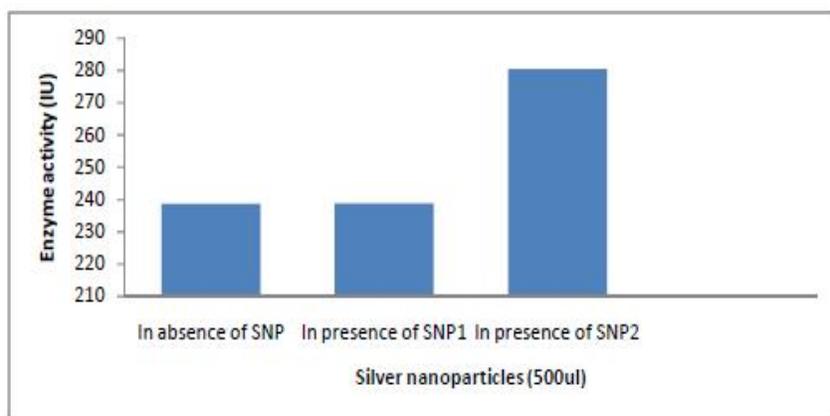


FIGURE 8. Effect of SNP 1 and SNP 2 on fungal pectinase activity

4. SUMMARY AND CONCLUSION

The present study summarizes the following:-

1. The fungal strain *Aspergillus niger* and *Aspergillus fumigatus*, are found to be pectinase degrading.
2. Pectinase production is maximum on 6th day in case of *Aspergillus niger* and 5th day in case of *Aspergillus fumigatus*.
3. Optimum substrate concentration for enzyme production is found to be 1% in *Aspergillus niger* and 1% in *Aspergillus fumigatus*.
4. Optimum inoculum concentration is found to be 10% in *Aspergillus niger* and 10 % in *Aspergillus fumigatus*.
5. The maximum pectinase production was found to be in shake flask method.
6. Biogenic synthesis of silver nano particles occur potentially using plant leaves extract of *Solanum nigrum* and *Sonchus asper*.
7. U.V visible spectrum analysis shows the maximum absorbance peak at 414 nm and 404 nm for the silver nano particles synthesized by the *Solanum nigrum* and *Sonchus asper* respectively.
8. The synthesized phyto silver nanoparticles of *Sonchus asper* revealed the enhancement in the enzyme activity of the fungal pectinase in the present work.

From the present study it may be concluded that the fungal strains screened for pectinase production are potential pectin degrading strains. The synthesized phyto silver nanoparticles using *Sonchus asper* shows the enhancement in the enzyme activity hence can be used at commercial scale for various applications.

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