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Research Article

Production of Pectinase by *Aspergillus niger* Isolated from Different Sites of Kathmandu Valley and Compare the Activity of the Best Strain

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A B S T R A C T

With a view to explore the feasibility of pectinase enzymes which have optimal efficiencies at different temperatures and pH which thereby helping to increase enzyme efficiency. The mesophilic temperature range of *Aspergillus niger* was isolated from different soils and water samples of Kathmandu valley by incubating these in Potato Dextrose Aga for 4–7 days at 28°C after a series of dilution. *A. niger* was screened for the production of pectinase by using orange peel as the substrate. The amount of pectinase enzyme was evaluated using the biuret method. The pectinase activity of a different sample of *A. niger* was evaluated using the DNS method, cup well agar and Sigma Aldrich protocol of pectinase activity analysis. The pectinase activity of the *A. niger* was 120 units as compared with standard pectinase activity 220 units, which shows it is a good producer of pectinase. Pectinase was subjected to varying conditions of pH and temperature. The optimum condition for the production of pectinase by *A. niger* is found to be pH 6.2 and temperature 50°C. Thus, obtained extracellular pectinase can be utilized for industrial processes using citrus peel as a substrate.

Keywords: *Aspergillus niger*, Pectinase, Orange Peel, Mesophile

Introduction

Pectinase is a generic name for a family of enzymes that catalyzes the hydrolysis of the glycosidic bonds in the pectic polymers. Pectinases include polygalacturonase (EC 446 3.2.1.15), pectin esterase (EC 3.1.1.11), pectin lyase (EC 4.2.2.10) and pectate lyase (EC 4.2.2.2), [Alkorta I., et al., 1998; Reid I., et al., 2000]. Pectinase is of great importance to several industries [Spanga G et al., 1995]. These enzymes are used to facilitate extraction, filtration and clarification and to increases yields in the production of fruit juices and beverages [Rombouts F.M., et al., 1988]. More than 30 different genera of bacteria, yeasts and molds have been used for the production of pectinase.Ernesto Favela-Torres, et al., [2006] revels that in last 15 years most frequently studied genera are *Rhizops, Penicillium, Fusarium, Aspergillus, Erwinia, Saccharomyces, Bacillus and Kluyvermyces*. However *Aspergillus, Erwinia and Penicillium* are mainly used for enzyme production studies. The mesophilic fungal pectinase mainly obtained from *Penicillium* and *Aspergillus* are used in industrial juice clarification processes [Aguilar G, et al.,

1987] and different range of fungal enzyme sources is being extended through various new technologies like recombinant and non- recombinant fungal strains. Temperature specific industrial fungal enzymes can be obtained from psychrotropic and thermophilic fungi, such as amylases, lipases, proteases, pectinase and xylanases. These potential application of these enzymes are in paper, food, detergent and pharma industries [Maheshwari R, et al., 2000]. The key features of the stains recognised for the pecinase enzyme production microbial souce depends on solide-state or submerged fermentation culture types, pectinase types produced, number, pH, genotypes of strains and thermal stability of the enzymes for insustrial production mostly use *A. niger* [Ernesto Favela-Torres et al, 2006].

Large scale production of the useful enzymes uses industrial fermentation of *A. niger* [Perrone G et al, 2006; Tjamos SE, et al., 2004; Abe J, et al, 1988].

Nowadays pectinase is one of the most important enzymes in food processing industries mainly for extraction and clarification of fruit juices and wines. Literature highlighting the optimization, biochemical characterization, genetics and strain improvement studies of pectinases from mesophilic fungi [Bartha JP et al., 1981; Fanelli C., et al., 1978; Marciano P, et al., 1982; Marcus L., et al, 1986] is available. The potential synthesis of pectinase is widespread among the microbial groups including bacteria [Reda, A.B., H.M. Yassin. M.A. Swelim and Z. Ebtsam, 2008] and fungus [Phutela, U., V. Dhuna, S. Sadhu and B.S. chadha, 2005]. Among several fungal strains *Penicillium, A. niger* and *Rhizopus* are mostly used for yielding extracellular enzymes as they are "Generally Regarded As Safe"(GARS), by processing the fermented medium [Blanco, P., C. Sieiro and T.G. Villa, 1999]. Thermally stable fungal strains are potential sources of industrially valuable enzymes such as proteases, lipases, amylases, pectinases and xylanases [Phutela, U., V. Dhuna, S. Sadhu and B.S. Chadha, 2005]. Based on the various substared used, orange peel contains large constituent of pectin, when feed to microorganisms as substrate they produce adequate amount of polygalacuronase.

Pectinase is widely used in the food industry especially in the processing of fruits and Vegetables since they decrease the viscosity and facilitate clarification of juices, mines, etc. [Kashyap, D.R., P.K. Vohra, S. Chopra and R. Tewari, 2001]. The microbial pectinase accounts approximately for 25% of the total worldwide enzyme sale [Jayani, R.S., S. Saxena and R. Gupta, 2005, Voragen, F., H. Schols and R. Visser, 2004].

The purpose of our study was to evaluate the activity of the enzyme isolated from the different regions of Kathmandu valley of mesophilic climatic conditions. The activity of psychrophilic enzymes produced at low and moderate temperatures offers potential economic benefits, through substantial energy savings in large-scale processes that would not require the expensive heating of reactors and cold-active pectinases can help to reduce viscosity and clarify fruit juices at low temperatures.

General Objective

The general objective of this study is to explore the feasibility of pectinase enzymes which have optimal efficiencies at different temperatures and pH which thereby helping to increase enzyme efficiency.

Specific Objective

- Isolation of pectinolytic *Aspergillus niger* from soils.
- Use easily available raw material, citrus peels.
- Determine activity at the optimized condition.
- Scale-up in 5L fermenter.

Literature Review

Fungal Pectinase

Pectinases are mainly used in the food industry to clarify fruit juices and other beverage. The chemical stracture of these polysaccharids are formed with (1-4) linked D-galacturonic acid residues in main chain with minute number of rhamnose residues and xylose, arabinose and xylose attached on its side chain [Deul and Stutz, 1958; Singh *et al.,* 1999; Kapoor *et al.,* 2000; Lang and Do-Renberg, 2000]. The fungal pectinase has great importance in the field of biotechnology. The production of fungal pectinase is easier and feasible than that of the production of pectinase from other sources.

Biotechnological Application of Microbial Pectinase

From several years, pectinases plays a great role in several industrial process in product extraction and purifications such as waste water treatment insustries, oil extraction, tea, coffee, textile, plant fiber processing, etc. Some of the research also indicate its uses in viruses purification system viruses [Salazar and Jayasinghe, 1999] and in conventional paper production process [Reid I. and Richard 2004; Viikari et al., 2001].

Pectinases are among the most important industrial enzymes. The pectinolytic enzymes produced from microbes contains potential biotechnological importance, so fungal pectinase is center for research throughout the globe as biological catalysts in various industrial production processes. Also, alkaline enzyme have tremendious uses in the textile, fiber degumming, waster water treatment and tea, coffee fermentation process.

Fruit Juice Extraction

Pectinase plays great role in fruit juice extraction and clarification process. It catalyses the pectins and provides the fruit juice turbidity and viscosity. For the fruit juice clarification a mixture of amylase and pectinase enzymes is used. This treatment of fruit pulps with enzymes reduces

the juice filteration time by upto 50% [Blanco et al., 1999] and increases the juice volume from apple, grapes and banana [Kaur and Kumar, 2004]. Different combination of other enzymes like cellulases, xylanase and arabinases with pectinases have been used for industrial treatment of juice extraction [Gailing et al., 2000]. The new techniques also incorporate pectinase as softening of citrus fruits peel for easy removal [Baker and Wicker, 1996].

Waste Water Treatment

For waste water treatment, pectinase is essentilally required in citrus fruit processing industries as the industrial waste water decomposition process (activated-sludge treatment) by micro organisms barely decompose the pectin substrates, [Tanabe *et al.*, 1986; Tanabe *et al.*, 1987] have tried to develop a new waste water treatment process by using an alkalophilic micro organism. Their soil isolate of an alkalophilic *Bacillus sp*. [GIR 621], produces an extra cellular endopectatelyase in alkaline media at pH 10.0.

Using these recombinant and non-recombinant strains provides better results for removal of pectin from waste water. There are several other techniques thast have been used in citrus fruit processing plants such as spray irrigation, physical dewatering of waste, chemical coagulation treatment, chemical hydrolysis and direct activeated sludge treatment process. The early treatment of waste sludge with the pectinase enzymes increases the removal of pectin substrate waste and prepare it for decomposition process followed by active sludge treatment [Hoondal et al., 2000].

Animal Feed

Pectinases enzyme cocktails used to breakdown the larger complex nutrients in smaller molecules for animal feed. This breakdown by hydrolysis process of fibers reduces the viscosity of feed and liberates nutrients which helps the animals to absorb more nutrients from feed and decrease the feces production amount [Hoondal et al., 2000].

Methodology

Collection of Soil Samples

For the collection of soil samples, different sites were selected with rich vegetation composting sites and waste dumping sites.

Kalimati Vegetable Market (PE.KVM) and Fruits Market was Selected (PE.KFM)

For *A. niger,* the plate exposure method was done. Three PDA plates were exposed for 5 minutes. The plates were sealed with paraffin tape and kept at 25-28°C for growth.

For plate exposure plates coding of Kalimati vegetable market was given as [PE.KVM.1, PE.KVM.2, PE.KVM.3].

For plate exposure plates coding of Kalimati Fruits market was given as [PE.KFM.1, PE.KFM.2].

Dumping site Teku was selected (PE.TDS)

The dumping site's soil was collected from the dumped wastes. It was kept in a plastic bag, made airtight by a rubber band and was stored at room temperature.

And the plate exposure method was done. Three PDA plates were exposed for 5 minutes. The plates were sealed with paraffin tape and kept at 25-28°C for growth.

For plate exposure plates coding was given as [PE.TDSP.1, PE.TDSP.2, PE.TDSP.3].

Roadside near Gwarkho was selected (PE.RSG)

The site was selected as the ongoing road expansion provided appropriate soil as 2-3 feet from the surface. It was kept in a plastic bag, made airtight by a rubber band and was stored at room temperature.

Phulchowki near the base camp was selected (PE.PS)

The sampling site was selected in the jungle of the Phulchoki at the shaded area where there is no human flow. The soil sample was taken by digging 1 feet deep. The sample was kept in a plastic bag and made airtight by a rubber band. The collected soil sample was stored in refrigerated conditions.

Mango Seed kernel was selected (PE.MSK)

Mature ripen mangoes were selected and the inner kernel was washed with distilled water. Karnen was dried in hot air oven and left in an open envioronment to decompose. Black spores of A.niger grown and spores were further grow on PDA plates for isolation of strain.

Identification

The fungus, *A. niger* were identified from among the isolated colonies from different sites by:

(a) Plate morphology observation (Table 1)
(b) Microscopy observation by Lactophenol cotton blue staining process. Spores/ sporangium/ spoangiophore; Mycelium – septate/ aspeted were observed.

Screening

For the screening of the best Pectinase producing fungus (*A. niger,* collected from different sites), pectin containing screening media (0.5% pectin + 1.5% agar) was selected.

Screening Methods	Annex 3
Screening using pectin containing media	

Media Selection

We had chosen only one media basis of their previous productive results. The media was commercially available PDB of HIMEDIA and 0.5% commercial Pectin of HIMEDIA.

Three best samples of Pectinase producing *A. niger* from different soil samples were selected and inoculated in the

above-mentioned media (broth) and shake flask culture was performed.

Production media	Annex 4

Optimization

Shake flask experiments

25 ml of the production media was prepared and distributed in the 50ml Erlenmeyer flasks. The medium to the flask ratio was 1:2. After the preparation of the medium, the pH was adjusted to 4.2 using 1N NaOH and 1N HCL and all the parameters were maintained constant. The medium was then sterilized in an autoclave at 121^0C, 15 lbs pressure for 20 minutes. The sterilized media allowed to cool before inoculating the selected *A. niger.*

Inoculum preparation for shake flask experiments

The pure colony, which was maintained in PDA plates, was inoculated in the PDB broth (25ml in 50 ml Erlenmeyer flasks) and incubated for 72 hours for the preparation of the inoculum. The spores of the fungus were used as inoculum for the shake flask experiments. Spores were counted by the Hemocytometer and maintained 5×10^7 spores from each of the Erlenmeyer flasks, were inoculated in the production medium respectively using a micropipette. Then the flask was incubated in the water bath shaker at 30^0C.

Determination of Enzymatic Assay of Pectinase

The activity of the enzyme was assayed using sigma protocol [The standardization procedure is described in Reagent Chemicals, (1981) 6^{th} ed., American Chemical Society Specifications, 551-552.] (Where Sigma Product or Stock numbers are specified, equivalent reagents may be substituted).

Unit activity

One unit will liberate 1.0µmol of galacturonic acid from polygalacturonic acid per minute at pH 4.0 at 25˚C.

Pectinase Assay by DNS Method

Preparation of DNS reagent (1000ml)

300gm Sodium potassium tartarate was dissolved in 500ml distilled water with stirring .10g of 3,5-dinitrosalicylic acid dissolved in 200ml of 2mol/lit sodium hydroxide. These two solutions were mixed and the final volume was made 1 liter by adding distilled water.

Pectinase assay

Pectinase assay was determined on the basis of breakdown of pectine substrate due to enzymetic hydrolysis process and producing the amount of reducing sugar produced by DNS (dinitrosalicylic acid) reagent metnod [Miller, 1959]. For this, 1 ml of 0.5% pectin, 0.5ml sodium acetate buffer (0.1M, pH 4.2) and 0.5ml crude enzyme was added in the test tube. The reaction mixture was incubated at 30˚C in the water bath for ten minutes. After 10 min, 2 ml of DNS reagent was added to stop the hydrolysis and the test tube was shaken to mix the contents. The test tube was heated to boiling on the boiling water-bath for 15 minutes. Then the tube was cooled and the absorbance was measured in a spectrophotometer at 575 nm. The enzyme and substrate blanks were run parallel. The blank containing 1ml of 0.5% pectin, 1ml of sodium acetate buffer (0.1M, pH 4.2) and 2ml of DNS reagent was used as a control. The standard curve was prepared for reducing sugars with galacturonic acid.

One unit of the enzyme was defined as the amount of enzyme needed to catalyze the reaction releasing 1mg of galacturonic acid per hour.

Enzyme production

Production media (commercially available PDB of HIMEDIA and 0.5% commercial Pectin of HIMEDIA) was used for pectinase production. The pH was adjusted to 4.2 with 1.0M NaOH and 1.0M HCL. The media were sterilized by autoclaving at 121˚C for 15 min at 15 lbs. pressure. The media (100ml in 250ml conical flasks) were inoculated with 5×107 spores. The enzyme assay was performed by the DNS method [Miller, 1959]. Mishra A.K, 2018; Mishra and Malik, 2017; Mishra A.K., 2019 ware assessed for adopting way of data presentation.

Extraction of the enzyme produced

For the production of pectinase enzyme from the isolated strain (PE.KVM), pectinase producing liquid media was used. 7 days old spores in counting 5×10^7 were used as inoculums and incubated in 150ml pectinase producing media. After 72 hours the fungal culture was separated from the crude enzyme by means of filtration using Whatman No.1 filter paper. The crude enzyme thus obtained through filtration was stored in a sterile container at 4˚C for further use. The filtration process was carried out in cold conditions in the ice water to prevent enzyme degradation.

Partial purification of the enzyme

The crude enzyme thus obtained from Whatman No.1 filtration stored at 4˚C was mixed with three volumes of ice-cold acetone and allowed to stand for 15 minutes [Rajendran et al., 2011]. The entire content was centrifuged at 4000 rpm for 20 min. The supernatant was discarded and the enzyme precipitation was dissolved in a minimal volume of sodium acetate buffer (0.1M, pH 4.2) and this partially purified enzyme was stored at 4˚C for further use.

Protein estimation by Biuret method

Quantitative estimation of the amount of proteins in the pectinase sample was performed by Biuret method using Biuret reagent [3 g of copper sulphate pentahydrate and

9 g of Potassium tartarate was dissolved in 500 ml of 0.2 mol/liter sodium Hydroxide and added 5 gram of Potassium iodide and made up to 1 liter with 0.2 mol/liter Sodium Hydroxide. Bovine Serum Albumin (BSA) was used as a protein standard.

Firstly, 3 ml of the biuret Reagent was added to the 2ml of Protein solution and mixed well and warmed at 37˚C for 10 minutes. The solution was cooled and the absorbance was taken at 540nm.

Characterization of Partially Purified Pectinase

The partially purified enzyme obtained after the precipitation with acetone was subjected to different enzymatic parameters like pH and temperature.

Effect of temperature on pectinase activity

Temperature dependence on pectinase activity was studied within the temperature range of 30-100˚C. In 5 units/ml enzyme, sodium acetate buffer (0.1M, pH 4.2) required to make volume up to 1 ml was added. Then each temperature labeled tube was incubated at respective temperature for 10 minutes. This process was followed by the addition of 1 ml 0.5% pectin in each test tube and incubated at 30˚C for 10 minuntes immediately after incubation 2 ml of DNS reagent was added and boiled in boiling water for 15 minutes. This was then allowed to cool and the absorbance at 575nm was taken. The mixture of 1ml sodium acetate buffer (0.1M, pH 4.2), 1 ml of 0.5% pectin and 2 ml DNS added in respective steps as above was taken as a control.

Effect of pH on pectinase activity

For the study of pH on the enzyme activity, the buffer solutions of different pH range from 3.3 to 10.2 were prepared. The 0.5% pectin solutions were also prepared by dissolving the pectin in buffer solutions of different pH. Buffer solutions of pH 3.2, 4.2 and 5.2 were prepared by using 0.2M acetic acid and 0.2M sodium acetate. The buffer solution of 6.2, 7.2 and 8.2 was prepared by using 0.2M monobasic sodium phosphate and 0.2M dibasic sodium anhydrous. The buffer solution of pH 9.2 and 10.2 was prepared by using 0.2M tris (hydroxymethyl) aminomethane and 0.2M hydrochloric acid. Then, 5units/ml of the enzyme was taken for the study of the effect of pH on enzyme activity. The buffer of the respective pH was added in the respective test tubes to make the volume 1 ml. To this solution 1 ml of 0.5%, pectin solution prepared in respective pH was added and the tubes were incubated at 30˚C for 10 min. Immediately after this 2 ml of DNS reagent was added and boiled in boiling water for 15 minutes. Then absorbance at 575 nm was recorded after cooling. The 1 ml respective pH buffer, 1 ml 0.5% pectin in a respective buffer and 2 ml DNS reagent following the above procedure were taken as a control.

Upscaling on the Bioreactor

The sample having the best Pectinase activity was designed to Upscale in 5-liter Bioreactor. The parameters like temperature and pH were kept constant during the experiment. The temperature of the Bioreactor was maintained at 30˚C and the pH was maintained at 4.2 during the experiment. The enzyme produced during the experiment was kept at the refrigeration temperature for future use. The sample of *Aspergillus niger* having the best enzyme activity was kept for enzyme production for 7 days.

Result

Isolation of *Aspergillus niger* from various sites

The *A. niger* fungi were isolated from different soils and water samples.

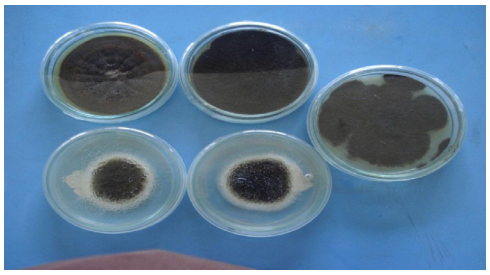


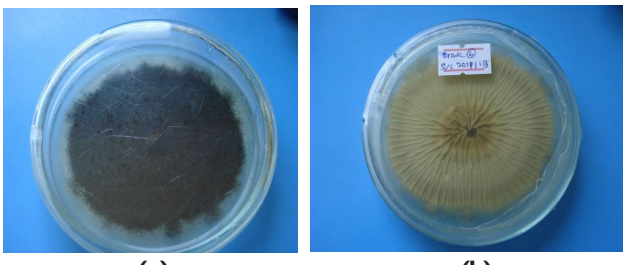
Figure 1.Isolated *A. niger* from different soil samples

Identification of the *Aspergillus niger*

The identification of *Aspergillus niger* was performed by the following methods.

- **Colony Morphology**

The colonies on PDA at 28 were white initially and becoming greenish-black with conidial production. The reverse was white to pale Yellow and growth produced was radial in the agar as shown in Figure 2.



(a) **(b)**

Figure 2.Pure culture of *Aspergillus niger* on PDA (front View) B: Pure culture of *Aspergillus niger* (backward View)

- **Microscopic Observation**

It was observed that spore-bearing heads were large, globular and were tightly packed. The chains of conidia were also observed. The conidia were greenish in color. The feature resembles that of *A. niger* and therefore, it was selected as the target genera.

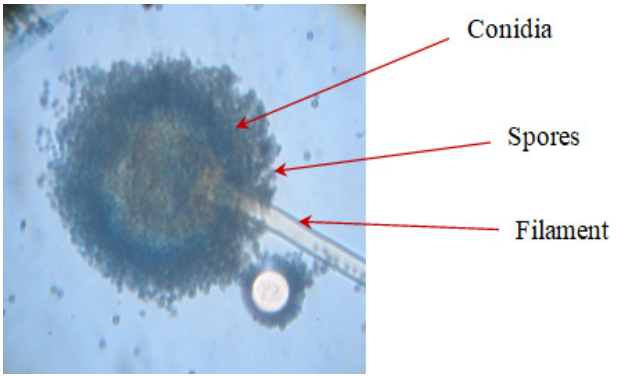


Figure 3.Pure culture of *Aspergillus niger* on PDA (front View) B: Pure culture of *Aspergillus niger* (backward View)

- **Enzyme estimation by Biuret Method**

The graphical presentation of the BSA standard curve was presented below:

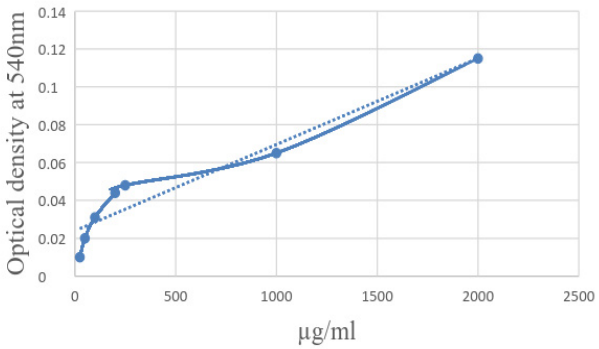


Figure 4.Optical density vs µg/ml at 540 nm using the biuret method

The different grades of the sample have the following range of Enzyme concentration at 540 nm:

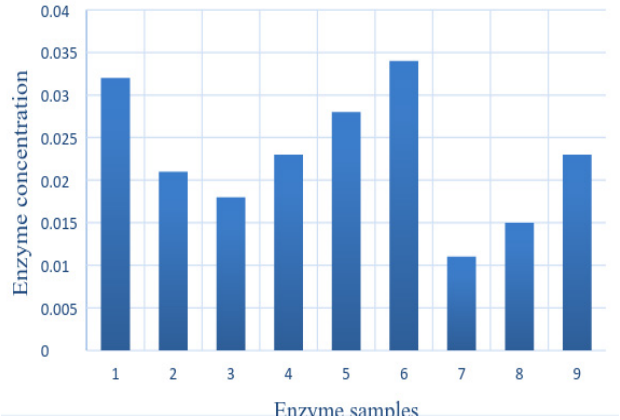


Figure 5.Enzyme concentration against Enzyme samples obtained at 540 nm using Biuret method

- **Pectinase Assay by DNS method**

The calibration curve of reducing sugar was presented below:

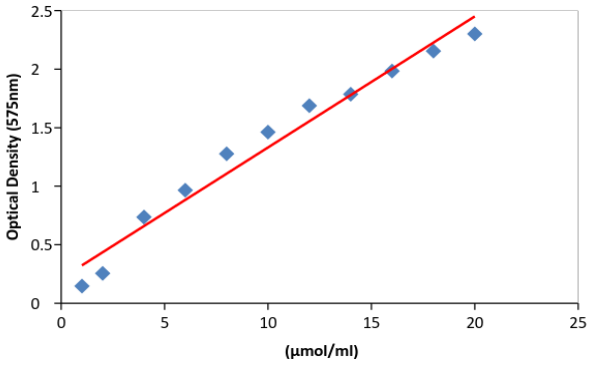


Figure 6.Calibration Curve of Reducing sugar at 575 nm by DNS method

- **Effect of temperature on pectinase production**

The graphical presentation of the Enzyme activity against temperature calculated using the DNS method was presented as:

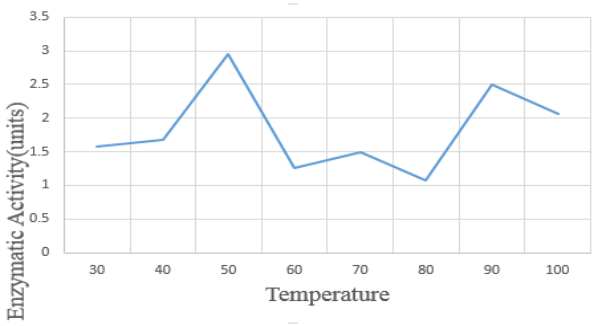


Figure 7.The graphical representation of Enzyme activity against temperature

- **Effect of pH on pectinase production**

The enzymes prepared from the 4.11.2 procedure were evaluated for the measurement of absorbance. The absorbance was taken at different pH ranges and the values were predicted at those pH. pH is one of the factors, that greatly affects the pectinase activity. The partially purified enzyme was assayed for the activity in varying pH ranges ranging from 3.2 to 10.2 pH ranges. It has been observed that the maximum enzymatic activity was observed from the *A.niger* at pH 6.2.

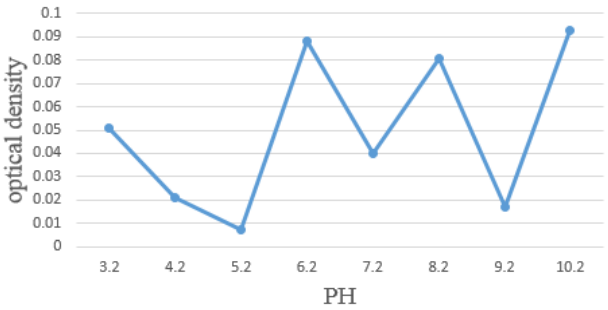
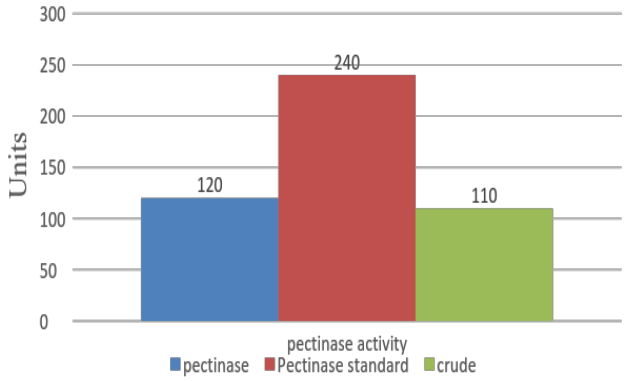


Figure 8.The graphical representation of optical density against pH

- **Determination of the pectinase activity (sigma protocol)**

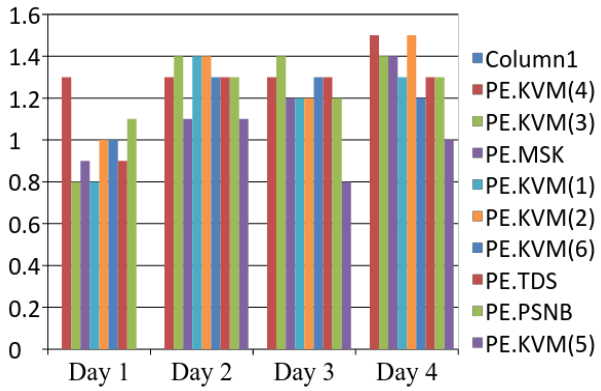
The determination of pectinase activity was measured using the sigma protocol. The pectinase activity of the mesophile obtained from these procedures had given the value of 120 units per ml. It suggests that the pectinase activity is good for the mesophile *A. niger.* The enzymatic activity of 120 units means 1 µmol of pectinase can degrade the 120 µmol of polygalcturonic acid into 120 µl of galacturonic acid.



- **Pectinase Activity testing with the measurement of clearance zone**

The clearance zone of the Mesophilic *A. niger* around the plate containing 0.5% pectin and agar (1.5%) having pH 4.5 have the following results.

Table 1: Clearance zone of different samples.



Discussion

Nine samples of *A. niger* were isolated from different sites and screened for the best pectinase producer. The primary criteria for screening were the secretion of pectinase in the culture medium. This was screened by measuring their clearance zone in the substrate containing medium.

The pure culture of, best enzyme producer, *A. niger* was used for the pilot-scale production of the enzyme. Potato Dextrose Broth containing 0.5% pectin media and in one flask in place of pectin citrus peel powder was used as a pectin source, in the production media. The pH of the media was maintained at 4.2 (0.1M NaOH, 0.1M HCl). To confirm the production of the enzyme in the broth 100 µl of the broth was drawn using a micro pipette and poured in the well bored in the media comprising pectin and agar. After the incubation of 24 h at 28˚C, the zone of clearance was observed. This method was performed on the second day.

The result obtained showed that the strain (PE.KVM) which was isolated was a good producer of the enzyme extracellularly as the standard pectinase activity was found to be 220 µl of galacturonic acid and the PE.KVM strain activity was found to be 120µl of galacturonic acid.

The other paper [S. Mrudula., R. Anitharaj., 2011] showed *A. niger* strain produced 232 U per ml of culture broth and we obtained 120 µl of galacturonic acid. Their result shows 23.2 µl of enzyme activity it shows that in our study the pectinase production is higher compared to them.

The citrus peel extracted pectin activity and the citrus peel powder used in the production media had the same concentration of pectin therefore, we had used peel extracted pectin instead of peel powder. The adopted steps for Extraction of Pectin from the Orange peels Powder.

Procedure

- The orange Peels were made dry.
- Then powdered by grinding and added to the 500ml of the water in 1000 ml of the beaker.
- Then 2.5 ml HCL was added to maintain pH 2.2.
- Then boiled for 45 minutes and filtered it using muslin cloth of 4 layers.
- Then filtered by whattman No.1 filter paper.
- The filtered cake was washed with 250 ml of boiled water and again filtered it and cooled to 25 ˚c (to minimize heat degradation of pectin).
- The extracted filtered pectin was precipitated by adding 200 ml of 95% acetone to 100 ml of extracted pectin with thorough stirring.
- The solution was left for 30 minutes to float the pectin on the surface then the pectin was skimmed off.
- Extracted pectin was purified with 200 ml acetone and then passed on a nylon and muslin cloth (to remove the residual and universal salt).
- Then the Extracted pectin was weighted and air-dried.
- Finally, the dried pectin was further reduced into small pieces using pestle and mortar and weighted using a Digital weighing balance.
- The percentage yield was calculated.

The pectinase enzyme obtained from the PE.KVM strain was partially purified in which pectinase activity 120 µl of galacturonic acid (One unit will liberate 1.0 µmole of galacturonic acid from polygalacturonic acid per minute

at pH 4.0 at 25°C), by this we had predicted the isolated strain was good pectinase producer.

The incubation temperature has been found to be a significant controlling factor for enzyme production [Kitprechavanich et al., 1984]. In order to find out the effect of the temperature, the enzyme obtained from the 4.11.1 procedure was subjected to various temperatures. The enzymatic activity was measured as the amount of reducing sugar liberated which will give rise to the different absorbance. The maximum absorbance was observed at 50˚C which indicates the maximum enzymatic activity. Enzyme activity was calculated as 2.93 units per 100 µl of an enzyme. This value is equal to 29.3 units. The absorbance increases from 30˚C–50˚C indicating the degradation of the pectin and release of reducing sugar. The absorbance again starts to decrease until 70˚C. It means the pectinase enzyme can't function properly and the conformation of the substrate changes.

Again, the absorbance starts to increase until the 100˚C. The increase in absorbance may be due to the inactivity of the pectinase or maybe by some other reasons. From 70˚C–100˚C the enzyme denatured and the absorbance gradually increased.

The maximum absorbance was observed at 50˚C which indicates the maximum enzymatic activity. The absorbance increases from 30˚C–50˚C indicating the degradation of the pectin and release of reducing sugar. The absorbance again starts to decrease until 70˚C. It means the pectinase enzyme can't function properly and the conformation of the substrate changes. The stability is a good attribute as it makes the enzyme less susceptible to thermal inactivation and is of great industrial value. This good stability could be due to the slow denaturation of the enzyme. Industrial processes are generally carried out at a high temperature at which most of the enzymes are deactivated.

Such drastic change in the values of the readings of Enzyme activity of DNS method and Sigma protocol was due to denaturation of the Enzyme at refrigeration temperature.

The other paper [Mrudula S, Anitharaj R, 2011] result showed at 50 °C the enzyme activity is high as compare to this result our result shows the same result. At higher temperatures, the activity was less. This might be due to the denaturation of the enzyme. pH is one of the factors which greatly affects the pectinase activity. The testing of partially purified enzyme for pectinase activity in various range of pH from 3.2 to 10.2, show higher activity of enzyme at pH 6.2 confirming that the enzyme works best at neutral pH line(Figure 5.5.). In other work [Mrudula S, Anitharaj R, 2011], it was reported as the maximum activity was observed at pH 5, it shows that this enzyme is alkaline in nature but our enzyme shows activity around neutral pH 6.2.

Conclusion

The industrially important enzyme Pectinase was successfully produced in the pilot-scale by using the mesophilic *A. niger* best strain PE.KVM. The plate morphology observation and the microscopic study was performed to identify the organism as *Aspergillus niger*. On the basis of a literature review, only one production media was chosen for the production of the enzyme. From these results, we can conclude that the isolated PE.KVM *A. niger* is a good producer of the enzyme pectinase.

Finally, we can conclude that the production of pectinase from *A. niger* can be industrialized using the microbial sources as a major attribute in the context of Nepal. The pectinase produced from the *A. niger* has a good enzymatic activity that will be helpful for various aspects and dimensions of biotechnology. Biotechnology is a growing field having the great potential required to build a better nation. The commercial production of pectinase from the fungal strains like *A. niger* species is economic and beneficial using citrus waste as the substrate with few modifications in the context of Nepal with moderate environmental conditions prevailing at the valley.

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