Exploitation of Vegetable and Fruit Waste for Cellulase and Pectinase Production Using Aspergillus sp.

Malarvizhi V[1] Menaka R[2] Vidhya N[3]
[1,2]PG Student [3]Professor
[1,2,3]Department of Microbiology
[1,2,3]Dr. N.G.P Arts and Science College Coimbatore-641048, Tamil Nadu, India

Abstract — Fermentation technology plays a very important role for production of microbial enzymes, antibiotics, single cell proteins, various food products. Use of Microbes as bioreactors for the developmet of the industrially important enzymes from microbial origin include Lipases, Amylases, Proteases, Cellulase and Pectinases. The current work involved for the production pectinase and cellulase enzyme using decaying fruit and vegetable waste. The organisms were isolated from the waste and were cultured onto SDA agar Plate as the isolation is of Fungi. The organism was screened for enzymatic activity using Carboxyl Methyl Cellulose and Pectin agar medium. The result revealed a clear zone of inhibition in the agar plates. Microscopic identification and Morphology study revealed that the Fungi isolated was Aspergillus niger. Aspergillus niger produced significant quantities of enzyme when grown in production medium under submerged condition and incubating for 10 days. The isolate showing maximum activity was identified and subjected to growth parameters optimization in attempt to increase the enzyme production ability of the isolate in larger scale. The activity of pectinase and cellulase production by fungus at various optimum conditions of pH 7 temperature 37°C were assessed. The enzymes was determined by titrartion method and used for Quantitative estimation of protein was done using Lowry's method at 660nm to know the concentration of protein and antioxidant test were done. Thus the enzyme was used for fruit juice extraction production. The amount of juice extracted in the control samples was less compared to the samples extracted using Pectinase and Cellulase enzyme. Concluded that Aspergillus niger from fruit and vegetable waste has ability to produce pectinase and cellulase and it is highly beneficial, it may consider as a source for production of industrial enzyme.

Keywords: Pectinase, Cellulase, Aspergillus Niger, Fruit and Vegetable Waste, Lowery Etal Method, Antioxidant Activity, Optimization of PH and Temperature

I. Introduction

Enzymes are delicate protein molecules necessary for life they are biocatalysts that catalyze and accelerate various biological reactions. Enzymes are the bio-active compounds that regulate many chemical changes in living tissues. The primary source of industrial enzymes is microorganisms, out of which, 50 % originate from fungi and yeast, 35 % from bacteria, while the remaining 15 % are either of plant or animal origin[1]. Vegetable wastes include the rotten, peels, shells, and scraped portions of vegetables or slurries. These wastes can be treated for bio-fuel production through fermentation under controlled conditions or else used for composting. The natural decomposition of wastes by microbes generates products with high humus content.

Pectinase is an enzyme found in plant cell walls that breaks down pectin. Commonly referred to as pectic enzyme one of the most studied and widely commercially used pectinases present in a component of the cell wall in fruits such as apples. Pectinase enzyme is used for extracting juice from fruit. By enzymatically breaking down the cell wall of fruit, pectinase releases the juice from within the cells. Pectinase is a group of enzymes that break down the pectin commonly found in plant sources. Pectin occurs naturally in all plants. Pectinase is a natural resource that may be derived from bacteria and fungi. This enzyme is commonly used in food industry that involves degradation of plant materials to speed up extraction of juices from fruits[2]. The primary source of industrial enzymes is microorganisms, out of which, 50 % originate from fungi and yeast, 35 % from bacteria, while the remaining 15 % are either of plant or animal origin.

Cellulase is a class of enzymes produced by the fungi, bacteria and protozoans that generate cellulolysis. This process is actually the hydrolysis of cellulose. The enzymes are also produced by certain termites. There are various types of cellulases. The name is also used for any naturally occurring mixture or complex of various such enzymes, that act serially or synergistically to decompose cellulosic material. Cellulases break down the cellulose molecule into monosaccharides ("simple sugars") such as beta-glucose, or shorter polysaccharides and oligosaccharides [3]. Cellulase plays important role in the process of fermentable sugars production from cellulose, which is the primary polysaccharides inlignocellulosics. Cellulase production is the key phase of the enzymatic cellulose hydrolysis process. Fungi are well known as decomposing agents of organic matters. Lignocellulose-rich materials, such as leaf litter, compost soil, decayed wood and lignocellulosic waste are potential substrates for lignolytic fungi[4].

Fruit juices are extracted by mechanical grinding of the fruits that result in the jelly like products. Due to complex pectin structure of fruits, it is difficult to extract juice from this highly viscous jellified pulp Pectinase enzyme acts on the pectin and breaks the glycosidic bonds present between the galacturonic acid monomers thereby decreases the water holding capacity of pectin. Hence high yield of juice is obtained

Application of cellulase can degrade cellulose fibrils which in turn causes reduction in wall strength. It also solubilizes the cell wall polysaccharides resulting in almost complete (95%) liquefaction. Fibers in juices are another great problem faced by the industries. They get clogged in the manufacturing line, affecting productivity and causing huge loses to the manufacturers. Most of the fibers in fruit juice are cellulosic in nature and therefore, filtering of juices becomes easier on application of cellulases. Macerating enzymes are used for complete liquefaction of fruit pulp after crushing the

fruit and also for better clarification and reduced viscosity of the final product[3].

The microorganism, *Aspergillus sp*., is a part of kingdom fungi, phylum Ascomycota, class eurotiomycetes, order eurtotiales, family trichocomaceae and genus *Aspergillus*. *Aspergillus niger* or A. *niger* is a fungus and one of the most common species of the genus *Aspergillus*. Filamentous fungi are the preferred source of industrial enzymes because of their excellent capacity for extracellular protein production. It is possible that large-scale commercial production of cellulolytic enzymes could benefit from the utilization of spent hydrolysates or similar residues as nutrient source. This study therefore aimed at the production of pectinase ad cellulase enzyme from fruit and vegetable waste from A.niger, for assaying enzyme activity and increasing fruit juice extraction by using this enzymes.

II. Materials and Methods

A. Collection of Sample

Vegetable and fruit waste was collected from local market located at Avinashi, Tirupur. Sample was collected using air tight plastic container and sample were transported to the laboratory, prior to its use.The collected sample were serially diluted for the isolation process and the pure culture were obtained under microscopy.

B. Pure culture preparation:

The collected samples were waved one gram of vegetable waste was weighed aseptically into 9ml of sterile distilled water separately and shaken thoroughly. From these, dilution were subsequently made up to 10-4 and pour plating of 10-2, 10-3 and 10-4 was done in sterilized potato dextrose agar (PDA).Streptomycin(100mg/L) was added to the PDA after sterilization to prevent bacterial growth. Plates were incubated in an inverted position at 30oC for 7days[5].

C. Colony Morphology of the Culture and Microscopy Examination:

After incubation, the plates were observed for fungal growths. A small portion of the mycelia growth was carefully picked with the aid of a pair of sterile inoculating needles and placed in a drop of lactophenol cotton blue on a microscope slide and covered with a cover slip.

D. Screening of Enzyme:

1) Screening Aspergillus niger for Pectinase:

Pectinase Screening Agar Medium (PSAM) is used for the selective growth of those microbes which release pectin. It was the prepared as per following composition: Pectin (0.5g), K2HPO4 (0.05g), MgSO4.7H2O (0.01g), NaCl (0.02g), CaCl2.2H2O (0.02g), FeCl3.6H2O (0.001g), Yeast extract (0.1g), Agar (2g), Distilled water(100ml) pH-5.0. The screening of the pectinolytic activity was conducted based on a clear zone formation on pectic agar medium after it was stained by Cetyl trimethyl ammonium bromide. Fungal isolates were inoculated on pectic agar medium and had been incubated at a room temperature for 96 hours.

2) Screening Aspergillus niger for Cellulase:

Cellulolytic activity was screened based on clear zone formation on Carboxy methyl cellulose (CMC) media. The media comprised Carboxy Methyl Cellulose (1g), FeSO4 (0.02g), K2HPO4 (0.3g), MgSO4.7H2O(0.01 g), CaCl2 (0.04g) and Agar (2.5 gm) in 100 ml, the initial pH of medium was adjusted to 7. Fungal isolates were inoculated at CMC media and incubated at 24 oC for 96 hours. To visualize the clear zone, the plates were flooded with an aqueous solution of Phenol red 1% for 30 minutes and washed with 1 M NaCl [6].

E. Enzyme Production:

1) Pectinase enzyme production by Solid State Fermentation:

Aspergillus niger was placed in a basal medium used for pectinase production, the medium consist of NaNO3(0.2g); K2HPO4 (0.1g); MgSO4(0.05g); KCl,(0.05g); FeSO4 (0.001g); Pectin (10g);Distilled water (100ml).The culture was grown for 7days at 25°C[7].

2) Cellulase Enzyme Production by Solid State Fermentation:

Aspergillus niger was placed in a basal medium used for cellulase production just like the modified medium of Deacon (1985) containing (in g/100ml), Yeast extract(0.2g); NaNO3(0.5g); KH2PO4(0.1g); MgSO4.7H2O(0.05g); and FeCl3(0.0001g). Carboxymethyl cellulose (CMC) was added at 1% concentration[8].

F. Crude Enzyme Extraction by Filtration:

To separate the liquid enzyme mixture and the mycelia mat along with the spore bodies filtration technique was employed. Filtrate broth through Whatman No.1 filter paper and then centrifuge at 10,000 rpm for 5 minutes. This crude enzyme extract is used for measuring Pectinase activity[8].

G. Enzyme Assay:

Enzyme activity is measured by increase in free Carboxyl group by titrating against NaOH in the present of ph indicator like phenolphthalein.

For assaying enzyme activity 20 ml of 1% pectin was dissolved in 0.15M NaCl pH-7 and 4ml of crude enzyme extract is take in beaker and incubated for 1 hour. After incubation the solution is titrated against 0.02 N NaoH to reach pH 7 using phenolphthalein as indicator (colour change from colourless to pink) the heated crude enzyme extract is used as control.

$$Enzyme\ activity\ = Vs - Vb\ (Normality\ of\ NaOH)\ \times 100/Vt$$

Enzyme activity is expressed as milli equivalents of NaOH consumed min -1ml-1 of crude enzyme extract under the assay conditions.

1) Estimation of the Protein Concentration by Lowry's et al Method:

Lowry assay (Lowry et al., 1951) was used to estimate the amount of protein present in biological samples. First, the protein was pre-treated with copper ion in alkali solution and then the aromatic amino acids in the treated sample reduce the phosphor molybdate phospho tungstic acid present in folin reagent. The end product has a blue color.1ml of test sample was treated with 1 ml of Lowry reagent and incubated at room temperature for 30 min. After incubation, 1ml of 1N Folin reagent was added, kept for 30 min and the absorbance was measured at 640 nm.

The activity was measured by the cell free supernatant was used to study the enzyme activity. To 2 ml

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of pectinse and cellulase 2.0% (w/v) in Tris – HCl pH 8.0, 0.5 ml of culture supernatant was added and incubated at 37˚C for 10 min. The reaction was terminated by the addition of 2.5 ml of 0.1M trichloroacetic acid (TCA) and further incubated at 37˚C for 30 min. It was centrifuged at 10,000 rpm for 10 min at 4˚C. To 2.5 ml of supernatant, 3 ml of 0.5M Na2CO3 and 0.5 ml of 1N FolinCiocalteu reagent were added, mixed well and incubated for 10 min. Absorbance was measured at 640 nm. The standard curve was prepared using different concentration of tyrosine. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1μmol of tyrosine per min under the defined assay conditions. Enzyme assay was done for both crude sample and partially purified fractions. The absorbance was measure at 660 nm and the standard graph was plotted. The amount of protein present in the given sample was estimated from the standard graph[8].

H. In vitro antioxidant assay:

1) DPPH assay:

The free radical scavenging activity of enzyme extracts of pectinase and cellulase enzyme was measured by using DPPH (22-diphenyl- 1-picrylhydrazyl). The scavenging activity for DPPH free radicals was measured according to the procedure. About 1ml of 0.1mM DPPH solution (<1.00 at 517nm in UV-spectrophotometer) was mixed 10μl, 20μl, 30μl,40μl and 50μl of lipase enzyme. Flowingly equal volume of methanol was added and kept at dark condition at room temperature for 30minutes. Standard ascorbic acid and absorbance adjusted DPPH solution was used as positive and negative controls in the assay. Spectrophotometer absorbance of the mixtures was measured at 517nm.

The percentage inhibition of DPPH radical quenching by the sample as well as standard was calculated as follows:

$$\text{DPPH scavenging in \%} = \frac{\text{OD of control} - \text{OD of sample}}{\text{OD of control}} \times 100$$

2) Optimization of temperature:

The optimum temperature of Enzyme from *Aspergillus niger* was determined by using four 250 ml conical flasks were taken. In each, 50 ml production medium was prepared and added. These flasks were autoclaved for 30 minutes and then 3 loops full of the culture was added in a sterile atmosphere to each conical flask. The incubation period, pH and inoculum size were used from the readings noted above. The varying factor here was the temperature. The four flasks were marked from 1-4 and kept for incubation with temperature values at 15 degrees Celsius (cold water bath), 37 degrees Celsius (shaker) and 45 degrees Celsius (autoclave). At the end of each day, the respective flask was taken and the enzyme activity of the content was checked and noted. The temperature giving the highest enzyme activity was noted.

3) Optimization of pH:

The optimum pH was determined by using four 250 ml conical flasks were taken. In each, 50 ml production medium was prepared and added. These flasks were autoclaved for 30 minutes and then 3 loops full of the culture was added in a sterile atmosphere to each conical flask. The incubation period was used from the period noted above, the inoculum size was 1% and the flasks were kept at room temperature. The varying factor here was the pH. The four flasks were marked from 1-4 and kept for incubation at pH 5.0, 7.o,10.0. At the end of each day, the respective flask was taken and the enzyme activity of the content was checked and noted. The pH value giving the highest enzyme activity was maintained as a constant.

4) Application in Fruit Juice Extraction:

To determine the enzyme activity by measuring the amount of apple juice released by pectinase and cellulase use and compare it with the normal protocol in the absence of the enzyme. Apples were chopped into cubes that are roughly 5 mm on a side. It is important to chop the apple into very small pieces added surface area helps the enzyme break down the pectin in the plant cell walls, releasing more juice. Macerating enzymes are used for complete liquefaction of fruit pulp after crushing the fruit and also for better clarification and reduced viscosity of the final product The chopped apples were divided equally between the two beakers. Sensitive balance was used to weigh equal amounts of chopped apple (20gms.) into each beaker. 10 ml of the enzyme was added to one beaker, and 10 ml of water to the other. The beakers were labeled as "Enzyme" and "water" respectively. Chopped apple pieces were stirred in each beaker with a glass-stirring rod. Be sure to wet all of the pieces.The beakers were covered with aluminum foil and were incubated at a 40°C water bath for 1 hour.After removing jars from water bath, a wooden spoon was used to gently stir/squeeze the apple pieces in each. To filter the juice from the filter paper was used in the funnels. A graph was made using the total volume of juice produced by each treatment and the weight of apples used.

III. RESULT AND DISCUSSION

A. Isolation of fungi:

The Sample was serially diluted at 1ml of dilution rate at 10-3 to 10-7 plated with PDA medium as shown in (Fig.1)

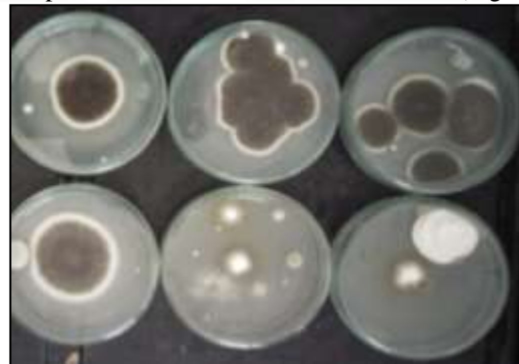

Fig. 1: Serial Dilution

B. Colony Morphology and Isolation of Aspergillus niger:

Aspergillus sp., filamentous fungi, which means that they tend to form filaments (hyphae) and thus resemble the structure of a plant (Fig.2).When viewed under the microscope, *A. niger* consists of a smooth and colorless conidiophores and spores. The mycelia thus obtained was used for the staining with Lacto phenol Cotton Blue and observed under 40X magnification. Based on the Colony Morphology the Mycelia were identified as *Aspergillus niger* (Fig.3).

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Fig. 2: *Aspergillus sp.,*

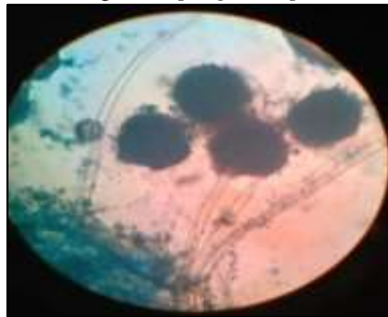


Fig. 3: Under LPCB staining

C. Screening of Enzyme:

1) Screening Aspergillus niger for Pectinase:

The PSAM medium allowed selective growth of Pectinolytic microbes, microscopy on cells from each zone revealed the presence of *Aspergillus niger.*

These isolates were screened in pectinolytic activities based on clear zone formation on pectic agar medium which is stained by cetyl trimethyl ammonium bromide also known as cetrimide or CTAB. The screening result showed that fungal isolates which showed pectinolytic activity by clear zone formation around colony.

Fig. 4: PSAM Medium shows clear zone formation

D. Screening Aspergillus niger for Cellulase:

The cellulolytic activities was measured based on secondary test. The culture were tested based on the clear zone formation on the minimal media containing CMC. The culture was strained on phenol red agar and left for 3 days at room temperature. After 3 days, it was found that clear zones were formed around the culture, thus indicating the presence of *Aspergillus niger* which degraded the phenol red. The result of the screening showed that had cellulolytic activities indicated by the clear zone formation around the colony So that *Aspergillus niger* had cellulolytic activities(Fig.5)

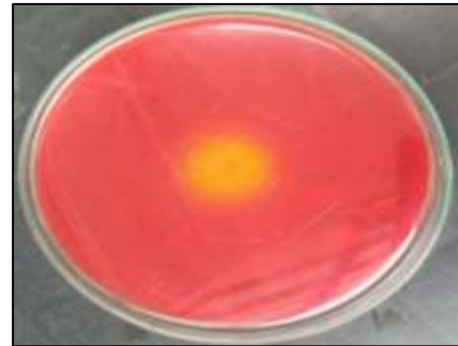


Fig. 5: CMC medium after colouring with Phenol red.

E. Estimation of the Protein Concentration by Lowry's Method:

The total protein concentration of Pectinase and Cellulase enzyme was estimated by UV –Visible Spectrophotometer. The concentration of Pectinase enzyme was found to be 0.1329 and cellulase enzyme concentration is 0.1220 (Table 1).

BSA Solution added	OD at 595 nm
0.1	0.0032
0.2	0.1109
0.3	0.1211
0.4	0.1249
0.5	0.1285
0.6	0.1315
0.7	0.1335
0.8	0.1368
0.9	0.1386
1ml	0.1417

Table 1:

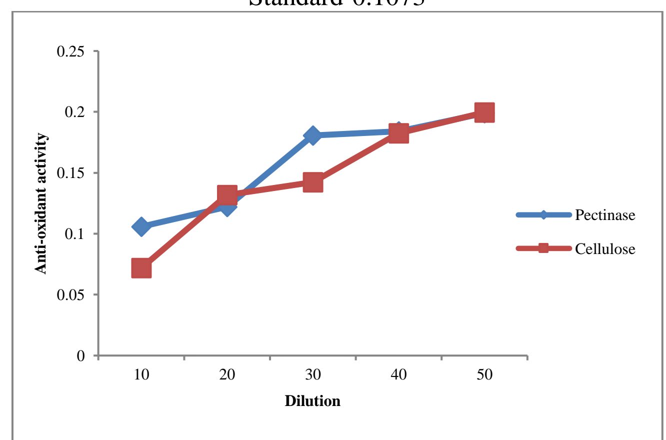
F. In vitro Antioxidant Activities:

The *in vitro* antioxidant activities of pectinase and cellulase enzyme are shown in table 2.

Dilution	OD at 517 nm Pectinase	OD at 517 nm Cellulase
10µl	0.1058	0.0718
20µl	0.1220	0.1318
30µl	0.1807	0.1423
40µl	0.1841	0.1824
50µl	0.1990	0.1996

Table 2:

Standard-0.1073



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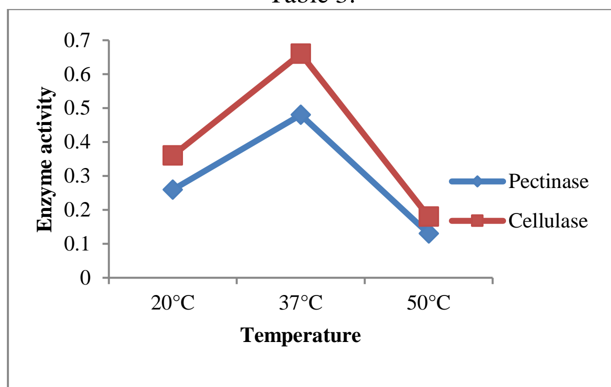
G. Optimization of Temperature:

The Optimum temperature of 37˚C gave the highest enzyme activity of Pectinase and cellulase.

Temperature	Pectinase	Cellulase
20	0.26	0.36
37	0.48	0.66
50	0.13	0.18

Table 3:

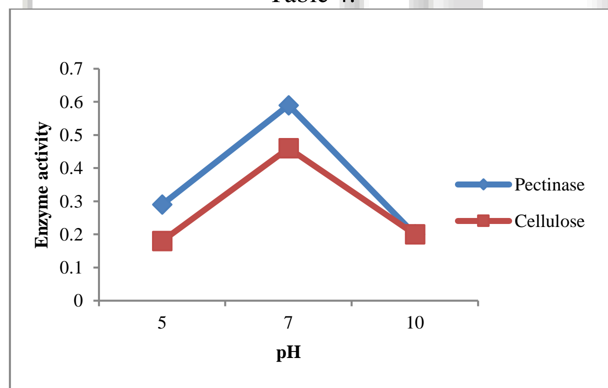


H. Optimization of pH:

The Optimum Ph of 7 gave the highest enzyme activity of Pectiase and Cellulase.

pH	Pectinase	Cellulase
0.5	0.29	0.18
0.7	0.59	0.46
10	0.20	0.20

Table 4:



I. Application in fruit juice extraction:

There is an extensive increase in the volume of the juice extracted with the use of enzymes when compared to the control (water)

Fig. 6: Cutted Apples with enzymes and control

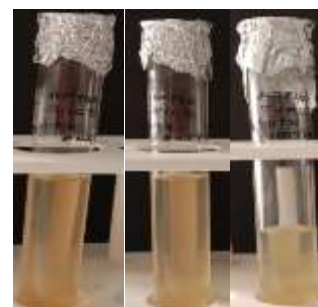


Fig. 7: Extracted Juice) i) pectinase ii) cellulose iii) control

IV. CONCLUSION

In this study a group of pectinolytic fungi were isolated from cheap sources like spoiled fruits and vegetables *was* found to be the potent source for pectinase. Although the *Aspergillus niger* produced comparable amounts of enzymatic activity the Optimum temperature is 37oC and pH is 7 gave the highest enzyme activity of Pectinase and Cellulase. By immobilising enzymes, they can be reuse again and again. It makes the easy removal of the enzymes from the juices. As different fruits requires different processing treatments like different enzymes for example cellulases, amylases and pectinases in optimized concentration can be immobilized according to the specific fruit and the optimized process to reduce the enzyme cost and incubation time. It is evident from this study that there is a possibility of utilizing *Aspergillus niger* isolated from waste to produce cellulase and pectinase enzymes in a cost effective and eco-friendly method, which can be used in production of ethanol, detergent, weave, textile, coffee, pulp and paper and pharmaceutical industries. The overall goal is to make valuable products in a cost effective way that are easily accepted by the consumers and benefit them in their health.

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