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Isolation, Purification And Assay Of Pectinase Producing *Aspergillus Niger* From Onion Peels In An Economically Feasible Way By Using Sabouraud Agar Medium

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ABSTRACT

This study was conducted to screen for pectinase production by Aspergillus niger isolated from soil samples collected from three different locations within Ahmadu Bello University, Zaria, (botanical garden (BG), refuse dump (RD) and sheep pen (SP) sites). Fifteen (15) soil samples were collected from different locations and used for isolation by cultural method. Isolates suspected to be Aspergillus niger were further identified by microscopic examination using lactophenol cotton blue stained-preparation and slide culture technique. The isolates were then screened in a pectin-containing medium for their pectinase activity. The isolates were further subjected to pectinase production using citrus pectin as the substrate under submerged fermentation conditions. Seven (7) isolates were confirmed to be Aspergillus niger with percentage occurrence of 60% each from sheep pen, refuse dumpsites and 20% from botanical garden.

Keywords: Aspergillus niger, Pectinase, Production, Soil, Screening etc.

INTRODUCTION

Aspergillus niger Pectin is a polymeric material having carbohydrate group esterified with methanol. It is an important component of plant cell wall with highest concentration in the middle lamella (1). This acts as a connecting substance between adjacent cells. Pectinases consist of a mixture of complex enzymes that specifically catalyzes the hydrolysis of pectin-containing substrates (2) These enzymes are divided into three main classes that catalyzes depolymerization, demethylation and de-esterification reactions. (Polygalacturonase is a depolymerizing enzyme that catalyzes the breakdown of 1,4-glycosidic bonds between two galacturonic acid residues in the smooth region of the pectin molecule. Whereas, pectin lyase (EC 4.2.2) and pectin esterase (EC 3.1.1) are demethylating and deesterifying enzymes respectively, these acts as the elimination reaction between two methylated residues and splitting of methoxyl groups releasing methanol Pectinases are derived from different sources such as bacteria, fungi, plants, insects, protozoans and nematodes (3) Several bacterial and fungi species have for long been known to produce pectinolytic enzymes and it is widely believed that the production of these enzymes is a major means by which these microorganisms invade the host tissue (4). Even though the occurrence of pectinolytic enzymes have been reported in a large number of bacteria and fungi, almost all the commercial preparations of pectinases are produced from fungal sources, with Aspergillus niger being the most commonly used fungi species for industrial production of pectinolytic enzymes (5). Aspergillus niger are widely distributed geographically and have been observed in

broad range of habitats because they can colonize wide variety of substances.

METHDOLOGY

Extraction of pectin

Orange peels were cut into small pieces and these were kept for air drying; 100g of dried orange peels were weighed and transferred into 1000ml beaker containing 400ml warm ethanol. Keep it aside for few mintues, after trhat peels were separated from ethanol by filtration process. These peels were again washed with sufficient quantity of distilled water. Again filter the solution to separate peels from water. To that peels add 800ml of distilled water and 12g of sodium hexametaphosphate and adjust pH with HCL to 2.2+0.1. this mixture was heated on water bath at 70^0 C for 1 hr with continous stirring to avoid sticking of peels to the bottom of the beaker. The extract was filtered through a muslin cloth and the residue were washed with 200ml of distilled water. The washings were added to the filtrate. The filtrate was concentrated on a hot plate to get $1/5^{th}$ of the initial volume. The concentrated pectin was cooled, and it was poured into a volume of 0.5M HCL–sprit(1:3) ratio solution. The mixture was stirred for 30 min and allowed to stand for 1 hr. the precipitate was centrifuged and washed with HCL-sprit solution again was it centrifuged and finally washed with acetone to remove traces of HCL and sprit. Again, it was centrifuged to remove acetone. This pectin was spreaded in to petri plates. The extracts was dried in oven at 40^0C for a few hours to constant weight. Finally it was stored in refrigerator. (6).

Pectinolytic activity test

25 ml of production agar medium pectin is sole carbon source was prepared and sterilized autoclave. Simultaneously, petri dish were sterilized by hot air oven(160^0 for 2 hours). The medium was poured in petridesh aseptically, leave for solidification. After solidification fresh mixture *Aspergillus niger* was stabbed at the center of petri desh. Incubate for 3 days at room temperature. Results were observed.

PRODUCTION OF PECTINASE ENZYME

In the production of Pectinase enzyme it mainly contains 2 steps:

1. Inoculum medium
2. Production medium

INOCULUM MEDIUM

Prepare SAB broth medium and sterilize for 15 min by using autoclave at 121^0 C, 15 lb pressure. Cool the medium for few minutes and add inoculum aseptically in laminar air flow chamber. Keep the mixture on rotary shaker for 24 hrs

PRODUCTION MEDIUM

Prepare production medium (pectin sole carbon source), up to 100ml with distilled water. This medium is sterilized by autoclave at 121^0 C at 15 lb pressure for 15 min. Cool the medium, then inoculum is added aseptically in laminar air flow chamber. Keep the mixture on rotary shaker for 4 days. Medium is centrifuged, then supernatant was collected and it is further proceed for purification process.

PARTIAL PURIFICATION OF PECTINASE ENZYME

Supernatant was precipitated by adding ammonium sulphate, upto saturation level and freeze it over night at 4^0 C. After freezing the precipitate is centrifuge for 10 min at 1000rpmthen separation of precipitate take place. The superintant layer was discarded and the remaining purified enzyme was collected. These were stored by adding sodium acetate buffer (*7*)**.**

STANDARDIZATION PROCEDURE

Prepare different volumes of 0.001N galacturonic acid from stock solution. To each add 1ml of sodium acetate buffer. Incubate for 15 min at 30^0 C, Then add 4ml DNS reagent to each tube and boil for 15 min. Measure the absorbance at 575nm, galacturonic acid omitted solution was taken as blank.(8)

ASSAY PROCEDURE

Polygalatcuronase activity was determined by measuring the amout of realsed reducing groups by pectin hydrolysis within 3,5 dinitrosalicyclic acid reagent. Different volumes(100, 300)partially purified enzyme was taken. To each stock tube add 1ml of pectin solutions and 1 ml of sodium acetate buffer. Incubates the tube for 15 mints at 30^0 C, after incubation add 4 ml of DNS reagent for boil for 15 mints at 40^0 C. then cool it for few mints and measure absorbance at 575nm.galacturonic acid was taken as standard. Pectinase omitted was taken as blank . one unit of polygalacturonase activity was defined as the amount of enzyme required to release to 1mmol of galacturonic acid per minte under standard assay conditions.(9)

Microbiology

Freshly sub cultured and fully sporulated slants of isolate was taken 2ml spore suspensions was prepared by inoculating a loopful of fungal isolate to sterile water . 3ml of sterile SAB agar medium was poured in petri dish after solidification 0.5ml of culture suspensions was poured over the medium the plate was inoculated. after proper incubation growth morphology was observed under microscope.(10)

RESULTS AND DISCUSSION

ISOLATION OF FUNGI FROM ONION PEELS

Block molds containing onions were collected from market and it was transported to laboratory. By using inoculating loop streak the organism in a test tube containing SAB agar medium aseptically in laminar air flow chamber. Incubate at room temperature for 3 days, these slants were further used for pectinase production.

Fig 1: Black molds containing onions

Fig 2: Strains of *Aspergillus niger*

COLLECTION OF ORANGES

- ✓ Fresh Oranges were collected from local market, perecherla
- ✓ Orange peels were cut in small pieces and they were kept for shade drying.
- ✓ Further these were used for the extraction of pectin.

Fig 3: Fresh oranges

Fig 4: Shade dried orange peels

EXTRACTION OF PECTIN FROM ORANGE PEELS

100g of dried orange peels were weighed And these were transferred in to 1000ml beaker containing 400ml of warm ethanol, then keep it aside for few minutes. After that peels were separated from ethanol by filtrations process. These peels were again washed with sufficient quantity of distilled water, again filter the solution to separate the peels from water. To that peels add 800ml of distilled water and 12g of sodium hexameta phosphate and adjust the pH with 3N HCl to 2.2 ± 0.1.The mixture was heated on water bath at 70^0 C for 1 hr with continous stirring.

The extract was filtered through a muslin cloth and residue was washed with 200ml of distilled water. The filterate was

concentrated on hot plate to get 1/5th of the initial volume. Concentrated pectin was cooled and it was poured into a volume of 0.5M HCl-Spirit (1:3) ratio solution. This mixture was stirred for 30min and allowed to stand for 1 hr. The precipitate was centrifuged, and washed with HCl and spirit solution. Again it was centrifuged and finally washed with acetone to remove traces of HCl and spirit. Again it was centrifuged to remove acetone. Finally pectin was spread into petri plates, and it was dried in an oven at 40^{o} C for a few hours to constant weight. Finally it was stored in Refrigerator.

Fig 5: Pectin extracted from orange peels

PRODUCTION OF PECTINASE ENZYME

In the production of Pectinase enzyme it mainly contains 2 steps:

1. Inoculum medium
2. Production medium

INOCULUM MEDIUM

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PRODUCTION MEDIUM

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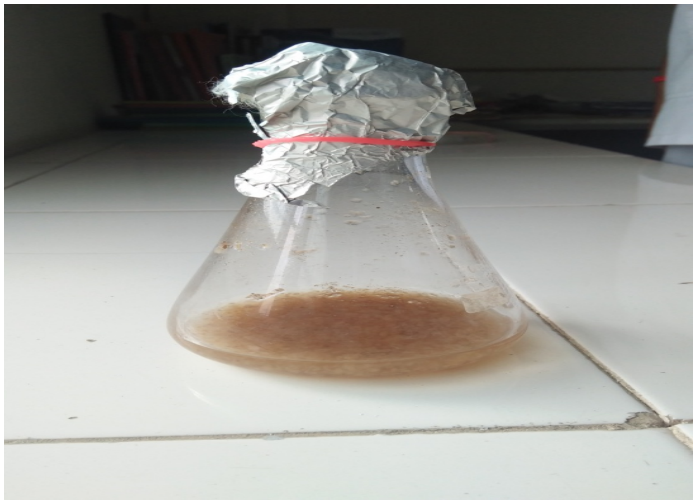

Fig 6: Production medium

PARTIAL PURIFICATION OF PECTINASE ENZYME

Supernatant was precipitated by adding ammonium sulphate, upto saturation level and freeze it over night at 4^{0} C.After it centrifuge for 10 min at 1000rpm,crystal form of purified enzyme gets separated to it add sodium acetate buffer.

Fig 7: Partially purified enzyme

STANDARDIZATION

Prepare different volumes of 0.001N galacturonic acid from stock solution.To each add 1ml of sodium acetate buffer. Incubate for 15 min at 30^0 C, Then add 4ml DNS reagent to each tube and boil for 15 min. Measure the absorbance at 575nm, galacturonic acid omitted solution was taken as blank.

Fig 8: Standardization of Galacturonic acid

Table 1: Standardization of Galacturonic acid

S.No	Vol of Galacturonic acid (µl)	Amount of galacturonic acid (µg)	Absorbance at 575nm
1	100	20	0.158
2	200	40	0.22
3	300	60	0.318
4	400	80	0.392
5	500	100	0.48

Table 2: Estimation of enzyme activity

S.No	Vol . of pectinase (µl)	Absorbance at 575 nm	Pectinase activity IU/min
1	100	0.126	13.95
2	300	0.260	46.63

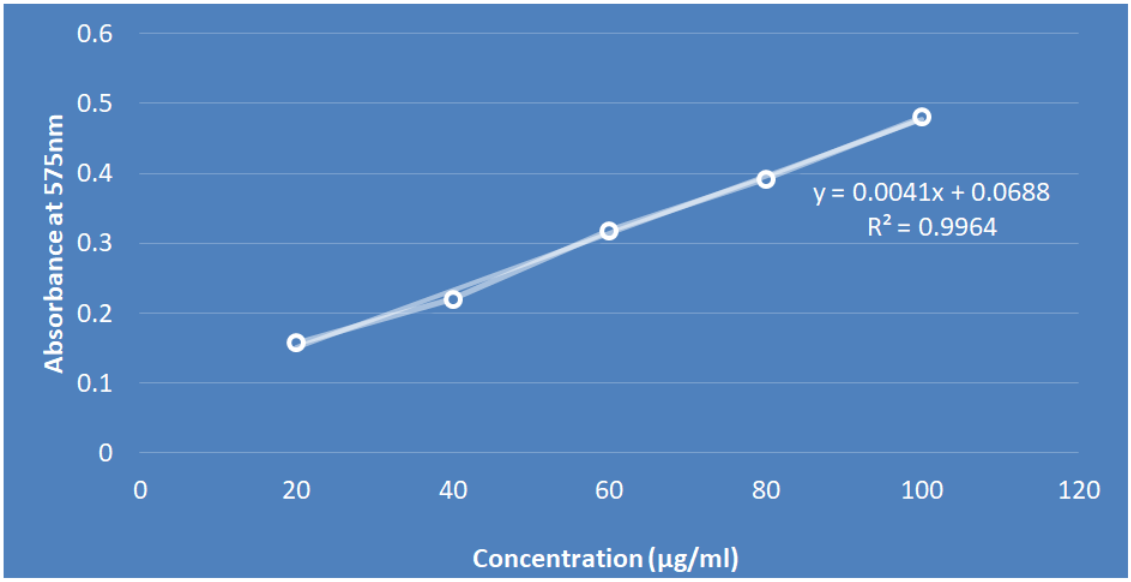

Fig 9: absorbance vs concentration of enzyme activity

By performing these studies we were confirmed that 100µl of partially purified enzyme produce 13.95µmol of galacturonic acid per minute this revealed that it contain 13.95 international units of pectinase whereas, 300µl of partially purified enzyme produce 46.63µmol of galacturonic acid per minute this revealed that it contain 46.63 international units of pectinase. One unit of pectinase activity was defined as the amount of enzyme required to release 1 µmol of galacturonic acid per minute.(12)

ASSAY

Different volumes (100,300µl) of partially purified enzyme was taken.To each tube add 1ml of pectin solution, 1ml of sodium acetate buffer solution. Incubate the tubes for 15min at 30^0 C. After incubation add 4ml of DNS reagent and boil for 15min at 40^0 C. Then measure the absorbance at 575nm. Pectinase omitted solution was taken as blank.
(13)

Fig 10: Partially purified emzyme

MORPHOLOGY

- ✓ 3ml of sterile SAB agar medium was poured in to petri plates.
- ✓ Allow to solidification.
- ✓ 2ml of spore suspension medium was prepared
- ✓ After that 0.5ml of culture suspension was added over the medium, incubate for 3-4 days.
- ✓ Morphology was observed under Trinocular microscope.

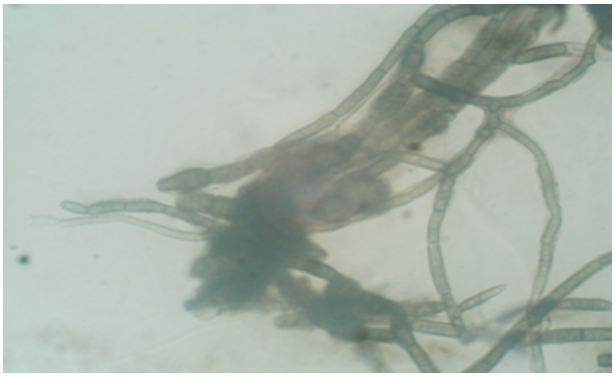

Fig 11: Microscopic image of *Aspergillus niger*

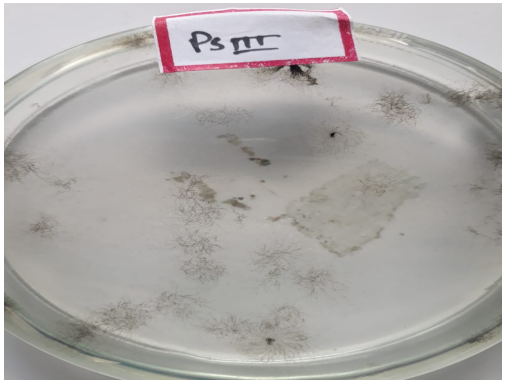

Fig 12: Macroscopic image of *A. niger*

CONCLUSION

It was concluded that orange peel could be an attractive and most useful substrate for the production of pectinase enzyme by using Aspergillus niger. From this work we want to explore the possibilities of converting waste to wealth. By this process we are readily accessible as waste with little or no cost and it also contain an appreciable amount of pectin, which is regarded as low cost substrate for efficient and economical production of pectinases using Aspergillus niger. Pectinases has wide applications in industries, which is produced with low cost through waste. It produce markable amount of pectinase while compared with marketed product. In this study we were isolated Aspergillus niger from onion peels.

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