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Optimization of Condition for Pectin's Production by *Aspergillus niger* in a Citrus Pectin based Submerged Fermentation Medium

Chukwunonso Anthony Nsude[1]*, Innocent Izuchukwu Ujah[1], Sopuruchukwu Simeon Agbowo[1] and Ferdinand C. Chilaka[2]

[1]Department of Applied Biochemistry, Enugu State University of Science and Technology, Nigeria

[2]Department of Biochemistry, University of Nigeria Nsukka, Nigeria

Abstract

Pectinases are one of the commercial enzymes that are extensively used in wine and fruit juice industries for clarification, and paper industry for bleaching up pulp. This work was focused on optimization of culture conditions for production of pectinase by submerged fermentation using citrus pectin as an inducer. Pectinolytic *Aspergillus niger* isolated from citrus pectin culture medium was used to determine the best initial pH, temperature, time, and nitrogen source and pectin concentration for pectinase production. Studies carried out on the pectin-depolymerizing capacity of the organism indicated 1.2 cm clearance zone. Temperature of 30°C, pH of 5.0, 4th day of fermentation, $(NH_4)_2SO_4$ and 1.0% pectin concentration were the optima for pectinase production with specific activities of 1.7 U/mg, 1.78 U/mg, 4.23 U/mg, 3.03 U/mg and 3.01 U/mg respectively. Results of this work will serve as reference material to researchers whose interest are on producing quality fungal pectinases at cheaper cost.

Keywords: Submerged fermentation; *Aspergillus niger*; Citrus pectin; Pectinase; Ripened orange fruits

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Correspondence:
Chukwunonso Anthony Nsude, Department of Applied Biochemistry, Enugu State University of Science and Technology, Nsukka, Nigeria, Tel: 2348063387246; E-mail: chukwunonso.nsude@esut.edu.ng

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Introduction

Pectins are a family of complex polysaccharides, primarily composed of α-1→4 linked D-galacturonic acid residues with a small number of rhamnose residues in the core chain and galactose, arabinose and xylose on their side chains Rangarajan et al. [1]. The highest concentrations of pectin are found in the middle lamella of cell wall, with a gradual decrease as one passes through the primary wall toward the plasma membrane [2]. Pectins and complex hemicelluloses contribute to the structural integrity and consistency of plant tissues as well as for the quality of vegetables and fruits [3]. Pectic polysaccharides such as, homogalacturonan, rhamnogalacturonan-1 and substitutes galacturonans, have been isolated from primary plant cell wall. Homogalacturonan (HG) is a linear polymer consisting of 1,4-linked α-D-galacturonic acid, in which some of the carboxyl groups are methyl-esterified at C-6 and carry acetyl group on O-2 and O-3 [4].

Pectinases are group of enzymes which cause degradation of pectin. They catabolize pectic substances by degradation reactions through depolymerization (hydrolases and lyases) and de esterification (esterases) reactions [5]. Various microorganisms like bacteria, fungi and yeast are responsible for their production. However, fungi species especially *Aspergillus* species are widely utilized in production of pectinases which are used in extraction, clarification and removal of pectin in fruit juices, in maceration of vegetables to produce pastes and purees and in wine making [6].

Pectinases are commercially produced using either submerged or solid state fermentation cultures. Enzyme producing microorganisms are isolated from different sources, such as soil, decayed plant parts and contaminated host tissue of plants. In solid state fermentation culture, microbial growth and product formation usually occur at or near the surface of solid substrate particles with low moisture content [7], whereas in submerged fermentation culture, production of pectinases generally depends on medium compositions such as pectin concentration, nitrogen source, pH of the medium, temperature and fermentation time. Semi-solid or submerged fermentation medium is more favorable to fungal growth since in the medium, the moisture content, agro-waste (substrate) and pH are the main factors determining enzyme yield. Thus, the aim of this research is to determine

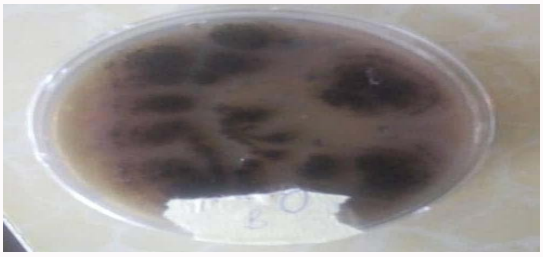

Figure 1: Pure culture of *Aspergillus niger.*

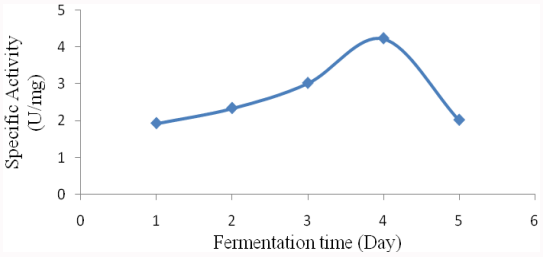

Figure 2: Effect of fermentation period on pectinase production.

the optimal biotechnological conditions for production of pectinase by *Aspergillus niger*, using industrial citrus pectin as a carbon source.

Materials and Methods

Microbial culture

Ripened orange fruits were purchased from different fruit markets in Eke Agbani in Nkanu West Local Government Area of Enugu, Nigeria. The fruits were exposed in an open space behind Biochemistry Laboratory in Enugu State University of Science and Technology and allowed to decay to serve as source of *Aspergillus niger*. Approximately 5 g of the rotten orange fruit were cut using sterile blade and homogenized in sterile medium of 1% citrus pectin; containing 0.14% of $(NH_4)_2SO_4$, 0.2% of K_2HPO_4, 0.02% of $MgSO_4.7H_2O$, 0.1% of nutrient solution containing; 5g/L $FeSO_4.7H_2O$, 1.6 mg/L $MnSO_4.H_2O$, 1.4 mg/L $ZnSO_4.7H_2O$, 2.0 mg/L $CoCl_2$ and pH 5.0, using a modification of Udewobele et al [8]. The pH of the medium was adjusted to pH 5.0 by using 1.0N HCl/1.0N NaOH. The mixture was incubated at room temperature (30°C) for 24 h. A loop of the homogenized medium was then streaked onto the same medium containing 3% agar-agar (the gelling agent) and incubated at 30°C for 4 days. All morphological contrasting colonies were purified by repeated culturing and sub culturing. Pure fungal isolates were maintained on Potato Dextrose Agar (PDA) slants for further experiment.

Fungal identification

Identification was carried out by relating features and the micrographs to "Atlas of mycology" by Barnett and Hunter [9]. Species identification was by examining both macroscopic and microscopic features of a three day old pure culture. Color, texture, nature of mycelia and/or spores produced, growth pattern in addition to microscopic features such as separation, spore shapes and so on were examined and confirmed *Aspergillus niger*.

Screening of fungal isolates for pectinolytic activity

The pectinolytic activity was first assayed by well diffusion method. The citrus pectin agar plate was prepared and three wells were made with sterilized cork borer in the Petri plate under aseptic conditions. The wells were filled with culture filtrate and incubated at room temperature overnight. The substrate utilized zone around the colony was observed using iodine solution Bijesh et al. [10]. The diameter of colonies and clear zones were measured. The strains were classified as very good producers of pectin depolymerizing enzymes when presented clear halos around colonies of at least 1.5 cm, good producers when the halos were of at least 1 cm, weak producers when halos were at least 0.5 cm and poor producers when no pectinolytic activity and no clearly is zones were observed Marcia et al. [11].

Secondary, a modified pectin liquid media was employed using 250 ml Erlenmeyer flasks containing 100 ml of sterile cultivation media optimized for pectinase production with 0.1% NH_4NO_3, 0.1% $NH_4H_2PO_4$, 0.1% $MgS0_4.7H_2O$, 0.5% citrus pectin and pH adjusted to 5.0. The flasks were stoppered with aluminum foil and autoclaved at 121°C for 15 min. Using a flamed and cooled cork borer (10 mm) and transfer needle, one disc of fungal hyphae from edge of actively growing citrus pectin agar culture was introduced into the liquid media. The flask was covered with sterile foil and incubated at 30°C for 4days. After incubation, the culture was harvested by filtration through what man No.1 filter paper and used for quantitative pectinolytic activity screening of the fungal isolate Adeleke et al. [12].

Pectinase assay

Pectinase activity was evaluated by assaying for Polygalacturonase (Pg) activity of the enzyme. This was achieved by measuring the release of reducing groups from citrus pectins using a modification of the 3,5-Dinitrosalicylic acid (DNS) reagent assay method described by Miller et al. [13] as contained in Wang et al. [14] with little modifications. The reaction mixture containing 0.8 ml of 0.2% citrus pectin in 0.05M sodium acetate buffer of pH 5.0 ml and 0.2 ml of enzyme solution were incubated for 20 min. 1 ml of DNS Reagent was added and there action was stopped by boiling the mixture in a boiling water bath for 10 min. The mixture volume was made up to 4 ml with 1 ml of Rochelle salt solution and 1 ml of distilled water. There action mixture was allowed to cool and then absorbance read at 575 nm. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the release of one micromole of galacturonic Acid per minute.

Protein determination

Protein content of the enzyme was determined by the method of Lowry [15], using Bovine Serum Albumin as standard. For protein standard curve, there action mixture contained 0.0 ml to 1.0 ml of protein stock Solution (2 mg/ml BSA) in test tubes arranged in triplicates. The volume was made up to1ml with distilled water. But for the test mixture, 0.1 ml of sample enzyme was mixed with 0.9 ml of distilled water. In either case, 5 ml of solution E was added and an allowed to stand at room temperature for about 10 min. 0.5 ml of solution C (dilute Folin-Ciocalteau phenol reagent) was added with rapid mixing. After standing at room temperature for 30 min, absorbance was read at 750 nm using Spectrophotometer. Absorbance values were converted to protein concentration by extrapolation from the standard curve.

Fermentation experiments

Submerged Fermentation (SmF) technique was employed using the method described by Ezugwu et al. [16], with the following modifications; 250 ml Erlenmeyer flask containing 100 ml of sterile cultivation media made up of 0.1% NH_4NO_3, 0.1% $NH_4H_2PO_4$, 0.1%

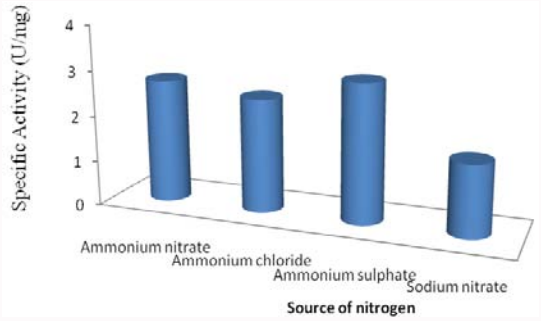

Figure 3: Effect of inorganic nitrogen sources on pectinase production.

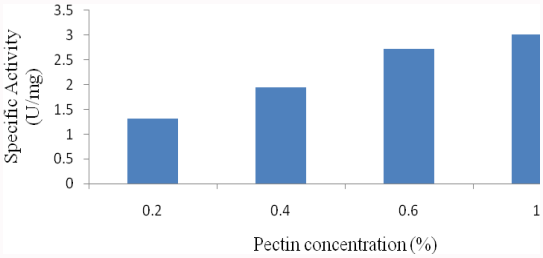

Figure 4: Effect of pectin concentration on pectinase production.

$MgS0_4.7H_2O$ and 0.5% citrus pectin, adjusted to pH 5.0 by using 1.0N HCl/1.0N NaOH. Inoculation was carried out as in quantitative pectinolytic activity screening. The culture was incubated for 4 days at 30°C and the mycelia biomass separated by filtration through what man No.1 filter paper. The filtrate was stored at 4°C and used as the crude enzymes for pectinase (polygalacturonase) activity and extracellular protein concentration.

Optimization of pectinase production by submerged fermentation technique

Optimization of physico-chemical parameters such as the incubation period, pH, temperature, pectin concentration and nitrogen source was performed to determine the best fermentation condition for enhanced pectinase production. Temperature range of 30°C to 50°C (interval of 10°C) and pH range of 3.5 to 6.0 (at interval 0.5) was screened for optimization of temperature and pH parameters. Incubation time of day 1, day 2, day 3, day 4, and day 5 were used to study the optimal incubation time for pectinase production. Effects of various citrus pectin concentration (0.2, 0.4, 0.6 and 1.0%) and inorganic nitrogen compounds (ammonium nitrate, ammonium sulphate, sodium nitrate and ammonium chloride) on pectinase production were also examined. Enzyme activity was determined using a modification of the 3,5-Dinitrosalicylic acid (DNS) reagent assay method described by Miller [13] as contained in Wang et al. [14] with little modifications. Also protein content of the enzyme was determined in each step by the method of Lowry et al. [15].

Results and Discussion

Screening of Isolate: In this study, attempt was made to isolate pectinase producing *Aspergillus niger* responsible for fruit decay. Fungal species isolated from rotten orange fruit sample was examined based on its morphological characteristics and confirmed *Aspergillus niger* (Figure 1). Pectinase activity of the organism was determined using agar well diffusion assay method where the fungal strain presented a clearance zone of 12 mm (1.2 cm). This result was

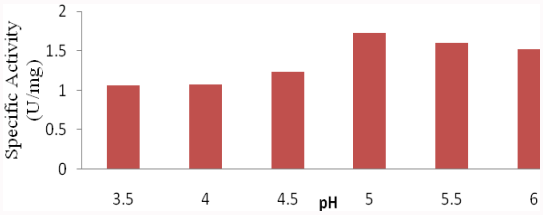

Figure 5: Effect of pH on pectinase production.

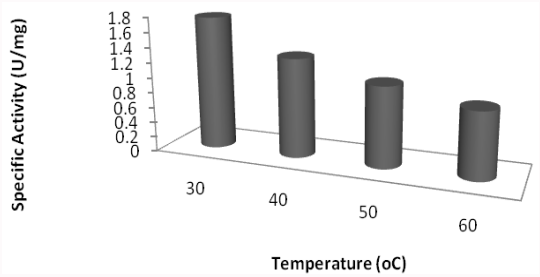

Figure 6: Effect of temperature on pectinase production.

in agreement with the statement of Marcia et al. [11] who said that pectin depolymerizing enzymes when presented clear halos around colonies of at least 1 cm are classified as good producers of pectinase. Also quantitative secondary screening of pectinase activity of the organism on 4th day of production presented a specific activity of 4.23 U/mg. This culture was used for optimization of pectinase production using submerged fermentation process.

Optimization of culture conditions

Pectinase production by filamentous fungi varies according to the strain, culture condition such as pH temperature time of incubation, nitrogen and carbon sources [17]. Effect of time on pectinase production was carried out over a period of five days by determine pectinase daily activity and protein concentration of crude enzyme filtrate. The result indicated the highest specific activity on 4th day of fermentation (Figure 2). Reported maximal pectinase activity from *Aspergillu niger* at the end of a fermentation period of 4 days [18]. Hence 4th day was considered the maximum day of pectinase production in this work.

Studies on the effect of inorganic nitrogen sources such as ammonium chloride ammonium nitrate, ammonium sulphate and sodium nitrate indicated ammonium sulphate best for pectinase production by *Aspergillus niger* and sodium nitrate least with specific activities of 3.03 u/mg and 1.57 u/mg respectively (Figure 3). This result is in agreement with the work of Abbasi et al. [19] who reported maximum production of *exo* and *endo* pectinase in ammonium sulphate than sodium nitrate as nitrogen source by *Aspergillus niger*. Also four days submerged fermentation with 0.2%, 0.4%, 0.6% and 1.0% of citrus pectin showed 1.0% citrus pectin best and 0.2% least for pectinase production with specific activities of 3.01 u/mg and 1.3U/mg respectively (Figure 4). Ezike et al. [20] produced pectinase with 1.0% orange pectin under submerged fermentation condition.

Furthermore, optimization studies at pH ranges of 3.5 to 6.0 (at internal 0.5) revealed pH 5.0 best for pectinase production with specific activity of 1.73 U/mg (Figure 5). Also studies at temperature ranges of 30°C to 60°C (at interval of 10°C) for 2 days revealed temperature of 30°C best for pectinase production with a specific activity of 1.78

u/mg (Figure 6). Gummandi et al. [21] analyzed the biochemical properties of microbial pectinase extracted from *Aspergillus niger* U-86 and found that the enzyme was stable at 30°C and at a pH of 3.0 to 6.0. According to Palaniyappan et al. [22] *Aspergillus niger* MTCC 281 showed highest pectinase activity of 5.15 u/mg at 30°C and 5.5 u/mg at pH of 5.5. The results of pH and temperature in this research are comparable with that of the above two authors [23].

Conclusion

This research work has shown that on day 4, at 30°C and pH 5.0, 1% concentration of citrus pectin and with ammonium sulphate, pectinase production by *Aspergillus niger* is maximum. The pectinase needed in fruit juice and textile industries can be produced commercially when the resources are highly made available.

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