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Pectinase production from a local isolate of *Aspergillus niger* using orange bagasse as a carbon source

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Abstract. *Timothy M, Mayel MH, Yohanna ER, Adondua MA, Chinekwu UK, Binunga BB, Janet T. 2022. Pectinase production from a local isolate of* Aspergillus niger *using orange bagasse as a carbon source. Asian J Nat Prod Biochem 19: 81-86.* Pectinases are a group of enzymes that catalyze the breakdown of pectin substances. The largest industrial applications of pectinases are in fruit juice extraction and clarification. However, it is in short supply, but due to the gradual increase in global population, fruit juice industries will need to increase the production of fruit juices, hence the need for mass production of pectinase. Little or no work has been done on producing pectinase from the locally sourced fungus *Aspergillus niger* in Wukari using orange bagasse as a carbon source. The present investigation was carried out to produce pectinase from a locally sourced fungus, *A. niger*, in Wukari, Taraba State, Nigeria. *A. niger* was identified morphologically and screened for pectinase production. Submerged fermentation was carried out, and the crude pectinase was harvested, with maximum enzyme production at 72 hrs. The effect of pH on pectinase activity was assessed, and different temperatures were used to test for pectinase activity. The crude enzyme's kinetic constants (K_m and V_{max}) were also determined. The optimal pH of pectinase was alkaline, at pH 8.0. The temperature at the crude enzyme expressed the highest activity was 50ºC. The crude enzyme activity was tested with the effect of substrate concentration; the optimum substrate concentration was discovered to be 2.0%. At the end of this research, the results showed that pectinase could be mass-produced to serve the needs of industries in demand to make good products from fruits available, considering the population increase.

Keywords: *Aspergillus niger*, catalytic activity, concentration, pectinase, substrates

INTRODUCTION

Pectin is a type of structural fiber found in the primary cell wall and an intracellular layer of plant cells, mainly in fruits, such as apples, oranges, and lemons, among others, where they contribute to the firmness and structure of plant tissues (Sathyanarayana and Panda 2003; Shaibu et al. 2022). They are high molecular weight acid polysaccharides primarily made up of α (1→4) linked D-galacturonic acid residues (Kashyap et al. 2001). For example, citrus fruit contains 0.5%-3.5% pectin, which is largely present in the peel portion of the fruit (Sathyanarayana and Panda 2003).

Pectinases are a group of enzymes that catalyze the breakdown of pectins. They are classified according to their way of an attack on the galacturonan part of the pectin molecule. They can be distinguished between pectin methylesterases (EC 3.1.11.1) that deesterify pectins to low methoxyl pectins or pectic acid, and pectin depolymerases, which split the glycosidic linkages betweengalacturonosyl (methyl ester) residues. Polygalacturonases (PGs) split glycosidic linkage next to free carboxyl groups by hydrolysis, while pectate lyase (Pl) split glycosidic linkages next to free carboxyl groups by β-elimination. Both endo types of PGs and PALs (EC 3.2.1.15 and EC 4.2.2.2, respectively) are known by splitting the pectin chain randomly. ExoPGs (EC 3.2.1.67) release monomers or dimmers from the non-reducing end of the chain, whereas exo-PALs (Pectate disaccharide-lyase) (EC 4.2.2.9) release unsaturated dimmers from the reducing end. Highly methylated pectins are degraded by endo-pectin lyases (PL; EC 4.2.2.10) and also by a combination of PE (Pectinesterase) with PG or PAL (Sarkanen 1991). Pectinases have various sources, including bacteria, fungi, plants, insects, protozoans, and nematodes. Even though many fungi and bacterial species are known to be producers of pectinase, the fungi *Aspergillus niger* is the most commonly used in the industrial production of pectinase because they have a wide range of carbon sources and can easily be handled (De Vries and Visser 2001).

Considering the rising population globally, there is a concurrent rapid growth in food demand. The fruit juice industry constitutes a major part of the food industry, and pectinase is a key ingredient in producing food materials like wines and juices of fruit origin. According to Abdel-Moshen et al. (2016), pectinases take 25% of the ever-increasing global demand for enzymes in the food industry. Pectinase can be produced using submerged or solid-state fermentation (El Enshasy et al. 2018). They are known to have a wide range of applications in the biotechnology industry, from vegetable oil and fruit juice extraction and clarification to cotton scouring, wastewater treatment, and degumming of plant fibers (Abdel-Moshen et al. 2016). In tea fermentation, pectinase acts as an accelerator in breaking down the pectin present in the cell walls of tea leaves and also destroys the foam-forming

component of instant tea powders by hydrolyzing the pectins. The change in color of tea during the fermentation also results in the development of a characteristic aroma (Garg et al. 2016). Another use of pectinase is in biorefineries for hydrolyzing pectin present in pectin-rich agro-industrial wastes (Biz et al. 2014). These agro-industrial wastes are processed into simple sugars to be converted into bioethanol or used as fermentable sugars (Collares et al. 2012).

The value of pectinases in the food industry has made them an overly expensive commodity that is not affordable by many, despite their importance. Should pectinases be made more available at a less expensive rate, the fruit juice and wine industries will surely do better in producing more juice with no clouds or haze. However, the reverse is the case, in that there are fewer producers of the enzyme, and it is less available than expected, especially in Wukari, Taraba State, or even Nigeria as a whole. To meet the high pectinase demand, discovering new microbial sources capable of producing higher yields is imperative. Unfortunately, little or no work has been done on producing pectinase from locally sourced fungus, *A. niger,* in Wukari using orange bagasse as a carbon source. The present investigation was carried out to produce pectinase from a locally sourced fungus, *A. niger,* in Wukari, Taraba State, Nigeria.

MATERIALS AND METHODS

Study area and collection of orange bagasse samples

The present study was conducted at Federal University Wukari Central Research Laboratory, Wukari, Taraba state, Nigeria. Samples of orange bagasse were collected from Wukari new market, along Wukari-Jalingo Road, and brought to Central Research Laboratory in Federal University Wukari, Taraba state. The collected samples were washed with distilled water, minced into small particles, and sun-dried (approximately 30°C) for 5 days.

Sample preparation

Samples of orange bagasse were dampened and allowed for 5 days to decay. One gram of the decaying sample was weighed aseptically into 9 mL of sterile distilled water separately and shaken thoroughly. The dilutions were subsequently made up to 10^{-4} and poured plating of 10^{-2} and 10^{-4} was done in sterilized potato dextrose agar (PDA). After sterilization, streptomycin (100 mg/L) was added to the PDA to prevent bacterial growth. Plates were incubated in an inverted position at room temperature for 7 days. After incubation, the plates were observed for fungal growth, as Yu and Xu (2018) reported.

Morphological identification

The plates with colonies of fungal growth were identified by observing their microscopic characteristics, such as color, texture, appearance, and diameter of colonies compared to the atlas, and suspected colonies of *Aspergillus* species were subcultured and allowed to grow for 4 days.

Screening of fungal isolates

The screening method of Fahmy et al. (2008) was used. Five milliliters of iodine solution was flooded on one agar plate containing selected fungal colonies and incubated at room temperature for two days. The zone of hydrolysis around the colonies indicated the pectinolytic activity of *A. niger* and the colonies were preserved for further study. The primary screened fungal isolates were inoculated on pectinase screening agar medium (PSAM) and incubated at room temperature for 42 hrs. The PSAM contained as follows (in g/100 mL): $(NH_4)_2HPO_4$, 0.3; KH_2PO_4, 0.2; K_2HPO_4, 0.3; $MgSO_4$, 0.01, pectin, 1.0; and agar, 2.5.

Fermentation for pectinase production

Submerged fermentation (SmF) was carried out using the method of Tobechukwu et al. (2014). Four 250 mL Erlenmeyer flasks containing 50 mL of sterile cultivation medium made up of 0.1% NH_4NO_3, 0.1% $MgSO_4.7H_2O$, 0.1% $NH_4H_2PO_4$ and 1% ground orange bagasse were autoclaved at 121ºC for 15 min. To every sterile flask, one disc of actively growing *Aspergillus* species from a 4 days agar medium was added using a cork borer of diameter 10 mm and then plugged properly. The culture was incubated for 4 days at room temperature. Then, a flask was selected at 24 hrs intervals, and the mycelia biomass was separated by filtration using a muslin cloth. The filtrates were analyzed daily for pectinase activity by taking the absorbance using a spectrophotometer at 540 nm.

Polygalacturinase assay

One (1) mL of 1.0% sodium acetate buffer (pH 5.5) was added to each of the three test tubes labeled Tube 1, Tube 2, and Blank. Afterward, 1 mL of the enzyme (Pectinase) was added to Tube 1 and 2. The mixtures in the three test tubes were incubated for 5 min at 50ºC so that the enzymes in the mixtures could acclimate under this condition. To each of the three test tubes, 1 mL 1% pectin, which served as the substrate, was added and incubated for 30 min at 50ºC. 1.5 mL 3, 5 dintrosalicyclic acid reagent was added to each test tube. Pectinase activity was ascertained by taking the absorbance of the mixtures in the test tubes using a spectrophotometer at 540 nm.

Effect of pH on pectinase activity

The effect of pH on enzyme activity was determined using the method of Yu and Xu (2018). **S**odium acetate buffer (1M; pH range, 4.5-5.0), sodium phosphate buffer (1M; pH range, 6.0-7.0) and Tris-HCl buffer (1M; pH range, 8.0-9.0) were used. Tubes containing 0.5 mL of the respective buffers were mixed with 0.5 mL of the enzyme. Then, 1 mL of 0.5% (w/υ) pectin solution was added and all the tubes were then incubated at 30ºC for 10 min, after which the residual activity of the enzyme was assayed to obtain the optimum pH.

Effect of temperature on pectinase activity

The method of Tobechukwu et al. (2014) was used to assay for pectinase activity. Different temperatures range from 25-50ºC at 5ºC intervals, taking 1 mL of the crude enzyme and 1 mL of pectin into 12 test tubes (each

temperature with a test tube and an enzyme-blank test tube) in a water bath and the temperature at which the enzyme expressed maximum activity was taken to be its optimum temperature.

Effect of substrate concentration on pectinase activity

The method of Tobechukwu et al. (2014) was used to assay for the effect of substrate concentration on pectinase activity. The substrate (pectin) was prepared in different concentrations ranging from 0.5-2.0% at intervals of 0.5% in 8 test tubes to evaluate substrate concentration on pectinase activity. One millimeter of enzyme suspended in acetate buffer (0.1M, pH 5.4) was mixed with 1 mL of the various substrate concentrations, and the absorbance was taken using a spectrophotometer at 540 nm. The substrate concentration that showed the highest activity of the enzyme was taken to be the optimum concentration for the enzyme activity.

Kinetic parameters of pectinase

The method reported by Tobechukwu et al. (2014) was used in the determination of the kinetic constant and maximum velocity (K_m and V_{max}) of pectinase.

Statistical analysis

The results observed are the mean of three independent experimental replicates (n=3), and values are represented as the mean ± standard error.

RESULTS AND DISCUSSION

Screening of fungal isolates

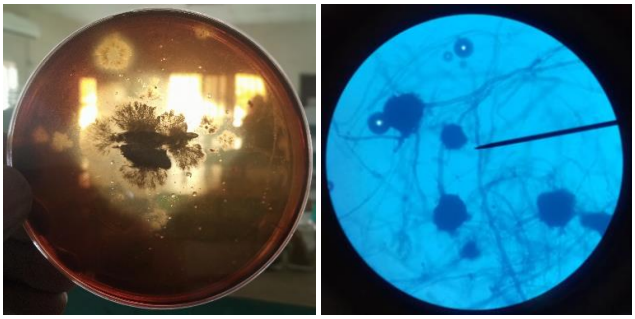
The ability of some fungal isolates to produce pectinase was tested by propagating on Pectinase Screening Agar Medium (PSAM). The result shows that the primary screened fungal isolates upon inoculation on PSAM and incubation at room temperature for 42 hrs showed the appearance of a clear zone which is an indication of pectinases production (Figure 1A). In addition, the morphological appearance of fungal isolates was identified by observing their macroscopic characteristics, such as color, texture, and appearance, under a microscope. The result revealed the following morphological characteristics: Shape-filamentous; Surface-suede-like surface; Opacity-opaque; color-blue-green (Figure 1B).

Fermentation for pectinase production

The selected isolates from the primary screening method were subjected to solid-state fermentation in a suitable medium and observed. The isolates were found to be able to consume pectin and produce pectinase. Pectinase production increased gradually over 24 hours of incubation until optimum pectinase production was achieved. After the optimum incubation period, pectinase production began to decrease. The result also revealed that pectinase production after 4-day fermentation gave maximum production on day 3 (Figure 2).

Effect of pH on pectinase activity

This study investigated the effect of various pH values on pectinase activity. At pH 2.0, pectinase activity was observed to be 2 µ/L. At pH 4.0, pectinase activity was 4 µ/L. At pH 4.5, pectinase activity was seen to be 6.9 µ/L. A decrease in pectinase activity was observed at pH 7.0 and 9.0, recording 5.0 µ/L and 5.1 µ/L, respectively. Maximum pectinase activity (7 µ/L) was recorded at pH 8.0. Varying the pH level affected pectinase activity (Figure 3).



A **B**

Figure 1. A. Screened fungal plate showing the appearance of a clear zone. B. Morphological appearance of fungal growth under the microscope

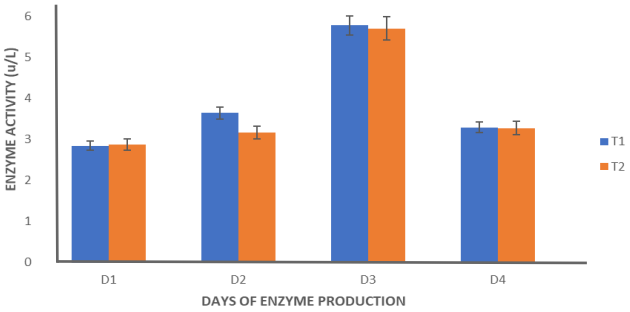


Figure 2. Pectinase production from submerged fermentation. The data represents mean ± standard error of replicates (n = 3). *T1- Tube one; T2- Tube two; D1-day one; D2- day two; D3- day three; D4-day four

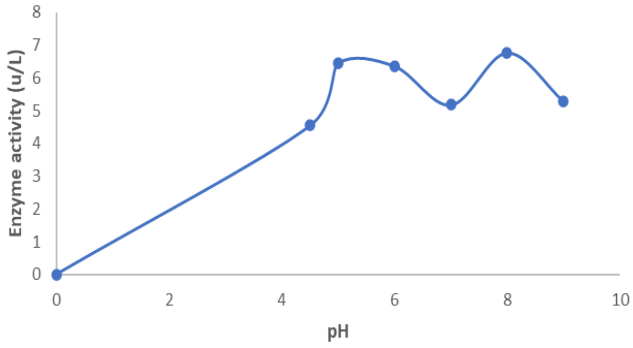


Figure 3. Effect of pH on pectinase activity

Effect of temperature on pectinase activity

The present study revealed that temperature change showed an effect on pectinase activity. At 10°C, pectinase activity was observed to be 1.8 µ/L. At 30°C, pectinase activity was observed to be 3.2 µ/L. A sharp decrease in pectinase activity was seen at 40-45°C, after which a sharp increase in pectinase activity was observed as the temperature kept increasing. The maximum temperature for pectinase activity observed was 50°C (Figure 4).

Effect of substrate concentration on pectinase activity

The result revealed that enzyme activity changed as substrate concentration varied. An increase in pectinase activity was observed as substrate concentration increased. Pectinase activity was quite stable from 0.5 substrate concentration to 1.0 substrate concentration. After which, an increase in pectinase activity was observed. At 0.5 substrate concentration, pectinase activity was observed to be 2.5 µ/L. At 1.0 substrate concentration, pectinase activity was observed to be 2.5 µ/L. At 1.5 substrate concentration, pectinase activity was observed to be 3.0 µ/L. Pectinase activity was revealed to be highest at 2.0 substrate concentration. At this substrate concentration, pectinase activity was 3.3 µ/L (Figure 5).

Kinetic parameters of pectinase

In this study, the kinetic parameters of pectinase were investigated. At 0.5 substrate concentration, the rate of reaction was found to be 0.18. At 1.0 substrate concentration, the rate of reaction was 0.32. At 1.5 substrate concentration, the rate of reaction was found to be 0.47. At 2.0 substrate concentration, the reaction rate was 0.59. A progressive increase in the reaction rate was observed as substrate concentration for the reaction increased. The K_m and V_{max} values for pectinase were found to be 5.93 and 2.22, respectively. The kinetic data and reciprocal plot (Lineweaver-Burk plot) for pectinase is presented below (Table 1; Figure 6).

Discussion

Pectinases have various screening assays that can be used to ascertain their presence in a pectin-containing medium. In this study, the screened fungal plate showed the appearance of a clear zone, connoting pectinase activity. Sudeep et al. (2020) reported that PSAM was used to grow the microorganism for 48 hrs. Iodine was used to flood the plates, and the appearance of a clearance zone affirmed pectinase activity. Fahmy et al. (2008) also reported using iodine to flood plates to observe the clearance zone on a plate.

The pectin extracted from orange bagasse was used to induce pectinase production in *A. niger* which had more pectinase activity under submerged fermentation. The entire fermentation process was carried out at a temperature of 50°C. The incubation period that gave peak production was 72 hrs, an average of 96 hrs, as reported by Tobechukwu et al. (2014) and 48 hrs reported by Sudeep et al. (2020). Chowdhury et al. (2017) reported maximum polygalacturonase activity on the 5th day of fermentation with *Penicillium chrysogenum*. Various factors can influence the production of pectinases. These include; concentration of nutrients, pH, temperature, moisture content, and influence of extraction parameters on the recovery of pectinases.

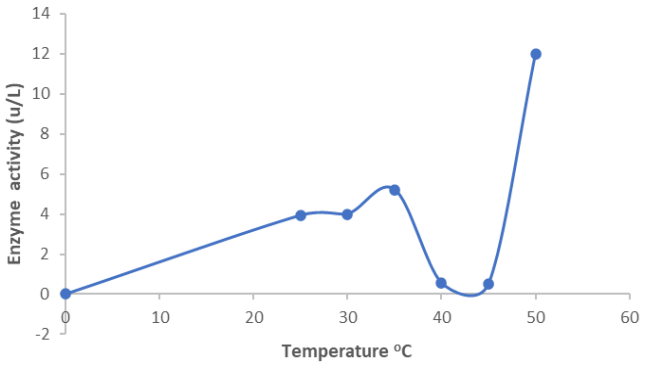


Figure 4. Effect of temperature on pectinase activity

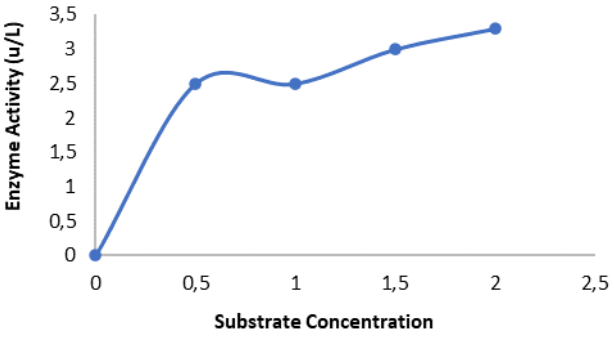


Figure 5. Effect of substrate concentration on pectinase activity

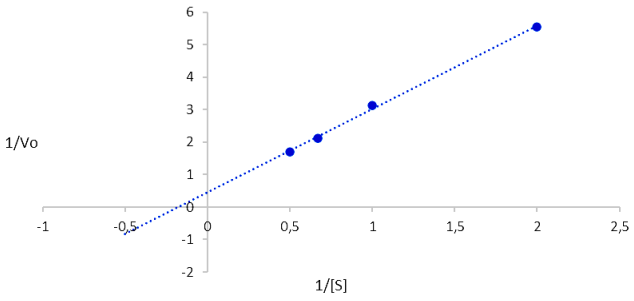


Figure 6. Lineweaver-Burk plot used for determination of Enzyme K_m and V_{max}. Intercept = 0.4499; V_{max} = 2.22

Table 1. Kinetic data for pectinase

[S]	V_o	1/[S]	$1/V_o$
0.5	0.18	2	5.56
1	0.32	1	3.13
1.5	0.47	0.67	2.13
2	0.59	0.5	1.7

One of the key factors that affect enzyme activity is pH. Some enzymes are more active in an acidic medium, while others prefer the neutral range and alkaline medium. The effect of pH on pectinase activity was determined at a pH range of 4.5-9.0 at intervals of 1.0 from pH 5.0 to check whether the enzyme activity is best expressed in an acidic, neutral, or alkaline medium. However, the pH that showed maximum enzyme activity was 8.0 (Figure 3), showing that the enzyme is more active in an alkaline medium. This is contrary to the report by Yu and Xu (2018), which showed maximum enzyme activity at pH 5.0, and De Vries and Visser (2001), which reported maximum activity at pH 6.5, both acidic media. Sudeep et al. (2020) reported that an acidic pH of 4.0-4.5 supports high pectinase activity. The pH optima for 30 fungal pectinases reported by Niture and Pant (2004) ranged from 2.5 to 6.0. According to Jayani et al. (2005), most microbial pectinases have an optimal pH of 3.5-5.5. However, Shet et al. (2018) reported that certain pectinolytic enzymes are more active in an alkaline medium; hence, this crude enzyme can be classified under such alkaline pH pectinases. According to Debing et al. (2006), pectinases are denatured at pH 8-9.5.

Temperature also greatly affects pectinase activity as it may reduce, give stable activity or express the maximum activity of the enzyme. The effect of temperature on pectinase activity in this study was assayed by subjecting the enzyme to different temperature values ranging from 25ºC-50ºC. The temperature at which the crude enzyme was most active was 50ºC (Figure 4). Khatri et al. (2015) and Sudeep et al. (2020) reported maximum pectinase activity at a temperature range of 30-50ºC. Jubayer et al. (2017) also reported that a temperature range of 40-45°C supports high pectinase activity. According to Jayani et al. (2005), most microbial pectinases have an optimal temperature range of 30-50ºC. Hence, the optimal temperature for pectinase activity obtained in this work agrees with the values obtained in the mentioned literature. These reports show that the enzyme can act optimally at temperatures between 30-50ºC.

Different substrate concentrations from 0.5-2.0% substrate (pectin) were used to test for the effect of substrate concentration (at intervals of 0.5) on the enzyme activity. As a result, the concentration of the crude enzyme was observed to express the highest activity at 2.0% pectin (Figure 5). On the other hand, Jubayer et al. (2017) reported maximum pectinase activity at 1.0% pectin concentration.

The kinetic parameters (K_m and V_{max}) of the enzyme were calculated by taking the double reciprocal of the plot. K_m and V_{max} of pectinase obtained were 5.93 and 2.22, respectively (Figure 6). Pectinases share a high degree of sequence homology, but their rate of pectin hydrolysis and hence, their kinetic constants can differ (Jubayer et al. 2017). Yu and Xu (2018) reported a K_m of 1.0 mg/mL and a V_{max} of 85 U/mg protein for pectinase isolated from *P. chrysogenum*. Siddiqui et al. (2012) reported a K_m value of 0.22 mg/mL for polygalacturonase from *Rhizomucor pusillus* isolated from decomposing orange peels. K_m values less than 0.15 and up to 5.0 mg/mL (<0.15-5.0 mg/mL) and specific activities 8.8-7000 U/mg were reported for some fungal pectinases by Sharma and Giridhar (2011).

In conclusion, the mass production of crude pectinase from a local isolate of *A. niger* in Wukari, using orange bagasse as a carbon source, is achievable under the following conditions; an incubation period of 72 hrs, pH of 8.0, a temperature of 50ºC, and substrate concentration of 2.0%. Depending on conditions that affect the enzyme activity per location, the aforementioned parameters can be adjusted to favor the mass production of pectinase for industrial use. The results obtained in this research can guide producers of pectinase on what parameters to vary for mass production of this enzyme.

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