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Comparative Studies of Agro Wastes for Pectinase Production and Characterization by Strains of *Aspergillus niger*

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ABSTRACT

Pectinase (EC 3.3.1.15) are often described as enzymes that breakdown pectin to give glucose and galaturonic acid. It is an inducible extracellular enzyme often produced by microorganisms. This investigation was done to compare three agro wastes (Orange peel, pineapple peel and corn pomace) for microbial pectinase production by solid state fermentation. Pectinase was precipitated with ammonium sulphate and characterized. Optimum incubation period of both strains of *Aspergillus niger* were 92 hrs with each agro wastes. Among the agro wastes, orange peel had the highest activity of 101.372±0.042 and 108.820±0.127 U for *A. niger* strain A1 and A2 respectively. Ammonium sulphate concentration of 80% indicates maximum specific activity for pectinase produced from both *A. niger* strains. Optimum pH and temperature of 5 and 50 ºC were observed for pectinase produced by both strains. Cu^{2+}, Zn^{2+}, Mg^{2+}, Fe^{2+}, Na^{2+}, Ca^{2+} and Mn^{2+} moderately activate pectinase activity. K^{+} tends to exert inhibitory effect on pectinase from both *A. niger* strains

Keywords: Pectinase, pineapple peel, corn pomace, orange peel, *Aspergillus niger*.

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Introduction

The word Pectinase is made up of pectin with suffix "-ase", to indicate enzyme (protein) that breakdown pectin. Pectin is a structural polysaccharide found in the middle lamella of plants, fruits and vegetables. Pectinase (EC 3.3.1.15) are enzymes that catalyze the breakdown of pectin or pectic substances, found in the middle lamella, which contribute to the structure and firmness of plant cell walls[1].

Microbial world is extremely diverse; microorganisms' generally secret pectinase to breakdown plant cell wall (microbial pectolysis) so that they can extract their own nutrient, at the detriment of the plant[1]. The ability to produce pectinase is common among groups of microorganisms. Microbial pectinase is most preferred in the industries because of their biochemical diversity, amenability to genetic manipulations and ease of cultivation[1].

Over the years, pectinase have been used in several industrial process, they are of great significance with a wide range of applications. They are commonly used in processes involving the degradation of plant materials, extraction of juice from fruits, wine production, treatment of pectic waste water, coffee and tea fermentation, oil extraction, animal feed, retting and degumming of plants fibers[2].

Utilization of agro industrial waste for enzymes production minimizes environmental pollution and enhances cost effective production of high value-added products.

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Various substrates like banana waste, sugarcane bagasse, wheat bran, rice bran, saw dust, apple pomace, lemon peel, orange peels, etc., can be employed for the pectinase production[2]. Keeping this in view, this study was aimed to compare different agro wastes as substrate for pectinase production from two strains of *Aspergillus niger* by solid state fermentation and characterize the enzyme produced.

Materials and Methods

Microorganisms

Two different strains of *Aspergillus niger* was obtained from the culture bank of Federal Institute of Industrial Research, Oshodi, Lagos Nigeria (6º32′47.9″N 3º21′04.5″E). The microorganisms were sub cultured and young cultures were screened for pectinolytic activity.

Screening for pectinase activity

The screening methodology involves the use of Pectinase Screening Agar Medium (PSAM) containing: 1 g, pectin; 0.3 g, Disodium hydrogen orthophosphate; 0.2 g, KH_2PO_4; 0.3 g, K_2HPO_4; 0.01 g, $MgSO_4$ and 2.5 g, agar in 100 mL of distilled water. The initial pH of medium was adjusted to 4.5. This medium was sterilized and distributed aseptically in petri dishes. The petri dishes containing PSAM were inoculated with *Aspergillus niger* and incubated (MINI75, Genilab, United Kingdom) at 30 °C for 48 hrs. At the end of the incubation period, the plates were stained with iodine solution[3].

Production media

The agro wastes employed for the pectinase production media were corn pomace, orange (*Citrus sinensis*) and pineapple (*Ananas comosus*) peels. Each of the agro waste were dried to less than 10% moisture content and milled to 300 millimicron particle size using Hammer mill. Exactly 70 g each of the three agro-wastes and 2 g pectin were moistened with 50 mL of mineral water (pH 4.5) containing 2 g Urea and 7 g ammonium sulphate. The three media were sterilized at 121 °C for 15 mins and allowed to cool under aseptic condition. The fermentation medium was inoculated with crude spores of each strain of *Aspergillus niger*. These were mixed thoroughly under sterile condition, covered with sterile aluminum foil and incubated at room

temperature for 6 days. At the interval of 24 hrs samples were withdrawn and the activities of pectinase were determined[4].

Pectinase extraction
The fermentation media were dissolved in 300 mL of sterile sodium acetate buffer for 60 mins. The mixture was sieved thrice with muslin cloth, centrifuged (2-16KL, Sigma, Germany) at 10,000 rpm for 15 mins and filtered using Whatman No 1 filter paper. The filtrate was used as crude pectinase in further studies[5].

Pectinase assay
The pectinase activity was measured according to the method described by Miller[6], with some modifications. The reaction mixture contained 0.5mL of crude pectinase, 0.5 mL of 1% pectin prepared in sodium acetate buffer (0.05 M; pH 4.5). The mixture was incubated at 50°C for 15 mins. The reaction was stopped with 1.0 mL dinitrosalycyclic acid solution after which the mixture was boiled for 10 mins and cooled. Spectrophotometer (VWR6300PC, ETL Inter-tek, USA) preset at 540 nm was used in the determination of pectinase activity, while the reducing sugar release was measured using galactouronic acid as standard.

Protein content
Protein content of the pectinase was determined by following the method of Lowry *et al*[7], with Bovine serum albumin as standard. The reaction mixture containing 0.2 mL, pectinase extract, 0.8 mL, distilled water, 5 mL alkaline solution and 0.5 mL Folin-C was incubated at 27ºC for 30 mins. The standard was prepared with Bovine Serum Albumin (10 mg/mL). After incubation, optical density of the colour development was read at 600 nm wavelength in a spectrophotometer.

Ammonium sulfate fractionation
Crude pectinase were first brought to 20% saturation with solid ammonium sulfate (enzyme grade). The precipitated proteins were regimented by centrifugation for 15 min at 500 rpm. The resulted pellet was dissolved in 5 mL of 0.2 M phosphate buffer at pH 6.2. The left supernatant was applied again with ammonium sulfate to achieve 40, 60, 80, and 100% saturation. Both enzyme activity and protein content were determined for each separate fraction[8].

Optimum pH of pectinase
The optimum pH of pectinase was studied by incubation of the enzyme with 50 mM of sodium acetate buffers (pH 3 to 8) in the presence of pectin and then residual activity of pectinase was measured[1].
Optimum temperature of pectinase
The optimum temperature of pectinase activity was determined by incubating pectinase extract in the presence of pectin at various temperatures (30 to 80 °C). Determination of the residual pectinase activity was done using the method employed for pectinase mentioned earlier[1].

Effect of activators and inhibitors
Effect of metal ions on pectinase activity was investigated using the following chemicals in concentrations of 10 mM: Cu^{2+}, Zn^{2+}, Mg^{2+}, Fe^{2+}, Na^{2+}, K^{+}, Ca^{+2} and Mn^{+2}. The samples in the presence of various metal ions were incubated for 30 mins at 50 °C and then the residual pectinase activity was determined[9].

Statistical analysis
Analysis of variance was done using the EXCEL 2010 software and Duncan Multiple Range Test (DMRT). The level of significance was set at $p \leq 0.05$.

Results and Discussion

All experiments were conducted in triplicate and results were the mean of data obtained. Experiment was analyzed using Analysis Of Variance (ANOVA) to determine the mean. Means followed by the same letter in the same column are not significantly different ($p \leq 0.05$). The ability of microorganisms to produce clear zone of pectin hydrolysis against the opaque background on pectin agar medium indicates pectinase production[1] as shown in Figure 1 and Figure 2 respectively. Among the *Aspergillus niger* strains, A2 had the highest zone of pectin hydrolysis of 31±0.500 followed by A1 with 27.0±0.500 mm on PSAM as shown in Table 1. Colonial morphology of these two strains was similar but differences in their spore color and pectinase producing ability indicate that they may be from the same specie but they are from different strain type. Appearance of spore head, color of mycelium, septation, type and color of spore of the *Aspergillus niger* are revealed in Table 2. The structure, spore color, type of hypae and fruiting body of the fungi can be used for fungi probable identification[4].
The activity of pectinase from each strain differs with the three agro wastes, orange (*Citrus sinensis*) peel, pineapple (*Ananas comosus*) peel and corn pomace at varied incubation period. Highest activity was observed from microbial pectinase produced using orange peels as substrate followed by pineapple peels while corn pomace had the lowest activity as shown in Table 3. In the recent work done by Praveen and Suneetha[10], the authors reported that orange peels contain great quantity of pectin. This often makes orange peel a suitable substrate for the production of microbial pectinase. Ezugwu *et al*[11]., who compared microbial pectinase produced from *Aspergillus species* (*A. niger*) using orange peels (*Citrus sinensis*), *Mangifera specie* (mango) and *Ananas specie* (pineapple) as substrate observed that pectinase obtained from *A. niger* using orange peels exhibited the highest activity when compared with the values obtained using other substrates.

Figure 1: Zone of pectin hydrolysis of *Aspergillus niger* strain A1

Figure 2: Zone of pectin hydrolysis of *Aspergillus niger* strain A2

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Table 1: Screening of microorganisms for pectinase production

Isolate codes	Zone of pectin hydrolysis (mm)
A1	27.0±0.500[b]
A2	31.0 ± 0.500[a]

A1- *Aspergillus niger* strain A1; A2 – Aspergillus niger strain A2. Mean values with different superscripts are statistically and significantly different at $p < 0.05$.

It was also observed that as the incubation period increased, the activities of pectinase produced from the two strains also increased. Highest pectinase activities were observed at 96 hrs for the three agro wastes. Pectinase from strain A1 had 101.372±0.042, 80.279±0.226 and 93.825±0.297 U while strain A2 had 108.320±0.127, 87.147±0.255 and 101.462±0.000 U using orange, corn pomace and pineapple peel as substrate respectively (Table 3). Udenwobele *et al*[12]., reported optimum incubation period of 96 hrs for crude pectinase from *A. niger*.

This is similar to the earlier work done by Mrudula and Anitharaj[13]., the authors equally reported optimum incubation time of 96 hrs for pectinase production from *A. niger* using orange peel as substrate. Decrease in pectinase activity after 96 hrs may be due to nutrient reduction in the fermentation medium as the microbial population was increased.

The crude pectinases were precipitated using ammonium sulphate at various concentrations. It was observed that 80% ammonium sulphate concentration had the highest specific activities of 15.852±0.197 U/mg/mL for pectinase from strain A1 and 16.801±0.2927 U/mg/mL for pectinase from strain A2 respectively as shown in Table 4. Effect of pH and temperature showed that pectinase activities increased as the pH and temperature was raised. Both strains had optimal pH of 5 and temperature at 50 ºC as illustrated in Figure 3 and Figure 4 respectively. These findings revealed that temperature and pH can either increase or decrease the enzyme activity. Mrudula and Anitharaj[13]., observed that partially purified pectinase from *Aspergillus niger* on orange peel as substrate shows optimum activity at 50ºC and pH 5.

The effect of metal ions on pectinase activities showed that the enzymes were moderately enhanced in the presence of divalent ions as shown in Table 5. K^+ strongly inhibited the pectinase activity. According to Eleni *et al*[14]., divalent ions act directly on the pectin molecule, stabilizing the negatively charged carboxyl groups and indirectly stimulate pectinase activity.

Table 2: Colonial morphology of microbial isolates

Strain code	Color of spore	Type of spore	Septation	Color of mycelium	Appearance of spore head	Suspected microbes
A1	Dark brown	Conidia	Septate	Black with no reverse color	Large tightly packed conidia chain	*A. niger*
A2	Black	Conidia	Septate	Black with no reverse color	Large tightly packed conidia chain	*A. niger*

A1- *Aspergillus niger* strain A1; A2 – *Aspergillus niger* strain A2.

Table 3: Effect of different agro waste on pectinase production at various incubation period

Agro waste/Day	Pectinase from A1 Activity(U)±SD	Pectinase from A2 Activity(U)±SD
	0 Hour	
OP	46.117 ± 0.085[a]	48.035 ± 0.084[a]
CP	31.834 ± 0.212[c]	42.286 ± 0.085[b]
PP	42.166 ± 0.170[ab]	46.029 ± 0.127[ab]
	24 Hours	
OP	46.119 ± 0.085[a]	48.125 ± 0.098[a]
CP	31.834 ± 0.212[b]	42.286 ± 0.085[b]
PP	42.166 ± 0.170[ab]	46.029 ± 0.127[ab]
	48 Hours	
OP	48.335 ± 0.085[a]	60.106 ± 0.460[a]
CP	44.142 ± 0.169[c]	47.945 ± 0.211[b]
PP	52.308 ± 0.028[a]	56.810 ± 0.217[ab]
	72 Hours	
OP	93.885 ± 0.042[a]	96.802 ± 0.288[a]
CP	56.960 ± 0.170[b]	60.404 ± 0.212[b]
PP	92.088 ± 0.127[a]	94.753 ± 0.000[a]
	96 Hours	
OP	101.372 ± 0.042[a]	108.320 ± 0.127[a]
CP	80.279 ± 0.226[b]	87.147 ± 0.255[b]
PP	93.825 ± 0.297[ab]	101.462 ± 0.000[ab]

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	120 Hours	
OP	60.284 ± 0.212[a]	65.225 ± 0.000[a]
CP	56.811 ± 0.075[ab]	57.769 ± 0.042[b]
PP	58.368 ± 0.127[a]	59.535 ± 0.085[b]
	144 Hours	
OP	48.335 ± 0.085[a]	60.105 ± 0.466[a]
CP	44.143 ± 0.169[b]	47.946 ± 0.211[b]
PP	52.408 ± 0.170[a]	56.810 ± 0.127[a]

OP- Orange peel, CP- Corn pomace, PP-Pineapple peel. A1- *Aspergillus niger* strain A1; A2 – *Aspergillus niger* strain A2. Mean values with different superscripts are statistically and significantly different at $p < 0.05$.

Table 4: Partial purification of pectinase using ammonium sulphate

Strain/Ammonium sulphate conc.	Pectinase from A1			Pectinase from A2		
	Activity (U) ± S.D	Protein conc. (mg/ml) ± S.D	Specific activity (U/mg/ml) ± S.D	Activity (U) ± S.D	Protein conc. (mg/ml) ± S.D	Specific activity (U/mg/ml) ± S.D
Crude	101.372 ± 0.042[a]	14.912 ± 0.291[a]	6.802 ± 0.141[c]	108.32 ± 0.127[a]	15.067 ± 0.052[a]	7.1895 ± 0.0162[d]
60 %	93.825 ± 0.296[ab]	9.9435 ± 0.101[ab]	9.437 ± 0.145[ab]	101.462 ± 0.022[ab]	10.671 ± 0.701[ab]	9.529 ± 0.626[c]
80%	80.319 ±0.169[c]	5.067 ± 0.052[c]	15.852 ± 0.197[a]	94.6935 ± 0.084[c]	5.637 ± 0.093[c]	16.801 ± 0.2927[a]
100%	60.107 ± 0.463[d]	4.9675 ± 0.156[c]	12.1045 ± 0.287[ab]	77.147 ± 0.254[d]	5.3185 ± 0.101[c]	14.5055 ± 0.026[ab]

A1- *Aspergillus niger* strain A1; A2 – *Aspergillus niger* strain A2. Mean values with different superscripts are statistically and significantly different at $p < 0.05$. SD - Standard Deviation

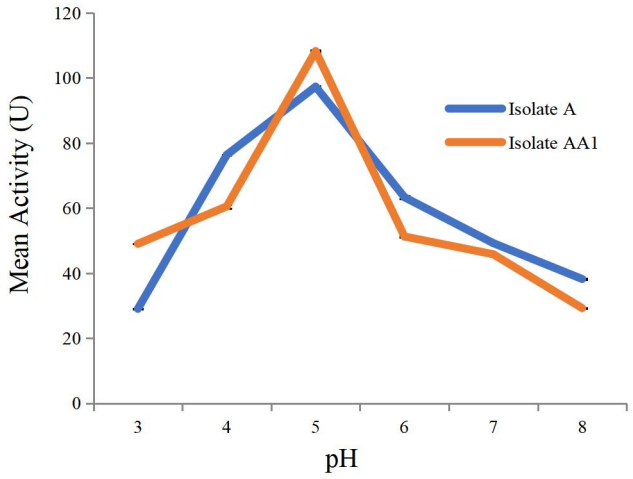

Figure 3: Effect of pH on pectinase activity.

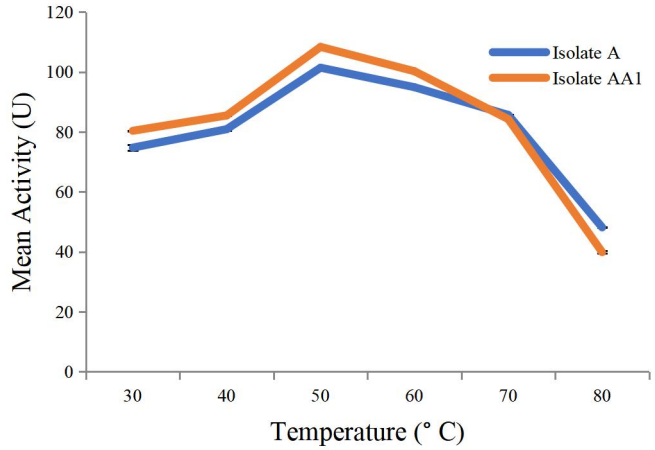

Figure 4: Effect of temperature on pectinase activity

Table 5: Effect of metal ions on pectinase activity

Metal ions	Pectinase from A1 Activity (U)±SD	Pectinase from A2 Activity (U)±SD
Cu^{2+}	104.846 ±1.652[a]	117.900 ± 0.779[a]
Zn^{2+}	102.330 ± 0.550[a]	111.794 ± 0.211[c]
Mg^{2+}	84.990 ± 0.170[ab]	113.695 ± 0.444[c]
Na^{2+}	101.881 ± 0.169[a]	115.058 ± 0.677[ab]
K^{+}	40.609 ± 0.169[c]	54.025 ± 0.762[d]
Ca^{2+}	102.001 ± 0.170[a]	115.807 ± 0.042[ab]
Mn^{2+}	105.324 ± 1.650[a]	117.723 ± 0.974[a]

A1- *Aspergillus niger* strain A1; A2 – *Aspergillus niger* strain A2. Mean values with different superscripts are statistically and significantly different at $p < 0.05$.

Conclusion

Billion tons of agro industrial wastes are generated globally every year. The disposal of these wastes is a serious problem in industries, as it often leads to environmental pollution. Citrus peels constitute a major waste in juice industries. As an alternative to disposal, this study showed that citrus peels can be utilized as a substrate for the production of biologically important industrial product such as pectinase. The use of orange peels, corn pomace and pineapple peel as agro wastes for pectinase production will reduce waste disposal menace, create ecofriendly environment in industries and improved pectinase yield in enzyme technology.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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