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Full Length Research Paper

Pectinolytic activity of wild-type filamentous fungi fermented on agro-wastes

U. A. Okafor[1], V. I. Okochi[1], Shalom Nwodo Chinedu[2]*, O. A. T. Ebuehi[1] and B. M. Onygeme-Okerenta[1]

[1]Department of Biochemistry, College of Medicine, University of Lagos, P. M. B. 12003 Idiaraba, Lagos, Nigeria.
[2]Department of Biological Sciences, College of Science and Technology, Covenant University, KM 10, Idiroko Road, Canaan Land, P. M. B. 1023 Ota, Ogun State, Nigeria.

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Five filamentous fungi (*Aspergillus clavatus*, *Aspergillus niger, Fusarium* sp., *Penicillum chrysogenum* and *Trichoderma* sp.) isolated from agrowaste samples in Lagos metropolis, Nigeria, depolymerized citrus pectin. Best pectolytic activity, as indicated by the diameter of clear, hydrolyzed zones on the medium plates containing commercial citrus pectin as sole carbon source, was obtained with *A. niger*, closely followed by *P. chrysogenum*. The two fungi also produced pectinases with different agrowastes (pineapple peel, orange peels, sawdust, sugarcane pulps and wheat bran) as the sole carbon source. The highest pectinase activity by both fungi was produce with wheat bran as the sole carbon source. Peak pectinase activity of 350.28 ± 2.82 and 478.25 ± 3.04 IU mg^{-1} protein was respectively obtained by submerged fermentation (SmF) at 48 h for *A. niger* and *P.chrysogenum* in media containing wheat bran as the sole carbon source. Solid-state fermentation (SSF) yielded higher levels of pectinase activity than the SmF. The strains of *A. niger* and *P. chrysogenum* have good prospect for pectinase production. Wheat bran is a good low-cost fermentation substrate for pectinase production by the investigated fungi.

Key words: Filamentous fungi, agro-wastes, pectinolytic activity, fermentation, pectinase production.

INTRODUCTION

Pectins are complex heterogeneous polysaccharides found in cell walls of higher plants. They consist of a backbone of α- 1, 4-linked D-galaturonic acid residues in which the carboxyl group at C-6 is esterified with a methyl group in some residues (Grant and Long, 1981). Most pectin contains smooth linear homogalacturonan regions and ramified hairy regions (Schols and Voragen, 1996). Together with cellulose and hemicelluloses, they build up the plant cell wall and contribute to the large quantities of plant fibers available, mainly as agricultural and municipal wastes, in developing countries (Schols and Voragen, 2002).

*Corresponding author. E-mail: sncresearch@gmail.com. Tel: +234 (0)806 5940 573.

A group of pectinolytic enzymes known as pectinases hydrolyzes pectins. Pectinases are complex hydrolytic enzymes that function as estarases and depolymerases. They include pectin esterases (EC. 3.1. 1. 11) which catalyse the hydrolysis of methylated carboxylic ester groups in pectin into pectic acid and methanol, pectin lyases (E.C. 4. 2. 2. 10) which cleave α (1, 4)-glycosidic linkages by transelimination resulting in galacturonide with a double bond between C-4 and C-5 at the non-reducing end and polygalacturonases which hydrolyze the α(1, 4)-glycosidic linkages in homogalacturonans (Call et al., 1985; Delgado et al., 1992; Soares et al., 1999). Pectinases have important applications in fruit processing industries such as in wine and fruit juice production (Kashyap et al., 2001).

Solid-state fermentation (SSF) and submerged fermentation (SmF) are important fermentation methods

employed for the production of microbial enzymes (Kavitha et al., 2000; Martin et al., 2000). Microbial growth and product formation usually occur at or near the surface of solid substrate particles with low moisture content; hence SSF appear to be advantageous for microbial enzyme production. The advantages of SSF over the SmF process include higher yield of products (Pandey, 1994), generation of less effluent and requirement of simpler equipment (Bennett, 1998). Reports are very few on the comparison of SmF and SSF for the production of pectinases. The present study involves screening of wild strains of filamentous fungi for pectinolytic activity using different agrowastes as fermentation substrates and the evaluation of pectinase production by SSF and SmF.

MATERIALS AND METHODS

Isolation and identification of pectinolytic microfungi

The filamentous fungi were isolated from decaying agrowaste matter collected from vegetables/fruits markets in Lagos metropolis using a basal medium containing commercial pectin as the sole carbon source. The solid medium contained (gL^{-1}): 10.0 pectin, 3.0 peptone, 2.0 yeast extract, 0.5 KCl, 0.5 $MgSO_4.7H_2O$, 0.01 $MnSO_4.5H_2O$, and 2.0 $(NH_4)_2SO_4$ and 20.0 agar. A supplement of 0.1% ampicilin and 1.0 mlL^{-1} of trace mineral solution (composed of 0.04 g $CuSO_4.5H_2O$, 0.08 g $FeSO_4$, 0.08 g Na_2MoO_4, 0.8 g $ZnSO_4$, 0.04 g $Na_2B_4O_7$, and 0.008 g $MnSO_4$ in 100 ml of distilled water) was added to the medium. pH value was adjusted to 5.5 before autoclaving at 121°C for 15 min. Inoculated plates were incubated at 30°C for 5 to 7 days. Pure cultures (one strain per plate) were obtained by repeated sub-culturing on PDA plates and maintained at 4°C on PDA slants. The isolates were examined and identified in the Deapartment of Botany and Microbiology, University of Lagos based on colonial and cultural characteristics and the morphology of their sporing structures. The microscopic structures of the isolates were studied using a light microscope (Carl, Zeiss, Germany) and by the description given by Thom and Raper (1945), Alexopoulus (1952), Talbot (1972) and Deacon (1980).

Screening of fungal isolates for pectinolytic activity

The isolates were cultivated on modified Czapek-Dox agar, with commercial citrus pectin as the sole carbon source, and screened for pectinolytic activity by a modified plate method of Phutela et al. (2005). The clearance zone formed around the colonies was determined using Potassium iodide – Iodine solution (5.0 g potassium iodide and 1.0 g iodine in 330 ml of distillled water).

Pectinase production with different agro-wastes

Two of the fungal isolates, *Aspergillus niger and Penicillum chrysogenum*, were studied for pectinase production using the different agrowastes, including pineapple peel, orange peels, sawdust, sugarcane pulps and wheat bran, as the sole carbon sources. The modified Czapek-Dox media contained 10 g L^{-1} of the different agrowastes as sole carbon source. One hundred milliliter (100 ml) of the sterile medium (pH 5.5) was inoculated with 2.0 ml of spore suspension (10^6 spores ml^{-1}) of the organism and incubated at 30°C with agitations (100 Osci min^{-1}) using Griffins Shaker. Cultures were harvested at 24-h intervals by centrifugation at 4000 g for 10 min. The culture supernatants were used as the crude enzyme sources.

Solid-state fermentation versus submerged fermentation

The comparative study of the solid-state fermentation (SSF) and submerged fermentation (SmF) was carried out using wheat bran as the sole carbon source. The medium for SmF contained per Liter of distilled water: wheat bran 10.0 g, $(NH_4)_2SO4$ 6.0 g, K_2HPO_4 6.0 g, KH_2PO_4 6.0 g and $MgSO_4.7H_2O$ 0.1 g. pH value was adjusted to 5.5 before inoculating with 2.0 ml of spore suspension containing 10^6 cells ml^{-1} of each fungus. The medium for SSF contained 5 g of wheat bran and 10 ml of the mineral salt solution: 6.0 g $(NH_4)_2SO_4$, 6.0 g K_2HPO_4, 6 g KH_2PO_4 and 0.1 g $MgSO_4.7H_2O$. Cultures were incubated at 30°C with agitations at 100 Osci/min using Griffins Shaker and harvested at 48 h by centrifugation at 4000 g for 10 min. The SSF culture was harvested after addition of 100 ml of sodium acetate buffer (0.05 M, pH 5.5) to the content of the flask. The culture supernatant were used as the crude enzyme sources.

Protein assay

The protein content of the crude enzyme was determined by the Folin ciocalteau method of Lowry et al. (1951) using Bovine Serum Albumin (BSA) as standard.

Pectinase assay

Pectinase activity was determined using citrus pectin as substrate. The reaction mixture, containing equal amounts of 1% pectin prepared in sodium acetate buffer (0.05 M; pH5.5) and suitably diluted crude enzyme, was incubated at 50°C in water bath for 30 min. The reaction was stopped with 1.0 ml dinitrosalycyclic acid solution (Miller, 1959) after which the mixture was boiled for 10 min and cooled. The colour was read at 540 nm using a spectrophotometer. The amount of reducing sugar released was quantified using galactouronic acid as standard. The specific enzyme activity (IU mg^{-1} protein) was calculated as the amount of enzyme required to release one micromole (1 µmol) equivalent of galactouronic acid per minute per mg protein under the assay condition. The results are expressed as Mean ± standard error of mean (SEM). Significant difference between values was determined by Fisher's protected least significant different *t*-test with two-tail probabilities of less than 0.05 considered significant.

RESULTS

Five filamentous fungi (*Aspergillus clavatus*, *A. niger, Fusarium* sp., *P. chrysogenum* and *Trichoderma* sp.) were selected and isolated from agricultural wastes collected from fruits/vegetables markets in Lagos metropolis, Nigeria, based on their ability to grow on the basal medium containing citrus pectin as the sole carbon source. Plate 1 (A – E) shows the relative pectinolytic activity of the fungal isolates as indicated by their relative clearance zones. *A. niger* (B) and *P. chrysogenum* (D) gave the highest pectinolytic activity as shown by the large clearance zones (over 70%).

Studies on pectinase production by *A. niger* and *P. chrysogenum* was carried out using different agrowastes, including pineapple peel, orange peels, sawdust,

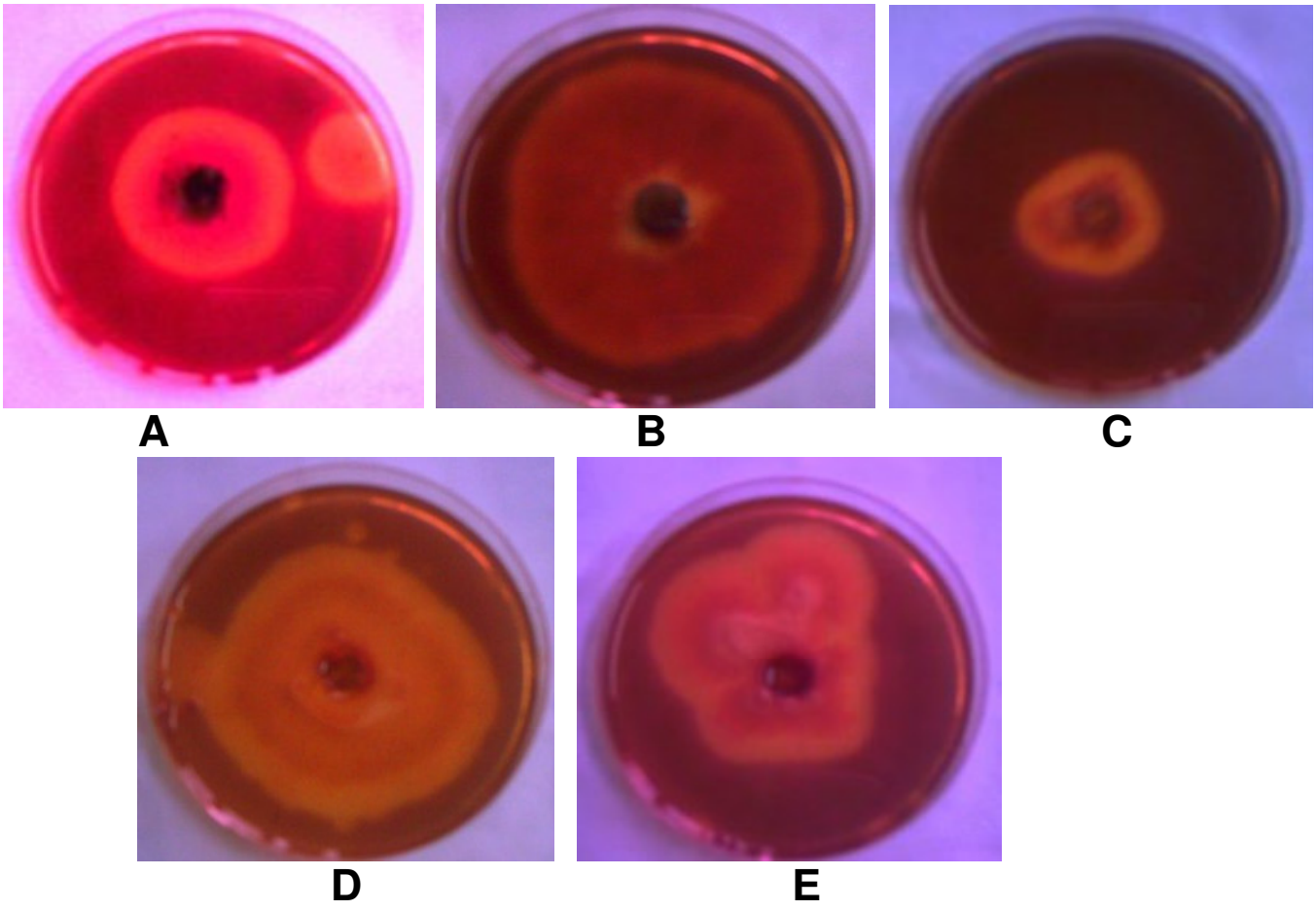

Plate 1. Preliminary screening of isolates for pectinolytic activity. A: *A. clavatus*; B: *A. niger*; C: *Fusarium* sp; D: *Penicillum chrysogenum,* and E: *Trichoderma* sp.

sugarcane pulps and wheat bran, as the sole carbon sources. Figures 1 and 2 respectively showed the pectinase activity of culture supernatants of *A. niger* and *P. chrysogenum* cultivated in modified Czapek-Dox media containing the different agrowastes. Both organisms significantly ($P < 0.001$) produced the highest pectinase activity at 48 h with wheat bran as sole carbon source. The maximum pectinase activity obtained with wheat bran was 350.28 ± 2.82 and 478.25 ± 3.04 mg^{-1} protein, respectively for *A. niger* and *P. chrysogenum*. Sugarcane pulp yielded the second best value of 282.13 ± 5.23 mg^{-1} protein at 72 h for *A. niger* whereas the second best value of 220.30 ± 3.05 mg^{-1} protein was obtained with pineapple peels at 48 h for *P. chrysogenum*. The least pectinase activity was obtained with orange peels for both organisms. The maximum pectinase production by *A. niger* using the different agro-wastes was in the order: wheat bran > sugarcane pulp > pineapple peels > sawdust > orange peels. For *P. chrysogenum*, the pectinase production using the agro-wastes followed the order: wheat bran > pineapple peels > sugarcane pulp > sawdust > orange peels.

Figure 3 is the plot of pectinase activity of the culture supernatants of *A. niger* and *P. chrysogenum* by solid state fermentation versus submerged fermentation. Pectinase activity of the supernatants from SSF cultures of both organisms was significantly higher ($P < 0.001$) than that obtained by SmF. The increase in pectinase production by SSF over SmF was more profound with *A. niger* (77.1%) compared to *P. chrysogenum* (15.0%). The maximum pectinase activity obtained for *A. niger* using SSF was 620.20 ± 5.16 IU mg^{-1} protein as against the value of 350.28 ± 2.82 IU mg mg^{-1} protein obtained by SmF. *P. chrysogenum* yielded a maximum pectinase activity of 550.07 ± 3.36 and 478.25 ± 3.04 IU mg^{-1} protein respectively by SSF and SmF.

DISCUSSION

Five filamentous fungi, which exhibited pectinolytic activity, were isolated from plant wastes in Lagos metropolis. The fungal isolates were identified as *A. clavatus*, *A. niger, Fusarium sp.*, *P. chrysogenum* and

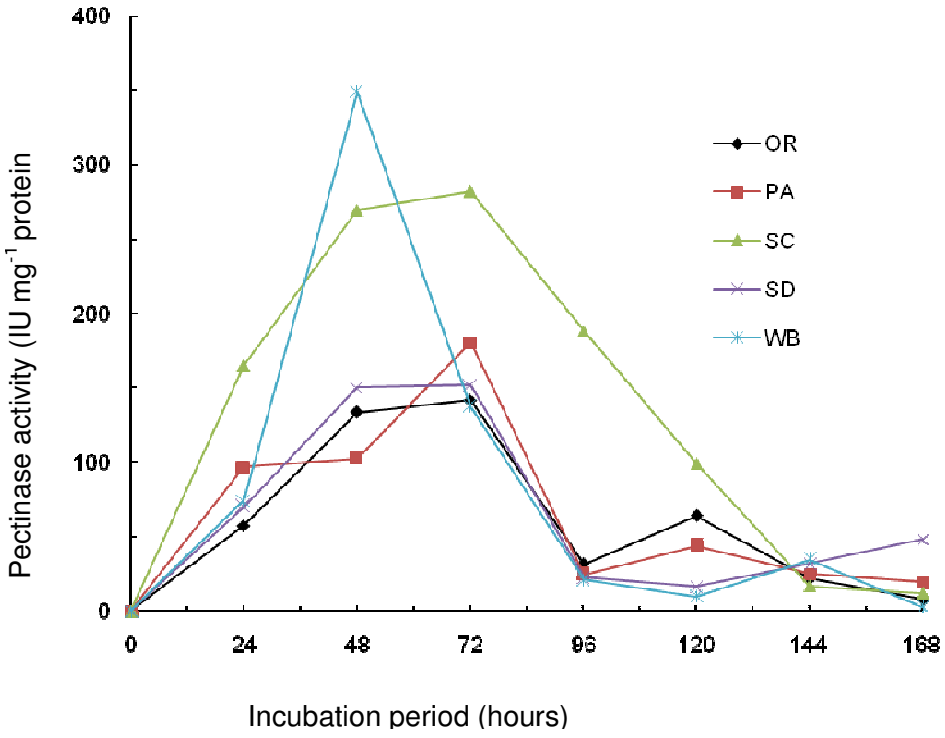

Figure 1. Pectinase activity of *A. niger* fermented on different carbon sources (OR = Orange peels; PA = Pineapple peels; SC = sugarcane pulp; SD = Sawdust; WB = Wheat bran).

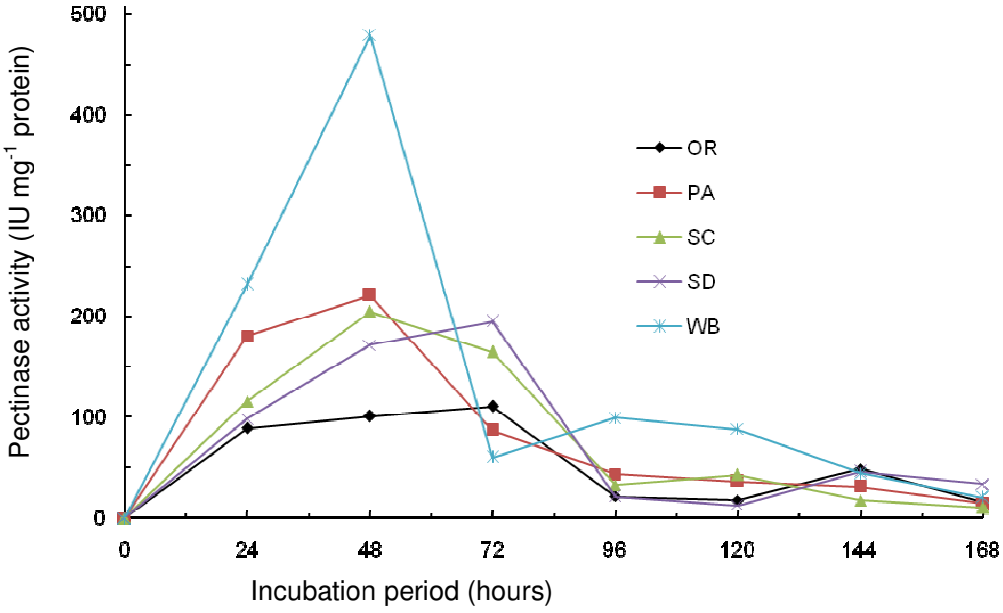

Figure 2. Pectinase activity of *P. chrysogenum* fermented on different carbon sources (OR = Orange peels; PA = Pineapple peels; SC = sugarcane pulp; SD = Sawdust; WB = Wheat bran).

Trichoderma sp. The best pectinolytic activity based on plate assay method, was given by *A. niger*, closely followed by *P. chrysogenum*. The present study shows that the wild strains of *A. niger* and *P. chrysogenum* produce extracellular pectinases which hydrolyze pectins. Cellulosic component of urban refuse appear to be good

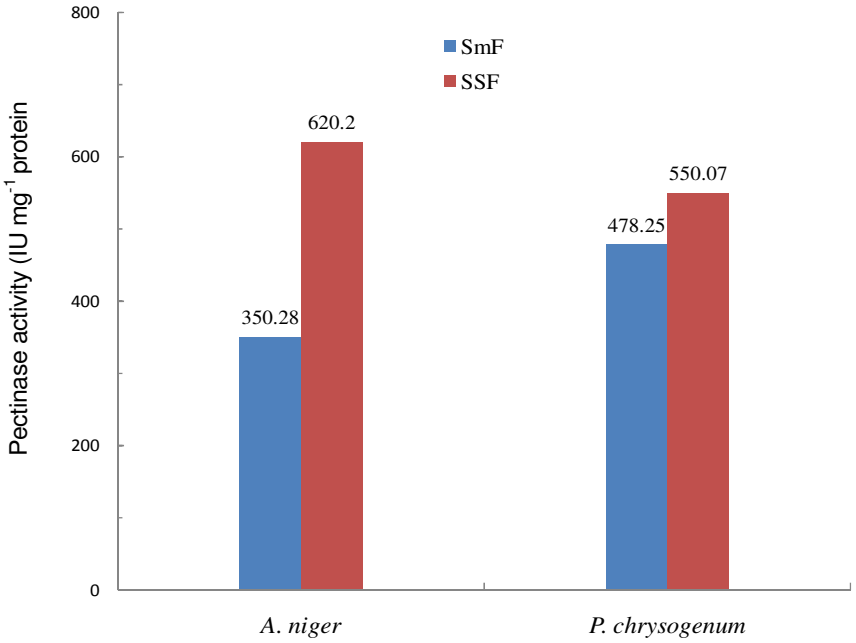

Figure 3. Pectinase production by *A. niger* and *P. chrysogenum* fermented on wheat bran (SSF = Solid state fermentation; SmF = Submerged fermentation).

sources of fungi with plant cell wall hydrolyzing activity. Nwodo-Chinedu et al. (2005) isolated some cellulolytic fungi from wood-wastes in Lagos, Nigeria. The strains of *A. niger* (ANL 301) and *P. chrysogenum* (PCL501) isolated by the group were reported to produce cellulases (Chinedu et al., 2008 a; Nwodo-Chinedu et al., 2007a) and xylanases (Chinedu et al., 2008b; Okafor et al., 2007a, b). Thus, these fungi produce the full complement of enzymes required for the hydrolysis of cellulosic biomass. This explains why the fungi thrive on waste plant matter and are capable of utilizing such waste materials as carbon sources in their culture media (Nwodo-Chinedu et al., 2007 b).

The strains of *A. niger* and *P. chrysogenum* produced the highest pectinase activity with wheat bran as sole carbon source. This implies that wheat bran is the most suitable of the five agrowastes (pineapple peels, orange peels, sawdust, sugarcane pulp and wheat bran) studied as potential substrates for pectinase production by the organisms. In an earlier work done in our laboratory, wheat bran was found to be the best 'agrowaste' substrate for xylanase production by strains of *A. niger* ANL301 and *P. chrysogenum* PCL501 (Okafor et al., 2007 a, b). The agrowaste is therefore well suited for the production of plant cell wall hydrolyzing enzymes by the fungi.

The incubation period for pectinase production by the two fungi using wheat bran as sole carbon source was 48 h. Siad et al. (1991) and Phutela et al. (2005) reported an incubation period of 48 h for optimal pectinase production for some fungal isolates while Fujio and Eledago (1993) reported a 72 h incubation time for polygalacturonase production by *Rhizopus oryzae*. This is ideal from an economic viewpoint is based on the enzyme production rate and the low-cost of the substrates.

Pectinase production from SSF culture of both organisms was significantly higher than that obtained by SmF. The higher level of pectinase activity by SSF compared to SmF varied with the organisms. For instance, it was more profound with *A. niger* compared to *P. chrysogenum*. Several workers proposed the use of SSF for pectinase production, using different solid agricultural and agro-industrial residues as substrates such as wheat bran and soy bran (Castilho et al., 1999, 2000; Singh et al., 1999). The present result clearly supports the use of SSF over SmF for pectinase production by filamentous fungi.

In conclusion, strians of pectinolytic fungi including *A. niger* and *P. chrysogenum*, have been isolated from fruit and vegetable wastes in lagos metropolis, Nigeria. Wheat bran had been identified as a suitable low-cost substrate for pectinase production by the strains of *A. niger* and *P. chrysogenum*. Higher levels of pectinase activity were obtained by SSF compared to SmF. The use of wheat bran for pectinase production will not only reduce the production costs of the enzyme but also help decrease pollution-load due to the agro-industrial waste.

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