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Full Length Research

Pectinase production by fungal-based solid-state fermentation of selected agricultural wastes

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ABSTRACT: Agricultural waste is the residues from growing and processing raw agricultural products. They can be properly managed using fungi to produce economical materials such as pectinase enzymes that act as biological agents which are applicable in many areas. This research was aimed at producing pectinase by fungal-based solid-state fermentation of agricultural wastes. Fungi were isolated from dump sites soil using the pour plate method. These fungal isolates were inoculated into the pretreated agricultural waste, including watermelon, mango, and pineapple peels. The degradation period was 15 days on mineral salt solid media using separate flasks for each waste and fungus. They were incubated at room temperature (28°C). The pectinase was assayed using pectin and DNSA (dinitrosalicyclic acid). Fungal isolates were identified and screened for pectinase production using standard methods. The pH and temperature were optimized to study the effect on pectinase activities using standard procedures and pectinase was purified using column sephadex G-100. Molecular identifications were carried out on the isolates using 18srRNA gene primers. The fungi were identified as *Aspergillus niger* strain AGF3 (OM273994), *Penicillium Chrysogenum* strain AGF2 (OM273982) and *Cladosporium tenuissimum* strain AGF1 (OM273981). *Aspergillus niger* strain AGF3 (OM273994) produced the highest specific pectinase activities in mango waste (2400 U/mg. protein/ mL and it can be compared to what was produced by *Cladosporium tenuissimum* strain AGF1 (OM273981) (1590 U/mg. protein/mL). High specific pecrtinase activity was also obtained from pineapple peels (2200 U/mg. protein/mL) produced by *Cladosporium tenussimum* strain AGF1 (OM273981). It can be concluded in this study that *Aspergillus niger* strain AGF3 (OM273994) and *Cladosporium tenuissimum* strain AGF1 (OM273981) were the best producers of pectinase using mango and pineapple peels, while *Penicillium chrysogenum* strain AGF2 (OM273982) produced substantial pectinase in all the three agricultural wastes but not at higher levels.

Keywords: Agricultural wastes, column sephadex G-100, fungal isolates, pectinase, solid-state fermentation.

INTRODUCTION

Biodegradable materials are substances, chemicals or wastes that can be changed to harmless states (which will not damage the environment) by the action of microorganisms such as bacteria and fungi. Biodegradation is also defined as the biologically catalyzed reduction in complexity of chemical compounds, i.e. the process by which organic substances are broken down into smaller compounds by living microbial organisms. When biodegradation is completed, the process is called “mineralization” (Marinescu *et al.*, 2009; Lima *et al.*, 2018). Agricultural wastes are the left over or residues from the growing and processing of raw agricultural products which include fruits, vegetables, meat, poultry, dairy products, cash crops and non-cash crops (FAO, 2015; Wagh *et al.*, 2022). Due to the influence of new global energy and environmental policies developed during the last decade, the international regulatory framework on sustainable development has been transforming the role of agriculture and especially the policies and strategies on the circular economy and bio-economy. New and better techniques for the recovery of agricultural wastes have been developed, based on industrial innovation and high technology, which

had contributed to guaranteeing resource efficiency, sustainable production and consumption and the reduction of negative environmental impact.

The development of enzymes was possible due to the availability of microbial sources. Many enzymes are produced economically from microorganisms which have led to genetic improvement (Banu*et al.*, 2010). Microbial enzymes have replaced many plant and animal enzymes. Enzymes have been found useful in many industries such as foods, beverages, pharmaceuticals, detergents, textiles, leather, chemicals, biofuels, animal feed, personal care, pulp and paper, diagnostics and therapy and many other areas or fields (Haile and Ayele, 2022). The food and beverage industries are among the major industries that make use of enzymes and manufacture a wide range of food and drinks to cater for the need of the growing world population (Demain and Vaishinav, 2009).

Enzymes technology is primarily engaged in the production, isolation, purification and use of enzymes either in soluble or immobilized form for the benefit of humankind (Khan, 2018). With advancements in recombinant DNA technology, enzyme engineering produces a more effective and diverse group of enzymes with useful applications in microbiology, biochemistry, diagnostics, therapeutics, biocatalysis and other areas such as structural biology. The purpose of this emerging technology is to produce unique sustainable products with specific functions to fulfil human needs (Bucholz *et al*., 2012). Process parameters like pH, temperature, moisture, oxygen transfer, and aeration can be controlled easily (Samanta, 2021). Solid-state fermentation (SSF) is suitable for the less moisture content requirement by the microorganisms involved. In SSF, nutrient rich waste materials like bran, bagasse and paper pulp can be used as substrates for the microorganisms and they are consumed very slowly and constantly. Hence, there is no need to supply the substrate for a longer time. The major merits of SSF include the fact that it is easy to handle, involves the recovery of higher concentrations of products and the generation of lesser effluent. Therefore, SSF is considered a promising method for commercial enzyme production. SSF was previously reported to be well-suited for developing countries due to its cost effectiveness. For instance, pineapple fruits are available throughout the year with the highest rate obtained during the rainy season, and thereafter, the waste is typically scattered into the environment. Generally, the higher the production of agro crops, the higher the waste generation, both from individuals, retailers and food industries. Such wastes may include husks, bagasse and pup residues. These wastes must be taken to a suitable location, which in turn adds to the industrial cost implication. Apart from the cost of treating the waste, there are still the risks of continuous environmental pollution (Lima *et al.*, 2018). This study therefore aimed at producing the enzyme pectinase from selected agricultural wastes using fungi in solid-state fermentation.

MATERIALS AND METHODS

Micro-organisms and sample collection

Three fungal species used in this investigation were isolated from fresh dump site soils in Ilorin metropolis, Kwara State. One gram (1g) of each soil sample from the dump sites was homogenized in sterilised distilled water and 1 mL was inoculated into potato dextrose agar and Saboraud agar by streaking method. After autoclaving each medium at 121°C for 15 min, gentamycin was added to prevent bacterial growth. The plates were incubated at room temperature (30°C) for 48 to 72 h, after which morphologically different colonies were purified using repeated streaking. The specific strains were identified by morphological and biochemical characteristics and maintained as stock cultures on potato dextrose agar slants (Martin *et al.*, 2004; Janani *et al*., 2011). From the stock culture, isolates were tested for their ability to produce extracellular enzymes using Azodyes (Sidiqui *et al.,* 2015). Pineapple, watermelon and mango fruits were purchased from the local shops in Ipata, Sango and Oyun in Kwara State. After washing the fruits, the outer layers were peeled and oven dried at 65°C for 18 to 24 h. The dried wastes were then ground into powder using an electric blender for 10 min (AOAC, 2019).

Processing of wastes, media preparation and fermentation

The dried wastes were pretreated using the chemical and steam method (AOAC, 2019). Solid state fermentation (SSF) was employed using mineral salt (ms) g/l) and was prepared as stated below.

Twenty grams (20 g) of each waste was added into the mineral salt media in separate Erlenmeyer Flasks (250 mL) following the modified methods of Husseiny *et al*. (2008) and Algahtani *et al.* (2022). The addition of the substrates into the mineral media, became mango mineral salt medium, pineapple mineral and water melon mineral salt medium.

Ten milliliter (10 mL) of mineral salt media composed of 0.1% of KH_2PO_4; 0.1% $(NH_4)_2\ SO_4$, 0.1% $MgSO_4.7H_2O$, 0.004% $ZnS0_4.7H_2O$ and 0.013% $FeSO_4.7H_2O$, were added to each of the flask containing 20 g of each waste plus 150-200 mL of distilled water. These were sterilized at 120°C for 15 min. Ten (10 mL) aliquots of conidia suspension containing a drop of tween 80 were poured/ inoculated into each Erlenmeyer flask and incubated at room temperature (30°C).

Pectinase extraction and enzyme assays

At the end of biodegradation (15 days), enzyme extraction was conducted using the modified method of Martin *et al.* (2004), Famotemi *et al.* (2015) and Abd-El-Aal *et al*.

(2022). The solid biodegraded substrate from each Erlenmeyer flask was mixed properly with distilled water. The quantity or volume of distilled water for mixing depended on the concentration or gramme of fermented materials (e g. 10 g to 200 mL, 5 g to 100 mL and 1 g to 20 mL of distilled water) (Husseiny *et al*., 2008). In this study, 1 g of fermented material was mixed with 20 mL of distilled water and stirred for 30 min. The materials were filtered using doubled muslin cloth and were centrifuged at 3,000 rpm for 20 min. At the end of centrifugation, the supernatants were removed and served as crude enzyme solutions with buffer and carefully stored at refrigerated condition (4°C) for further analysis. The same quantity of buffer solution (pH5.0) (1 mL) was added to the crude enzyme (1 mL) for preservation till further analysis.

Purification of enzyme (Ammonium salt precipitation)

The method of Siddiqui *et al*. (2015) was modified, 60% of Ammonium Sulphate was measured and added to 10 mL of crude enzyme sample and stirred slowly. It was carried out at room temperature for 30 min and later kept overnight, then, the samples were centrifuged at 3,000 rmp for 30 min. After the centrifugation, the filtrate was collected and kept for further analysis at low temperature (4°C).

Measurements of (Pectinase) enzyme activity

The activity of the enzyme obtained from biodegraded material was determined by measuring the release of reducing groups from the waste substrates. The method of Miller (1959) as cited by Martin *et al.* (2004), Husseiny *et al*. (2008) and Famotemu *et al.* (2015) was employed. The measurement was carried out using 3, 5-dinitrosalicylic acid (DNSA) reagent assay.

One millilitre (1 mL) of enzyme solution was added to 1 mL of pectin and mixed with 1 mL of buffer solution. The mixture above was mixed with 1 mL of DNSA reagent and boiled for 10-15 min. Then, the mixture was cooled to room temperature and absorbance was measured using a spectrophotometer at 540 nm (Spectum lab 7525 Spectrophotometer). Martău *et al.* (2021) method was also modified in this process.

One unit definition of enzymatic activity (U) was defined as the amount of enzyme which released one µmol of galacturonic acid per minute. Also, one unit of enzymatic activity (U) was defined as the amount of enzyme which released 1 µmol of uronide per min. The enzyme production was expressed in units per gram of the initial dry solid substrate (Ug-1) (Martin *et al.,* 2004).

Enzyme assay

One millilitre (1 mL) of the enzyme solution was added to 1 mL of pectin, then mixed with 1 mL of buffer solution. Subsequently, 1 mL of DNSA reagent was added to the mixture, which was then boiled for 10-15 minutes. Thereafter, the mixture was cooled to room temperature and the absorbance was measured using a spectrophotometer at 540 nm (Spectumlab 7525 Spectrophotometer), as modified from Martău *et al.* (2021).

Purification of Pectinase by gel filtration

The semi-purified pectinase was subjected to gel filtration chromatography using Sephadex -G100. The semi-purified pectinase was introduced into the column and eluted with Tris buffer. The flow rate was maintained at 0.2 mL/min. Fractions of 5 mL each were collected from Sephadex-G 100 column. The pectinase activity and protein concentration were determined for each separate fraction according to Husseiny *et al.* (2008), Sharma *et al. (*2013) and Abd-El-Aal *et al*. (2022).

Enzyme kinetics

Effect of pH and temperature on enzyme activity

Effect of temperature: There is an optimum temperature at which enzymes are highly active and after the optimum temperature, there is an upper limit after which the enzyme becomes ineffective and denatured. In this study, seven different temperature ranges were selected (20, 30, 40, 50, 60, 70, and 80°C). The process of preparation of enzyme solution was the same as that of enzyme assay but at different temperature ranges (Siddiqui *et al.,* 2015; Kham, 2018).

Effect of pH: In this research work, pH 4, 5, 7, and 9 were studied and Acetate buffer of different pH was prepared. The preparation of the enzyme for the spectrophotometer (at 540 nm using Spectumlab 7525 spectrophotometer) reading was the same as in the enzyme assay (Kham, 2018; Martău *et al.,* 2021).

Determination of protein concentration (dye-binding method for protein determination)

The protein content in the enzyme was determined by Bio–Rad protein Assay (dye reagent) as adopted from Bradford (2001). Five millimetres of diluted dye reagent (Orange G at pH 2.2) was pipetted into 100 mL of sample solution. The mixture was then incubated at room temperature for 5 min. The absorbance was measured at 595 nm against the blank of deionized water.

RESULTS

Identification of Fungal Isolates for Extra-cellular Enzymes

Three fungal isolates were selected and tested for their abilities to produce extra–cellular enzymes (pectinase).

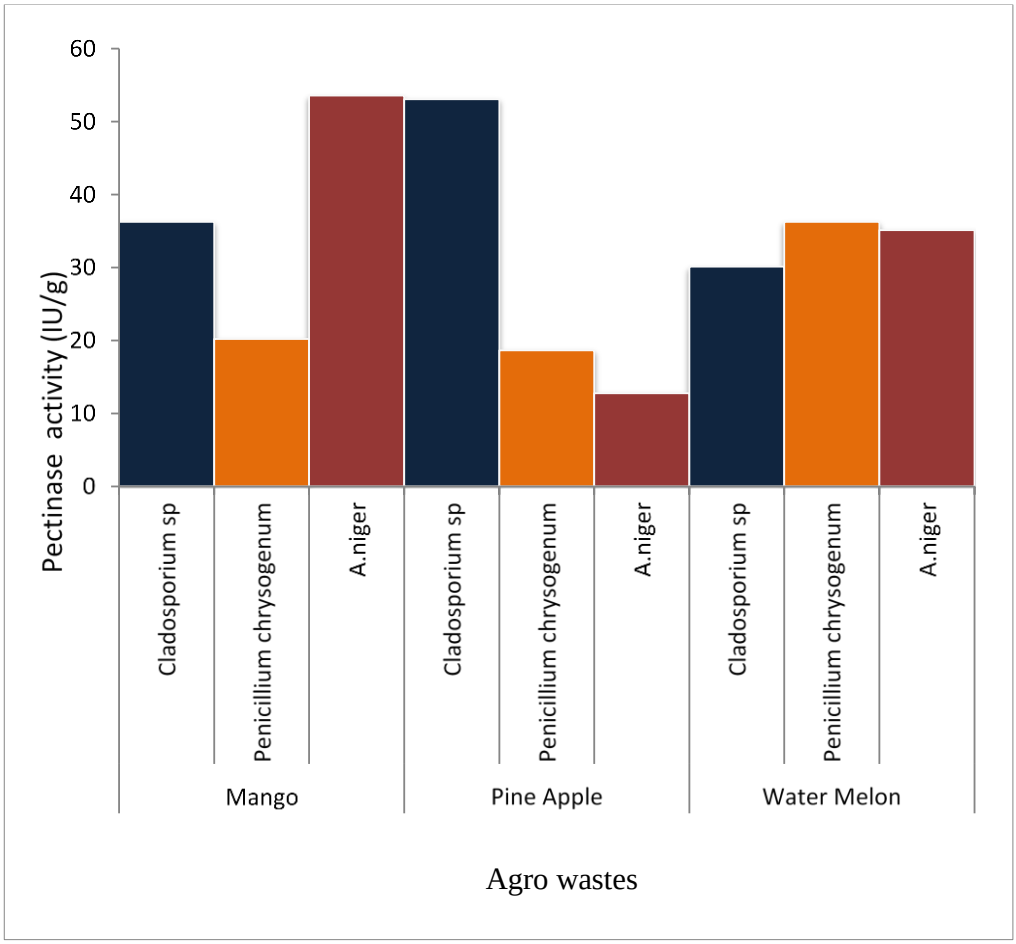

Figure 1. Production of Pectinase enzyme from selected waste using fungal isolates.

Aspergillus niger strain AGF3 produced the highest zone of clearance, indicative of the highest enzyme production amongst the isolates (mean diameter of zone of clearance = 36.47mm), followed by *Penicillium chrysogenum* strain AGF2 (32.80 mm) and then *Cladosporium tenuissimum* strain CZBC AGF1 (28.67mm). Figure 1 showed the mean pectinase production from the mango, pineapple and watermelon wastes after degradation by the fungal isolates. The activities of the pectinase attained its peak in the mango waste fermentation setup using *Aspergillus niger strain* AGF3 (53.60 U/mg.protein/mL) while in pineapple waste, the maximum production was exhibited by *Cladosporium tenuissimum* strain AGF1 (53.49 U/mg.protein/mL). Conversely, *Penicillium chrysogenum* produced substantial pectinase in watermelon wastes (39.8 U/mg.protein/mL).

Characteristics of the partially purified pectinase

Effect of pH on activities of pectinase.

The results shown in Table 1 indicated that the activity of the pectinase produced by *A. niger* strain AGF3 reached the maximum at pH 5 and at pH 4, and was reduced to the minimum after the peak.

On the other hand, Table 1, revealed that with regards to *Penicillium chrysogenum* strain AGF2, the pectinase enzyme produced was very active at pH 7, while at pH 9, the activity dropped down to its minimum.

Moreover, it can be seen from Table 1 that the activity of pectinase produced from the waste degraded by *Cladosporium tenuissmum* strain AGF1 peaked at pH 7, and decreased to a minimum at pH 4.

Effect of temperature (thermal stability) on pectinase enzyme

The graph in Table 2 showed that at 50°C, the activity of the pectinase enzyme reached the peak, but at 80°C, the activity dropped down to the minimum level. This decrease in activity indicated that the enzymes are incapable of working at incubation temperatures from 60 to 80°C.

Similarly, the data presented in Table 2 confirmed that at 50°C, the pectinase activity was at maximum, however, the minimum activity was noticed at 80°C. The highest

Table 1. Effect of pH on Pectinase activity by the organisms (*Aspergillus niger*, *Penicillum chrysgenum* and *Cladosporium tennuisimum*) using agricultural wastes (water melon, mango and pineapple waste).

Organism	Agro waste	pH/Pectinase activity (IU/g)			
		4.0	5.0	7.0	9.0
Cladosporium tenuissimum (AGF1)	Mango	4.6	3.5	4.0	2.3
	Pineapple	2.3	5.5	10.4	8.7
	Watermelon	2.3	5.2	3.2	4.3
Penicillum chrysgenum (AGF2)	Mango	4.6	2.0	8.5	1.5
	Pineapple	3.2	5.7	3.8	1.7
	Watermelon	3.0	5.5	7.5	2.3
Aspergillus niger (AGF3)	Mango	2.6	2.3	3.2	2.0
	Pineapple	3.8	4.3	3.2	2.3
	Watermelon	3.3	6.0	3.3	1.7

Table 2. Effect of Temperature on Pectinase activity produced by the organisms (*Aspergillus niger*, *Penicillum chrysgenum* and *Cladosporium tennuisimum*) using agricultural wastes (water melon, mango and pineapple waste).

Organism	Agro waste	Temperature (°C)/Pectinase Activity (IU/g)						
		26	30	40	50	60	70	80
Cladosporium tenuissimum (AGF1)	Mango	10.60	13.30	13.51	14.27	12.16	6.51	5.30
	Pineapple	9.50	11.02	13.30	12.40	4.00	4.73	3.50
	Watermelon	2.41	3.20	3.20	3.47	1.80	1.50	1.10
Penicillum chrysogenum (AGF2)	Mango	3.33	3.00	7.20	10.50	6.40	4.22	2.21
	Pineapple	2.50	3.50	3.50	11.50	2.67	1.73	1.50
	Watermelon	0.87	10.00	10.00	6.23	4.30	2.10	1.10
Aspergillus niger (AGF3)	Mango	2.30	3.30	10.3	10.3	4.20	2.00	1.8
	Pineapple	4.50	7.13	9.50	9.50	6.2	2.5	1.0
	Watermelon	3.60	4.13	3.60	3.93	3.33	2.0	1.60

specific activity (2400 U/mg.protein/mL) was obtained from pectinase produced by *A. niger* growing on mango waste substrate. On the other hand, *Cladisporium tenuissimum* produced the pectinase with the highest specific activities from pineapple waste (2200 U/mg.protein/mL), and finally, the pectinase produced by *Penicillium chrysogenum* from watermelon had a specific activity of 1500 U/mg.protein /mL; and was thermally stable at 40 and 50°C.

Table 2 showed that the highest specific activity (2400 U/mg. protein/mL) was exhibited by the pectinase produced by *A. niger* from mango, and the enzyme was close to its maximum range of activity at 40 and 50°C, however, the activity was noticed to be minimum when the temperature was at 80°C. On the other hand, *Cladosporium tenuissimum* produced the highest specific activities of pectinase from pineapple substrate (2200 U/mg.protein/mL), and finally, *Penicillimum chrysogenum* had pectinase specific activity of 1500 U/mg.protein/mL), with a thermal stability range of 40 and 50°C.

Fractionation pattern of pectinase

The data in Figure 2a indicated the fraction pattern of pectinase produced by *Penicillium chrysogenum* strain AGF2 from water melon waste (WMW). The enzyme fraction activities began from 4 and continued to the 6th fraction, nevertheless, fraction 5 was observed to reach the maximum specific activity at 1500 U/mg.protein/mL.

Conversely, the results from Figure 2b revealed the pectinase activity produced by *Cladosporium tenuissimum* strain AGF1 in water melon waste, with the activities of the enzyme fractions starting to appear from fractions 6 up to 8. Fraction number 7 was observed to reach the maximum specific activity at 1100 U/mg.protein/mL.

The results from Figure 2c revealed the fractionation pattern of pectinase produced by *Aspergillus niger* strain AGF3 grown on watermelon waste (WMW). The enzyme fraction activities began to appear from fraction 3 and continued until fraction 6. Fraction number 5 was however

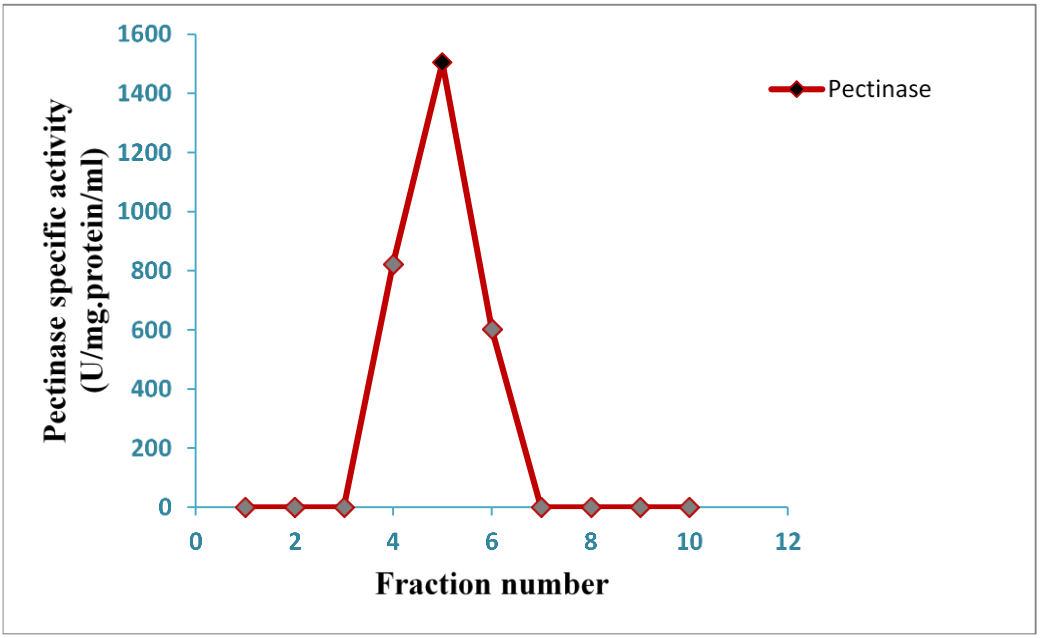

Figure 2a. Fractionation pattern of pectinase produced by *Penicillium chrysogenum* strain AGF2 from WMW using Sephadex-G100 column.

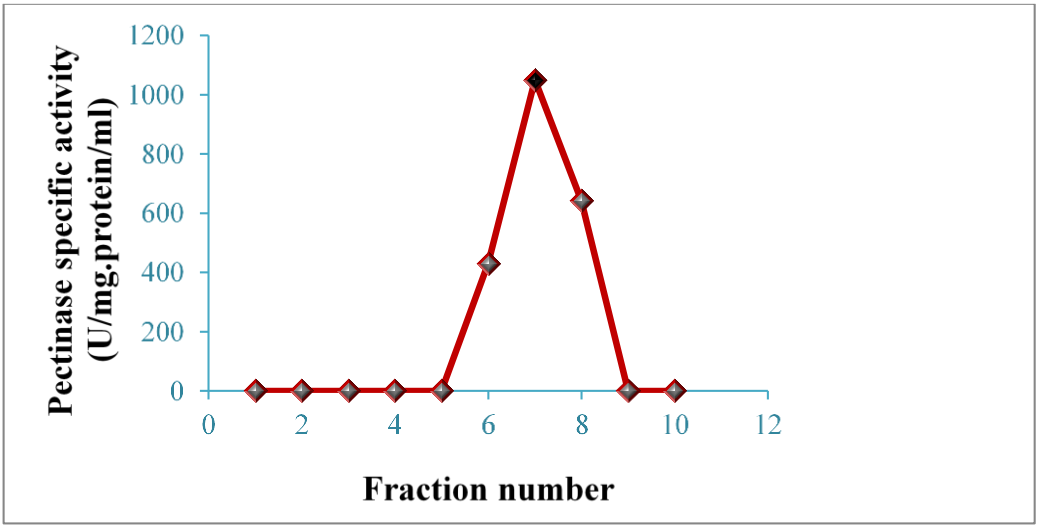

Figure 2b. Fractionation pattern of pectinase produced by *Cladosporium tenuissimum* strain AGF1 from WMW using sephadex-G100 column.

observed to have the maximum specific activity at 1350 U/mg.protein/mL.

In Figure 2d, the activities of the enzyme fractions produced by *Cladosporium tenuissimum* strain AGF1 grown on pineapple waste (PNPW) started appearing from the 4th until the 7th fraction. Fraction number 6 was however observed to reach the maximum specific activity at 2200 U/mg.protein/mL.

On the other hand, the results shown in Figure 2e revealed that the activity of the pectinase produced by *Cladosporium tenuissimum* strain AGF1 grown on water melon waste started appearing from the 4th and continued appearing up to the 7th enzyme fraction. Fraction number 5 (Figure 2e) was observed to reach the maximum specific activity at 1100 U/mg.protein/mL.

Finally, the enzyme activities of the pectinase produced by *A. niger* strain AGF3that utilised mango waste (MGW) as a substrate started to appear from the 3rd to the 7th fractions, with the 5th fraaction reaching the maximum specific activity of 2400 U/mg.protein/mL (Figure 2f)

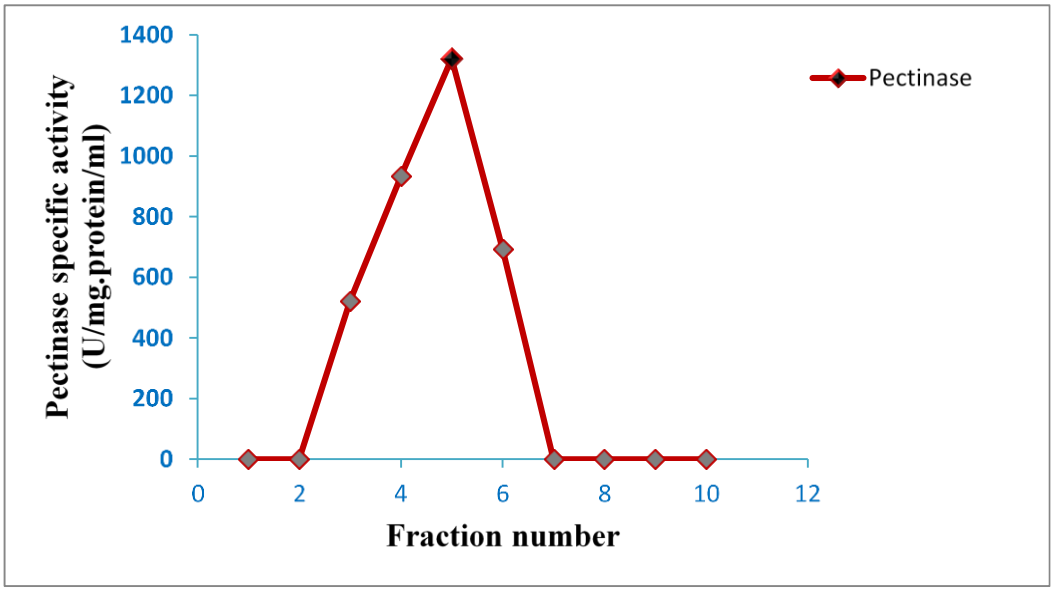

Figure 2c. Fractionation pattern of pectinase produced by *Aspergillus niger* strain AGF3.

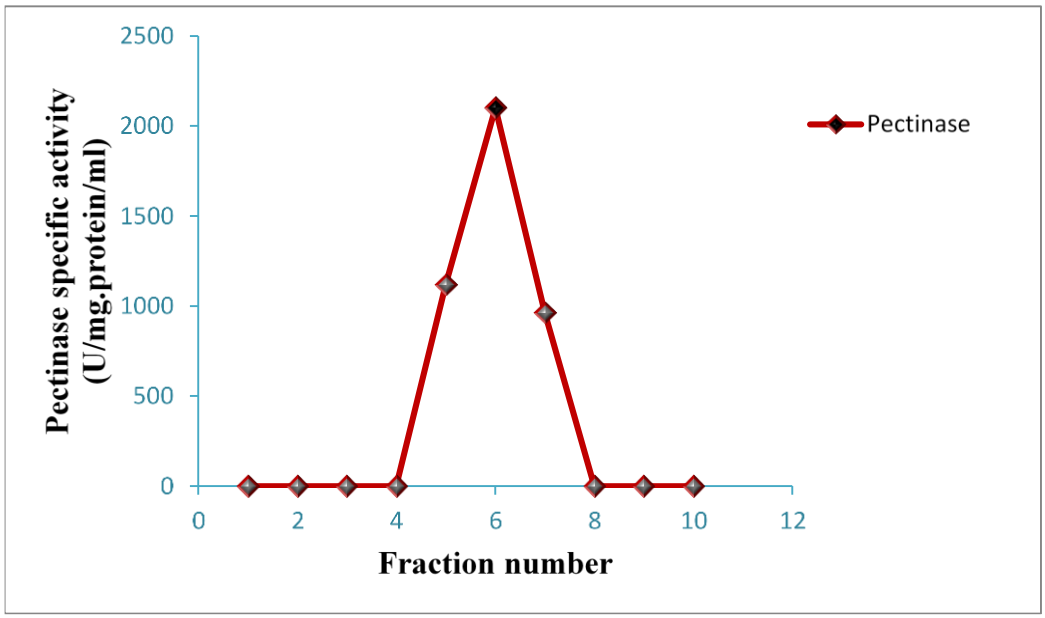

Figure 2d. Fractionation pattern of pectinase produced by *Cladsoporium tenuissimum* strain AGF1 from PNPW using Sephadex-G100 column.

DISCUSSION

This study revealed the usefulness of fungi for pectinase enzyme production and degradation of agricultural wastes. The bulk of agricultural wastes is not suitable as food for animal feed components because they cannot be digested directly by many animals e.g. mono gastric animals (Lima *et al*., 2018).

During degradation, many fungi are capable of utilizing the organic matter in wastes as a source of carbon to synthesize cellular biomass or for energy generation (Husseiny *et al*. (2008)). The findings in this work were similar to the report of Sudeep *et al*. (2020) that fermentation processes lead to the breaking down of organic matter by fungi. This work was similar due to the fact that fungi isolates employed produced extra cellular enzyme that degraded the waste.

The Pectinase from fungal strains *A. niger* strain AGF3,

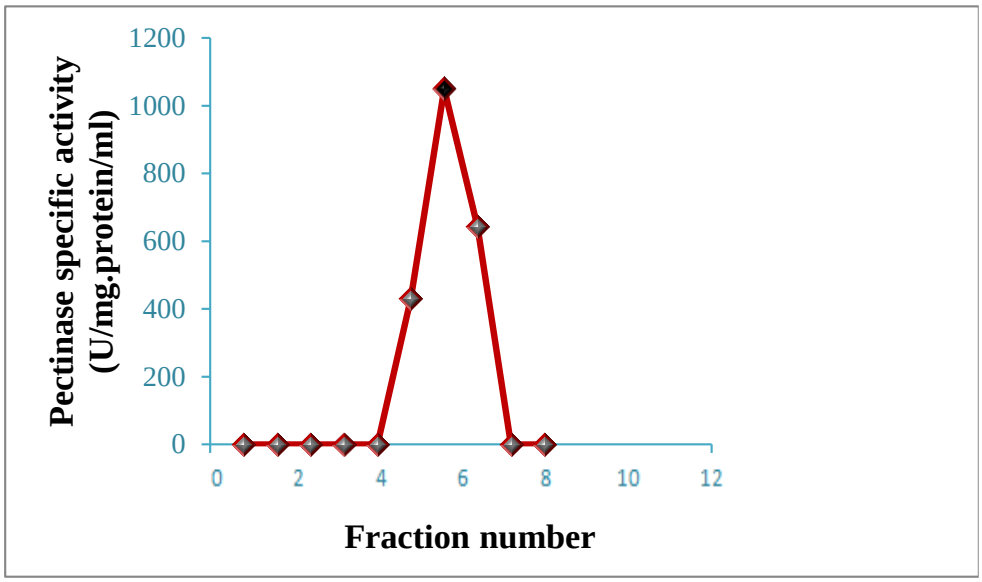

Figure 2e. Fractionation pattern of pectinase produced by *Cladsoporium tenuissimum* strain AGF1 from WMW using sephadex-G100 column.

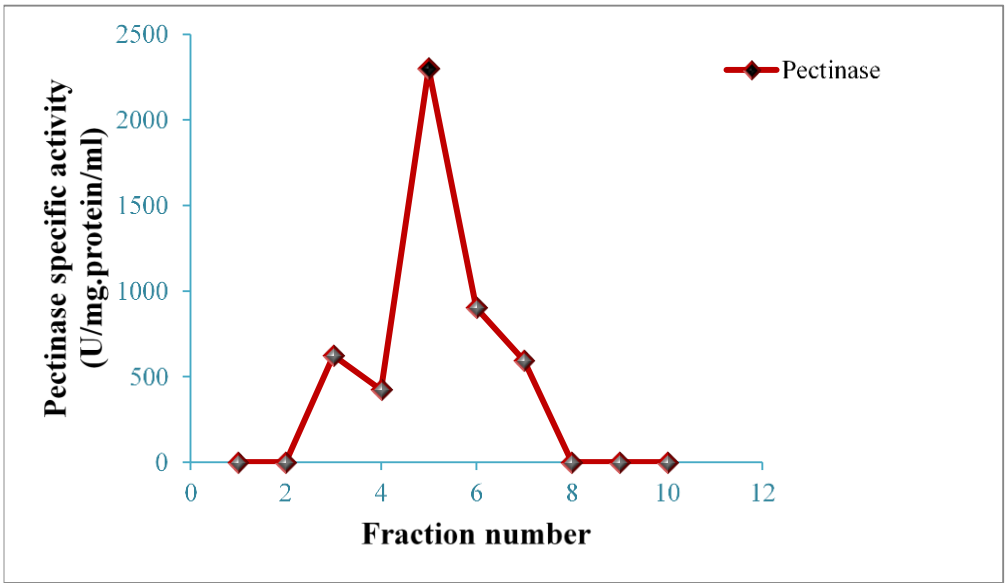

Figure 2f. Fractionation pattern of pectinase produced by *Aspergillus niger* strain AGF3 from MGW using Sephadex-G100 column.

Cladosporium tenuissmum strain AGF1 and *Penicillium chrysogenum* strain AGF2 produced higher specific activities, and this was similar to the findings of other authors who had used other strains of fungi for enzyme production (Banu *et al*., 2010).

The effects of pH ranges were also tested on the activities of pectinase produced by fungi strains in this study. The various pH of activities of pectinase produced by fungi were corroborated with the past researchers documentations especially those who had used fungi spp like other strains of *A. niger* and other fungi for pectinase enzyme production by solid state fermentation using citrus pulp and other wastes (Banu *et al.,* 2010; Rodríguez *et al.,* 2012). Pectinase activities from fungi in this study were observed at pH ranging from pH 4 to 9. Previous studies had reported a pH of 6.9 as the maximum for activities of pectinase (Famotemi *at al*., 2015). Other studies reported that pectinases reached their maximum activities at pH 4 (Martin *et al*., 2004).

The pH values and different peaks of enzymatic activities

observed in this present investigation were in line with the observations and reports of other researchers which implies that pH below 6.9 or above can affect the maximum activities of pectinase enzymes (Martin *et al.,* 2004; Husseiny *et al.,* 2008 Famotemi *et al.,* 2015).

The thermal stability of pectinase produced by fungi strains was between 40-50°C and these temperature stabilities were favourably compared to the report of Martin *et al*. (2004) and Husseiny *et al*. (2008) who reported that temperature stabilities of activity of pectinase and cellulase enzyme were 40, 50, 60°C and at a lower temperature than 40°C.

In view of the findings of other researchers on the thermal stability of pectinase, Famotemi *et al*. (2015) reported that the pectinase thermal stability in their studies showed that the pectinase obtained from the strains of *A. niger* used was stable at 50°C. On the other hand, Patil and Chaudri (2010) obtained pectinase from fungi in solid state degradation of agro waste and reported a temperature of 35°C as the maximum temperature for pectinase activity.

Moreover, Martin *et al*. (2004) reported that pectinase activity was maintained at 70°C during the initial stability of the enzyme. In this present investigation, the enzyme did not exhibit the ability to be active from 65-100°C which indicated that above 60°C, the pectinase activity was decreased i.e. high temperature affect the performance of pectinase.

Conclusion

It can therefore be concluded that fungi used in this study were very effective for the production of pectinase employing solid state degradation of the substrates used. The degradation of the agro wastes employed in this study can lead to a reduction in the environmental pollution caused by agricultural wastes that are very abundant during and after harvesting periods. Pectinase enzyme has potential applications in many sectors of the economy within and outside Nigeria such as the food and pharmaceutical industries.

Recommendation

It is recommended that the fungi employed in the study can be used to degrade agro wastes for pectinase production. In addition, mango, pineapple and watermelon wastes should be constantly employed for pectinase enzyme production, this will reduce the cost of importation and production, making this important enzyme to be available in the industries.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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