

Production of Pectinase by Fungi isolated from Degrading Fruits and Vegetable

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ABSTRACT: Peelings of fruit and vegetable waste are usually thrown away as waste and they constitute environmental nuisance because they are found in heaps littering our environment and contributing to environmental pollution. This work was therefore aimed at using these fruit peelings as substrates in solid state fermentation for production of fungal pectinase. Pectinase producing fungal isolates from spoiled fruit peels and pomace were selectively obtained among other fungal isolates by culturing in successive enrichment medium. The obtained isolates were screened on agar plates containing 0.5% pectin as the sole carbon source. Two fungal isolates *Rhodotorulla* spp and *Mucor mucorales* were selected due to the large clearance zones they had on the plates and were used in solid state fermentation for the production of pectinase. Substrates used for the production of pectinase were dried and milled orange and pineapple peelings and water melon pomace while incubation was for 14 days. Assay was carried out every other day. Crude enzyme extract from both isolates were characterized. Highest pectinase activity of 82.95U/dry weight of substrate (dw) was recorded for *Rhodotorulla* spp. using orange peelings as substrate on the 8th day while the least was 16.12U/dw on water melon pomace as substrate on the 2nd day. *Mucor* had its highest pectinase activity of 46.05U/dw on orange peelings as substrate on the 12th day. Optimum temperature and pH for pectinase by *Rhodotorulla* spp was at 35°C and 6.0 respectively while that of *Mucor mucorales* was 45°C and 5.8 respectively. Pectinase by *Mucor mucorales* was relatively stable even at 65°C. Michaelis Menten (K_m) constant value for pectinase by *Rhodotorulla* spp was 3.0 mg/ml while the maximum velocity (V_{max}) was 0.023364U/mg/min. K_m value of pectinase by *Mucor mucorales* was 15 mg/ml while the V_{max} was 0.043364U/mg/min. The molecular weights of pectinase by *Rhodotorulla* spp. were 35 and 45 kDa while that of *Mucor mucorales* was 60 kDa. Fruit waste therefore could be used as substrates for the production of microbial pectinases.

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INTRODUCTION

Pectinases hydrolyze pectin, the soluble complex polysaccharides that occur widely in plant cell walls. Pectinases are commercially used in many processes (Kashyap *et al.*, 2001) and nearly 25% of the global enzyme sales are attributed to pectinase (Kaur *et al.*, 2004). It degrades polygalacturonan by hydrolysis of the glycosidic bonds that link galacturonic acid residues.

Pectinase have been reported to be produced by a large number of bacteria and fungi such as *Bacillus* spp., *Clostridium* spp., *Pseudomonas* spp., *Aspergillus* spp., *Monilla laxa*, *Fusarium* spp., *Verticillium* spp., *Penicillium* spp., *Sclerotinia libertiana*, *Coniothyrium diplodiella*, *Thermomyces lanuginosus*, *Polyporus squamosus*, nematodes, yeasts and protozoa (Jayani *et al.*, 2005). The microbial world has shown to be very heterogeneous in its ability to synthesize different types of pectolytic enzymes with different

mechanisms of action and biochemical properties (Favela-Torres *et al.*, 2005; Gummadi & Panda, 2003). Pectic enzymes are produced by both prokaryotic microorganisms, which primarily synthesize alkaline pectinases, and by eukaryotic microorganisms, mostly fungi that synthesize acid pectinase (Kashyap *et al.*, 2001, Hoondal *et al.*, 2002; Jayani *et al.*, 2005). Furthermore, the production of these enzymes has also been described in yeast (Alimardani-Theuil *et al.*, 2011; Blanco *et al.*, 1999).

Pectinases are used in the textile industry as they are capable of depolymerising the pectin breaking it into low molecular water soluble oligomers improving absorbency and whiteness of textile material and avoiding fiber damage (Sonia *et al.*, 2009). Pectinase is used in juice clarification. There is use of xylanopectinolytic enzymes in paper and pulp industry. Pectinases are effective in biobleaching of mixed hard wood and bamboo kraft pulp, as pretreatment of kraft

pulp with xylano-pectinolytic enzymes from same alkalo thermotolerant isolate produced pulp with superior quality facilitating adaptation of environment friendly technology in paper pulp industry (Amanjot *et al.*, 2010). Pectinase also find application in the degumming of plant fibers, retting of plant fibers, pectinase from *Bacillus species* are used in waste water treatment. Pectinase are further used in coffee and tea fermentation by breaking pectin present in tea leaves, oil extraction by avoiding emulsification formation, improvement of chromaticity and stability of red wines (Botella *et al.*, 2005), pectinase improve wine characteristics of colour and turbidity, biscouring of cotton. Pectinases possess biological applications in protoplast fusion technology and plant pathology (Ernesto *et al.*, 2006).

It is known that the fruits and vegetables are highly perishable and a lot are wasted as a result of this. Also these fruits are not easily disposed off because a large number of them tend to spoil easily and they are found degrading in heaps in our environment. Instead of allowing them to constitute nuisance in the environment, the perished fruits and vegetables could be turned into wealth by utilizing them as substrates for certain enzyme production. Fungi and yeasts are common colonizers of degrading fruits and vegetables, therefore using both or either of them to produce microbial enzymes such as pectinase in solid state fermentation of degrading fruits and vegetables and their peelings will go a long way in bringing in wealth for the nation

Materials and Methods

Sterilization Process

All glass-wares were sterilized using hot-air oven (Gallenkampus Model NYC-101) at 180°C for three hours. All media were sterilized by autoclaving in an autoclave (Model YM50) at a temperature of 121°C and 15psi for 15minutes. Inoculating needles, cork borers and blades were sterilized by flaming until red-hot. All subculturing and inoculation was carried out in lamina flow chamber.

Enrichment for Isolation of Pectinase Producers

1gm of each sample was aseptically inoculated into each 100ml of 0.5% (w/v) pectin broth containing the following in g/l: CuSO₄ 5H₂O(0.5), KCl(0.5), MnSO₄ 7H₂O(0.01), FeSO₄ 7H₂O(3.00), NaNO₃(1.00), ZnSO₄(0.50) and Pectin(5) in 1000ml 0.1M Phosphate buffer (pH6.2) flasks and covered back. The flasks were incubated on rotary shaker (Model) at 30°C±2°C for 7 days. After 7 days. 1ml of each previously enriched medium was inoculated into 100ml of another newly prepared 0.7% (w/v) pectin broth in 250 ml flask and covered with aluminum foil. Each flask was incubated on rotary shaker (Model) at 30°C±2°C for 7 days. This was done four times consecutively and increasing the concentration of

pectin in the broth by 0.2g by each subsequent preparation isolation was done Nitinkumar and Bhushan (2010).

Plate Screening of Pectinase Producers

Pectin agar plates were prepared containing in g/l the following: CuSO₄ 5H₂O(0.5), KCl(0.5), MnSO₄ 7H₂O(0.01), FeSO₄ 7H₂O(3.00), NaNO₃(1.00), ZnSO₄(0.50), 1% (w/v) pectin and agar (15g) in 1000ml phosphate buffer (pH6.2) IN 1000ml Erlenmeyer flask. The medium was sterilized by autoclaving and allowed to cool down to about 40°C. The medium was then dispensed aseptically into sterile petri dishes and allowed to set in a lamina flow. 0.1ml of each of the last enrichment medium was inoculated on each pectin agar plate and incubated at 30°C±2°C for 5 days. After 5 days, the plates were each flooded with 1% acetyl trimethyl ammonium bromide (CTAB) to observe the zones of clearance which indicates production of pectinase by isolates Nitinkumar and Bhushan (2010).

Identification of Selected Isolates

Pure culture of selected isolates was obtained by subculturing the isolates on Potato Dextrose Agar plates consistently until pure culture of each isolate was obtained and later transferred to PDA slants. The culture slants were kept after wards in the refrigerator at 4°C. Pure cultures were subcultured every 2 weeks to maintain isolates. A composite of cultural and morphological characteristics were used to identify the fungi. Cultural characteristics included morphology on culture plates such as mycelia growth, size, type, pigmentation (surface and reverse) and sporulation were used (www.dr.fungus.com).

Production of Pectinase by Selected Isolates

Collection and Preparation of Samples

Fruit peels (pineapple, water melon and orange) and spoilt vegetables were collected from Orita market, Ibadan, Oyo State, Nigeria. The samples were collected in clean, properly labeled, polythene bags and taken to the laboratory for further work. Each substrate was milled into about 10mm size and dried in the oven at 60°C until the weight was constant. 5g of each substrate medium was weighed into each 100ml Erlenmeyer flasks and each was moistened with 10ml of a moistening medium consisting of CuSO₄ 5H₂O (0.5), MnSO₄ 7H₂O(0.01), FeSO₄ 7H₂O(3.00), NaNO₃ (1.00), ZnSO₄ (0.50) in 1000ml of 0.1M phosphate buffer (pH6.2) in a 1000ml Erlenmeyer flask, covered with aluminum foil and sterilized by autoclaving at a temperature of 121°C, 15psi for 15minutes. After sterilization, each flask was allowed to cool to room temperature. The pure culture of each isolate on slants was homogenized carefully in 10 ml of sterile distilled water and 2ml of the obtained homogenate was introduced into each flask containing the substrate. The flasks were incubated at 30±2°C for

14 days and assay was done every other day throughout incubation period.

Assay for Crude Pectinase

Extraction of crude pectinase was done by simple contact method (Krishna *et al.*, 1996). 0.5% (w/v) of pectin was prepared in phosphate buffer (pH 6.2) as the enzyme substrate. 1ml each of the crude enzyme extract was added to 1ml of the pectin solution in each McCartney bottle and was left for 10 minutes at room temperature. 1ml of DNSA was added to the mixture thereafter reaction was stopped by adding 1ml of Rochelle's salt and boiled in water at 90°C for 5 minutes. Then the mixture was diluted by adding 2ml of phosphate buffer. The absorbance was measured Spectrophotometrically at 595 nm in a spectrophotometer (752W-UV-VIS grating spectrophotometer) (Miller, 1959). A standard graph was generated using standard glucose solution. One unit of Pectinase activity was defined as the amount of enzyme which liberated 1 μ m glucose per min.

Enzyme characterization

Effect of pH on pectinase activity

The effect of varied pH on the enzyme produced by each isolate was determined. Conical flasks containing buffer solution of varied pH (5.8, 6.0, 6.2, 6.4, 6.6, 6.8, 7.0, 7.2 and 7.4) were prepared and 0.5%(w/v) of pectin was dissolved in each of them. Each bottle containing the enzyme substrate mixture was incubated at 30° ± 2°C for 10 minutes. 1ml of 3, 5 dinitrosalicylic acid (DNSA) was later added to each mixture to terminate the reaction and later boiled for 5 minutes. The absorbance was read at 595nm using a spectrophotometer (752W UV-VIS Grating Spectrophotometer) (Miller, 1959).

Effect of temperature on pectinase activity

The effect of different temperature on the enzyme produced by each isolate was determined. 1 ml of crude pectinase was introduced into each bottle containing 1ml of 0.5% (w/v) pectin in Phosphate buffer (pH6.2). Each bottle containing the reaction mixture was incubated at varied temperatures of 35°C, 40°C, 45°C and 50°C. 1ml of 3, 5 dinitrosalicylic acid (DNSA) was added to each bottle containing enzyme-substrate mixture to terminate the reaction and later boiled for 5 minutes. The absorbance was read at 595nm using a spectrophotometer (752W UV-VIS Grating Spectrophotometer) (Miller, 1959).

Determination of Thermostability of Pectinase

Thermostability of the crude pectinase was determined by exposing the enzyme to temperature ranges of 45°C – 70°C at different durations of 0.5hour, 1 hour, 1.5 hours, 2 hours and 2.5 hours. After each exposure enzyme assay was carried out to determine the activity of the pectinases.

Effect of Different Concentration of Pectin on Enzyme Activity and Determination of K_m and V_{max} of Pectinase

The effect of different pectin concentration on the activities of the enzyme produced by the isolates was studied. Different concentrations (0.025%, 0.075%, 0.05%, 0.10%, 0.125% (w/v) pectin in phosphate buffer (pH6.2). 1ml of was introduced into each McCartney bottle containing each concentration of pectin and incubated at 30° ± 2°C for (10 minutes). Thereafter, 1ml of 3,5 dinitrosalicylic acid (DNSA) was added to each reaction mixture to terminate the reaction and later boiled for 5 minutes. The absorbance was read at 595nm using a spectrophotometer (752W UV-VIS Grating Spectrophotometer) (Miller, 1959).

Determination of Molecular Weight of Crude Pectinase

Molecular weight of the crude pectinase was determined by the method of Laemli, (1970).

RESULTS

Four fungal isolates that were able to degrade pectin were obtained from the degrading fruit and vegetable waste. Two isolates were however selected for further work after the screening test because they had higher relative pectinase activity. These isolates were identified as *Rhodotorulla spp* (a yeast) and *Mucor mucorales*. Results of the screening test were presented on Table 1. From the results shown on Figs. 1 and 2, all the substrates supported the production of pectinase by the isolates but the amount of pectinase produced varied on each substrate and by each isolate. Pectinase production was highest by *Rhodotorulla spp* on the 8th day (82.95U/g dry weight of substrate (dw)) in Orange peelings as shown on Fig. 1 while maximum pectinase was produced by *Mucor mucorales* on the 12th day (46.05U/g dry weight of substrate (dw))) on the same substrate (Fig. 2). The least production of the enzyme by both isolates was on the 2nd day of incubation (Figures 1 and 2).

Optimum activity of the pectinase produced by *Rhodotorulla spp* was at 35°C (58.7U/ml) while the least activity of the enzyme was at 60°C (9.01U/ml). Highest activity of pectinase produced by *Mucor mucorales* was at 45°C (49.3U/ml) while the lowest was at 30°C (30.7U/ml). Comparing the activities of the pectinase by the isolates, pectinase by *Mucor mucorales* was able to withstand higher temperature than that produced by *Rhodotorulla spp* (Fig. 3).

Pectinase by *Rhodotorulla spp* and *Mucor mucorales* had optimum activities at pH values of 6.0 and 5.8 respectively and the least for each of them were pH values of 7.4 with activities of 25.6U/ml and 8.3U/ml respectively as shown on Figure 4.

Substrate concentration of 0.5% (w/v) prompted the highest activity of the pectinase produced by *Mucor mucorales* and *Rhodotorulla spp* respectively while the least was at concentrations of 1.0% (w/v) for *Mucor mucorales* and 0.75% (w/v) for *Rhodotorulla spp*. This is presented on Figure 5. The maximum velocity for pectinase by *Mucor mucorales* was 0.043 U/mg/min while that of *Rhodotorulla spp* was 0.023 U/mg/min Michaelis Menten constant for pectinase by *Mucor mucorales* was 15.0 mg/ml while it is 3.5 mg/ml for *Rhodotorulla spp* as obtained from the double reciprocal curve plotted for the enzymes (Figure 6).

The molecular weight profiles of the crude pectinases from the isolates were 90kDa *Rhodotorulla spp* and 35, 45 and 60 kDa for *Mucor mucorales* as shown on Plate 1.

Discussion

Fungal isolates used in this work for the production of pectinase were *Mucor mucorales* and *Rhodotorulla spp*. Fungal isolates have been successfully used for the production of pectinase by different researchers (Silva *et al.*, 2002; Suresh and Viruthagiri, 2010; Banu *et al.*, 2010), infact the most common source of microbial pectinase is from *Aspergillus niger* (Castilhoa *et al.*, 1999). Agro industrial wastes have been considered as for solid state fermentation and production of microbial enzymes because they serve as cheaper substrates for microbes (Pandey *et al.*, 2002). Orange bargaisse and other fruit waste have been used by other researchers as substrate for the production of microbial enzymes and in all cases have served as good sources of the substrate needed to induce the synthesis of such enzymes (Silva *et al.*, 2002; Seyis and Aksoz, 2005). The composition of the different substrates used may have affected the amount of pectinase produced by each of the isolates. On the other hand, the consistency of the substrate particles may have interfered in their packing during the fermentation thereby affecting the gas and heat exchange within the system (Mitchell *et al.*, 2000). Low pectinase production observed in both isolates on all the substrates on the 2nd and forth days may be because the microorganisms were trying to adapt to the new substrates as sources of carbon since they were subcultured from PDA slants whose carbon source is simpler than the new substrates. The relatively low production of pectinase by these isolates after an optimum production on the 8th day for *Rhodotorulla spp* and 12th day for *Mucor mucorales* may be because fungal pectinase are subject to catabolite repression by high free sugar concentration affecting inducible and constitutive enzymes (Aguillar & Huitron, 1987 and Guevara *et al.*, 1997).

Silva *et al.*, (2002) found orange bargaisse to be the best substrate for polygalacturonase production by

Penicillium sp which is in agreement with what was obtained in this work in which orange peelings supported best pectinase production by *Rhodotorulla spp*. In the production of alkaline pectinase by bacteria isolated from decomposing fruit materials, bacterial isolate from orange waste showed the maximum pectinase enzyme activity (Bhardwaj *et al* 2010). This may be because orange peels have very high pectin content and is more acidic than the other substrates making it a conducive condition for yeasts (Okafor, 2007). The optimum production of pectinase by the bacteria isolate was found to be at 72 hours in submerged fermentation (Bhardwaj *et al* 2010) which is in variance with what was obtained in this work.

Every enzyme has its optimal activity and stability up to a certain temperature and get denatured at higher temperature (Lehninger *et al.*, 1992). Optimum temperature of pectinase produced by *Mucor mucorales* was 45°C while that of *Rhodotorulla spp* was 35°C, while this results agrees with that of some researchers it is in various with that of others. In screening of pectinase producing bacteria and their efficiency in biopulping of paper mulberry bark (Poonpairaj *et al.*, 2001), pectinase activity from bacterial strains N05 and N10 had the optimum activity at pH 10 and temperature 35°C. Blanco *et al.*, (1999) reported that some yeasts (*Tephrosia candida* and *Kluyveromyces fragilis*) produced pectinase with maximal activities at temperatures up to 60°C.

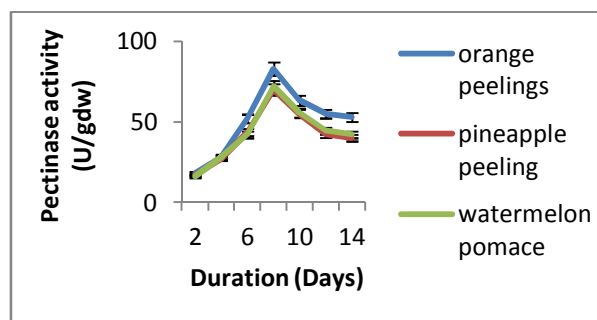


Figure1:Time course for the production of pectinase by *Rhodotorulla spp*

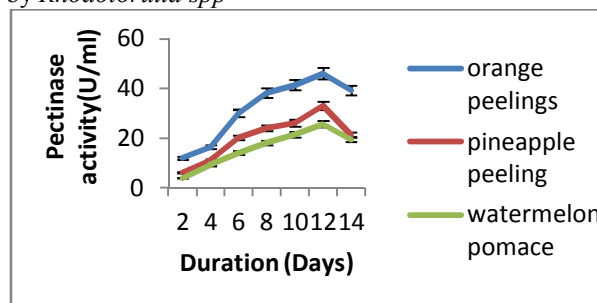


Figure2:Time course for the production of pectinase by *Mucor mucorales*

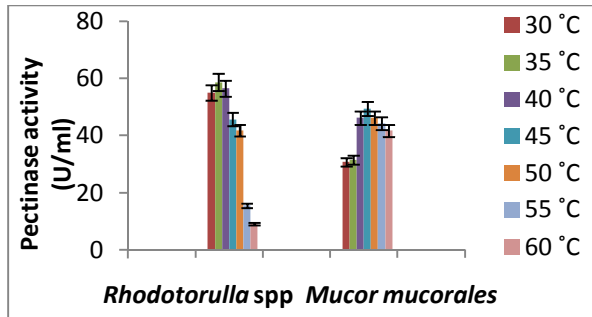


Figure 3: Effect of temperature on pectinase by the fungi

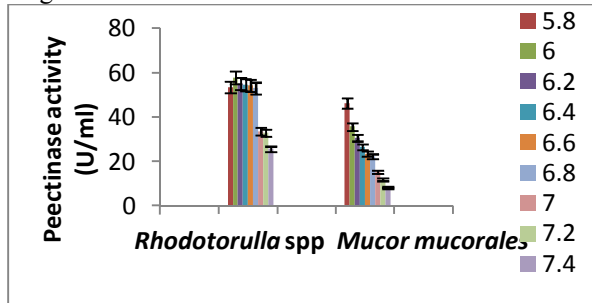


Figure 4: Effect of different pH on pectinase by the fungi

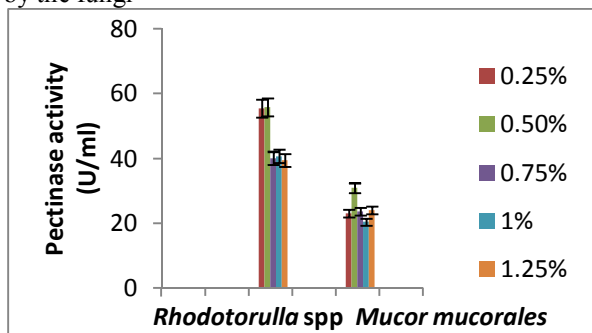


Figure 5: Effect of different substrate concentration on pectinase by the fungi

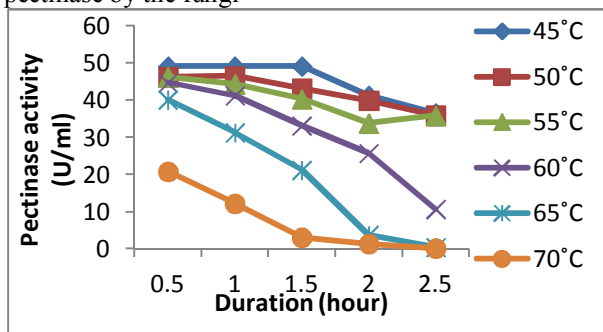


Figure 8: Thermostability of pectinase synthesized by *Mucor mucorales*

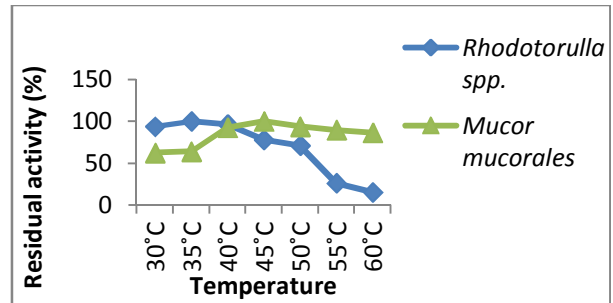


Figure 6: Residual pectinase activity of isolates at varied temperature

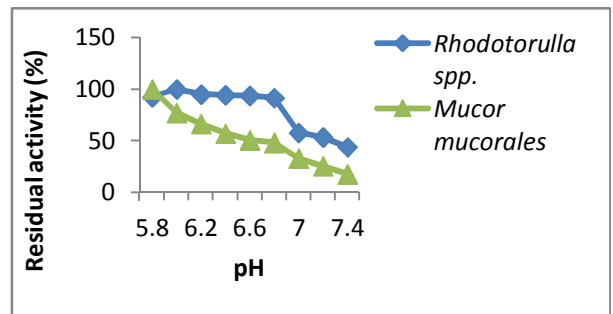


Figure 7: Residual pectinase activity of isolates at different pH

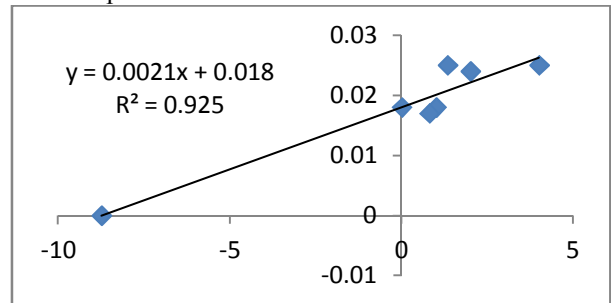


Figure 8: Lineweaver-Burk graph showing reciprocal of K_m and V_{max} values of pectinase by *Rhodotorulla spp*.

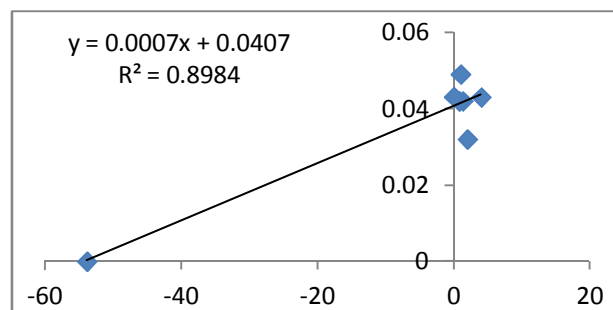


Figure 9: Lineweaver-Burk graph showing reciprocal of K_m and V_{max} values of pectinase by *Mucor mucorales*.

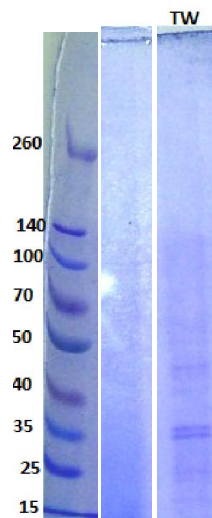


Figure 10: Molecular weight of pectinases produced by *Rhodotorulla spp* and *Mucor mucorales*
KEY:

First Lane: Molecular weight Marker

Second Lane: Pectinase by *Rhodotorulla spp*.

Third Lane: Pectinase by *Mucor mucorales*

Also Kluskens *et al.*, (2005) reported the optimal temperature for a polygalacturonase from *Streptomyces sp.* QG- at 60°C and the hyperthermophilic bacterium *Thermotoga maritima* was at 80°C. One of the pectinase characterized in this work had its maximum activity at 35°C which is within the mesophilic range since the isolate is generally a mesophilic fungus (Alexopoulos *et al.*, 1980).

Marcia *et al.*, (1999) recorded a maximum activity of the pectinase produced by *Penicillium spp.* to be at pH 6.0, Pereira *et al.*, 2002 also reported that the optimum pH for polygalacturonase production by *Penicillium sp* was found to be at pH 6 these results are within the range of the pectinase produced by *Rhodotorulla spp* which had maximum activity at pH 6.0. However Banu *et al.*, 2010 obtained a result that is a little deviated from the result obtained in this work recording maximum activity at pH6.5 for *Penicillium spp* they worked with. Much work has not been done on production of pectinase by *Mucor spp.* and *Rhodotorulla spp.* Maximal activity of pectinase synthesized by *Mucor mucorales* used in this work was at pH 5.8, Alexopoulos *et al.*, (1980) reported that fungi prefer acidic pH range for growth and so the enzymes synthesized by them maintain activity within this range too. This result is however close to what was obtained in this study and. Fungal pectinase with high affinity for alkali pH had however been reported by Denis *et al.*, (2005) in *P. viridicatum* with maximal activity at pH 8.0 and able to maintain 80% of its activity at pH 9.

Reda *et al.*, 2008, reported the maximum production of pectinase from *Bacillus sp* at pectin concentration of 1% while Pereira *et al.*, 2002, reported the optimum pectinase production from *Penicillium sp* obtained from soil at pectin concentration of 1.5%. Both results were in variance with results obtained in this work.

Conclusion

The exploration of microbial biodiversity in decaying plant matter has allowed, especially in recent years, to identify and characterize new pectin-enzyme-producing microorganisms. Also, it has been technically possible on the one hand to select wild strains and constitutive mutants that produce a single enzyme, and on the other hand, the heterologous expression in bacteria and yeast of numerous genes which encode pectin enzymes, obtaining producing strains of interest.

The result from this research work shows that pectinase was produced by *Rhodotorulla spp* and *Mucor spp* used in this work. *Mucor spp* produced pectinase at an optimum temperature of 45 °C which is a desirable property for microbial pectinase that is targeted for industrial use. This is because most of the industrial processes in which these pectinases are used are carried out under these environmental conditions. Thus, the pectinase obtained from this work has a potential for industrial use. It therefore obvious from the results obtained in this work that vegetable and fruit waste can be used as substrate for solid state production of microbial pectinase which can be a source of income for Nigeria if more work is done on the economics of the production process by optimizing the production conditions.

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