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Comparative Studies on Pectinases obtained from *Aspergillus fumigatus* and *aspergillus niger* in Submerged Fermentation System using Pectin Extracted from Mango, Orange and Pineapple Peels as Carbon Sources.

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Abstract
Pectinase was produced from *Aspergillus species* (*A. fumigatus,* and *A. niger*) in a submerged fermentation system after 4 and 5 days of fermentation, respectively using pectin extracted from different agro-wastes (mango, orange and pineapple peels) as the carbon sources. The pectin was extracted from mango, orange and pineapplepeels with extractionyields of 17%, 15.5% and 8.33%, respectively. The specific activitiesof the crude pectinases using mango, orange and pineapplepectin extract were found to be 11.20U/mg, 52.9U/mg and 11.14U/mg protein, respectively. The specific activities of precipitated and dialyzed pectinase from *A fumigates* were found to be 11.78U/mg and 34.60U/mg, respectively, whilethatfor the enzyme from *A niger* using orange and pineapple pectin extract were found to be 33.56U/mg, 92.08U/mg, and 17.26U/mg, 49.42U/mgprotein,respectively. The optimum pH for pectinase from *A. Fumigates* (mango)and *A. niger* (orange) were found to be 5.5 and 5, respectively with common optimum temperature of 40˚C while pectinase from *A. niger* (pineapple) had optimum activity at 55˚C, pH 5.5. The maximum velocity, V_{max} and Michealis constant, K_m obtained from Line weaver-Burk plots of initial velocity data at different concentrations of pectin were found to be625µmol/min and 45.5mg/ml for pectinase from *A fumigatus*, 200 µmol/min and 18mg/ml for pectinase from *A. niger* on orange pectin and 5000µmol/min and 89.5mg/ml for pectinase from *A. niger* using pineapple pectin, respectively. The result suggests that peels of mango, orange and pineapple can be used for value added synthesis of pectinase, an important enzyme in fruit juice clarification.

Keywords: Agro-wastes, pectinase, pectin, *Aspergillus fumigatus*, *Aspergillus niger*, submerged fermentation, partial purification and characterization

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Introduction

Pectin is high molecular weight acid polysaccharide, primarily made up of α-(1-4) linked D-galacturonic acid residues with a small number of rhamnose residues in the main chain and arabinose, galactose and xylose on its side chain (Galiotou-Panayotou*et al.,* 1993). Pectin is found in the plant cell wall where it contributes to cell wall rigidity (Steven *et al.,* 2000; Zykwinska *et al*., 2005). Three major pecticpolyssacharide groups are recognized with all containing D-galacturonic acid to a greater or a lesser extent (Rouse and Crandall, 1976). They are homogalacturonan (HG) which is a linear polymer formed by D-galacturonic acid which can be acetylated and/or methyl esterified.Rhamnogalacturonan I (RG I) is composed of the repeating disaccharide rhamnose-galacturonic acid. Rhamnogalacturonan II **(**RGII) is a homogalacturonan chain with complex side chains attached to the galacturonic residues. Industrially extracted pectin is used for pectinase production using *Aspergillus species.* Pectinase is a generic name

for a family of enzymes that catalyze hydrolysis of the glycosidic bonds in the pectic polymers (Yogesh *et al*., 2009; Vivek *et al*., 2010). Pectinases are classified into Pectin Methyl Esterases (PME) or pectinesterases, Polymethylgalacturonases (PMG), Polygalacturonases (PG), Pectatelyases (PGL) and Pectin Lyases (PL) (McCready, 1970, Daniel, 2009, Eleonora*et al.,* 2009). Pectinase is sourced from different genera of bacteria, yeasts and moulds but *Erwinia, Bacillus, Saccharomyces, Kluyveromyces, Aspergillus, Penicillium, Fusarium* and *Rhizopus*are the genera most frequently used over the years, with strains of *Aspergillus*, *Penicillium* and *Erwinia*mainly used for enzyme production studies. The choice for microbial source for pectinase production depends on the type of culture required for their production, (solid-state or submerged fermentation), number and type of the produced pectinases (esterases, hydrolytic depolymerases and eliminative depolymerases), pH and thermal stability of the enzymes, and genotypic characteristic of the strain (wild type, mutagenized strain, and homologous or heterologous recombination) (Chadha *et al.,* 2005; Ernesto *et al.,* 2006; Reda *et al*., 2008;Yogesh *et al.,* 2009;Eleonora *et al.,* 2009; Pilanee *et al.,* 2010; Bhaskara *et al.,* 2011, Udenwobele *et al*., 2014). Pectinases are applied in several conventional industrial processes, such as textile, plant fibre processing, oil extraction, treatment of industrial waste water, containing pectinacious material etc. Pectinases have also been reported to work on purification of viruses and in making of paper (Reena *et al.,* 2005). The frequent use of fruits such as mango, orange and pineapple for production of juices, nectars, concentrates, jams, jelly powders and flakes generate lots of wastes in the form of peel wastes and seed kernels which could bring about environmental pollution (Bali, 2003; Ian, 2006; Fowomola, 2010; Ashoush and Gadallah, 2011). The current study is focused on harnessing the wastes generated from fruits juice industry in the form of peels into pectin. The pectins extracted from these peels were used as the only carbon source for *Aspergillus fumigates* and *Aspergillus niger* in submerged fermentation system for pectinase production. The pectinase activities were compared by determining their optima pH, temperature, and concentration for industrial applications.

Materials and Methods

Collection and processing of sample: Mango, orange and pineapple were collected from Obollo market in Udenu L.G.A. and Ogige market, in Nsukka L.G.A, of Enugu State. They were washed with water, peeled and treated with 96% ethanol. These peels were washed again with water and dried under the sun for 7 days. The dried peels were ground to powder with a milling machine and stored inside a clean container at room temperature for extraction.

Extraction of Pectin: Pectin was extracted using the method described by McCready (1970). A quantity 100g of ground peels were weighed into a 2000ml beaker containing 800ml of distilled water. Freshly ground sodium hexametaphosphate (12g) was added and the initial pH was adjusted with 3N HCl to 2.2 ± 0.1. The mixture was heated in a water bath at 70°C for 1hr and stirred with a propeller typed stirrer and the pH checked at intervals of 15min. The extract was vacuum filtered through a muslin cloth and the residue was washed with 200ml of distilled water, and the washings were added to the filtrate. The filtrate was concentrated by evaporation on a hot plate to approximately 1/5 of the initial volume. The concentrated pectin was cooled to 50°C and poured into a volume of ethanol in the ratio of 1:3 the ethanol contained 0.5M HCl. The mixture was stirred for 30min and allowed to stand for 1 hr. The precipitate was vacuum filtered and washed with ethanol-HCl (0.5M) solution. The extract was further washed with acetone to remove traces of HCL and ethanol. The extract was dried in an oven at 40°C for few hr to constant weight and ground finely.

$$\%\ \text{yield} = \frac{\text{mass of extracted pectin (g)}}{\text{total mass of ground peels(g)}} \text{x100}$$

Media for Isolation of Microorganisms: Soil samples collected from the site of decaying mango, orange and pineapple peels were pooled together and homogenized in sterile media containing 1% pectin; 0.14% $(NH_4)_2\ SO_4$, 0.2% K_2HPO_4, 0.02% $MgSO_4.7H_2O$, 0.1% nutrient solution containing; 5g/l $FeSO_4.7\ H_2O$, 1.6mg/l $MnSO_4.H_2O$, 1.4mg/l $ZnSO_4.7H_2O$, and 2.0mg/l $CoCl_2$. The mixtures were incubated

at 30^{o}C for 24 hr. Solid media containing1% mango, orange and pineapple pectins, 0.14% $(NH_4)_2SO_4$, 0.2% K_2HPO_4, 0.02% $MgSO_4.7H_2O$, 0.1% nutrient solution containing; 5mg/l $FeSO_4.7\ H_2O$, 1.6mg/l $MnSO_4.H_2O$, 1.4mg/l $ZnSO_4.7H_2O$, 2.0mg/l $CoCl_2$ and 3% agar-agar (the gelling agent) (w/v) were prepared and used to enhance physical identification of the *Aspergillus strains* present and to fully acclimatize the organisms to the use of pectin as carbon source. The media were autoclaved at 121^{o}C, 15psi for 15min. They were allowed to cool to 45^{o}C and then poured into Petri dishes and allowed to gel. The plates were then incubated in a B & T Trimline incubator at 37^{o}C overnight to check for sterility. A loop of homogenized extract from the liquid media was streaked onto the solid media under the flame of Bunsen burner. The plates were thereafter incubated at 35^{o}C till visible colonies were observed. All morphological contrasting colonies were purified by repeated streaking and sub-culturing on separate plates. This process was continued till pure fungal cultures were obtained. Pure fungal isolates were then maintained on potato dextrose agar (PDA) slants as stock cultures. PDA media were prepared according to the manufacture's description. Three day old pure cultures were examined. The colour, texture, nature of mycelia or spores and growth patterns were also observed. The three day old pure cultures were used in preparing microscopic slides. Identification was carried out by relating features and the micrographs to "Atlas of mycology" by Barnett and Hunter (1972). Submerged fermentation (SmF) technique was also employed using 250ml Erlenmeyer flasks containing 100ml of sterile cultivation media optimized for pectinase with 0.1% NH_4NO_3, 0.1% $NH_4\ H_2PO_4$, 0.1% $MgSO_4.7H_2O$and 1% mango, orange or pineapple pectin. The flasks were stoppered with aluminium foil and autoclaved at 121^{o}C 15psi for 15min.From the PDA slants, fresh plates were prepared and three day old cultures were used to inoculate the flasks. In every sterile flask, two discs of the respective fungal isolates were added using a cork borer of diameter 10mm and then plugged properly. The cultures were incubated for 4 days at room temperature (30^{o}C) using electrical shaker. At the fourth day of fermentation, mycelia biomass were separated by filtration and the filtrates were analyzed for pectinase activity and extracellular protein concentration.

Pectinase Assay: Pectinase activity was evaluated by assaying for polgalacturonase (PG) activity of the enzyme. This was achieved by measuring the release of reducing groups from mango pectin using a modification of the 3, 5-dinitrosalicylic acid (DNS) reagent assay method described by Miller (1959) as contained in Wang *et al*.(1997) with little modifications. The reaction mixture containing 0.5ml of 0.5% pectin in 0.05M sodium acetate buffer of pH 5.0 and 0.5ml of enzyme solution were incubated for 1 hour. DNS reagent (1 ml) was added and the reaction was stopped by incubating the mixture in a boiling water bath for 10min. The mixture volume was made up to 4ml with 1ml of Rochelle salt solution and 1ml of distilled water. The reaction mixture was allowed to cool and then the absorbance read using a JENWAY 6405 UV/VIS spectrophotometer (Beckman/Instruments, Inc., Huston Texas) at 575nm. One unit of enzyme activity was defined as the amount of enzyme that catalyses the release of one micromole of galacturonic acid(a reducing sugar) per min.

Protein determination: Protein content was determined by the method of *Lowry et al*. (1951) using bovine serum albumin as protein standard as contained in Udenwobele *et al.* (2014)

Ammonium Sulphate Precipitation: Ammonium sulphate precipitation was carried out at 80% ammonium sulphate saturation. This was allowed to stand at 4^{o}C for 30h. The precipitate was centrifuged using Cole-palmer VS-13000 microcentrifuge at 3500 rpm for 30min and pellets re-dissolved in 10ml of 0.05M acetate buffer pH 5.0. The pectinase activity was assayed using the method by Miller, (1959) and protein concentration by Lowry *et al*. (1951).

Dialysis: The ammonium sulphate precipitated protein was dialyzed for 12h against 0.05M sodium acetate buffer, pH 5.0. The dialyzed enzyme was also assayed for pectinase activity (Miller, 1959) and protein concentration (Lowry *et al*., 1951).

Effect of pH on Pectinase Activity: The effect of pH on the pectinase activity was determined at different pH values in 0.05M sodium acetate buffer (pH 3.5 - 5.5), phosphate buffer (pH 6.0 - 7.5) and Tris-HCl buffer (pH 8.0 - 9.5) and the enzyme activity was assayed as described above using pectin as the substrate.

Effect of Temperature on Pectinase Activity: The effect of temperature on pectinase activity was determined at various temperatures from 25-70^{o}C interval of 5^{o}C for 1h at the optimum pH5.5and the enzyme activity was assayed as described above using pectin as the substrate.

Effect of Substrate Concentration on Pectinase Activity: The enzyme activity was also determined at different substrate concentration from 5-50 mg/ml at pH5.5 and temperature 40˚C. The V_{max} and K_m values of the enzyme were determined using the double reciprocal plot of initial velocity data.

Results and Discussion

Pectin Extraction: Pectin was extracted from peels obtained from mango, orange and pineapple at pH 2.2, 70˚C for 1h with extraction yields of 17%, 15.5% and 8.33%, respectively. Extraction conditions of pH 1.5 to 3.0 and temperature of 60 to 100^{o}C for 0.5 to 6 hr are required for pectin extraction (Sharma *et al*., 2006).This result indicates that mango, orange and pineapple peels contain appreciable amount of pectin although mango peels was found to have highest pectin content follow by orange peels while pineapple peels had the least. The pectin was used as inducer for the production of pectinolytic enzymes. The selection of the peels for this study was not only based on the pectin content but also on the following factors; they represent one of the cheapest agro-industrial wastes, they are available at any time of the year, their storage constitutes no problem in comparison with other substrate. *Aspergillus sp*ecies were first isolated from decomposing agro-wastes and then sub-cultured to obtain the pure culture utilized in the submerged fermentation. *A. fumigatus* grew better with the mango extract, while *Aniger* utilized orange and pineapple extract and grew equally well.

Pectinase production: Three pectinolytic fungi, *Aspergillusniger, Aspergillusfumigatus* and *Aspergillusflavus* were isolated from natural source and 2.5L of crude pectinase was each produced from*Aspergillusfumigatus* and *Aspergillusniger* in submerged fermentation since the two organisms showed higher pectinolytic yield. The specific activity of the crude enzyme from *A. fumigatus* using mango pectin extract was found to be 11.20U/mg , while pectinase from *A. niger* using orange pectin extract gave specific activity of 52.94U/mg. Pectinase from*A niger* using pineapple pectin extract had specific activity of 11.14U/mg (Table 1). This shows that pectinase obtained from *A. niger* using pectin from orange peels exhibited the highest activity when compared with the values obtained for pectinases obtained from *Aspergillus fumigatus* and *Aspergillus niger* using pectin obtained from mango and pineapple peels, respectively.Pectinases obtained were subjected to80% ammonium sulphate saturation and subsequent dialysis and the activities were observed to increase as purification level increases (Table 1). The partially purified enzymes were characterized based on the effects of pH, temperature and substrate concentration.

Figure 1 shows that as pH increased from pH 3.5 to pH 5.5, the pectinase activity increased. Further increase in pH beyond 5.5, decreased pectinase activity making 5.5 the optimum pH for pectinases obtained using mango and pineapple pectin as carbon sources. Also, the optimum pH for pectinases obtained using orange pectin as the carbon source was found to be5.0. Similar result was also obtained by Jayani *et al*., (2005) for polygalacturonase from *Aspergillus awamori*, *Thermococcus auraniacus* and *Penicillium frequentans*. Ramakrishna *et al*., (1982) also reported that the optimum pH for pectinase activity from thermotolerant *Aspergillus sp*. N12 was 5.5.These results suggest that the enzymes do very well at pH range 5.0 to 5.5 and could be applied industrially at this pH range.

The optimum temperature for pectinase obtained from *Aspergillus fumigatus* using mango pectin as carbon source was found to be 40°C, while that from *A niger*using orange and pineapple pectin as carbon source) werefound to be 40˚C and 55˚C, respectively (Figure 2). The increase in the enzyme activity as temperature increased may be as a result of change in the enzyme conformation which brings the essential residues to close proximity for catalysis. The decrease in the activity could be as a result of thermal denaturation at high temperature.Similar results were also reported for polygalacturonase by *Aspergillus awamori* and *Aspergillus niger* (Jayani*et al*., 2005). Exo-polygalacturonase from *Monascus* and *Aspergillus* sp (Freitas *et al.,* 2006) exhibited maximum activity at 60 and 50°C. The endopolygalacturonase from *Mucourrou xii* NRRL 1894 exhibited maximum activity at 35°C (Saad*et al*., 2007).The maximum velocity, V_{max} and Michealis constant, K_m obtained from Lineweaver-Burk plots of initial velocity data at different concentrations of the pectin were 625µmol/min and 45.5mg/ml for (pectinase from *A fumigatus* using mango pectin extract), 200µmol/min and 18mg/ml for pectinase from *A. niger* (using orange pectin extract), 5000µmol/min and 89.5mg/ml for *A.niger* (using pineapple pectin

Table 1: Purification Table of pectinases obtained from *Aspergillus fumigatus* (using pectin from mango peels) and *Aspergillus niger* (using orange and pineapple peels) as the carbon source, respectively.

Enzyme	Volume (ml)	Protein (mg/ml)	Activity (µmol/min)	Total activity	Specific Activity (U/mg)	Purification fold
Crude						
Mango	1000.0	1.372	15.36	15360.00	11.20	1.00
Orange	1000.0	0.972	25.73	25730.00	26.47	1.00
Pineapple	1000.0	10.420	116.12	116120.00	11.14	1.00
$(NH_4)_2SO_4$						
Mango	65.0	6.206	73.08	4750.20	11.78	1.19
Orange	62.0	2.523	72.63	4503.06	28.78	1.08
Pineapple	64.0	15.820	272.98	17470.72	17.26	1.55
Dialyzed enzyme						
Mango	72.2	4.030	139.47	10032.00	34.60	2.90
Orange	70.0	2.083	95.86	6710.20	46.02	1.74
Pineapple	76.0	12.840	634.56	48226.56	49.42	4.43

µmole/min= Unit (U)

extract) (Figure 3). Banu*et al*. (2010) reported a K_m of 1.0mg/ml and $V_{max}$of 85U for pectinase isolated from *Penicillium chrysogenum*. K_m and V_{max} obtained in this work are comparable with those of above mentioned authors. The only difference may be as a result of environmental conditions. V_{max} and K_m indicates the level of affinity the enzyme has forits substrate. The pectinase from mango culture was used to degrade pectin extracted from orange and pineapple peels and its specific activities were 30U/mg and 28.39U/mg, respectively compared to 34.60U/mg which is the specific activity obtained when pectin extracted from dry mango peels was used. This may indicate why it is pertinent to use pectinase obtained from *A. fumigatus* (using mango pectin extract) to clarify mango juice and that from *A. niger* (using pectin from orange and pineapple peels) to clarify orange and pineapple juice, respectively.

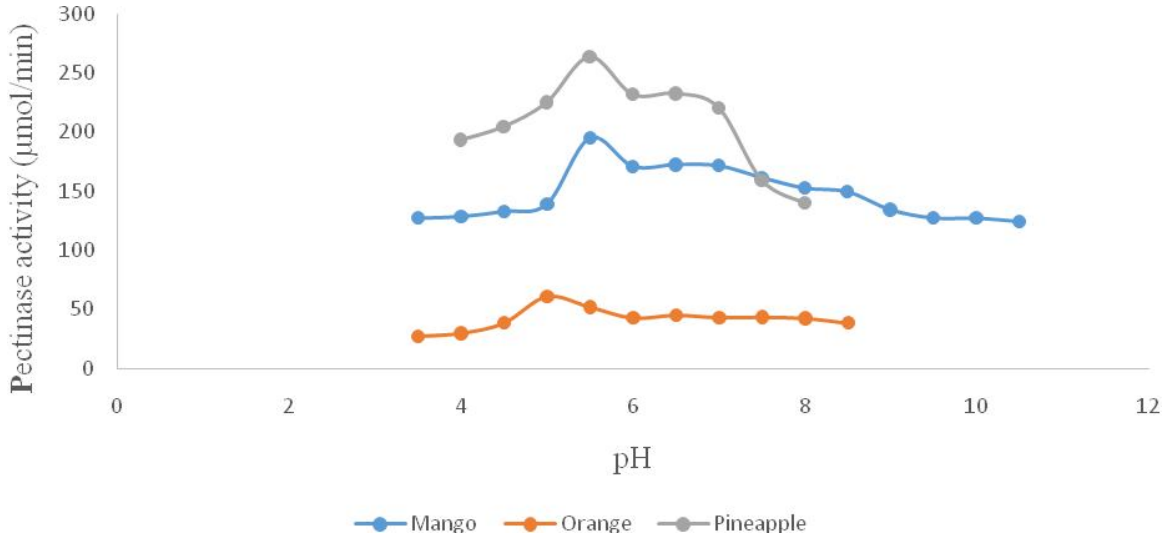

Figure 1: Effect of pH on activity of pectinases obtained from *Aspergillus fumigatus* (using pectin from mango peels) and *Aspergillus niger* (using orange and pineapple peels) as the carbon source, respectively.

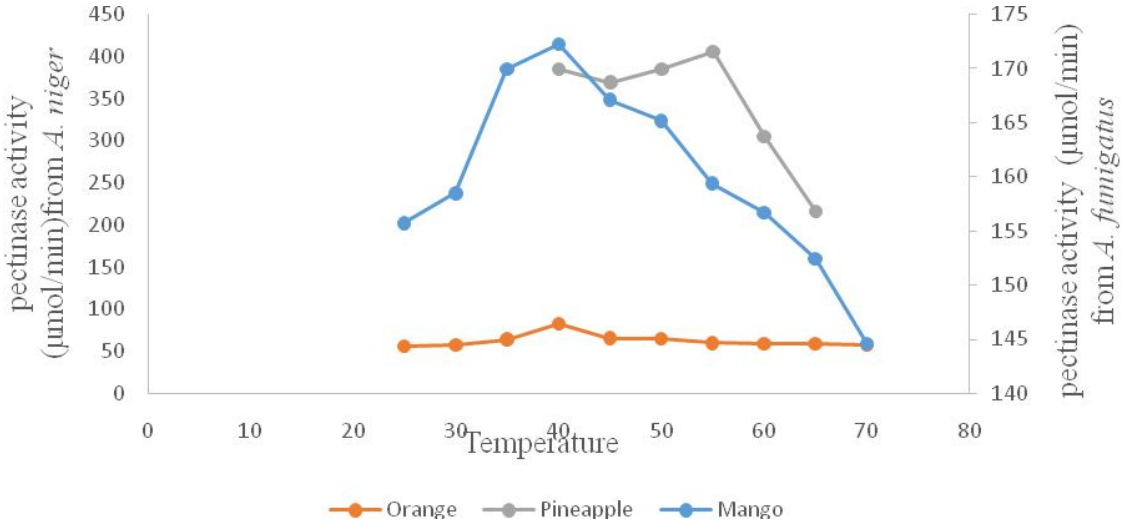

Figure 2: Effect of temperature on activity of pectinases obtained from *Aspergillus fumigatus* (using pectin from mango peels) and *Aspergillus niger* (using orange and pineapple peels) as the carbon source, respectively.

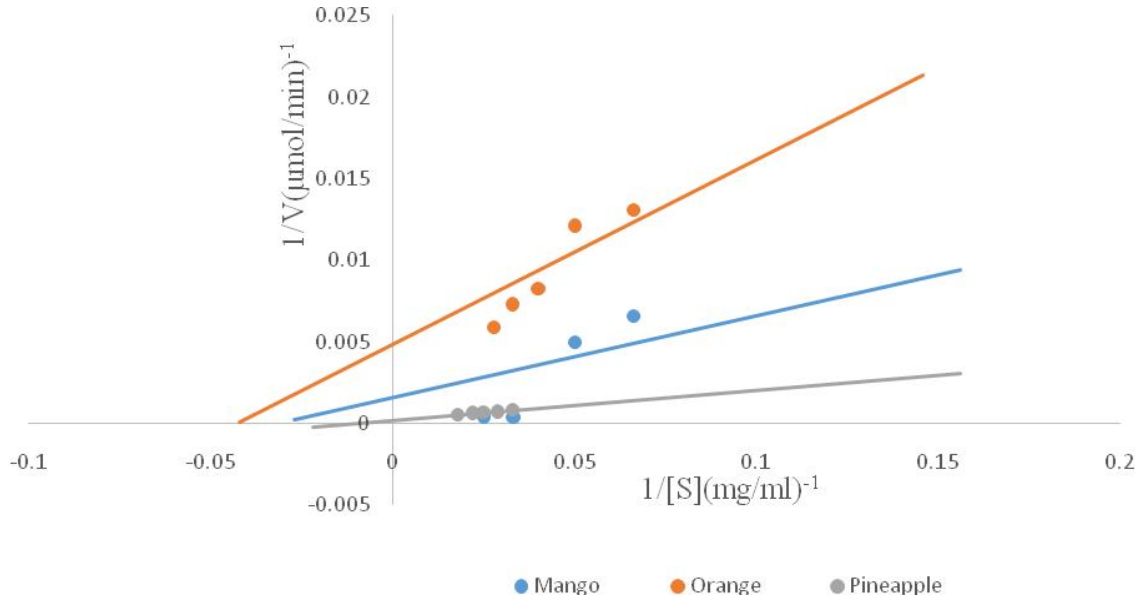

Figure 3: Lineweaver-Burk plot of initial velocity data for pectinases obtained from *Aspergillus fumigatus* (using pectin from mango peels)and *Aspergillus niger* (using orange and pineapple peels) as the carbon source, respectively.

Conclusion

The result suggests that mango, orange and pineapple peels can be used for value added synthesis of pectin which can be used to induce the production of pectinase (by *Aspergillus* strains), an important enzyme in fruit juice clarification and bio-washing of textile materials. Also, the enzymes obtained in this research have noble properties such as temperature, pH stability and high affinity for pectin extracted from local source which made them useful for biotechnological application. This research has demonstrated a simple way of converting waste to wealth.

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