CHARACTERISATION OF POLYGALACTURONASE PRODUCED BY
***ASPERGILLUS AWAMORI* CICC 2040 ON SELECTED PRETREATED FRUIT**
PEELS AND ITS APPLICATION IN JUICE PROCESSING

BY

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ABSTRACT

Utilisation of orange and plantain peels as substrates for production of polygalacturonase is increasing due to their high pectin content and availability. However, high ligno-cellulosic content of these peels limits their bio-conversion. Pretreatment of the peels for improved bio-conversion is sparsely reported. This study was therefore designed to evaluate effects of alkaline and microwave pretreatments of orange and plantain peels on the activity of polygalacturonase produced from *Aspergillus awamori* CICC 2040, and its applications in juice extraction and clarification.

Orange (*Citrus sinensis* L.) and plantain (*Musa paradisiaca* Linn.) peels were oven-dried at 60 °C for 48 h and milled into powders. The powders were subjected to alkaline and microwave pretreatments separately, using response surface methodology. Factors interacted for alkaline pretreatment included Particle Size, PS (<0.4250, 0.4250<PS<0.8025 and 0.8025<PS<1.1800 mm), NaOH molarity (0.010, 0.055 and 0.100 M) and time (1.0, 6.5 and 12.0 h), while those for microwave pretreatment were PS (<0.4250, 0.4250<PS<0.8025 and 0.8025<PS<1.1800 mm), microwave power (240, 480 and 720 W) and time (2.50, 6.25 and 10.00 min). These factors were interacted to determine combinations for Maximum Polygalacturonase Activity (MPA). Combinations that gave MPA from alkaline and microwave treatments were also interacted (combined treatment). Pretreated and untreated (control) powders were inoculated with 10^6 spore/mL *Aspergillus awamori,* incubated at 28 °C for 5 days, and crude polygalacturonase was extracted and its activity determined. Crude polygalacturonase was purified with activated charcoal (0.5%, w/v). Molecular weight, activation energy, catalytic and substrate affinity constants of crude and purified polygalacturonase and their activities at different pH (3-12) and temperature (25-75 °C) were determined. Applications of crude and purified polygalacturonase in mango juice extraction and apple juice clarification were evaluated and compared with a commercial polygalacturonase. Yield and Flow Behaviour Index (FBI) of mango juice, and colour intensity of apple juice were determined. Data were analysed using descriptive statistics and ANOVA at $\alpha_{0.05}$.

Alkaline pretreatment combinations that gave MPA were <0.4250 mm, 0.100 M and 1.0h, and 0.8025<PS<1.1800 mm, 0.010 M and 1.0h for orange and plantain peels, respectively. Same microwave pretreatment combination, 0.8025<PS<1.1800 mm, 720 W and 10.00 min gave MPA for orange and plantain peels. Activity of crude polygalacturonase obtained for the control, alkaline, microwave, and combined alkaline and microwave pretreated samples were 25.17, 38.46, 26.21 and 42.05 U/mL; and 26.04, 38.82, 26.72 and 44.98 U/mL for orange and plantain peels, respectively. Molecular weight, activation energy, catalytic and substrate affinity constants for crude polygalacturonase were 20.00-96.20 kDa, 2.26-3.07 kJ/mol, 29.41-40.00 U/mL and 0.206-0.320 mg/L, respectively, and 30.00-31.00 kDa, 1.23-2.15 kJ/mol, 55.55-90.91 U/mL and 0.722-0.909 mg/mL for purified polygalacturonase. Optimum pH and temperature for crude polygalacturonase were 5.5 and 55°C, respectively, and 4.5 and 40°C for purified polygalacturonase. Yield of mango juice was 58.20-87.60% and FBI was 0.377-0.627. Colour intensity of apple juice was 37.89±0.04-42.61±0.01. Yield of mango juice and colour intensity of apple juice increased significantly following treatment with polygalacturonase.

Alkaline and microwave pretreatments of orange and plantain peels enhanced the production of polygalacturonase by *Aspergillus awamori*. Polygalacturonase was effective for juice extraction and clarification.

Keywords: *Aspergillus awamori* CICC 2040, Fruit peels, Juice processing, Polygalacturonase

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DEDICATION

This work is dedicated to my late father, Mr. John Obisesan Ishola Adedeji for teaching me the principles of self-reliance and hard work.

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CERTIFICATION

I certify that this work was carried out by Mr. O.E. Adedeji in the Department of Food Technology, University of Ibadan.

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ACRONYMS AND ABBREVIATIONS

SSF	Solid state fermentation
SmF	Submerged fermentation
PG	Polygalacturonase
GRAS	Generally regarded as safe
MW	Microwave radiation
OPF	Orange peel flour
PPF	Plantain peel flour
FCCCD	Face-centered central composite design
RSM	Response surface methodology
CICC	China Centre of Industrial Culture Collection
MEA	Malt extract agar
v	Velocity of reaction
s	Substrate concentration
Vmax	Kinetic constant
Km	Kinetic constant
kd	Inactivation rate constant
ko	Arrhenius constant
Ea	Activation energy
T	Temperature
K_B	Boltzmann's constant
h	Plank's constant
ΔH	Enthalpy of activation
ΔG	Free energy of activation
ΔS	Entropy
SEM	Scanning electron microscopy
FTIR	Fourier transform infrared spectroscopy
SDSPAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
UV/VIS	Ultra violet/visible
BSA	Bovine serum albumin

ΔC	Chromaticity
ΔE	Colour intensity
τ	Shear stress
γ	Shear rate
n	Flow behaviour index
K	Consistency index
τ_o	Yield stress

CHAPTER ONE

1.0   INTRODUCTION

1.1   Background of the Study

Pectinases are a group of related enzymes that are involved in breaking down of pectin-rich materials that are major constituents of plant tissues (Dey *et al*., 2014). They modify cell wall structures through tissue maceration and cell lysis, which results in tissue degradation (Heerd *et al*., 2012). Pectinases are employed in food, paper and pulp, animal feed, waste management and pharmaceutical industries (Jayani *et al*., 2005). They represent 10% of estimated commercial enzymes (Anuradha *et al.,* 2014). Pectinases can be found in higher plants (Ubani *et al*., 2015), however, microbial sources are mainly used for industrial applications (Tapre and Jain, 2014). Production of enzymes from microorganisms is eco-friendly (Lakshmi *et al*., 2009). Pectinases are often produced from pectolytic microorganisms through solid-state fermentation (SSF) or submerged fermentation (SmF) processes (Garg *et al*., 2016; Reynolds *et al*., 2018).

Based on substrate preference, three classes of pectinases are known. These are: esterase, lyase and polygalacturonase (PG) (Jayani *et al*., 2005). Among these pectinases, PG finds higher industrial applications due to its stability at various pH values and temperatures (Anuradha *et al*., 2010). Polygalacturonase (E.C. 3.2.1.15) (Tapre and Jain, 2014) is a pectinase that is involved in degradation of polygalacturonan in plant's cell walls through hydrolytic breakdown of glycosidic bonds that bind galacturonic acid moieties (Heerd *et al*., 2012). Extraction and purification of PG from *Aspergillus niger, Aspergillus sojae, Aspergillus oryzae*

and *Aspergillus awamori* (referred to as the domesticated species of *A. niger*) are well documented (Juwon *et al*., 2012; Herd *et al*., 2012; Kant *et al*., 2013; Dey *et al*., 2014). *Aspergillus awamori,* also known as *Aspergillus luchuensis* (Ichishima, 2016), belongs to the *Aspergillus niger* group (Meyer *et al.,* 2011). Members of *Aspergilus niger* group are morphologically related moulds, and are characterised by brown to black-shaded conidiospores (Ichishima, 2016). Many studies have established optimisation of process conditions (such as pH, temperature, carbon source and nitrogen supplementation) for PG production from *A. awamori* (Botella *et* al., 2007; Anuradha *et al*., 2014; Zaslona andTrusek-Holonia, 2015; Divya and Padma, 2016). In addition, there have been considerable interests in the use of food wastes and agricultural residues as substrates for the production of bio-products, both from economic and environmental viewpoints. Utilisation of agricultural residues is increasing due to high cost of traditional feedstocks (Wadhwa *et al*., 2015). Environmental concern of un-utilised wastes stems from generation of hazardous materials that are released to nature as a result of their degradation. This results in environmental pollution, which has both short and long term effects (Prasertsan *et al*., 2014; Obi *et al*., 2016).

Different pectin-rich agricultural by-products such as orange peel, sweet lime peel, jack fruit rind, carrot peel, beet root peel, water melon rind, pumpkin pulp, plantain peel, sugarcane bagasse, tomato pulp, lemon peel and papaya peel have been used as substrates for PG production (Ptichkina *et al*., 2008; Anuradha *et al*., 2010; Anuradha *et al*., 2014). Among these, orange and plantain peels have enjoyed high preference due to their wide availability (Li *et al.,* 2015; Castillo-Isreal *et al.,* 2015). In recent times, several studies have demonstrated improvement in PG production through the use of mixed culture (Zhou *et al*., 2011); mutant strains (Latifian *et al*., 2007; Anuradha *et al*., 2010; Evstatieva *et al*., 2014); mixture of substrates (Taskin and Eltem, 2008); and pretreatment of substrates (Li *et al*., 2015; El-Shishtawy *et al*., 2015). However, only substrate pretreatment has been used on industrial scale (Wi *et al*., 2013; Montgomery and Bochmann, 2014).

1.2   Problem Statement

Utilisation of agricultural residues and fruit processing wastes as substrates for microorganisms for subsequent elaboration of bio-products are limited due to certain inherent problems. Agricultural residues and food waste have high concentration of complex organic molecules (Wang *et al.,* 2017). According to Song *et al.* (2016), physicochemical properties of plant tissues, which are a function of their composition in terms of lignin, cellulose and hemicelluloses determine their bio-conversion rate. This implies that lignin, cellulose and hemicelluloses are physical barriers that limit microbial and enzymatic hydrolysis of biomasses (Yu *et al.,* 2015). Specifically, lignin is known to adsorb enzyme thereby reducing its degradation efficiency (Ju *et al.,* 2013). Cellulose has been considered a factor that limits accessibility of microorganisms to agricultural residues. Severity of this occurrence is dependent on residue's surface area, crystalline and amorphous ratio of cellulose as well as its degree of polymerization (El-shishtawy *et al.,* 2015). Several studies have demonstrated that bio-conversion rate of these residues is dependent on properties of cellulose (Li *et al.,* 2015; Yang *et al.,* 2017; Lai *et al.,* 2017).

Properties of enzymes e.g. cellulase and xylanase produced from pretreated agricultural by-products are well documented (Zhao *et al*., 2008; Zhao *et al*., 2009; Rahnama *et al*., 2013; Salihu *et al*., 2015). Increased PG activity was reported for alkali-pretreated highly ligno-cellulosic materials (wheat straw and palm leaves) using *Trichoderma reesei* under SSF (El-Shishtawi *et al*., 2015). Findings of Li *et al*. (2015) also showed increased exo-pectinase activity of microwave pretreated orange peel using *Aspergillus japonicus* under SmF. However, information on the properties of PG produced from *Aspergillus* species using pretreated pectin-rich agricultural by-products under SSF is scanty. Furthermore, there is paucity of information on the optimisation of pretreatment operation conditions of agricultural residues and food wastes for improved PG production.

1.3   Justification for the Study

Total annual global output for enzymes is estimated at $ 4.4 billion (Jaramilo *et al.,* 2015) and food processing sector dominates the market (Miguel *et al.,* 2013).

According to Anuradha *et al.* (2014), pectinases account for 10% of this figure. In Nigeria, approximately ₦ 3.6 billion is spent annually on enzyme importation (FIIRO, 2016) and in a recent survey (FIIRO, 2018), pectinase was listed as one of the enzymes on high demand. Several studies have shown the production of PG from bacteria (Gupta *et al*., 2008; Rehman *et al*., 2012; Adiguezel *et al*., 2016). However, PG produced from fungi is preferred for many industrial applications due to its higher stability at various processing conditions (Rheman *et al*., 2012). Filamentous fungi especially the genus *Aspergillus* is often used for the production of acidic pectinases that are used in fruit processing (Yadav *et al*., 2015). Members of *Aspergillus niger* group such as *Aspergillus niger, Aspergillus awamori, Aspergillus ficuum, Aspergillus phoenicus* are known to be safe (Ichishima, 2016) and therefore regarded as GRAS (generally regarded as safe) (Rehman *et al*., 2012). A preliminary investigation carried out by Anuradha *et al*. (2010) on the yield and activity of PG from eight fungi isolates (from *Aspergillus, Penicillium, Fusarium* and *Saccharomyces* species) of high pectin hydrolytic potential showed that the genus *Aspergillus* (*A. awamori* MTCC 9166) had highest PG activity.

Solid-state fermentation and SmF permit the conversion of agricultural and agro-industrial residues into products of high commercial values (Kumar *et al*., 2014). SSF offers such advantages as low contamination risk due to low moisture content of substrate, higher yield and lower effluent volume (Taskin and Eltem, 2008; Ezekiel and Aworh, 2013; Zaslona and Trusek-Holownia, 2015). From the economic point of view, cost of production of enzyme is lower in SSF than in SmF (Sukumaran *et al*., 2009). Furthermore, enzymes produced from SSF have higher activity (Ortiz *et al*., 2016) and are more thermo-stable (Melikoglu *et al*., 2013) than those produced under SmF using the same microbial strain.

Orange and plantain peels are considered good candidates for microbial cultivation due to their inherent nutrients (Emega *et al.,* 2007; Romelle *et al*., 2016). In addition, large volume of wastes to the tune of 50% of fruit weight is generated during processing of orange and plantain fruits (Kamal *et al*., 2011). According to FAO (2014), approximately 1.5 million metric tons of orange fruit waste is

generated annually in Nigeria. Ayanwale *et al.* (2016) also reported an annual waste generation to the tune of 6.5 million metric tons in top plantain producing countries (Ghana, Nigeria, Cote d'voire) of West Africa.

Pretreatment of agricultural residues is required to enhance proliferation of microoganisms, thereby increasing bioconversion rate of agricultural residues, and consequently, improves the yield and activity of products (Li *et al*., 2015). Pretreatment causes disruption of highly organised lignin-carbohydrate composite of agricultural residues (Li *et al*., 2015). This results in lignin removal with attendant increase in surface area of carbohydrate amenable to hydrolysis (Rahnama *et al*., 2013). In addition, pretreatment enhances equalization of residues from different sources in order to ensure consistency in product quality (Kumar *et al.,* 2009). Overall, pretreatment reduces cost of downstream operations (Yang and Wyman, 2008; Chang *et al.,* 2017).

1.4   Objectives

The main objective of this study was to evaluate effects of alkaline and microwave pretreatments of orange and plantain peels on the activity of polygalacturonase produced from the peels by *Aspergillus awamori* CICC 2040*,* and its applications in juice extraction and clarification.

Specific objectives were to:

i.   optimise alkaline and microwave pretreatment conditions of orange and plantain peels for maximum PG activity,

ii.   determine the effect of substrate pretreatment methods on chemical composition and morphology of orange and plantain peels   ,

iii.   characterise crude and purified PGs in terms of stability, kinetic and thermodynamic properties,

iv.   evaluate the application of PG in mango juice extraction by determining its effect on yield, chemical composition, rheological and sensory properties of the juice, and

v.   evaluate the application of PG in apple juice clarification by determining its effect on chemical composition, colour order, rheological and sensory properties of the juice.

1.5   Research Hypotheses

i.   Alkaline and microwave pretreatments of orange and plantain peels enhance PG production by *Aspergillus awamori* CICC 2040.

ii.   Purification of PG improves its stability, kinetic and thermodynamic properties.

iii.   Application of PG in mango juice extraction increases the yield and improves flow behaviour characteristics of the juice.

iv.   Application of PG in apple juice clarification improves the chemical composition and colour order properties of the juice.

CHAPTER TWO

2.0   LITERATURE REVIEW

2.1   Fermentation Processes

Fermentation processes involve biological conversion of complex substances (substrates) into simpler ones through the activities of microorganisms under favourable environmental conditions (Subramaniyam and Vimala, 2012). Additional compounds (metabolites) are released consequent to the metabolic breakdown (Abdul Manan and Webb, 2017). Fermentation processes are categorized into: Transformation processes which involve the modification of food raw materials through the activity of microorganisms such that appropriate biochemical reactions cause desirable changes in the materials (Blandino *et al.,* 2003). Fermented foods are produced through transformation processes by spontaneous/wild microorganisms (as often practiced in many parts of Africa) or through the use of appropriate (backslopping) or defined starter cultures (Yamada *et al.,* 2011); and Other microbial processes including production of enzyme, biomass, metabolites and recombinant products (Renge *et al.,* 2012)

2.1.1   Types of fermentation

Products of fermentation are produced through solid state and submerged or liquid fermentation (Thomas *et al.,* 2013).

(a)   Submerged fermentation (SmF)

This involves fermentation of substrates in a free flowing medium (Perez-Guerra *et al.,* 2003). There is rapid utilization of substrates owing to large surface area and therefore, there is a need for constant replacement or supplementation of nutrients (Subramaniyam and Vimala, 2012). This fermentation type is most

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suitable for microorganisms, which require high moisture content for proliferation and do not require a solid support (Subramaniyam and Vimala, 2012). Submerged fermentation offers advantages such as easy process control and design; extraction of products, ease of determination of mass/energy transfer and short cultivation time (Perez-Guerra *et al.,* 2003).

(b) Solid state fermentation (SSF)

According to Thomas *et al.* (2013), SSF is defined as a heterogenous process that comprises of solid, liquid and gaseous phases with attendant likelihood for improved microbial proliferation and consequent increased yield of bio-products. SSF was defined by Mitchell *et al.* (2011) as a process of cultivating microbes on moistened solid in a continuous gas phase. These definitions imply SSF as a fermentation in the absence of free flowing water, which creates an environment which resembles a natural habitat of the selected organism (Abdul Manan and Webb, 2017). SSF offers advantages such as high yield, low incidence of contamination, efficient aeration, uniform dispersion of microbial spores, simple culture requirement and low effluent generation (Perez-Guerra *et al.,* 2003).

Disadvantages of SSF include: need for substrate pre-treatment which implies additional cost, difficulty in biomass determination, difficulty in process control, high requirement for inoculum volume and long cultivation times (Perez-Guerra *et al.,* 2003). In recent times, successes have been recorded not only in SSF research, but also in industrial applications due to its numerous advantages. Availability of cheap substrates such as food processing wastes and agricultural residues (with high nutrient density) and the need for their utilization has also influenced development in SSF (Panesar *et al.,* 2016; Saithi and Tongta, 2016; Behera and Ray, 2016). Solid state fermentation processes have enjoyed tremendous improvements, particularly, in the aspect of process control (Hanc and Dreslova, 2016). Recent interests in SSF have also triggered development in industrial applications in the areas of microbial growth kinetics and bioreactor

designs (Fanaei and Vaziri, 2009; Jiang *et al.,* 2012; Abdul Manan and Webb, 2016; Jimenez-Penalver *et al.,* 2016).

2.1.2 *Aspergillus* **species of industrial relevance**

Aspergillus is a class of fungi which has enjoyed most research and industrial attentions among moulds that have been documented (Meyer *et al.,* 2011). This stemmed from its ubiquity, ease of cultivation and hence, high economic advantage. *Aspergilli* produce characteristic asexual spores that resemble aspergillum (Bennete, 2018). *Aspergilli* grow as saprophytes by degrading nutrient dense vegetations and consequently, produce different metabolic products (Paredes *et al.,* 2015). *Aspergilli* are heterotrophic in nature (Bennette, 2018). They secrete enzymes and acids to their micro-environment which help to digest polymeric molecules into simpler ones which are re-absorbed into the cells (Bennette, 2018). *Aspergilli* are unique among fungi due to their ability to grow at extreme conditions. According to Meyer *et al.* (2011), *Aspergillus* can grow successfully at temperature 10 to $50^{o}C$, pH 2 to 11, 0 to 34% salinity and water activity of 0.6 to 1, on oligotrophic and nutrient- dense substrates. They are therefore easily adaptable to SSF and SmF for large scale industrial processes (Meyer *et al.,* 2011). They have been applied in many industrial applications such as enzyme production, industrial chemicals and fermented foods (Melikoglu *et al.,* 2013; Dey *et al.,* 2014; de Souza *et al.,* 2015; Abdul Manan and Webb, 2016). There are more than 250 known species of *Aspergillus* and this is expected to increase due to advances in molecular technologies in the study of phylogenic diversity (Yamada *et al.,* 2011). The most industrially exploited species of *Aspergillus* are *Aspergillus niger, Aspergillus oryzae, Aspergillus awamori, Aspergillus sojae* and *Aspergillus terreus* (Meyer *et al.,* 2011).

(a) Classifications of *Aspergillus*

Aspergillus awamori belongs to the *Aspergillus niger* group (Meyer *et al.,* 2011). Members of *Aspergilus niger* group are morphologically related molds and are characterised by brown to black-shaded conidiospores (Ichishima, 2016). Other members include: *Aspergillus ficuum, Aspergillus phoenicus, Aspergillus foetidus, Aspergillus pulveralentis, Aspergillus tubigensis, Aspergillus inuii, Aspergillus usamii* and *Aspergillus niger.* They are collectively regarded as *Aspergillus niger* van *Tighem* (Meyer *et al.,* 2011)*. Aspergillus awamori* also known as *Aspergillus luchuensis* (Ichishima, 2016) is regarded as the domesticated species of *Aspergillus niger* (Meyer *et al.,* 2011). It has important applications especially in the production of *awamori* (a Japanese alcoholic beverage) (Ichishima *et al.,* 2016), *koji* (Yamada *et al.,* 2011; Hong *et al.,* 2013; Hong *et al.,* 2014) and enzymes such as pectinase (Dey *et al.,* 2014), amylase (Melikoglu *et al.,* 2013) and xylosidase (Paredes *et al.,* 2015). The other group of *Aspergillus* with marked morphological differences includes *Aspergillus japonicus, Aspergillus ellipticus, Aspergillus heteromorphus* and *Aspergillus aculeatus* (Ichisima, 2016; Bennette, 2018).

(b) Safety aspects in the use of *Aspergillus* species

Aspergillus awamori is known to be safe (Ichishima, 2016) and therefore categorised as GRAS (Schuster *et al.,* 2002). Like other members of the *Aspergillus niger* group, it is non-pathogenic in nature as humans are frequently in contact with its spores without reported cases of intoxication. The few cases of *Aspergillus niger* pathogenicity were associated with opportunistic invasion due to previous history of immune-suppressive conditions in patients (Schuster *et al.,* 2002). Experimental studies using animal assay have also confirmed non-pathogenicity of *A. niger* (Bennette, 2018).

2.2 Microbial Products

Microbial products are organic materials or chemicals that are produced by microbial cells during their growth on appropriate culture media (Krivoruchko and Nielsen, 2015). In 2014, the global output for microbial products was estimated to be $143.5 billion and with an annual growth rate of 14.6%, it was projected to reach $306 billion by 2020 (McWilliams, 2015). They are produced in large quantities through microbial processes. Microbial processes are known to be very specific and can be easily manipulated to improve productivity (Sanchez and Demain, 2008). Microbial products include: microbial enzymes, metabolic products (primary and secondary), microbial biomass and recombinant products (Krivoruchko and Nielsen, 2015).

2.2.1 Microbial enzymes

Enzymes are referred to as bio-organic catalysts that speed up the rate of biochemical and metabolic processes by lowering the activation energy complex such that the rate of conversion of substrate to product is significantly increased (Nigam, 2013). Enzymes are a very important agent of food deterioration and spoilage; however, they have beneficial properties that are continuously being harnessed in various food, pharmaceutical, medical and agricultural applications (Thomas *et al.,* 2013; Anbu *et al.,* 2015). Common enzymes that are used in the food processing industry include: pectinases, hydrolases, lipases, proteases, amylases, cellulases, hemicellulases etc. (Sewalt *et al*., 2016). Their utilization in the food industry has been extensively reviewed (Gurung *et al.,* 2013; Anbu *et al.,* 2015). In 2015, total global output for enzymes was estimated at $ 4.4 billion (Jaramillo *et al.,* 2015) and the food processing sector dominated the market (Miguel *et al.,* 2013). Enzymatic applications remain an integral part of many food processes for the production of arrays of quality products (Jaramillo *et al.,* 2015). This is a complete deviation from the practice in the early stages where enzyme applications were limited to food fermentations (Li *et al*., 2012). Enzymes are categorized based on source i.e. plant, animal and microbial sources (Ubani *et al.,*

2015), however, microbial enzymes have enjoyed greater research and industrial attentions. According to Anbu *et al.* (2013), microorganisms can be cultured on appropriate culture media to produce a large quantity of enzymes in few days. The production of microbial enzyme is safe because non-toxic by-products are generated (Lakshmi *et al*., 2009). Furthermore, microbial enzymes are more stable and active at various process conditions (Gopinath *et al.,* 2013; Anbu *et al.,* 2015).

(a) History of enzymes

The word 'enzyme' was coined by a German Physiologist Wilhelm Kuhne 1878, subsequent to his discovery of alcohol from sugar as a result of the activities of yeast (Robinson, 2015). Enzyme was transliterated from Greek word '*en* (within) *zume* (yeast)'. Since this discovery, considerable successes have been recorded especially in the aspect of extraction, characterisation and application of enzymes, however, pure isolation and crystallization of enzymes did not materialize until 1920 (Robinson, 2015). It was in the 1980s that the catalytic activity of enzymes was correlated with proteins. In the same decade, catalytic activity of enzymes was correlated with RNA (rybozymes) which plays major role in gene expression (Tropeano *et al.,* 2013).

(b) Properties of enzymes

Enzymes carry out catalytic activities, which cause transformation of substrate to product without the enzyme being consumed (Nigam, 2013). Enzyme's catalytic potential is expressed in terms of 'turnover' rate, number or frequency. This indicates the number of mole of substrates that can be transformed into a product by one mole of enzyme (expressed as m^{-1} or s^{-1}). For example, turnover frequency of catalase, beta-galactosidase, chymotrypsin, tyrosinase are 93, 000, 200, 100 and 1, respectively (Robinson, 2015). In addition to their high catalytic potential, enzymes are also highly specific i.e. they only cause catalysis of specific reaction types on appropriate substrates to give desirable products (Sewalt *et al.,* 2016). Certain enzymes exhibit group specificity while others have much higher specificity i.e. absolute specificity (Robinson, 2015). A good example of the

former is alkaline phosphatase, which can cause removal of phosphate group from many substrates. Glucose oxidase has absolute specificity on only beta-D-glucose and does not have any activity on other monosaccharides (Robinson, 2015). Enzymes are protein in nature (Anbu *et al.,* 2015). The building blocks of enzymes are several (100 to over 2000) units of amino acid which are bonded to form globular protein. The amino acids are arranged as single or multiple polypeptide chains. The polypeptide chains are folded to give a specific three-dimensional structure. This structure has an active site that binds to the substrate. The properties of the active site in terms of its shape and size determine how easily it binds with the substrate's molecules. The other component of the polypeptide chain helps to stabilize the active site to provide enough room for active site-substrate interaction (Loperena *et al.*, 2012). Enzymes also contain a non-protein component called cofactor. A cofactor consists of an organic (coenzyme) and inorganic (usually Fe, Mn, Co, Cu, Zn) parts. The portion that binds permanently with protein is called the prosthetic group while the inactive component is called the apoenzyme. The combination of cofactor and apoenzyme is called holoenzyme (Margesin *et al.*, 2007).

(c) Sources of enzymes

Major sources of enzyme are plants, animals and microorganisms where it primarily involves in cellular metabolism (Gopinath *et al.,* 2013; Ubani *et al.,* 2015). Prior to 1970, most enzymes used in industrial applications came from plant and animal sources because it was believed that enzymes from these sources were less toxic and less liable to contamination compared with those form microorganisms. However, as technology advanced and coupled with increased demand of enzymes in many industries, competitive cost of microbial enzymes were given preference and their use became popular (Anbu *et al.,* 2015).

Advantages of microbial enzymes over other types can be summarized in terms of economic, technical and ethical reasons (Lakshmi *et al.,* 2009). High throughput of enzymes in few days in a small production facility is an economic advantage

(Anbu *et al.,* 2013). For example, 20 kg of rennin can be produced from recombinant *B. subtilis* in a 100-litre fermenter in less than 12 h. This is a high throughput compared to an average of 10kg rennin output from a calf's stomach in several months (Kumar *et al.*, 2011). Additionally, different facilities are required for rearing animals or for cultivation of plants. This along with cost of transportation to the site of enzyme extraction is an economic disadvantage (Robinson, 2015). Technical advantage of enzyme production from microorganisms can be judged from its high extraction rate. Microbial enzymes are secreted extra-cellularly and this implies ease of extraction and purification. Microbial intracellular enzymes are easier to extract compared to those from plant and animal sources because their extraction require less rigorous operations (Tropeano *et al.,* 2013). Enzymes from plant and animals are often localised in few tissues or organs, thus making other components to be wasted (Robinson, 2013). The use of animal for experimentation or commercial purposes is often faced with many ethical issues and as a result, it is guided by stringent local and international laws. Microbial enzymes therefore, are a good alternative in this regard (Kumar *et al.,* 2011).

(d) Classification of enzymes

Classification of enzymes is based on their catalytic reactions (Eleuche *et al.,* 2015) and therefore, they are broadly classified into six groups: oxidoreductase (oxidation/reduction reactions), transferases (atom/group transfer), hydrolases (hydrolysis), lyases, isomerases and ligases. Detailed classifications are well documented (Robinson, 2015). Table 2.1 shows selected enzymes employed in food processing.

(e) Pectinase enzymes

Pectinases are a group of related enzymes, which are involved in breaking down of pectin-rich materials that are major constituents of plant tissues (Dey *et al*., 2014). They modify cell wall structures through tissue maceration and cell lysis, which results in tissue degradation (Heerd *et al*., 2012). This is achieved through

depolymerization and demethoxylation of glycosidic linkages of pectin, polygalacturonic acid or pectic acids to yield different products (Heerd *et al.,* 2012). Pectinases are broadly divided into three based on their preference for substrates. These are: esterases, hydrolases and lyases. Hydrolases and lyases are both categorised as depolymerases due to their hydrolytic mode of action (Anand *et al*., 2016)

(i) Production output

Total annual global output for enzymes is estimated at $ 4.4 billion (Jaramilo *et al.,* 2015) and food processing sector dominates the market (Miguel *et al.,* 2013). According to Anuradha *et al.* (2014), pectinases account for 10% of this figure. In Nigeria, approximately ₦ 3.6 billion is spent annually on enzyme importation (FIIRO, 2016) and in a recent survey (FIIRO, 2018), pectinase was listed as one of the enzymes on high demand in the country.

(ii) Esterases

Estarases (EC 3.1.1.11) are also called polymethylgalacturonate esterases and function by randomly cleaving the methyl ester of galacturonate unit (Garg *et al.,* 2016). Esterases are further classified into pectin methyl esterase and pectin acetyl esterase (Sharma *et al.,* 2013).

(iii) Hydrolases

Hydrolases, also known as hydrolytic depolymerases are classified into polygalacturonases (PG) and polymethylgalacturonases (PMG) (Tapre and Jain, 2014). Polygalacturonases are pectinases that involve in the degradation of polygalacturonan in plants' cell walls through hydrolytic breakdown of glycosidic bonds that bind galacturonic acid moieties (Heerd *et al*., 2012).

Table 2.1. Selected Enzymes Employed in the Food Industry

Enzyme	Description	Source	Application in food industry
Pectinase Includes estarases, Polygalacturonases and lyases	Causes depolymerization and demethoxy-lation of pectin, pectic acid and and poly-galacturonic acid to yield different compounds (Rheman *et al.,* 2012)	Bacteria especially *Bacillus* spp. Moulds such as *Aspergillus awamori, A. niger, A. nidulans* (Dey *et al.,* 2014)	Fruit juice extraction and clarification (Joshi *et al.,* 2011). Vegetable retting and demucilisation (Murthy and Naidu, 2011)
Xylanase e.g. hemicellulase and hydrolases	Glycosidases (o-glycoside hydrolases). Catalyses endo-hydrolysis of 1,4-β-D-xylosidic linkages in xylan backbone thus converting xylan to xylooligo-saccharides and xylose (Verma and Satyanarayana, 2012)	Bacteria, algae, fungi, protozoa, gastropods (Knob and Carmona, 2010)	In brewing to increase e.g. reduction of haze in final product (Elleuche *et al.,* 2015). Coffee and fruit juice extraction (Knob *et al.,* 2014)
Cellulases	Divided into three based on substrate Specificity: endoglucanases, exo-glucanases and β-glucosidases (Haq *et al.,* 2015)	Bacteria e.g. *Bacillus, Clostridia,* and *Pyrococcus* (Kuhad *et al.,* 2016). Mesophilic fungi strains of *Trichoderma, Penicillium citrinum* (Dutta *et al.,* 2008)	Tissue softening, colour enhancement etc. (Salahuddin *et al.,* 2012).

Table 2.1. Selected Enzymes Employed in the Food Industry (Continued)

Enzyme	Description	Source	Application in food industry
Amylase include α-amylases, β-amylases and pullulanases	Break down starch to yield dextrin, maltose, glucose and maltooligo-saccharides (Adeniran *et al.,* 2010; Oziengbe and Onilude, 2012)	*B. licheniformis, B. circulans* (Joshi, 2011). Fungi: *Rhizomucor pusillus* (He *et al.,* 2014); *A. oryza* (Rana *et al.,* 2013)	Starch liquefaction (Joshi, 2011). Fructose and glucose production (Van Der Maarel *et al.,* 2002)
Xylosidase	Exo-type glucosidases that catalises the hydrolysis of 1,4-β-D-xylooligo saccharides (Terrasan *et al.,* 2013)	Yeast, *Bacillus* (Banka *et al.,* 2014); *Aspergillus awamori* (Paredes *et al.,* 2015)	Fruit juice processing (Paredes *et al.,* 2015)
Lipase (triacyl-glycerol acyl-dairyhydrolases)	Lipolytic enzymes which catalise long chain tryglicerides to diacyl-glycerides, monoglycerides, glycerol and free fatty acids (Dror *et al.,* 2014)	Bacterial, mould and yeast (Espinosa-Luna *et al.,* 2015)	Dressing of baked foods flavour enhancer in products, emulsifier in foods (Sharma *et al.,*2011)
Laccase	Copper containing enzyme with un-Usual substrate specificity. Exits as monomeric, dimeric and tetrameric glycoprotein (Lu *et al.,* 2013)	*Ascomycetes, Basidiomyces, Deuteromycetes* (Gochev and Krastanov, 2007)	Decolorization of pigments and in waste water treatment (Desai and Nityanand, 2011)

Table 2.1. Selected Enzymes Employed in the Food Industry (Continued)

Enzyme industry	Description	Source	Application in food
Protease Peptide hydrolases and peptidases	Lytic enzymes which proteins by hydrolyzing peptide linkages of proteins (Li *et al.,* 2013)	*Bacillus* spp, *Aspergillus, Rhizopus, Penicillium* (de Souza *et al.,* 2015)	Meat tenderization, hydrolysate production, emusilfication of foods (de Souza *et al.,* 2015)
Peroxidases	Oxidize wide range of reducing subsrates with the help of H_2O_2 and other peroxides (Fodil *et al.,* 2012)	*Streptomyces sp* (Fodil *et al.,* 2012). Fungi e.g. *Phlebia radiate, Dichomitus squalens* (Janusz *et al.,* 2013)	Food waste treatment and bioremediation (Santos *et al.,* 2014)

Polygalacturonases are further classified into three, these are: Endo-PG (E.C. 3.2.1.15), which randomly cleaves pectic acid to yield oligo-galacturonates (Garg *et al.,* 2016); Exo-PG1 (E.C. 3.2.1.67) attaches non-reducing end of polygalacturonic acid to produce monogalacturonates; Exo-PG2 (E.C. 3.2.1.82) initiate cleavage of polygalacturonic acid to produce Di-galacturonates (Garg *et al.,* 2016). Polymethylgalacturonases hydrolytically cleaves α 1,4 glycosidic linkage of pecin. It comprises Endo-PMG which cleaves α 1,4 glycosidic linkage of pecin randomly to yield oligo-methyl galacturonates; and Exo-PMG which cleaves pectin its non-reducing end to produce methylmono-galacturonate (Garg *et al.,* 2016).

(iv) Lyases

Lyases, also known as eliminative depolymerases are classified into polygalacturonate lyase and polymethylgalacturonase lyase (Heerd *et al*., 2012). Polygalacturonate lyases cause trans-eliminative cleavage of α 1,4 glycosidic linkage of pectic acid to produce un-saturated galacturonates. It is further divided into three: Endo-polygalacturonate lyase (EC 4.2.2.2) which cleaves α 1,4 glycosidic linkage of pectic acid to produce un-saturated galacturonates; Exo-polygalacturonate lyase (EC 4.2.2.9) cleaves pectic acid at its non-reducing end randomly to produce un-saturated di-galacturonates; Oligo-galacturonate lyase (EC 4.2.2.6) cleaves α 1,4 glycosidic linkage of pectic acid terminally (Garg *et al.,* 2016). Polymethylgalacturonate lyase causes transelimination of α 1,4 glycosidic linkage of pectin. It is divided into: Endo-polymethylgalacturonate lyase (EC 4.2.2.10) which randomly attacks α 1,4 glycosidic linkage of pectin by trans-elimination to yield un-saturated methyl oligo-galacturonate; Exo-polymethylgalacturonate lyase causes terminal trans-elimination of α 1,4 glycosidic linkage of pectin to produce un-saturated methyl mono-galacturonates (Garg *et al.,* 2016).

(v) Sources of pectinase enzymes

Pectinases are found in many plants and microoganisms (Ubani *et al*., 2015). They are present in many plants where they are involved in cell wall metabolism such as

ripening and pathogenesis (Sharma *et al.,* 2013). Reports have shown successful isolation of pectinase from plants such as citrus, pawpaw, pineapple, tomato etc. (Ubani *et al*., 2015). Pectinases from microbial sources are used for industrial applications because of high throughputs of microbial processes (Tapre and Jain, 2014). Also, production of enzymes from microorganisms is environmentally friendly (Lakshmi *et al*., 2009). Bacteria and fungi are major sources of microbial pectinases. Bacteria especially *Bacillus* spp. are major producers of alkaline pectinases (Patil *et al.,* 2012; Oumer and Abate, 2017) while acidic pectinases are produced from fungi (Dey *et al.,* 2014). Fungal pectinases have proven to be very useful because of their stability in many processing conditions (Rheman *et al*., 2012). Filamentous fungi especially the genus *Aspergillus* is often used for the production of acidic pectinases that are used in fruit processing (Yadav *et al*., 2015). Members of the *Aspergillus niger* group such as *Aspergillus niger, Aspergillus awamori, Aspergillus ficuum* and *Aspergillus phoenicus* are regarded as GRAS (Rehman *et al*., 2012). A preliminary investigation carried out by Anuradha *et al*. (2010) on the yield and activity of PG from eight fungi isolates (from *Aspergillus, Penicillium, Fusarium* and *Saccharomyces*) of high pectin hydrolytic potential showed that the genus *Aspergillus* (*A. awamori* MTCC 9166) had highest PG activity. Other sources of microbial pectinases are yeasts and actinomycetes (Sharma *et al.,* 2013).

(vi) Production of pectinase enzymes

Pectinases are often produced from pectolytic microorganisms through SSF (Dey *et al.,* 2014; Dey and Benerjee, 2014; Soccol *et al.,* 2017) or SmF (Qureshi *et al.,* 2012; Ezugwu *et al.,* 2014; Li *et al.,* 2015; Khatri *et al.,* 2015) fermentation processes. SSF and SmF permit the conversion of agricultural and agro-industrial residues into products of high commercial values (Kumar *et al*., 2014). Solid state fementation offers such advantages as low contamination risk due to low moisture content of substrate, higher yield and lower effluent volume (Taskin and Eltem, 2008; Ezekiel and Aworh, 2013; Zaslona and Trusek-Holownia, 2015). Cost of production of enzyme is lower in SSF (Sukumaran *et al*., 2009). Furthermore, enzymes produced

from SSF have higher activity (Ortiz *et al*., 2016) and are more thermo-stable (Melikoglu *et al*., 2013) than those produced from SmF using the same microbial strain. Increased activity of enzyme produced via SSF is attributed to adaptability of pectinolytic organisms especially filamentous fungi to substrate with low moisture content (Ortiz *et al*., 2016). Major disadvantages in the use of SSF are difficulty in measurement of growth parameters and energy/material balances, which result in difficulty in process control (Rodriguez-Fernandez *et al.,* 2011).

Activity of pectinases produced via SSF and SmF processes is dependent on the growth characteristics of organisms which are a function of availability of nutrients and favourable micro-environmental conditions (Divya and Padma, 2015; Divya and Padma, 2016). In view of this, process conditions are often optimised to determine the best condition that produces enzyme with the highest activity. Conditions that are often optimized include: substrate to inoculum ratio, temperature of chamber, fermentation period, air flow intensity, carbon source, nutrient supplementation, pH of the medium and so on (Rodriguez-Fernandez *et al.,* 2011; Anuradha *et al*., 2014; Zaslona andTrusek-Holonia, 2015; Divya and Padma, 2016).

(vii) Purification of pectinase enzymes

Purification of enzymes is done in order to remove impurities that are present in the crude enzymes, which would otherwise cause instability and affect the activity of enzymes. Commonly reported methods for enzyme purification include the use of activated charcoal (Dey and Banerjee, 2014), gel filtration (Chinedu *et al.,* 2017), ammonium sulphate precipitation (Ezugwu *et al.,* 2014; Khatri *et al.,* 2015), acetone precipitation (Anand *et al.,* 2016) and ultra-filtration (Poletto *et al.,* 2015). The choice of a method is dependent on its availability (Anand *et al.,* 2016). Degree of purification is evaluated by variables such as: specific activity defined as ratio of enzyme activity to protein content; rate of purification i.e. absorbance of the purified enzyme divided by the absorbance of un-purified enzyme; fold purification is the ratio of specific activity of purified enzyme to the un-purified one; enzyme recovery is obtained by dividing the activity of purified enzyme to the activity of the un-purified one (Mahesh *et al.,* 2016; Anand *et al.,* 2016).

Purification causes biochemical changes in protein content of enzymes. Protein content of PG from *Aspergillus niger* reduced after purification (Kant *et al*., 2013). In addition, Silva *et al.* (2007) recorded a reduction in protein content of PG elaborated by *Penicillium viridicatum*. This suggests that other components in the crude enzyme contributed to its total protein content. Joshi *et al.* (2011) reported reduction in PG activity of pectinase produced from *Aspergillus niger*. One major way employed for the determination of degree of purification is through the evaluation of enzyme's protein molecular weight and purity (Paudel *et al.,* 2015). This is usually done with the aid of an electrophoretic procedure especially sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Diversity in molecular weight of enzymes' protein fractions forms the basis for differentiating among microbial species (Ortiz *et al.* 2016). According to Ahmed *et al.* (2015), properties of enzyme's proteins are based on factors such as substrate composition, microorganism type and analytical methods. Heerd *et al.* (2014) reported differences in beet (substrate) sugar composition as significant factors that influenced the purity of pectinase enzymes.

The number of protein bands often differentiates crude and purified enzymes. Crude enzymes are characterized by many protein bands as a result of presence other proteins and impurities (Paudel *et al.,* 2015). Purified enzymes usually consist of monomer (a single band) or heterodimer (2 or more) bands of protein (Kant *et al.,* 2013). Findings by earlier workers showed variation in molecular weight of PG. Ahmed *et al.* (2015) reported molecular weight of 30 kDa for purified pectinase produced from *Aspergillus niger*. Also, Kant *et al.* (2013) recorded a molecular weight of 34 kDa for a purified PG produced from *Aspergillus niger* MTCC 3323. Kant *et al.* (2013) further reported the production of a heterodimer PG that showed 2 protein bands of 34 and 69 kDa.

(viii) Stability of pectinase enzymes

Stability of pectinase enzymes is conducted at various pH to determine optimum pH of the enzymes. Studies had shown optimum activity of fungal PG to range between 4.0 and 6.0 (Suresh and Viruthagiri, 2010; Kant *et al.,* 2013; Ahmed *et al*., 2015). However, Joshi *et al.* (2011) reported a lower optimum pH 3.5 for PG produced from

Aspergillus niger. Neutral or alkaline pectinase is often produced by bacteria especially *Bacillus* spp. (Qureshi *et al.,* 2012; Paudel *et al.,* 2015). A recent study by Khatri *et al.* (2015) showed the pH optimum of PG produced from *Aspergillus niger* MCAS2 to be 8.2. Ahmed *et al.* (2015) reported maximum PG activity at 55 ºC. However, this value contradicted 30 ºC maximum activity reported for PG produced from *Aspergillus niger* (Joshi *et al.,* 2011). Also, Gomes *et al.* (2011) reported maximum activity at 37 ºC for PG produced from *Aspergillus niger* ATCC 9642. These variations could be because fungal strains produce proteins with different biochemical properties, hence different properties (Zaslona and Trusek-Holownia, 2015). Activity of pectinase enzymes reduces with increasing temperature. According to Zohdi and Amid (2013), denaturation of enzymes' proteins occurs at 50 ºC and above. Joshi *et al.* (2011) recorded a reduction in activity of PG produced from *Aspergillus niger* at temperature above 50 ºC. Nevertheless, certain PGs showed thermostability at 65 ºC and above. Khatri *et al.* (2015) reported that 82% activity of pectinase from *Aspergillus niger* MCAS2 was retained when subjected to a temperature of 100 ºC. Microbial strains produce enzymes with different cysteine moiety in their amino acid sequences (You *et al.,* 2010). Cysteine promotes formation of disulphide bonds, which have strong hydrophobic effect that results to high stability of bio-products (Singh *et al.,* 2012a).

The major factor which determines thermostability of pectinase is the source i.e. microorganism. Due to the wide applications of thermostable pectinase enzymes, efforts are constantly geared towards isolation of microorganisms that can produce thermostable pectinases (Zohdi and Ahmid, 2013). Pectinases differ in their stability in the presence of metal ions (Dogan and Tari, 2008) and this is attributed to the type of microbial strain used (Zaslona and Trusek-Holownia, 2015). Review of earlier studies showed variations in PG properties in the presence of metal ions. Khatri *et al.* (2015) reported inhibition of PG produced from *Aspergillus niger* MCAS2 in the presence of sodium and calcium ions. Also, Kant *et al.* (2013) observed an inhibition of PG in the presence of K^+, Ca^+ while Zn^{2+} while Mg^{2+} and Cu^{2+} stimulated it. Enzyme inhibition occurs by the presence of some metals. The inhibition could be due to denaturation of proteins (Zohdi and Ahmid, 2013) and protein

polymerisation, which forms bridges between peptide chains, and blockage of thiol groups of proteins (Silva *et al.,* 2007).

Mechanisms of enzyme enhancement or inhibition vary among compounds. Surfactant such as Tween 20 might cause an increase in the affinity of PG's active sites to the substrate, which would culminate in reduction in surface tension (Paudel *et al.,* 2015). Ethylene di-amine tetra acetic (EDTA) acts by chelating metal ions from enzymes (Juwon *et al.,* 2012). Published data have shown variation in the stability of PGs in the presence of different compounds. Dogan and Tari (2008) reported improved PG stability by EDTA and glycerol while Tween 80 inhibited it. Paudel *et al.* (2015) reported that Triton-X-100 inhibited pectinase enzyme. Juwon *et al.* (2012) also reported that PG produced from *Aspergillus niger* CSTRF was inhibited by EDTA. Kant *et al.* (2013) recorded higher activity of PG on pectin compared to other non-pectic polysaccharides. The affinity of PG to pectin is stemmed from its high hydrolytic capability on glycosidic bonds that link the galacturonic acid residues of pectin (Heerd *et al.,* 2012). This implies that properties of pectinases are dependent on the nature of substrates (Kant *et al.,* 2013). Kinetics of enzymes are generally described by the use of Michalis-Menten equation or a modified Line-Weaver Burke equation (Dogan and Tari, 2008). From the characterisation, kinetic constants: Vm which measures catalytic activity of enzymes and Km which is an indication of enzyme's affinity to the substrate are determined (Kant *et al.,* 2013; Adiguezel *et al*., 2016). Suresh and Viruthagiri (2010) determined the Vmax and Km of pectinase to be 2.33 U/mL and 0.294 mg/mL, respectively. According to Dogan and Tari (2008), these parameters vary among pectinase enzymes based on microorganism type and purification method.

(ix) Uses of pectinase enzymes

Pectinase enzymes are frequently used in the food industry. According to Anuradha *et al*. (2014), 10% of estimated commercialized enzymes are pectinases. Some of the applications include: fruit juice extraction and clarification (Dey *et al.,* 2014; Cereti *et al.,* 2016; Cereti *et al.,* 2017); demucilisation of coffee (Murthy and Naidu, 2011; Oumer and Abate, 2017); production of pectin hydrolysate (Sharma *et al.,*

2013); waste water treatment (Poletto *et al.,* 2015; Mahesh *et al.,* 2016); degumming/maceration of vegetable fibers (Reynolds *et al.,* 2018) and extraction of vegetable oils (Sharma *et al.,* 2013).

(d) Standards and safety considerations in the use of enzymes in the food Industry

The use of enzymes in the food industry has witnessed tremendous acceptance within the last few decades (Robinson, 2015). This is consequent upon the discovery of enzyme use as an alternative to otherwise harsh chemical and physical means of accomplishing processing operations (Schweigert *et al.,* 2000). Consequently, enzyme application in food processing is perceived as a means of achieving sustainability in industrial production and conservation of inherent food nutrients (Ramos and Malcata, 2011). Increasing utilisation of enzymes in food processing has attracted interests of food standard regulators and governments, especially in developed countries (Spok, 2006). Today, newly produced enzymes require authorization by Food and Drug Administration (FDA) in the United States (US) and European Food Safety Authority (EFSA) (EFSA, 2005).

Legislations on enzyme usage are put in place to ensure safety during enzyme production, and utilisation, and often contain specifications on enzyme activity and purity (Tuomi, 2000). Possibility of allergy and irritation associated with occupational hazards during enzyme production or application and risk of oral toxicity in consumers are major safety concerns in enzyme regulations (Schweigert *et al.,* 2000). Occupational hazards may be due to inhalation of dust/aerosol during enzyme production or application in food systems, and this may result in respiratory allergy or skin irritation especially with enzymes that have catalytic property. High incidence of occupational hazards associated with enzymes' aerosols is found in bakery, animal feed and enzyme production industries (Spok, 2006). Risks associated with toxicity are seldom associated with enzymes, but rather with by-products or contaminants in enzyme preparations (Spok *et al.,* 2005). These by-products and contaminants are often elaborated as parts of products during enzyme production from microorganisms (producers) or other microorganisms that are phylogenetically related to the producers (Vanhanen *et al.,* 2001). The Association of Manufacturers and Formulators of Enzyme Products (AMFEP) have conducted

over a thousand toxicity tests and there was no corresponding evidence of enzyme toxicity associated either with occupational hazards or with the consumers (AMFEP, 2001). Nonetheless, efforts are still made to ensure safety in enzyme preparation, particularly in the areas of microorganism identification for toxin production; regulation of processing conditions to mitigate toxin elaboration because of changes in pH, temperature etc.; and animal testing for possible toxicity (Scientific Committee on Food (SCF), 2001).

Based on food legislation considerations, enzymes are categorised into food additives and processing aids (Spok *et al.,* 2005). Additives are known to perform technological functions in foods while processing aids do not (Spok, 2006). These definitions vary in different quarters. For example, enzymes are regarded as additives in the US, Japan and Canada while they are referred to as processing aids in Australia. The JECFA (FAO/WHO Expert Committee on Food Additives) see no difference between the two (Spok *et al.,* 2005). Major food enzyme legislative bodies around the globe include the European Union (Council Directive 89/107/EEC), FDA, JECFA and AMFEP (Pariza and Johnson, 2001). Standards and specifications of each body are well documented (Spok, 2006). Each of these bodies has distinctive regulations; however, their structures are similar. Majority of them including the AMFEP believe that safety of enzymes largely depends on their sources (AMFEP, 2001), therefore, enzymes must be produced from non-pathogenic and non-toxigenic organisms. Criteria for microorganism selection for enzyme production are well documented (AMFEP, 2001). As discussed in Section 2.1.2b, *Aspergillus awamori* as well as other members of the *Aspergillus niger* group have obtained the GRAS status which guarantees its use in enzyme production (Schuster *et al.,* 2002; Ichishima, 2016).

2.2.2   Metabolic products

Metabolic products are organic materials and chemical compounds that are elaborated by microorganisms during their growth on an appropriate culture media (Tamano, 2014). They enjoy wide applications in many manufacturing processes. Metabolic products are classified into primary and secondary based on the phase of

production during the growth of microorganisms (Pickens *et al.,* 2011). Primary metabolites are products that are elaborated during the log or exponential phase of microbial growth and play a huge role in sustenance of life of the producing microorganism (Sanchez and Demain, 2008). Primary metabolites are either produced via anabolic or catabolic process (Sanchez and Demain, 2008). Products of anabolic metabolism include: vitamins, amino acids, nucleotides etc. Those of catabolic metabolism are organic acids such as succinic, lactic, citric, acetic acids etc. Production of primary metabolites is dependent on microbial growth rate and nutrient availability (Tamano, 2014). Secondary metabolic products are generated during the decline phase of growth of microoganisms (Ruiz *et al.,* 2010) and unlike the primary metabolites, they do not contribute to the sustenance of life of the producers but rather, inhibit it (Pickens *et al.,* 2011). Secondary metabolites are of low molecular mass (Tamano*,* 2014) and are stable because they are not easily degraded by the producing microbe. Also, they can be easily over-produced since they are produced outside the cells, thus, the producing organism is not stressed by their accumulation (Tamano, 2014). Common examples of secondary metabolites in the food industry inclue: antibiotics, pigments, gums, phenolic compounds etc. (Martins *et al*., 2011).

2.2.3 Microbial biomass

Microbial biomasses are microbial cells that are specifically cultivated as foods for humans and animals. In this form, they are referred to as single cell proteins (Nasseri *et al*., 2011). Microbial biomasses are also cultivated for use as defined starter cultures employed for fermented foods. Production and application of microbial biomass are well documented (Ahmed *et al.,* 2010; Rajoka *et al.,* 2012; Vieira *et al.,* 2013; Ahmed *et al.,* 2017; Ritala *et al.,* 2017).

2.2.4 Recombinant products

Metabolic products' production processes are often faced with certain limitations. These among others include: low yield or bio-activity, production of inhibitory products, complexity in product recovery etc (Cheng *et al.,* 2013). Metabolic engineering are therefore required to manipulate the processes in order to ameliorate

the anomalies. Genetic engineering or recombinant DNA technology involves manipulation of genetic make-up of microorganisms for the improvement of the production process. Many studies have demonsrated improvement of microbial metabolic processes using recombinant DNA technology (Zhang *et al.,* 2009; Dai *et al.,* 2013; Zhao *et al.,* 2013; Lian *et al.,* 2014).

2.3   Substrates for the Production of Microbial Products

Substrates are organic materialsm, which serve as a source of nutrients for the proliferation of microorganisms. They form part of the essential elements required for fermentation or bioconversion (Pant *et al.,* 2009). Substrates are modified through structural changes of inherent chemical molecules to give value added products known as fermented foods. They also provide required nutrients for microorganisms for onward production of metabolic products and enzymes. Substrates, in addition to provision of nutrients also serve as a support or anchorage for microorganisms especially in SSF processes (Kareem *et al*., 2011).

A substrate must contain a carbon source as well as protein and minerals; however, carbon source is the most important component. Accumulation of products is often dependent on carbon source, therefore, selection of easily metabolisable carbon source is highly imperative (Vandenberghe *et al.,* 2018). A substrate is said to be ideal if it contains all the required nutrients that are necessary for maximum growth of organisms and subsequent optimum yield or activity of bio-products (Kareem *et al.,* 2011). This may appear not feasible as no single material may contain all the nutrients required for optimum microbial growth. In practice, many substrates are screened and the best are selected and supplemented with deficient nutrients (Anuradha *et al.,* 2010). Also, substrates are selected based on the concentration of inducing chemical compounds for a given product (Sanchez and Demain, 2008). For example, pectin is an inducing nutrient for pectinase enzyme production (Li *et al.,* 2015). There are two sources of substrate used in bioconversion processes. These are traditional feedstock and food/agricultural residues.

2.3.1 Traditional feedstock

Traditional feedstocks are food materials that are purposely used for the propagation of microorganisms (Barros *et al.,* 2013). They also include energy crops that are cultivated. Recent reports on the production of bio-products using traditional substrates include the production of succinic acid from glucose (Barros *et al.,* 2013), citric acid production by *A. niger* and *Candida* spp. on sucrose and starch (Vandenberghe *et al.,* 2018), and fermentation of refined sugar in lactic acid production (Hu *et al.,* 2016). The use of traditional feedstocks is no longer feasible due to their high cost (Hu *et al.,* 2016). Another controversy that has bewildered the use of traditional feedstock is associated with competition of interest in the use of foods for human consumption and as substrates for microbial products (Tabil *et al.,* 2011). A certain school of thought argued that the major problem of the world is the provision of food for her teeming 7 billion population, majority of whom are impoverished due to food insecurity (Tabil *et al.,* 2011). Therefore, efforts should be geared towards production of food to combat food insecurity while an alternative source of feedstock should be harnessed for the production of bio-products.

2.3.2 Food processing wastes and agricultural residues

Any material that is generated, but not utilised at any point within the food value chain is known as waste (Vidhyalakshmi, 2012). Wastes are removed from the production process because they are undesirable and could pose a detrimental effect on the process if not removed (Oreopoulou and Tzia, 2007). Wastes accumulate during the conversion of organic raw materials and subsequent extraction of valuable materials as finished products (Russ and Schnappinger, 2007). They are rich in macro and micronutrients which are characterized by high biochemical oxygen demand (BOD) and chemical oxygen demand (COD) (Wadhwa *et al*., 2015).

Valorization of wastes has both economic and environmental merits. From the economic perspective, use of wastes for production of bioproducts is cheaper compared to traditional feedstocks (Wadhwa *et al*., 2015). Environmental concern of un-utilized wastes stems from generation of hazardous materials that are released to the environment as a result of bio-degradation. This results in environmental pollution which has both short and long term effects (Prasertsan *et al*., 2014; Obi *et*

al., 2016). Food wastes have been employed as substrates for microorganisms in the production of valuable materials such as enzymes and metabolites (Bari *et al*., 2010; Gassara *et al*., 2011; Tao *et al*., 2011; Silva *et al*., 2013). Chemically and physically induced extraction of materials such as essential oils, sugars, protein isolates, pectin, cellulose and neutraceuticals from wastes have also been reported (Domınguez-Perles *et al*., 2010; Domınguez-Perles *et al*., 2011; Papaioannou and Liakopoulou-Kyriakides, 2012; Rudra *et* al., 2015; Sancho *et al*., 2015). Food processing wastes and agricultural residues have also served as an alternative source of energy generation through direct combustion or biogas production (Liu *et al*., 2015; Jung *et al*., 2015). Wastes from orange, onions, peach, mango etc have been used as dietary fibre supplements in refined foods (Mateos-Aparicio *et al*., 2010; do Espirito *et al*., 2012). Aqueous or ethanolic extract from fruits and vegetables which consists active compounds such as furfural and phenolic compounds has been applied in bio-preservation against spoilage and pathogenic microorganisms (Khan and Hanee, 2011). Food wastes are also used in single cell protein, a rich source of protein, fat, nucleic acid, vitamins, minerals and essential amino acid lysine (Mondal *et al.*, 2012; Oboh *et al*., 2012). Food wastes have also found application in adsorption of heavy metals in waste water treatment (Iqbal *et al*., 2009a, b; Ahmad *et al*., 2012). Composts produced from fruit and vegetable wastes are used to supplement nitrogen up to 6-22% in fertilizer production (Jardia *et al*., 2008; Sarkar *et al*., 2010).

2.4   Citrus

Citrus originated from South East Asia and was first cultivated in China (Olife *et al*., 2015). Today, it is grown in both pacific and tropical areas of the world (Obi *et al*., 2016). The sweet orange (*Citrus sinensis*) is the most cultivated tree crop in the world (Padharipande and Makode, 2012). According to FAO (2014), the production volume of orange in the world in 2016 was 47.08 million metric tons with Brazil being the highest producer followed by United States of America and China. This figure is projected to reach 49.3 million metric tons by 2018 (Statista, 2018). Information on production output of orange in Nigeria is scarce; however, Akpan *et al.* (2014) reported that Nigeria produces a large quantity of orange annually which represents 3% of the entire world production. Overall citrus production volume in

Nigeria based on the survey conducted by Food and Agriculture Organisation (FAO) in 2014 is 3.9 million metric tons (FAO, 2014). Citrus is largely cultivated in the southern and middle-belt regions of Nigeria (Olife *et al*., 2015). The production volume is expected to increase due to Federal Government's drive to diversify Nigeria's economy, and ban on importation of fruit and vegetable products (Obi *et al*., 2016).

2.4.1   Wastes generated from orange processing

Wastes in the tune of 50% of the original weight of orange fruit in forms of peel, pulp and seeds are generated during processing to juice and other products (Kamal *et al*., 2011). According to a recent survey (Statista, 2018), 23.5 million metric tons of waste is generated annually from citrus fruits. Wastes from orange have been reported to have significant quantity of nutrients. Proximate analysis of the fruit peel showed that it contains 9.73, 8.70, 5.17, 14.19 and 53.27% of crude protein, lipid, ash, crude fibre and carbohydrate, respectively (Romelle *et al*., 2016). Romelle *et al*. (2016) further reported high contents of minerals such as Ca, Zn, Fe and Mn in tune of 162.03, 6.84, 19.95, 1.34 mg/100g dry peel respectively. According to Zaslona and Trusek-Holownia (2015), orange peel on dry basis contains 23.02% pectin, 37.08% cellulose, 11.04% hemicellulose and 9.06% sugar.

2.4.2   Orange peel utilisation

Due to the richness of orange peel, it has enjoyed wide applications in numerous food and non-food systems. Chief among the components of orange peels are the phenolic compounds which comprise of polyphenols, phenolic acid and flavonoids (Cheynier, 2012; Kodal and Aksu, 2017). They are applied in functional foods due to their numerous health benefits (Gosslau *et al.,* 2014; Nakajima *et al*., 2014; Rafiq *et al*., 2016). Due to its high pectin content, orange peel flour can be incorporated into foods to increase their physicochemical properties such as water absorption capacity, gelling capacity, viscosity etc (Rwubatse *et al*., 2014). Improved rheological and sensory properties were recorded in biscuit when orange peel flour was incorporated into wheat flour (Nassar *et al*., 2008). Another component of

orange peel with immense food application is its essential oil. Giwa *et al.* (2018) reported an essential oil yield of 4.4% from orange peel with the aid of steam distillation. Essential oil, which has an active component referred to as limonene has been shown to have prophylactic property, which confers radical scavenging ability on it (Rezzoug and Louka, 2009). This is particularly applicable in bio-preservation of foods. Aboudaou *et al.* (2018) reported significant reduction in lipid oxidation of liquid egg that was treated with 0.1-0.5% essential oil of orange peel. Pathogens such as *Listeria monocytogens*, *Bacillus cereus, Campylobacteria jejuni, Escherichia coli* etc. were inhibited by the action of the essential oil from orange peel (Fisher and Phillips, 2006). Antibacterial and antioxidant properties of orange peel's essential oil were also applied for the control of spoilage organisms in stored fish (Djenane, 2015), tofu, strawberry (Rahmawati *et al.*, 2017), orange slices, cakes (Radi *et al.*, 2017) etc.

Orange peel is a good candidate for valorization because of its high nutrient density. Torrado *et al.* (2011) reported the production of citric acid to the tune of 193mg/g dry weight by *Aspergillus niger* on orange peel via SSF. Huge success was also recorded on the production of citric acid by the same microoganism via SmF on orange peel (Rivas *et al.,* 2008). Kumar *et al.* (2016) reported 1.796g/L yield of lipopeptide, a bio-surfactant with an emulsion capacity of 75.17% from *Bacillus licheniformis* on orange peel. Also, ethanol has been produced from different microorganisms that were cultivated on orange peel. Oberoi *et al.* (2010) recorded an ethanol yield of 0.25g/g from a 2-stage hydrolysis and fermentation procedures. Orange peel is a good good raw material for pectinase production because of its high pectin content, which serves as a good inducer substrate (Jaswail and Ravindra, 2016). Li *et al.* (2015) produced exo-pectinase from *Aspergillus japonicus* on orange peel under submerged fermentation. They further reported the elaboration of other enzymes such as carboxylmethy cellulase, xylanane and filter paper cellulase. Also, reports abound on process optimisation for the production of pectinases using orange peel under SSF or SmF from *Aspergillus sojae* (Demir *et al.,* 2012; Gogus *et al.,* 2014), *Aspergillus niger* (Mrudula and Anitharaj, 2011), *Penicillium pinophlilum*

(Ahmed and Mostafa, 2013) and *Trichoerma viridi* (Irshad *et al.,* 2014) on orange peel.

2.5 Plantain

Banana (*Musa* spp.) is a general name given to all herbaceous plants that belong to genus and family *Musa* and *Musaceae*, respectively (Lee *et al*., 2010). *Musa* comes in variety of shapes and colours and are classified as *Musa paradisiaca*, *M. acuminate, M. balbisiana* and *M. sapientum* (Vu *et al.,* 2018). According to FAOSTAT (2013), bananas are important and popular fruit crops and account for 16.8% of global fruit output. Plantain and bananas originated from Southern Asia and as of today, they are cultivated in many parts of the world (Emega *et al.,* 2007). According to FAOSTAT (2012), China is the topmost producer of plantain in the world while Angola is the topmost producer in Africa. According to FAO (2018), 117.9 million metric tons of bananas were produced in the world in 2017. Top producers of plantain in West Africa are Ghana, Cameroon, Nigeria and Cote d'voire. In 2011, these countries produced about 12.4 million metric tons which was equivalent to 32% of total global output (Ayanwale *et al.,* 2016). In Nigeria, plantain production increased from 1.9 million metric tons in 1999 to 2.78 million metric tons in 2013 (Ayanwale *et al*., 2016) and 3.09 million metric tons in 2017 (FAO, 2018). The increase is due to increasing awareness of the benefits of plantain as a functional food and its applicability in numerous food systems.

2.5.1 Plantain peel and its composition

According to Castillo-Isreal *et al.* (2015), wastes such as peel, stalk and pseudo-stem which account for over 40% of total fruit weight are generated during plantain processing into products such as chips, puree, flour, starch etc. (Abiodun-Solanke and Falade, 2010; Suyatma *et al.,* 2015). According to FAO (2018), approximately 47.2 and 1.2 million metric tons of banana wastes were generated in 2017 globally and in Nigeria, respectively. Composition of plantain peel varies among varieties (Khawas *et al.,* 2014) and with maturity (Emega *et* al., 2007), however, wastes from different sources are often equalized prior to valorization (Lin *et al.,* 2013).

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Chemical characterisation of plantain peel by Emega *et al.* (2007) showed that it contains 8 to 11% protein, 40 to 50% dietary fibre and 2.2 to 10.9% lipid. They further reported that plantain peel contains polyunsaturated fatty acids (linoleic and α-linolenic acids), starch and potassium. It contains 17.05% pectin (Castillo-Isreal *et al.*, 2015), 12.45% ash, 19.68mg/100g Ca, 1.72mg/100g Zn, 15.15mg/100g Fe and 9.05mg/100g Mn (Romelle *et al.,* 2016). Analysis of plantain peel by Igbokwe *et al.* (2016) showed that it contains 46% cellulose and 25.7% lignin. Plantain peel is a rich source of starch (392.9g/kg), total dietary fibre (376.4g/kg), soluble dietary fibre (73g/kg), and insoluble dietary fibre (303.4g/kg) (Arun *et al.,* 2015). Plantain peel also contains phenolic compounds. According to Passo-Tsamo *et al.* (2015), plantain peel contains approximately 40 phenolic compounds, which are categorized into hydroxycinnamic acid, catecholamines, flavonols and flavan-3 ols.

2.5.2   Plantain peel utilisation

Due to the richness of plantain peel in important nutrients, it is employed in numerous applications. Plantain peel is considered a functional food due to its richness in phenolic compounds which have many health benefits and a good radical scavenging candidate for the control of oxidation (Idoko and Oladiji, 2014; Vu *et al.,* 2018). Lee *et al.* (2010) produced a functional jelly with high anti-oxidative property from plantain and banana peels. Plantain peel oil (amyl acetate) is a good flavoring agent (Mohapatra *et al.,* 2010), substrate for wine (Faturoti *et al.,* 2006) and ethanol production (Mohapatra *et al.,* 201). Pectin extract from plantain peel was also used as a coating agent on banana chips for quality improvement (Suyatma *et al.,* 2015). Inclusion of 10% plantain peel into wheat flour resulted in a significant increase in dietary fibre of cookie without affecting its sensory properties (Arun *et al.,* 2015). Plantain peel flour was used as a binding agent in meat sausage and this resulted in improved functional properties of the sausage (Chasoy and Cock, 2017).

Plantain peel is also a good substrate for microorganisms for the production of bio-organics and enzymes (Jamal *et al.,* 2012). Kannahi and Elangeswari (2015) reported production of cellulase from *Aspergillus niger* and *Trichoderma viridae* on banana peel. Solid state production of α-amylase by *Bacillus subtilis* on banana peel

was also reported by Paul and Sumathy (2013). Sun *et al.* (2011) reported plantain peel as a suitable feedstock for the production of carboxylmethyl cellulase, β-glucosidase, filter paper cellulase by *Trichoderma viride*. Ogunyemi *et al.* (2015) discussed that degradability of plantain peel by strains of *Klebsiella, Enterobacter* and *Aspergillus* yielded cellulolytic enzymes of high activities. Plantain peel is a good feedstock for α-amylase production by bacteria especially *Bacillus subtilis* (Unakal *et al.,* 2012). Banana peel, when used as a feedstock for ethanol production from *Saccharomyces cerevisiae* yielded 35.5g/L of ethanol (Singh *et al.,* 2014a). Singh *et al.* (2014a) further reported bio-ethanol production by *Aspergillus niger* on plantain peel. Glucose production by enzymatic hydrolysis of plantain peel by pectinase enzyme was reported by Corona *et al.* (2015) and Ajayi (2016) reported a glucose yield of 0.87mg/mL from a 7-day *Aspergillus niger* fermented plantain peel. Valorization of plantain peel by *A. niger, A. flavus* and *Penicillium* spp. resulted in significant increase in protein and sugar contents, thus enhancing its digestibility and nutrient density for animal feeding (Akinyele and Agbro, 2007). Ravi *et al.* (2011) reported production of single cell protein from *Trichoderma harzianum* using plantain peel as substrate. Glutathione was also produced from *Candida utilis* SZU 07-01 on plantain peel (Xue *et al.,* 2011).

2.6   Lignocellulosic Materials

Plants' cell walls consist of three main components: lignin, cellulose and hemicelluloses and are therefore, regarded to as lignocellulosic biomass (Kumar *et al.,* 2009). Other components include pectin, extractives (non-structural materials e.g. proteins, sugars and pigments) and ash (Cara *et al.,* 2007).

2.6.1   Structural pattern of lignocellulosic materials

Cellulose consists between few hundreds to over 9, 000 D-glucose units that are linked by β(1-4) to form long chains that are held together by hydrogen and van der waals bonds to constitute what is known as microfibrils (Kumar *et al.,* 2009; Tabil *et al.,* 2011). The microfibrils are covered by hemicelluloses and lignin (Jorgensen *et al.,* 2007). Cellulose consists of two fractions: a linear and well-organised crystalline fraction that constitutes the higher proportion and the un-organised

amorphous fraction with significantly lesser proportion (Tabil *et al.,* 2011). The crystallinity of cellulose stemmed from its linear configuration and makes it recalcitrant to digestion and degradation (Tabil *et al.,* 2011). On dry weight basis, a typical plant material contains about 40 to 60% of cellulose. It is tough and insoluble in aqueous solutions (Perez *et al.,* 2007). Hemicelluloses consist of chains of numerous hetropolymers, which are otherwise called matrix polysaccharides (Kumar *et al.,* 2009). Hemicellulose matrices contain several random sugars such as glucose, mannose, arabinose, rhamnose etc. and uronic acid such as 4-o-methylglucuronic, D-glucuronic and D-galacturonic acids, and are linked by β-(1,4)-glycosidic or β-(1,3)-glycosidic bonds (Alizadeh *et al*., 2005). These backbones have several branching and high degree of acetylation that make hemicelluloses less rigid compared to cellulose (Cara *et al.,* 2007). Hemicellulose has strong adhesive property and this is responsible for the bonding of lignocellulosic materials (Tabil *et al.,* 2011). Lignin is a biopolymer that forms an integral component of plant tissues and lacks definite primary structure and thus, has many unusual properties (Tabil *et al.,* 2011). It has a phenolic monomer which is made up of three phenyl propionic alcohols. These are: coniferyl, coumaryl and sinapyl alcohols. The are called guaiacyl propanol, p-hydroxyphenyl propanol and syringyl alcohol, respectively. They are linked by alkyl-aryl, alkyl-alkyl and aryl-aryl ester bonds, respectively (Kumar *et al.,* 2009). Lignin occupies the spaces between cellulose and hemicellulose and thus, confers structural support and impermeability on plant cells (Perez *et al.,* 2007).

2.6.2 Challenges in the utilisation of food processing wastes/agricultural residues

Utilisation of agricultural residues and fruit processing wastes as substrates for microorganisms for subsequent elaboration of bio-products are limited due to certain inherent problems. Agricultural residues and food waste have high concentration of complex organic molecules (Wang *et al.,* 2017). According to Song *et al.* (2016), physicochemical properties of plant tissues, which are a function of their composition in terms of lignin, cellulose and hemicelluloses determine their bioconversion rate. This implies that lignin, cellulose and hemicelluloses are

physical barriers, which limit microbial and enzymatic hydrolysis of biomasses (Yu *et al.,* 2015). Specifically, lignin is known to adsorb enzyme thereby reducing its degradation efficiency (Ju *et al.,* 2013). Also, cellulose has been considered a factor that limits accessibility of enzyme to substrate. The severity of this occurrence is dependent on substrate's surface area; crystalline and amorphous ratio of cellulose as well as its degree of polymerization (El-shishtawy *et al.,* 2015). Several research studies have demonstrated that substrate's bioconversion is dependent on the properties of cellulose (Li *et al.,* 2015; Yang *et al.,* 2017; Lai *et al.,* 2017). Plant biomasses are characterised with poor physical properties especially in terms of shape, weight and size. Consequently, they have low bulk density which makes preliminary processing operations (sorting, cleaning etc), distribution and transportation costly and difficult (Kashaninejad *et al.,* 2014). Food wastes especially fruit and vegetable by-products are characterized with varying chemical composition and physicochemical properties (Wadhwa *et al.,* 2015) due to changes in processing schedules, inconsistency in the quality of raw materials and seasonal variation (Lin *et al.,* 2013). Variability in biomass' quality results in inconsistency in the quality of bio-products (Lin *et al.,* 2013).

2.7   Pre-treatment of Food Wastes/Agricultural Residues

Pretreatment of substrates enhances proliferation of microorganisms on substrates; thereby increasing bioconversion of substrates and improves yield/activity of products (Li *et al*., 2015). Substrate pretreatment causes disruption of highly organised lignin-carbohydrate composite (Li *et al*., 2015). This results in lignin removal with attendant increase in surface area of carbohydrate amenable to hydrolysis (Rahnama *et al*., 2013). Pre-treatment of materials deconstructs them in order to break their recalcitrance (Lai *et al.,* 2017). Also, pretreatment of biomasses promotes densification which consequently, improves their physical properties and therefore, reduces cost of downstream operations (Yang and Wyman, 2008; Chang *et al.,* 2017). According to Kumar *et al.* (2009), pre-treatment of biomass should increase the formation of sugars or other inducer substrates required for subsequent hydrolysis and should not degrade sugars nor produce inhibitory compound, which may limit or reduce the rate of formation of bio-products.

2.7.1 Pretreatment methods

Methods that have been used for the pre-treatment of residues can be categorized as chemical, physical (or physicochemical), biological and combination of methods. The selection of a pre-treatment method is dependent on factors such as availability, cost and efficiency of operation. (Tabil *et al.,* 2011).

(a)   Chemical pretreatment methods

Chemical pre-treatment of food wastes and agricultural residue is a common practice in upstream bioconversion processes (Salihu *et al.,* 2015). Many findings abound on the use of acid, alkali, oxidizing agent, organosolv, ozone, ionic liquids etc. for pre-treatment of lignocellulosic materials.

(i) Acid pretreatment

Acid pretreatment employs the use of H_2SO_4 or HCl for the treatment of lignocellulosic materials for improved enzymatic activities. Acids have strong dissolution properties and cause de-crystallization of cellulose. In addition, less than 4% dilute acid is required for the hydrolysis of hemicelluloses fraction (usually xylan) of lignocellulosic materials to yield xylose. The efficiency of acid pre-treament is based on molarity or concentration of acid, operating conditions and substrate particle size (Kumar *et al.,* 2009). Several works have been done on the use of acids for substrate pre-treatment purposes. Salihu *et al.*(2015) reported improved cellulase production consequent to acid (1N H_2SO_4) pre-treatment of agricultural residues (finger millet, cassava peel, sugarcane bagasse, rice straw, sheanut cake, banana peels, sorghum and soybean hulls). In addition, the use of 0.1M HCl for the pre-treatment of rice bran and groundnut shell improved the growth of a mutant strain of *Aspergillus niger* and subsequently resulted in increased activities of FPase, CMCase and β-glucosidase (Narasimha *et al.,* 2016). According to Jonsson and Martin (2016), acid hydrolysis for the pre-treatment of lignocellulosic materials often results in sugar degradation and formation of inhibitory compounds

e.g. furfural and acetic acid. These may inhibit proliferation of producers (microoganisms) and hence, reduce bioconversion rate.

(ii) Alkaline pretreatment

Alkaline pretreatment is the most studied chemical pretreatment method due to its high lignin removal efficiency and low incidence of inhibitory compounds generation. Common alkali compounds used for this purpose include NaOH, $Ca(OH)_2$, CaO, KOH and $NH_3.H_2O$ (Kumar *et al.,* 2009), and among these, NaOH is most preferred because of its high efficiency (Wang *et al.,* 2017). Alkaline pre-treatment causes sulphonation of lignin, which makes it to solubilize. Consequently, hydrophilicity of lignin increases which makes it to be easily attacked by microganisms (Yang *et al.,* 2017). Common disadvantage of alkaline pre-treatment is high production of effluent as a result of washing of pre-treated materials. However, advantages of alkaline pre-treatment in terms of its efficiency supersede this demerit (Kumar *et al.,* 2009). Studies on the use of alkali for the pre-treatment of lignocellulosic materials for improved bioconversion are numerous (Cho *et al.,* 2013; El-shtawy *et al.,* 2015; Satale and Oh, 2015).

(iii) Pre-treatment with oxidizing agents

Pre-treatment with oxidizing agents e.g. hydrogen peroxide and peracetic acid at 160-290°C and 0.69-4.9MPa causes oxidative reactions, which digest and weaken lignocellulosic matrices and thus, improve their bioconversion rates (Dutra *et al.,* 2017). Salihu *et al.* (2015) reported improved cellulase production consequent to 1N H_2O_2 pre-treatment of agricultural residues (finger millet, cassava peel, sugarcane bagasse, rice straw, sheanut cake, banana peels, sorghum and soybean hulls). Also, the use of 0.1M H_2O_2 for the pre-treatment of rice bran and groundnut shell improved the growth of a mutant strain of *Aspergillus niger* and subsequently resulted in increased activities of FPase, CMCase and β-glucosidase (Narasimha *et al.,* 2016). The use of oxidizing agents for residue pretreatment purposes is limited because of the formation inhibitory compounds (Dutra *et al.,* 2017).

(iv) Ozonolysis

Pre-treatment of residues based on the principle of ozonolysis involves the use of ozone at ambient temperature and pressure. It is suitable for the improvement of rice straw (Kumar *et al.,* 2009). The methodology is expensive due to high cost of ozone (Kumar *et al.,* 2009).

(v) Organosolvation

Organosolvation involves simultaneous pre-hydrolysis and delignification of linocellulosic biomass with the aid of organic or aqueous solvent and inorganic acid (such as H_2SO_4 or HCl) to disrupt the structure of lignin (Kumar *et al.,* 2009). Common solvents used include methanol, ethanol, acetone and ethylene glycol. Pan *et al.* (2015) reported improved enzymatic hydrolysis of ethanol organosolvation of soft wood. Major disadvantage is high cost of solvent recovery (Kumar *et al.,* 2009).

(vi) Pre-treatment with ionic liquids

Pre-treatment with the aid of ionic liquids (also called green solvent) such as polyethylene glycol, sodium dodecyl sulphate, 1-Allyl-3-methyl imidazolium chloride etc. is gradually gaining wide acceptance due to their high efficiency (Chang *et al.,* 2017) and thermostability (Yang *et al.,* 2017).

(vii) Pre-treatment with surfactants

Pre-treatment of residues with the aid of surfactant such as Triton X, Tween 20, Tween 80 etc. are gradually gaining attention. Surfactant reduces hydrophobicity of substances by reducing the surface tension between the hydrophobic and hydrophilic phases (Chang *et al.,* 2017).

(b)   Physical pretreatment methods

Physical pretreatment methods include heat treatment, size reduction, microwave, steam explosion, ultrasound, extrusion etc. (Kumar *et al.,* 2009).

(i) Heat treatment

Pre-treatment of residues with heat treatment involves heating the lignocellulosic materials at temperatures above 300 °C. Severity of lignin disruption and cellulose de-crystallization increases with increasing temperature. Heat pre-treatment has been applied for improvement of spent grain for prior to ethanol production (Montgomery and Botchmann, 2014). Major disadvantages are: high energy requirement and inability to degrade high lignocellulosic materials (Montgomery and Botchmann, 2014).

(ii) Pre-treatment by size reduction

Pre-treatment of residues based on the principles of size reduction or comminution involves the use of equipment such as hammer mill, tub grinder, knife mill etc. (Tabil *et al.,* 2011) for chopping, grinding, milling or cutting residues such that their surface areas, pore sizes and inter-particle bonding characteristics are increased. These enhance subsequent delignification and densification operations due to improvement of residue's frictional properties, bulk and particle density (Mani *et al.,* 2004).

(iii) Pre-treatment by microwave radiation

Microwave pre-treatment involves the use of microwave radiation (a form of di-electric energy) with microwave power between 200-1000W to cause structural disruption of residues (Inan *et al.,* 2016). Microwave pre-treatment changes ligocellulosic biomass through disintegration of lignin and hemicellulose fractions (Li *et al.,* 2015). According to Normanbhay *et al.* (2013), microwave pre-treatment ensures uniformity of process, which results in consistency in product quality. It is also characterised with features such as short pre-treatment time; reduced energy consumption; ease of control and high efficiency due to even distribution of heat within the matrices of the material.

(iv) Pre-treatment by steam explosion

Steam explosion pre-treatment of biomass employs the use super-saturated steam at temperature 160-260 ^{o}C under high pressure (0.69-4.83 MPa). Explosive decompression is generated when the pressure is released and this causes hydrolysis of hemicellulose and transformation of lignin (Montgomery and Botchmann, 2014). Garmakhamy *et al.* (2013) reported improvement in delignification of steam-exploded canola straw.

(v) Ultrasound pretreatment

Ultrasound pretreatment causes deconstruction of lignocellulosic biomass by energy cavitation. When a material is subjected to ultrasound energy, bubbles are generated within the material and a collapse of cavitation bubbles generates significant shear stress in the medium, which subsequently disrupt the material (Wang *et al.,* 2017). Wang *et al.* (2017) demonstrated the use of ultrasound for the the improvement of grass clipping.

(vi) Extrusion cooking method

Extrusion cooking involves forceful passage of lignocellulosic materials through screw at high temperature. Frictional forces are generated which cause a disruption of the highly ordered lignocellulosic materials (Montgomery and Botchman, 2014). It is applicable in the pre-treatment of cereal straws. The major disadvantage is high downtime as a result of constant wear of screws (Montgomery and Botchmann, 2014).

(c) Biological methods of pretreatment

This employs rot fungi (soft, white, brown) for the degradation of lignin in agricultural residues (Tabil *et al.,* 2011). The fungi act by elaborating laccase and/or peroxidase, which have high lignocellulosic-degrading properties (Montgomery and Botchmann, 2014). It is also commonly used for the removal of hazardous materials from effluent. Okano *et al.* (2015) reported the use of white rot fungi (*Pycnoporus*

cinnabarinus and *Ceriporiopsis subvermispora*) to remove phenolic toxins from wastewater. Biological methods require less energy; however, they are very slow (Kumar *et al.,* 2009).

(d) Pretreatment by combination of methods

The major objective in the combination of methods for the pretreatment of residues is the synergistic merits of each of the methods and this results in improved efficiency of the process. Chemical pre-treatment may involve the use of more than one chemical reagent. For example, Dutra *et al.* (2017) reported better enzymatic hydrolysis of lignocellulosic materials that were subjected to alkaline and hydrogen peroxide pretreatment compared to hydrolysis when the chemicals were applied singly. Also, Irfan *et al.* (2011) reported improvement in percentage delignification of sugarcane bagasse that was pre-treated with a combination of 5% H_2O_2 and 2% NaOH to the tune of 88%, whereas, 51% delignification was recorded when 2% NaOH was used. Combination of physical and chemical pre-treatments is also frequently practiced. Wang *et al.* (2017) reported improvement in the rate of saccharification of grass clipping that was pre-treated with a combination of ultrasound and alkaline pre-treatment. Also, increase in activity of exo-pectinase was recorded when orange peel was pre-treated with microwave and surfactant. Combination of NaOH and microwave radiation for improved bioconversion is a common practice (Kashaninejad *et al.,* 2010; Tabil *et al.,* 2011; Normanbhay *et al.,* 2013; Inan *et al.,* 2016).

2.7.2 Properties of lignocellulosic materials

Properties of plant materials vary in terms of their concentration of lignin, hemicellulose, cellulose and polysaccharides. In addition, the properties are influenced by pretreatment methods (Raj *et al.,* 2016)

(a) Percentage solids recovery

Total solids recovered after a pretreatment operation is dependent on pretreatment method. Higher solids are obtained from physical pre-treatment methods compared to chemical ones. Raj *et al.* (2016) reported low solids loss of substrate as a result of high salvage capability of microwave pretreatment. This is similar to the low solids

loss of microwave pre-treated dragon fruit foliage (Ethaib *et al.,* 2016). According to Kataria and Gosh (2014), high (15.6 to 47.5%) solids were lost when Kans grass (*Saccharum spontaneum)* was subjected to NaOH pre-treatment. Low percentage solids recovery of chemically pre-treated substrates is due to dissolution of molecules such as lignin and sugars by the alkali (Cotana *et al*., 2015).

(b) Chemical composition of lignocellulosic materials

Properties of pre-treated residues in terms of its chemical composition are dependent on the methodology adopted. Li *et al.* (2015) had reported the stability of pectin in orange peel to microwave radiation. The finding of Koh *et al*. (2014) also showed stability of pectin extracted from microwave pre-treated jack fruit rind. Pre-treatment often results in an increase in cellulose content of pre-treated agricultural residues and food wastes due to cellulose bulging, a phenomenon which occurs as a result of cellulose condensation owing to lignin and hemicelluloses removal (Pandey and Negi, 2015). Cellulose comprises of crystalline and amorphous fractions (Brodeur *et al*., 2011). While the former contributes to the crystallinity of cellulose and is recalcitrant to breakdown, the later is susceptible to breakdown through chemical, physical and biological means (Brodeur *et al*., 2011). Therefore, an objective of pretreatment is to reduce cellulose crystallinity, which invariably increases the amorphous fraction. The degree at which this occurs is a function of pre-treatment method, structural properties and chemical composition of substrates (Raj *et al*., 2016).

Pre-treatment usually causes reduction in hemicelluloses and lignin content of residues. Maeda *et al.* (2011) recorded a marked reduction of hemicelluloses in banana pseudo-stem subjected to chemical pre-treatment. Microwave pre-treatment is also known to be very efficient in the disintegration of hemicelluloses (Kumar *et al*., 2009). A remarkable decrease in hemicellulose of wheat straw pre-treated with microwave radiation was also reported by Kashaninejad *et al*. (2010). Reduction of lignin as a result of pre-treatment is advantageous because low lignin contents of pre-treated residues will increase their accessibility to microbial attack for subsequent hydrolysis (Yang *et al*., 2017). NaOH pretreatment is an efficient method

for lignin removal because of its high polarity, which causes increased lignin solubilization (Ethaib *et al*., 2016). Combination of NaOH and microwave is also an efficient method for lignin removal. Studies have shown that better lignin removal was achieved when microwave and NaOH were combined for the pre-treatment of switch grass (Keshwani *et al*., 2007), wheat straw (Kashanenejad *et al*., 2010) and barley straw (Inan *et al*., 2016). In the presence of alkali, microwave increases degradation of lignin fibres through the dissolution of phenolic hydroxyl group and β-carbons that are adjacent to α-carbonyl groups, which are the major building blocks of lignin (Starr *et al*., 2015).

Chemical pretreatment is efficient for the removal of extractives and ash due to their high dissolution capabilities (Liu *et al.,* 2017). Chemical pre-treatment involves washing of pre-treated substrates to a neutral pH in order to remove adhering chemicals. According to Yu and Chan (2010), loss of ash often accompanies substrate washing after pre-treatment. Since mineral elements contribute to instability of pH in solutions, low ash content in pre-treated biomasses improves their stability of pH during downstream processing (Liu *et al*., 2017). Chang *et al.* (2017) reported an increase in total sugar of alkaline pre-treated rice straw. This could be due to increased concentration of cellulose in pre-treated substrates, which breaks down to yield more sugar (El-Shishtawy *et al*., 2015). Inan *et al.* (2016) reported that microwave pre-treated barley straw gave a lower total sugar content compared to combined NaOH and microwave pre-treated ones.

(c) Morphology of lignocellulosic materials

Morphology of pre-treated residues are studied with the aid of scanning electron microscopy (SEM). Pre-treatment of residues results in increased surface area and porosity of surface structures (Han *et al.* 2012; Ethaib *et al.,* 2016; Yang *et al*., 2017). Han *et al*. (2012) observed that wheat straw became rough and soft/loose consequent to NaOH pre-treatment. According to Raj *et al.* (2016), the major objective of pre-treatment is to reduce the rigid nature of substrates thereby giving room for increased metabolic activity of microorganisms and/or enzymes for improved production of bio-products. Sahare *et al.* (2012) had attributed increased saccharification of pre-

treated corncob to the elimination of rough external surface and expansion of lignin fibre. During the pretreatment of rice straw, Chang *et al.* (2017) demonstrated that the structural properties of pre-treated rice straw varied depending on the pre-treatment type. This suggests that different degrees of pre-treatment of feedstock can be achieved by different methods and no two pre-treatment methods will give the same substrates' morphological properties.

(d) Structure of lignocellulosic materials

Structural characterisation of un-treated and pre-treated residues in terms of types of functional group is conducted with the aid of Fourier Transform Infrared Spectroscopy (FTIR) (Tabil *et al.,* 2011). The principle is based on Bouguer, Beer and Lambert law which states that the intensity of an absorption band is directly correlated with the weight of homogenous compound in a solution (Adapa *et al.,* 2009). Biomass, which consists of lignin, cellulose and hemicelluloses are easily identified by designated wave numbers on the FTIR spectra. According to Adapa *et al.* (2011), lignin fraction is represented by spectra 1430, 1370, 1340, 1320 and 1200 cm^{-1}; hemicellulose fraction by 1600, 1460, 1250, 1210, 1165 and 105 cm^{-1}; and cellulose fraction by 1430, 1370, 1340, 1329 and 1200 cm^{-1}. Pre-treatment of residues usually results in changes in wave number at a given peak. According to Santale and Oh (2015), this can be measured by calculating percentage relative change where a positive value is an indication of reduction in the wave number of a band in relation to the control band (band of un-treated biomass).

2.8   Fruit Juice

Fruits are cherished and relished all over the world for their aesthetic, nutritional and health benefits (Mohamed *et al.,* 2014; Mustafa *et al.,* 2016). According to FAO (2010), 609 million metric tons of fruits are produced globally on an annual basis. Like other categories of food, high (roughly 40%) is lost as a result of improper postharvest handling. Therefore, fruits are often processed into more stable forms such as juice, jam, preserves, nectar, toffee etc. (Sharma *et al.,* 2014).

The juice, which is the major component of fruits, varies both in quantity and in composition based on the type of fruit, cultural practice employed for cultivation, age of plants, degree of ripeness etc. (Joshi *et al.,* 2011).

2.8.1 Extraction of juice from fruits

The major objective in juice extraction is to ensure maximum yield of juice (Cereti *et al.,* 2016). Irrespective of the method employed, the principle lies in depectinization and rupturing of cells for the release of water (Lee *et al.,* 2006). Mechanical/physical method of juice extraction employs operations such as heating, freezing, pressing, filtration, ultrafiltration, microfiltration etc. to expel water from cells (Seyedabadi *et al.,* 2016). Several reports and studies abound on the extraction of juice by physical or mechanical means (Seyedabadi *et al.,* 2016; Cereti *et al.,* 2016; Cereti *et al.,* 2017). Mechanical method of juice extraction is less employed in the fruit industry due to high-energy requirements, lower yield, high solids waste and increased mechanical damage because of fouling of filter beds (Oszmianski *et al.,* 2009). Enzymatic extraction and clarification of juice are common operations in fruit processing (Padma *et al.,* 2017). It has advantages such as better yield, lower energy consumption and better nutrient retention when compared to mechanical methods. Enzymes that are often employed for juice extraction and clarification include pectinases, cellulases, proteinases and hemicellulases (Sharma *et al.,* 2014).

2.8.2 Yield of fruit juice

Earlier studies showed increase in fruit juice as a result of enzymatic extraction: 17.5% for bael (*Aegle marmelos*) (Singh *et al.,* 2012b); 26% in plum fruit (Joshi *et al.,* 2011); 11.46% in carrot juice (Kaur and Sharma, 2013); 13.5% in apple juice (Oszmianski *et al.,* 2009). The increase in yield in enzymatically extracted juice is attributed to loss of water holding potential of pectin consequent to its degradation by pectinase. Therefore, more water containing less complex materials such as sugars is liberated and this increases the yield (Lee *et al.,* 2006).

2.8.3 Chemical composition of fruit juice

According to Cerreti *et al.* (2017), properties that are related to enzyme such as concentration, types, purity are important factors that influence juice extraction and clarification. Reports on the influence of enzymatic extraction on the composition of fruit juice showed increase in sugars and soluble solids. This is due to increased degradation of complex polysaccharides to sugars (Cerreti *et al.,* 2016). Abbes *et al.* (2011) reported reduction in pH in enzymatically extracted date syrup. This is advantageous because low pH is instrumental to better shelf stability of juice (Bahramian *et al.,* 2011). Enzymatic juice extraction is also characterised with reduction in titratable acidity and ascorbic acid. Vijayanand *et al.* (2010) had attributed reduction in vitamin C in enzymatically extracted umbu (*Spondias tuberose*) juice to oxidation of ascorbic acid during extraction operation.

2.8.4 Rheological properties of fruit juice

For most fruit juices, shear stress increases with an increasing shear rates. This pattern suggests a pseudoplastic or shear-thinning non-Newtonian behaviour. Gouvea *et al.* (2017) reported pseeudoplastic behaviour for umbu juice extracted with pectinase enzymes- rapidase and pectinex. Pseudoplastic behaviour of fluids also entails reduction in viscosity with increasing shear rates. Gouvea *et al.* (2017) reported reduction in viscosity of umbu juice extracted with pectinase enzyme. Reduction in viscosity is attributed to breakdown of polysaccharides such as pectin, which has high jelly-forming capability (Sharma *et al.,* 2014). According to Singh *et al.* (2012b), one of the objectives of enzymatic juice extraction is to disintegrate the jelly nature of fruit pulp for easy juice recovery. Also, rheological properties of juices are characterized with the aid of flow behaviour parameters such as flow behaviour index, consistency index and yield stress (Sharoba and Ramadan, 2011). According to Deshmukh *et al.* (2015), flow behaviour properties are dependent on solids/solute (mostly sugars and organic acids) content. Solids and solute contents determine their level of interaction and this interaction (i.e. water-solute interaction) is based on the nature of solute in terms of its shape, size and molecular weight; and its degree of hydration in the solvent (Diamante and Umemoto, 2015). High soluble

solids content increases water and solute interaction, which results in increased molecular hydration (Deshmukh *et al.,* 2015; Sakhale *et al.,* 2016).

Generally, the flow characteristics of juices increase with increase in temperature. This stems from reduction in viscosity as a result of enhanced mobility of molecules which led to increased inter-molecular collision as temperature increases (Rao, 2007). Deshmukh *et al.* (2015) reported higher flow characteristics of sapota (*Achras sapota*) juice with increasing temperature. Enzymatic juice extraction reduces the incidence of yield stress because of reduced viscosity (Sharoba and Ramadan, 2011). Yield stress is a form of potential energy, which must be overcome before flow of liquid can commence, therefore, its reduction is advantageous because less energy will be required for equipment design (Rao, 2007). Activation energy is a thermodynamic tool that is used to measure the degree of temperature dependency of juices (Deshmukh *et al.,* 2015). The higher the activation energy, the higher the rate of viscosity reduction (Manjunatha *et al.,* 2012). Sharoba and Ramadan (2011) reported that the activation energy of golden berry juice reduced as a result of enzymatic extraction.

2.8.5   Enzymatic clarification of fruit juice

Clarification of juice is accompanied with increase in juice clarity and reduction in turbidity (Sharma *et al.,* 2014). According to Tribess and Tadini (2006), apple juice is cloudy due to the presence of pectic and non-pectic polysaccharides; cellulose, proteins, tannins, metals etc. Success has been recorded on the use of PG for the clarification of apple (Joshi *et al.,* 2011; Dey *et al.,* 2014; Dey and Banerjee, 2014) and guava (Kant *et al.,* 2013) juices. Enzymes such as pectinases, cellulases, hemicellulases and proteinases degrade complex polysaccharides especially pectin which leads to crystal formation. The crystals are easily removed via centrifugation, decantation, and filtration or by any other mechanical separation method (Sharma *et al.,* 2014). Performance of enzymes in juice clarification is dependent on enzyme related properties such as activity, purity, catalytic activity etc. (Levaj *et al.,* 2012; Diamante and Umemoto, 2015). Colour order properties in terms of L*, a*, b* and colour intensity are parameters that are employed for the evaluation of extent of

enzymatic clarification of juice (Umsza-Guez *et al.,* 2011). Studies have shown improvement in colour order properties of fruit juices extracted enzymatically (Umsza-Guez *et al.,* 2011; Kaur and Sharma, 2013; Deshmukh *et al.,* 2015) . The improvement is indication of increased clarity (Kaur and Sharma, 2013) or colour degradation as a result of auto-oxidation of phenols (Deshmukh *et al.,* 2015).

CHAPTER THREE

3.0   MATERIALS AND METHODS

3.1   Materials

Peels of orange (*Citrus sinensis* L. Osbeck) and plantain (*Musa paradisiaca* Linn.) were obtained from small-scale food processing factories in Ibadan, Nigeria. Fungal strain, *Aspergillus awamori* CICC (China Centre of Industrial Culture Collection) 2040 was obtained from China National Research Institute of Food and Fermentation, Beijing, China. Mango (*Mangifera indica* L.) fruits, *Ogbomosho* cultivar were obtained from Oje Market, Ibadan, Nigeria, and Apple (*Malus domestica* Borkh) fruits, Green Trucape, South Africa, were obtained from Shoprite Ltd., Ibadan, Nigeria. Pectinase enzyme (17389-10G, Sigma, Switzerland), standard protein marker (Amersham Biosciences, UK), polygalacturonic acid (Oxoid, England), activated charcoal (Xiamen ACC, China), and pectin (Oxoid, England) were procured from Bristol Scientific Company Ltd., Lagos, Nigeria. Acetic acid, Tween 20, glycerol (Klincent Laboratory, India), n-hexane, methanol, sodium hydroxide (Loba Chemie, India), malt extract agar (MEA), potato dextrose agar (Oxoid, England), glucose, sucrose, starch, isopropanol (ACROS Organics, USA), sodium acetate, 3, 5 di-nitro salicylic acid (DNS), citric acid (Guangdong Guanghua, China) were procured from Ezek Robert Global Ltd., Ibadan, Nigeria. All reagents used were of analytical grade.

3.2   Methods

Methods employed in production and pretreatment of orange and plantain peels, production, purification and characterisation of PG, and processing of mango and apple juices are discussed in this Section.

3.2.1 Production of orange and plantain peel flours

Five hundred kilogrammes each of orange and plantain peels were blanched (80 ^{o}C for 3 min), rinsed and dried in a hot air oven (NL9023A, England) at 60 ^{o}C for 48 h. The dried peels were milled into powders in an attrition mill (BLG-402, China) (Kashaninejad and Tabil, 2011), and sieved into 3 different particle sizes with the aid of 0.4250, 0.8025 and 1.1800 mm sieves (United States Pharmacopoeia Standard Sieves). The powders were packaged in polyethylene containers (ZipLock, China) and stored at -20±2°C in a freezer for subsequent analyses.

3.2.2 Pretreatment of orange and plantain peel flours

Orange and plantain peels were subjected to alkaline and microwave pretreatment, separately using response surface methodology, and in combination.

(a) Alkaline pretreatment of orange and plantain peels

Alkaline pretreatment method outlined by Salihu *et al*. (2015) was adopted with little modification in molarity of NaOH. Fifty gramme of flour sample was treated with 1 L NaOH using varying pretreatment conditions. Pretreated samples were rinsed with distilled water to pH 7, dried in a hot air oven (NL9023A, England) at 60 ^{o}C for 24 h and stored (-20±2 ^{o}C) in a freezer for subsequent analyses.

(b) Microwave pretreatment of orange and plantain peels

Microwave pre-treatment was done based on the procedure reported by Inan *et al.* (2016). Fifty gramme of flour sample was added to 1 L distilled water in a container and the mixture was treated in a laboratory microwave oven (NX-802 with 25 L capacity, 800W power output and frequency of 2450MHz) at varying power level for different pre-treatment time. Thereafter, the residue was oven-dried (NL9023A, England) at 60 ^{o}C to constant moisture content and stored (-20±2°C) until required for use.

(c) Experimental design for pretreatment of orange and plantain peels

Face centered central composite design (FCCCD) under the response surface methodology (RSM) was used for the evaluation of three independent variables each of alkaline and microwave pretreatment methods. Factors interacted for alkaline pretreatment were Particle Size, PS (<0.4250, 0.4250<PS<0.8025,

Table 3.1. **Experimental Design of Factors for Alkaline Pretreatment of Orange and Plantain Peels**

Run	Substrate's particle size (mm)	NaOH Molarity (M)	Time (h)
1	<0.425 0	0.010	1.0
2	0.8025<x<1.1800	0.010	1.0
3	<0.425 0	0.100	1.0
4	0.8025<x<1.1800	0.100	1.0
5	<0.425 0	0.010	12.0
6	0.8025<x<1.1800	0.010	12.0
7	<0.425 0	0.100	12.0
8	0.8025<x<1.1800	0.100	12.0
9	<0.4250	0.055	6.5
10	0.8025<x<1.1800	0.055	6.5
11	0.4250<x<0.8025	0.010	6.5
12	0.4250<x<0.8025	0.100	6.5
13	0.4250<x<0.8025	0.055	1.0
14	0.4250<x<0.8025	0.055	12.0
15	0.4250<x<0.8025	0.055	6.5
16	0.4250<x<0.8025	0.055	6.5
17	0.4250<x<0.8025	0.055	6.5
18	0.4250<x<0.8025	0.055	6.5
19	0.4250<x<0.8025	0.055	6.5
20	0.4250<x<0.8025	0.055	6.5

0.8025<PS<1.1800 mm), NaOH molarity (0.010, 0.055 and 0.100 M) and pretreatment time (1.0, 6.5 and 12.0 h) (Table 3.1) while those for microwave pretreatment were PS (<0.4250, 0.4250<PS<0.8025, 0.8025<PS<1.1800 mm), microwave power (240, 480 and 720 W) and pretreatment time (2.50, 6.25 and 10.00 min.) (Table 3.2). The factors were interacted to determine combination for maximum polygalacturonase activity. Experiments were conducted in triplicates and means of measured values were used to generate the response (PG activity). A linear equation was fitted to the data by multiple regression procedure (Equation 3.1)

$$Y = \alpha_0 + \sum_{i=1}^{n} \alpha_i X_i + \sum_{i=1}^{n} \alpha_{ii} X_i^{2} + \sum_{i=1}^{n-1} \sum_{i=1}^{n} \alpha_{ij} X_i X_j \qquad (3.1)$$

Where Y represents predicted response, PG activity (U/mL), X_1, X_2, X_3.......Xn are independent variables, α_0 is a constant, and αi, αii and αij are linear, squared and interaction effects, respectively. Multiple regression model were evaluated with the aid of analysis of variance (ANOVA) and quality of fit was tested by determining coefficient of determination (R^2). These were achieved using the Minitab software, version 16.2.1 (Stat-Ease Inc., USA).

(d) Combined alkaline and microwave pretreatment of orange and plantain peels

Combined (microwave radiation and NaOH) pretreatment was carried out based on the method outlined by Peng *et al*. (2014). This was achieved by interacting alkaline and microwave pretreatment combinations that gave maximum PG activity from alkaline and microwave pretreatments. Fifty gramme of flour sample was added to 1 L NaOH in a beaker and the mixture was treated in a laboratory microwave oven (NX-802 with 25L capacity, 800W power output and frequency of 2450MHz). The sample was neutralized to pH 7, dried in a hot air oven (NL9023A, England) at $60^{o}C$ for 24 h and stored ($-20\pm2^{o}C$) for subsequent analyses.

Table 3.2.   Experimental Design of Factors for Microwave Pretreatment of Orange and Plantain Peels

Run	Substrate's particle size (mm)	Microwave power (W)	Time (min)
		Independent variables	
1	<0.425 0	240	2.50
2	0.8025<x<1.1800	240	2.50
3	<0.4250	720	2.50
4	0.8025<x<1.1800	720	2.50
5	<0.425 0	240	10.00
6	0.8025<x<1.1800	240	10.00
7	<0.4250	720	10.00
8	0.8025<x<1.1800	720	10.00
9	<0.425 0	480	6.25
10	0.8025<x<1.1800	480	6.25
11	0.4250<x<0.8025	240	6.25
12	0.4250<x<0.8025	720	6.25
13	0.4250<x<0.8025	480	2.50
14	0.4250<x<0.8025	480	10.00
15	0.4250<x<0.8025	480	6.25
16	0.4250<x<0.8025	480	6.25
17	0.4250<x<0.8025	480	6.25
18	0.4250<x<0.8025	480	6.25
19	0.4250<x<0.8025	480	6.25
20	0.4250<x<0.8025	480	6.25

3.2.3 Polygalacturonase production

(a) Culturing of microorganism

Fungal strain *Aspergillus awamori* CICC 2040 was maintained on malt extract agar at 28°C for 6 days. Inoculums for the experiments were prepared from heavily sporulated MEA slants.

(b) Solid state fermentation and extraction of crude polygalacturonase

Solid state fermentation procedure described by Dey *et al.* (2014) was adopted. Substrate was mixed with Czapek-dox medium (2.5 g/L $NaNO_3$, 1 g/L KH_2PO_4, 0.5 g/L KCl and 0.5 g/L $MgSO_4.2H_2O$) at pH 4.0 in ratio 1:2 (w/v) in a 250 mL Erlenmeyer flask and autoclaved (121°C, 15 psi) for 15 min. Subsequently, the substrate was inoculated with 10^6 spore/mL of the culture and incubated in an incubator (CLN115 Pol Eko Aparatura, Poland) at 28 °C for 5 days. After this, fermented mass was suspended in distilled water to form a 50 g/L suspension. The suspension was placed in an incubator (CLN115 Pol Eko Aparatura, Poland) at 30 °C for 1 h and centrifuged (K24IR, Centurion Scientific Ltd, UK) at $2200 \times$ g for 10 min. The supernatant was separated by using Whatman No. 1 filter and PG assay conducted. Enzyme was stored at -20°C in a freezer until required (Melikoglu *et al.*, 2013).

3.2.4 Purification of polygalacturonase

Crude PG was purified with the aid of activated charcoal as described by Dey and Banerjee (2014) with modification in contact time. A 0.5 g activated charcoal was added to 10 mL cell-free crude PG and the mixture was incubated (CLN115 Pol Eko Aparatura, Poland) at 28 °C for 3 h. Subsequently, it was centrifuged (K24IR, Centurion Scientific Ltd, UK) at $6000 \times$ g for 10 min.

Specific activity SU, rate of purification LCI, fold purification FP and percentage enzyme recovery PR were calculated using Equations 3.2, 3.3, 3.4 and 3.5, respectively (Kumar and Parrack, 2003).

$$\text{SU} = \frac{Enzyme\ activity}{Protein\ content} \tag{3.2}$$

$$\text{LCI} = \frac{Absorbance\ of\ purified\ enzyme}{Absorbance\ of\ crude\ enzyme} \tag{3.3}$$

$$FP = \frac{Specific\ activity\ of\ purified\ enzyme}{Specific\ activity\ of\ crude\ enzyme} \qquad\qquad (3.4)$$

$$PR = \frac{Activity\ of\ purified\ enzyme}{Activity\ of\ crude\ enzyme} \times 100 \qquad\qquad (3.5)$$

3.2.5   Evaluation of enzyme stability

(a) Evaluation of polygalacturonase stability at different pH values

Activity of PG was determined at different pH values based on the procedure outlined by Dogan and Tari (2008). This involved conduction of PG assay at pH values that ranged from 3.0 to 12.0, using different buffers. A 0.5 mL each of PG and pH buffer solution was incubated at room temperature (28 ± 2 ^{o}C) for 2 h. Thereafter, aliquot was taken from the mixture and assayed for PG. Relative activity was obtained as the ratio of activity of PG at a given pH to the maximum activity obtained within the pH range considered.

(b) Evaluation of polygalacturonase stability at different temperatures

Activity of PG was carried out by conducting PG assay at different temperatures that ranged between 25 and 75°C at 10°C intervals, based on the procedure described by Dey *et al.* (2014). A 0.5 mL each of PG and 0.5% polygalacturonic acid solution was incubated in a water bath (NL9023A, England) at each temperature for 10 min and PG assay conducted. Relative activity was obtained as the ratio of activity of PG at a given temperature to the maximum activity obtained within the temperature range considered.

(c) Evaluation of polygalacturonase activity in the presence of selected chloride ions

Polygalacturonase activity in the presence of selected chloride ions was evaluated according to the method described by Goncalves *et al.* (2016). Polygalacturonase assay was conducted in the presence of chlorides of Na^+, Ca^{2+}, Mg^{2+}, K^+, NH_4^+ and Cu^{2+} at a final concentration of 0.5 mM in 0.1 M acetate buffer at pH 5.0. Relative activity was determined as the ratio of activity of PG in the presence of an ion to the activity of PG in the absence of the ion (Goncalves *et al*., 2016).

(d) Evaluation of polygalacturonase activity in the presence of selected compounds

Polygalacturonase activity in the presence of selected compounds was evaluated according to the method described by Kant *et al.* (2013). Polygalacturonase assay

was conducted in the presence of compounds i.e. ethylene diamine tetra acetic (EDTA), citric acid, glycerol and Tween 20 at a final concentration of 0.1 mM in 0.1 M acetate buffer at pH 5.0. Relative activity was obtained as the ratio of activity of PG in the presence of a compound to the activity of PG in the absence of the compound.

(e) Evaluation of substrate specificity and kinetic constants of polygalacturonase

Substrate specificity of PG was done according to the method described by Dogan and Tari (2008). Substrates used were pectin, starch, malt extract and potato dextrose. A solution that contained 0.5 mL each of PG and 0.5% substrate solution was prepared and PG assay was conducted. Furthermore, PG assay was conducted using different concentrations (0.1, 0.2, 0.3, 0.4, and 0.5%) of pectin and PG activities at these concentrations were determined. Reciprocals of PG activity (velocity) and substrate concentration were plotted, and kinetic constants (Vmax and Km) were determined using the Lineweaver-Burk double reciprocal plot (Equation 3.6) (Rehman *et al*., 2012).

$$\frac{1}{v} = \frac{1}{Vmax} + \frac{Km}{Vmax} + \frac{1}{s} \tag{3.6}$$

Where v and s are velocity of reaction and substrate concentration, respectively.

(f) Evaluation of thermodynamic properties of polygalacturonase

Inactivation kinetics of PG was determined according to the procedure outlined by Dogan and Tari (2008). This was achieved by incubating enzyme in a water bath (NL9023A, England) at temperatures 60, 70, 80 and 90 °C in the absence of the substrate. Aliquots were taken (every 20 min for 2 h), rapidly cooled in an ice bath and assayed for PG. Percentage residual activity was calculated as the ratio of enzyme activity at a given time to the initial activity. Inactivation rate constant (kd) was obtained from a semi-log graph of residual activity and time. Half life (t½) and decimal reduction time (D) were calculated as ratio of ln 2 and kd; and ln 10 and kd, respectively. Arrhenius equation (Equation 3.7) was used to evaluate temperature dependency of PG (Melikoglu *et al.,* 2013).

$$\ln kd = \ln(ko) - \left(\frac{Ea}{R}\right)\left(\frac{1}{T}\right) \tag{3.7}$$

Where Ea is activation energy (kJ/mol), ko is Arrhenius equation constant, R is the ideal gas constant = 8.314 J/molK and T is temperature (K). Eyring equation (Equation 3.8) was used for the estimation of thermodynamic data (Melikoglu *et al.,* 2013).

$$Kd = (\frac{K_B}{h}T)e^{(\frac{\Delta H}{RT})}e^{(\frac{\Delta S}{R})} \qquad (3.8)$$

Where k_B is Boltzman's constant, 1.38×10^{-23} J/K; h is Plank's constant, 6.63×10^{-34} Js.

The enthalpy of activation ΔH, kJ/mol; free energy of activation ΔG, kJ/mol and entropy ΔS, J/molK were obtained from Equations 3.9, 3.10 and 3.11, respectively (Melikoglu *et al*., 2013).

$$\Delta H = E_a - RT \qquad (3.9)$$

$$\Delta G = -RT \, ln\frac{k_d}{K_B T}h \qquad (3.10)$$

$$\Delta S = \frac{\Delta H - \Delta G}{T} \qquad (3.11)$$

3.2.6 Evaluation of polygalacturonase in mango juice extraction

Juice was extracted from mango fruits based on the method outlined by Anuradha *et al.* (2016). Mango fruits were washed in a running clean water, peeled and pulp scraped with the aid of a sharp knife. The pulp was blended with a waring blender (Sa1706, China) and divided into four equal parts. One percent (obtained from preliminary investigation) enzyme solutions (1:50, v/v) i.e. crude polygalacturonase, purified polygalacturonase and a commercial pectinase enzyme (with activity of 10.5 U/g, molecular weight of 34.5 kDa, optimum temperature and pH of 50 °C and 4.5, respectively) were added to 50 mL pulp in separate 250 cm^3 beakers. Juice sample without enzyme served as control. Subsequently, the pulps were incubated (CLN115 Pol Eko Apatura, Poland) at 40 °C for 4 h. After incubation, juice was extracted from the pulp by filtration using a 200 mesh sieve (0.075 mm) and pasteurized at 85 °C for 2 min. The pasteurized mango juices were cooled to room temperature (28±2 °C) and thereafter, kept at 4±2 °C in refrigerator (Hisense RS230S, China) for subsequent analyses.

3.2.7 Evaluation of polygalacturonase in apple juice clarification

Apple juice was prepared according to the methodology outlined by Zhang and Zhang (2014). Fresh apple fruits were washed with water, cut into cubes of 1 cm^3 and blended with a waring blender (Sa1706, China) to a pulpy consistency. The pulp was sieved with a 200 mesh sieve (0.075 mm) to obtain a cloudy juice. The juice was pasteurized at 95°C for 1 min and rapidly cooled to 50 °C. One percent (obtained from preliminary investigation) enzyme solutions i.e. crude polygalacturonase, purified polygalacturonase and a commercial pectinase enzyme (with activity of 10.5 U/g, molecular weight of 34.5 kDa, optimum temperature and pH of 50°C and 4.5, respectively) were added to 50 mL of prepared apple juices in separate 250 cm^3 beakers. Juice sample without enzyme served as control. Temperature of the juices was maintained at 50 °C in an incubator (CLN115 Pol Eko Apatura, Poland) for 5 h. After incubation, the juice was sieved with a 0.038 mm sieve and kept at 4±2°C in refrigerator (Hisense RS230S, China) for subsequent analyses.

3.3 Analyses of Peels, Polygalacturonase and Fruit Juices

3.3.1 Determination of chemical composition of pretreated and untreated orange and plantain peels

(a) Determination of pectin

Pectin content of orange and plantain peels was determined using the HCl-alcohol method described by Liu (2004). Sample (1 g) was dissolved in 10 mL conc. HCl and the pH adjusted to 2.3. The solution was filtered with the aid of Whatman number 1 filter paper. The filtrate was precipitated in isopropanol and the precipitate was obtained through filtration with Whatman number filter paper and dried in a hot air oven (NL9023A, England) at 50 °C for 6 h. Pectin content was determined using Equation 3.12.

$$\text{Pectin content (\%)} = \frac{(Sample\ weight - weight\ of\ dried\ precipitate) \times 100}{Weight\ of\ sample} \qquad (3.12)$$

(b) Determination of cellulose

Cellulose content was obtained based on the procedure reported by Irfan *et al.* (2011). Dried residue (1 g) was treated with 15 mL 80% acetic acid and 1.5 mL conc. HNO$_3$, while heating under reflux. After 20 min of heating, the mixture was filtered with the aid of Whatman number 1 filter paper and washed. The digest was dried in a hot ait oven (NL9023A, England) at 105 °C for 6 h and the residue was

incinerated in a muffle furnace at 550 °C in order to account for the ash content. Cellulose content was calculated using Equation 3.13.

$$Cellulose\ cotent\ (\%) = \frac{(weight\ of\ digest - weight\ of\ ash) \times 100\%}{weight\ of\ sample}$$ (3.13)

(c) Determination of hemicellulose

Hemicellulose content was obtained based on the procedure reported by Irfan *et al.* (2011). The sample was defatted prior to hemicellulose determination. Three gramme of sample was mixed with chloroform/methanol (2:1 v/v) at a ratio of 1:10 (w/v). The mixture was heated at 60°C with continuous shaking (200 rpm) for 30 min in a water bath (NL42OS, England). It was then cooled to room temperature (32±2°C) and centrifuged at 11 000 × *g* for 25 min at 15 °C in an Avanti J-E centrifuge (Beckman Coulter, Inc., Fullerton, CA, USA).The resulting pellet was air dried at room temperature (32±°C) for 48 h and free of solvent odour. A 1 g of defatted residue was weighed into a 250 mL Erlenmeyer flask, which contained 150 mL of 0.5 M NaOH and the mixture was boiled for 4 h. After heating, the mixture was cooled and filtered with the aid Whatman number 1 filter paper and the residue was washed till the pH became neutral. The residue was dried at 105 °C to a constant weight and hemicellulose content was obtained using Equation 3.14.

$$Hemicellulose\ cotent\ (\%) = \frac{(W_1 - W_2) \times 100\%}{W_3}$$ (3.14)

Where W_1, W_2 and W_3 are weights of defatted residue, dried residue and sample, respectively

(d) Determination of lignin

Lignin content was estimated based on the 72% sulfuric acid treatment method described by Chang *et al.* (2017). To a 0.3 g of residue in a test tube, 3 mL of 72% H_2SO_4 was added and incubated at room temperature (28±2°C) with periodic shaking every 30 min for 2 h. Subsequently, 84 mL of distilled water was added and the mixture was autoclaved at 121°C for 1 h. Thereafter, the mixture was filtered and the residue obtained was dried (NL9023A, England) at 105°C to a constant weight. The dried residue was ashed and acid insoluble lignin was calculated using Equation 3.15. Absorbance of filtrate was obtained at 320 nm in a UV/VIS spectrophotometer (Jenway 6850, China). Subsequently, acid soluble lignin was obtained by comparing the reading with the calibration curve prepared with standard

acid soluble lignin. Total lignin content was obtained by adding acid soluble and acid insoluble lignin.

$$Acid\ insolube\ lignin\ (\%) = \frac{(sample\ weight - weight\ of\ digest) \times 100\%}{sample\ weight} \qquad (3.15)$$

(e) Determination of extractives

Extractive was determined by the procedure outlined by Liu *et al.* (2017). Five milliliter each of n-hexane and methanol were added to 1 g of sample in a centrifuge tube and the mixture was heated at $60^{\circ}C$ in a water bath (NL42OS, England) with continuous shaking for 30 min. The mixture was cooled to room temperature ($28 \pm 2^{\circ}C$) and centrifuged (K24IR, Centurion Scientific Ltd, UK) at $3000 \times g$ for 25 min. Extractive was thereafter obtained as difference between sample weight and residue obtained after centrifugation.

(f) Determination of ash

Ash content was determined by the AOAC (2000) method. A known sample weight was taken into a crucible of known weight and taken into a muffle furnace at 550 $^{\circ}C$ for incineration for 5 h. Thereafter, the crucible was cooled in desiccators and ash content calculated using Equation 3.16.

$$Ash\ cotent\ (\%) = \frac{(W1 - W2) \times 100\%}{W3} \qquad (3.16)$$

Where W_1 = weight of crucible and residue after ashing, W_2 = weight of crucible and W_3 = initial sample weight.

(g) Determination of moisture content

Moisture content of samples was determined using the oven-dry method (AOAC, 2000). Sample (2.5 g) was weighed into a drying pan of known weight and dried in a hot air oven (NL9023A, England) at $105^{\circ}C$ to a constant weight. Moisture content was calculated using Equation 3.17.

$$Moisture\ cotent\ (\%) = \frac{(W1 - W2) \times 100\%}{W1} \qquad (3.17)$$

Where W_1 and W_2 are initial and final sample weight, respectively

(h) Determination of total sugar

Total sugar was determined using the method described by Irfan *et al.* (2011). A 1 g sample was poured into 10 mL distilled water, and the mixture was thoroughly mixed and centrifuged (K24IR, Centurion Scientific Ltd, UK) at $2000 \times g$ for 10 min. One milliliter of the filtrate was mixed with 1 mL of 5% phenol solution in a test tube and 5 mL of 96% H_2SO_4 was added. The mixture was shaken, incubated at room temperature ($28\pm2\ ^{\circ}C$) for 30 min and absorbance (UV/VIS spectrophotometer 6850, Jenway, China) was read at 490 nm. The reading was compared with the calibration curve with glucose as standard.

3.3.2   Determination of morphological changes in pretreated peels

Scanning electron microscopy was used to evaluate morphology of pretreated peels based on the method described by Agu *et al.* (2017). Morphological changes as a result of pre-treatment of orange and plantain were determined with the aid of scanning electron micrograph (Phenom ProX, USA). Prior to analysis, samples were dried in a hot air oven (NL9023A, England) at 60 $^{\circ}C$. Subsequently, gold sputter coatings (10-100 nm) were made on the samples and microscopic examination was conducted at 5 kV. Images were taken at $2900\times$ magnification.

3.3.3   Determination of structural changes in pretreated peels

Structural changes in samples was determined according to the method described by Nomanbhay *et al.* (2013). Fourier Transform Infrared Spectroscopic (FTIR) analysis was conducted with the aid of a spectrometer (Perkin Elmer Spectrum BX 80857, UK) with scan range, scan number, scan speed and resolution of 4000-6000 cm^{-1}, 64, 0.5 cms^{-1} and 41 cm^{-1}, respectively. Sample (3 mg) was mixed with 300 mg spectroscopic grade KBr on a disc fixed by an agar mortar. Thereafter, the disc was taken to the equipment operated at 10 MPa for 3 min and the spectra was displayed on a computer screen attached.

3.3.4   Polygalacturonase assay and characterisation

(a) Determination of polygalacturonase activity

Polygalacturonase activity was determined according to the method described by Heerd *et al.* (2012). A 0.5 mL each of PG and 0.5% polygalacturonic acid was prepared in acetate buffer (pH 5.0) and the mixture incubated in a water bath (NL 42OS, England) at 50 $^{\circ}C$ for 10 min. Thereafter, 3 mL of freshly prepared 3, 5 di-

nitro salicylic acid (DNS) solution was added and the mixture heated at 90 $^{\circ}$C for 15 min. The mixture was rapidly cooled and absorbance (UV/VIS spectrophotometer 6850, Jenway, China) read at 575nm. One unit of PG activity was calculated as the amount of enzyme required to release 1 µmol of D-galacturonic acid per minute of reaction (µmol/min). A blank was prepared by mixing buffer, DNS and distilled water, and subjected to similar treatment as the enzyme solution. Polygalacturonase activity was expressed in unit of activity per mL (U/mL).

(b) Determination of total protein content of polygalacturonase

Protein content of PG was determined according to the modified Bradford's method (Kant *et al.,* 2013) using bovine serum albumin (BSA) as standard. Sample (0.2 mL) was taken into a test tube with the aid of a micropipette and 4.5 mL of a solution that contained 2% Na_2CO_3 in 0.1N NaOH, 1% sodium potassium tartarate, 0.5% $CUSO_4.5H_2O$ was added and incubated at ambient temperature ($28\pm2^{\circ}$C) for 10 min. Subsequently, 0.5 mL of a mixture of 2N Folin-phenol and water (1:1) was added. After 30 min of incubation, absorbance (UV/VIS spectrophotometer 6850, Jenway, China) was read at 660 nm. Protein content was obtained by comparing the reading with the calibration curve prepared with BSA as standard.

(c) Determination of enzyme protein purity and molecular weight

Protein purity and molecular weight of PG were determined using SDS-PAGE, according to the method described by Khatri *et al.* (2015). Protein bands and molecular weight of crude and purified PGs were determined using 10 g/100 mL acrylamide. Protein and sample buffer (1:1) was loaded and SDS gel with 2cm buffer zone was cast and ran in a steady current of 20 mA/gel. Staining was achieved with the aid of colloidal coomassie, and protein bands of PGs were compared with bands of molecular weight of a standard protein marker.

3.3.5   Colour order analysis of crude and purified enzymes

L (degree of lightness), a (degree of redness and greenness) and b (degree of yellowness and blueness) and colour intensity of crude and purified PGs were measured with the aid of a colorimeter (Minolta CR-300, Osaka, Japan) according to the method described by Burdurlu and Karadeniz (2003). The equipment was calibrated according to manufacturer's instructions. A 10 mL sample was poured on

a white plate and placed close to the prism of the equipment. The colour order parameters were obtained from an attached printer.

3.3.6 Determination of chemical composition of mango and apple juices

(a) Determination of percentage transmittance

The procedure described by Baxter (1995) was used to determine percentage transmittance. Samples were diluted with distilled water to give a soluble solid of 11.2 °Brix before the percentage transmittance was read at 650 nm with the aid of UV/VIS spectrophotometer (6850 Jenway, China).

(b) Determination of soluble solids

Refractometric method which involves the use of an Abbe refractometer (ATR-W2, England) was used for the determination of total soluble solids of juices (AOAC, 2005). Standardization of the equipment was done with distilled water at 20 °C. Subsequently, a drop of the sample was introduced on the prism of the equipment and soluble solids was read off from the screen of the equipment.

(c) Determination of pH

The procedure described by Joshi *et al.* (2011) was employed for pH determination, which involves the use of a digital pH meter (Orion Benchtop). Prior to pH determination, the pH meter was standardized with buffer 4 and 7 solutions. Thereafter, the tip of the pH meter probe was dipped into the sample (10%) and reading was displayed on the pH meter screen.

(d) Determination of reducing sugar

The methodology described by Cerreti *et al.* (2016) was used for the determination of reducing sugar. Appropriate dilution of 1mL juice was taken into a test tube and 3mL of DNS solution was added. The solution was heated in boiling water for 10 min and rapidly cooled on an ice bath. Absorbance of the solution was read at 570 nm with the aid of UV/VIS spectrophotometer (6850 Jenway, China). The reading was compared with the calibration curve obtained from standard sucrose solution in order to obtain reducing sugar.

(e) Determination of total sugar

Total sugar content was determined using the Dubois method, which involves digestion of sample with phenol and sulphoric acid (Heerd *et al.*, 2015). One

milliliter of appropriate sample dilution was mixed with 1 mL of 5% phenol solution and 5 mL of 96% H_2SO_4 was added. The mixture was shaken, incubated at 28°C (CLN115 Pol Eko Aparatura, Poland) for 30 min and absorbance was read at 490 nm. The reading was compared with the calibration curve obtained from standard glucose solution in order to obtain total sugar.

(f) Determination of titratable acidity

The procedure described by Dey and Banerjee (2014) was used for determination of titratable acidity. Twenty five milliliter of 10% juice was taken and 2 drops of phenolphthalein indicator were added. The solution was titrated against 0.1 N NaOH that was running from a burette. Titration was halted as a permanent pink colouration was observed. Thereafter, percentage titratable acidity was calculated using formular in Equation 3.18.

$$\% \text{ Titratable acidity} = \frac{Titre\ value\ \times titratable\ acid\ factor^*\ \times 0.1}{Volume\ of\ sample\ taken\ for\ titration} \times 100 \qquad (3.18)$$

*Titratable acid factor = 0.064 for citric acid (mango juice) and 0.067 for malic acid (apple juice).

(g) Determination of ascorbic acid content of juice

Standard method, which involves the use of 2, 6 dichlorophenolindophenol (dye) titration was used for ascorbic acid determination (Diamante and Umemoto, 2015). Filtered dye solution (0.05%) was titrated against 10 mL of standard ascorbic acid solution (0.005 g of ascorbic acid in 60 mL 20% orthophosphoric acid) until a faint pink colour persisted for 15 seconds. Subsequently, 20 mL sample solution (10%) was titrated against the dye and ascorbic acid content of juice was expressed as mg of ascorbic acid in 100 mL of dye solution (Equation 3.19).

$$Ascorbic\ acid\ content\ (\frac{mg}{100} mL\ juice) = 20(V)(C) \qquad (3.19)$$

Where V = volume of dye used for titration, and C = ascorbic acid per mL of dye solution.

(h) Determination of specific gravity of juice

Specific gravity of juices was obtained with the aid of a hydrometer (GL Zeal, UK) (AOAC, 2005). The test sample (juice) was poured into a large glass jar, the

hydrometer was carefully inserted into the jar, and specific gravity was read off at room temperature (28 ± 2 °C).

3.3.7 Determination of colour order properties of mango and apple juices

Degree of lightness, a and b values of juices were measured with the aid of a colorimeter (Minolta CR-300, Japan) according to the method described by Burdurlu and Karadeniz (2003). The equipment was calibrated according to manufacturer's instructions. A 10 mL sample was poured on a white plate and placed close to the prism of the equipment. The colour order parameters were obtained from an attached printer. From the data obtained, deltachrome (ΔC), colour intensity (ΔE) and hue angle were obtained from Equations 3.20, 3.21 and 3.22, respectively

$$\Delta C = \sqrt{\Delta a^2 + \Delta b^2} \tag{3.20}$$

$$\Delta E = \sqrt{\Delta L^2 + \Delta a^2 + \Delta b^2} \tag{3.21}$$

$$Hue\ angle = Tan^{-1} b/a \tag{3.22}$$

3.3.8 Determination of rheological properties of mango and apple juices

Rheological characterisation of mango juice was carried out based on the method described by Sharoba and Ramadan (2011). This involved the use of concentric cylinder viscometer (Haake RVS Rotovisco, Germany) using share rates that ranged between 51.09 and 1021.8 s^{-1}. Juice (300 mL) was poured into the viscometer bottle, placed under the rotating spindle of the equipment and shear stress was read off. Relative viscosity of apple juice was calculated as the ratio of viscosity of juice and distilled water (Joshi *et al.,* 2011).

Power Law model (Equation 3.23) was used to generate mango juice flow characteristics namely: consistency index, K (Pa.s), and flow behavior index, n.

$$\tau = K\gamma^n \tag{3.23}$$

To establish the occurrence of yield stress (Pa) in the samples, Herschel-Bulkey (Equation 3.24) and Casson models (Equation 3.25) were used.

$$\tau = \tau_0 + K\gamma^n \tag{3.24}$$

$$\tau^{0.5} = \tau_0^{0.5} + K\gamma^{0.5} \tag{3.25}$$

Where τ_0 is the yield stress

Temperature dependency of mango juice was determined using the Arrhenius equation (Equation 3.26).

$$\ln \tau = \ln \tau_0 - \left(\frac{Ea}{R}\right)\left(\frac{1}{T}\right) \qquad (3.26)$$

Where Ea is the activation energy (kJ/mol), τ_0 is the Arrhenius equation constant, R is the ideal gas constant = 8.314 J/molK and T is temperature (K).

3.3.9 Sensory evaluation of juice samples

Sensory evaluation of mango and apple juices was conducted using a 9-point Hedonic scale described by Joshi *et al.* (2011). Juices were assessed by a 50-man panel that comprised of 62% female and 38% male postgraduate students of the Department of Food Technology, University of Ibadan. The panelists assessed the samples for colour, taste, consistency, clarity (for apple juice only), mouthfeel and overall acceptability. Examination of coded samples for the sensory attributes was evaluated in separate booths under white light. Samples were served at 8±2°C. Water and cracker biscuit (Yale, Nigeria) were provided between samples for palate cleansing.

3.3.10 Statistical analyses

Multiple regression models obtained for the optimisation of substrate pre-treatments were evaluated using analysis of variance (ANOVA) and t-test while the quality of fit of the models was expressed by the coefficient of determination (R^2) using Minitab software® version 16 (Minitab, Inc. Coventry, USA). One-way ANOVA was used for the analysis of data from enzyme characterisation and applications while Post hoc (Duncan Multiple Range) test was used to separate the means. These were achieved using the SPSS version 23 (Stat-Ease Inc., USA).

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Optimisation of Alkaline Pretreatment Conditions of Orange and Plantain Peels for Maximum Polygalacturonase Activity

Polygalacturonase activity obtained at different combinations of independent variables for alkaline pretreatment of orange (OPF) and plantain (PPF) peels are presented in Table 4.1. Polygalacturonase activity ranged from 11.05 (Run 2) to 38.46 U/mL (Run 3) and from 12.59 (Run 7) to 38.82 U/mL (Run 2) on OPF and PPF, respectively. For OPF, maximum PG activity of 38.46 U/mL was obtained at Run 3 i.e. <0.425 mm, 0.1 M NaOH and 1 h. However, maximum PG activity of 38.82 U/mL was obtained from Run 2 i.e. $0.8025 < x < 1.18$ mm, NaOH molarity 0.01 M and 1 h for PPF. Differences in chemical composition of orange and plantain peels might have been responsible for the variation. According to Li *et al.* (2015), chemical constituents such as pectin and sugars of substrates partly determine enzymatic activity. Earlier reports have also shown differences in activity of polygalacturonase obtained from different substrates (Anuradha *et al*., 2010; Padma *et al.,* 2012; Heerd *et al*., 2014).

PG activity varied significantly ($p<0.05$) depending on particle size of substrate, molarity of NaOH and pretreatment time. The variation clearly validated the need for optimization of substrate pretreatment prior to enzyme production. Low PG activity recorded for Runs 2, 6 and 10 for OPF and Runs 7 and 9 for PPF could be due to low proliferation of *A. awamori* under the prevailing conditions. Slow microbial growth that results from unfavourable nutrient and non-nutrient based

Table 4.1.   Activity of Polygalacturonase from *Aspergillus awamori* on Alkaline Pretreated Orange and Plantain Peels

| | | | | PG activity (U/mL) | | | |
| | | | | From orange peel | | From plantain peel | |
Run	Particle size (mm)	Molarity (M)	Time (h)	Experimental	Predicted	Experimental	Predicted
1	<0.425 0	0.010	1.0	35.23	36.82	21.97	23.57
2	0.8025<x<1.180	0.010	1.0	11.05	10.68	38.82	37.54
3	<0.425 0	0.100	1.0	38.46	38.10	21.62	18.82
4	0.8025<x<1.1800	0.100	1.0	16.73	17.52	36.73	38.73
5	<0.425 0	0.010	12.0	30.05	29.36	28.74	26.90
6	0.8025<x<1.1800	0.010	12.0	13.59	14.05	37.24	40.20
7	<0.425 0	0.100	12.0	33.53	34.00	12.59	14.03
8	0.8025<x<1.1800	0.100	12.0	25.74	24.24	34.71	33.27
9	<0.4250	0.055	6.50	34.09	33.07	15.23	16.87
10	0.8025<x<1.1800	0.055	6.50	14.50	15.12	35.68	33.44
11	0.4250<x<0.8025	0.010	6.50	26.47	25.48	31.40	29.96
12	0.4250<x<0.8025	0.100	6.50	30.62	31.22	23.31	24.12
13	0.4250<x<0.8025	0.055	1.00	29.01	27.36	28.74	29.22
14	0.4250<x<0.8025	0.055	12.00	25.74	27.00	29.28	28.16
15	0.4250<x<0.8025	0.055	6.50	26.35	27.02	25.75	25.87
16	0.4250<x<0.8025	0.055	6.50	26.11	27.02	25.50	25.87
17	0.4250<x<0.8025	0.055	6.50	26.78	27.02	25.61	25.87
18	0.4250<x<0.8025	0.055	6.50	27.01	27.02	25.89	25.87
19	0.4250<x<0.8025	0.055	6.50	27.57	27.02	25.90	25.87
20	0.4250<x<0.8025	0.055	6.50	27.50	27.02	25.29	25.87

factors reduces rate of metabolism and hence, yield of bioproducts (Akinpelu *et al.*, 2016). Experimental and predicted PG activity obtained at each pretreatment combination was significantly close. For example, experimental and predicted PG activity obtained from OPF at Run 8 were 38.46 and 38.10 U/mL, respectively. This is an indication of high degree of correlation. This implied suitability of response surface methodology (RSM) in the analysis of pretreatment variables for improved PG activity.

4.1.1 Significance of terms from alkaline pretreatment variables

The significance ($p<0.05$) of each variable as assessed by ANOVA is presented in Table 4.2. High F-values of 71.42 and 21.88 for PG activity produced from OPF and PPF, respectively showed the significance of the model. This is corroborated by a very low p-value of 0.00 and non significant ($p>0.05$) lack of fit. R^2 and R^2(adj) values of 98.47% and 97.09%, respectively for PG activity from pretreated OPF validated that the model was in good agreement with experimental data. In the same vein, R^2 and R^2 (adj) values of 95.17% and 90.82%, respectively also showed good agreement of the model with PG activity obtained from pretreated PPF. The findings suggested that 98.47% and 95.17% of the variations in the predicted and experimental data of PG obtained from pretreated OPF and PPF respectively were covered by the models. For PG activity produced from pretreated OPF, one term each of linear (X_1-particle size), quadratic (X_1^2 -particle size $\times$ particle size) and two cross product combinations of X_1X_2 (particle size $\times$ NaOH molarity) and X_2X_3 (NaOH molarity $\times$ time) were found to be significant. Therefore, the remaining non-significant ($p>0.05$) terms were deleted from the regression equation (Equation 4.1).

$$Y = 43.47 - 1.34X_3 - 20.47X_1^2 + 81.83X_1X_2 + 1.30X_1X_3 \qquad (4.1)$$

Significant ($p<0.05$) terms for PG activity obtained from pretreated PPF were X_3, X_3^2, X_1X_2 and X_2X_3. The regression equation is given in Equation 4.2.

$$Y = 15.31 - 0.80X_3 - 0.09X_3^2 + 87.42X_1X_2 + 7.38X_2X_3 \qquad (4.2)$$

Table 4.2.   Analysis of Variance of Fitted Models of Polygalacturonase from Alkaline Pretreated Orange and Plantain Peels

Source	DF	Orange peel Sum of square	Mean square	F-value	P-value	DF	Plantain peel Sum of square	Mean square	F-value	P-value
Model	9	994.58	110.51	71.42	0.00	9	880.10	97.79	21.88	0.00
X_1	1	805.51	0.31	0.20	0.67	1	689.40	13.63	3.05	0.11
X_2	1	82.31	6.25	4.04	0.072	1	85.32	14.19	3.17	0.11
X_3	1	0.33	18.74	12.11	0.006	1	2.83	6.63	8.48	0.03
X_1^2	1	20.68	23.40	15.12	0.003	1	13.89	1.47	0.33	0.58
X_2^2	1	6.22	4.89	3.16	0.106	1	15.90	3.76	0.84	0.38
X_3^2	1	0.07	0.07	0.05	0.832	1	21.94	21.94	5.91	0.04
X_1X_2	1	15.46	15.46	9.99	0.01	1	17.64	17.64	4.95	0.04
X_1X_3	1	58.64	58.64	37.90	0.00	1	0.22	0.22	0.05	0.827
X_2X_3	1	5.64	5.65	3.65	0.085	1	32.97	32.97	7.38	0.022
Residual error	10	15.47	1.55			10	44.70	4.47		
Lack of fit	5	13.71	2.74	7.79	0.121	5	44.42	8.88	2.65	0.193
Pure error	5	1.76	0.35			5	0.28	0.06		
Total	19	1010.65				19	9224.80			
R^2	98.47%					95.17%				
R^2 (adj)	97.09%					90.82%				

X_1- particle size; X_2- NaOH molarity; X_3- pretreatment time; DF- degree of freedom

4.1.2 Interactive effect of alkaline pretreatment variables on polygalacturonase activity

Alkaline pretreatment variables i.e. substrate particle size, NaOH molarity and pretreatment time had significant ($p < 0.05$) effect on PG activity. Figures 4.1a and b show contour plots for interactive effects of substrate particle size and molarity of NaOH on PG activity obtained from OPF and PPF, respectively. Maximum PG activity was obtained at lowest OPF particle size (<0.425 mm) and highest NaOH molarity (0.1 M) and vice versa. Maeda *et al.* (2011) reported increase in enzymatic hydrolysis of sugarcane bagasse pre-treated with 1-4% NaOH and a reduction at NaOH concentration of 0.5%. Particle size reduction increases substrate surface area which in turn increases bioavailability of nutrients for microbial proliferation, hence, improved yield of bioproducts (Salihu *et al*., 2015).

Low PG activity was obtained at low NaOH molarity probably due to non inhibition of chemical barriers at low NaOH concentration. For example, OPF is a rich source of limonene and essential oils which are inhibitory to enzymatic hydrolysis (Li *et al*., 2015; Wu *et al*., 2017). On the other hand, maximum PG activity was produced from PPF pre-treated with 0.01 M NaOH and particle size 0.8025<x<1.18 mm. This is advantageous because of reduction in NaOH consumption and energy required for size reduction. Low *et al.* (2015) also reported reduction in glucanase activity from banana pseudostem pre-treated with high concentration of NaOH. Low PG activity produced from PPF pretreated at high molar concentration of NaOH could be due to destruction of carbohydrates which might have resulted in the production of inhibitory compounds (Pandey and Negi, 2015).

Figures 4.2a and b suggest an interactive effect between substrate particle size and pretreatment time on the PG activity from pretreated OPF and PPF, respectively. This corroborated the finding of Han *et al.* (2012) who reported improved enzymatic hydrolysis of wheat straw as a result of synergistic effect of reduced particle size and pre-treatment time. Improved enzymatic activity is related to increase in adsorption of microorganisms on carbohydrate fibers, which is a
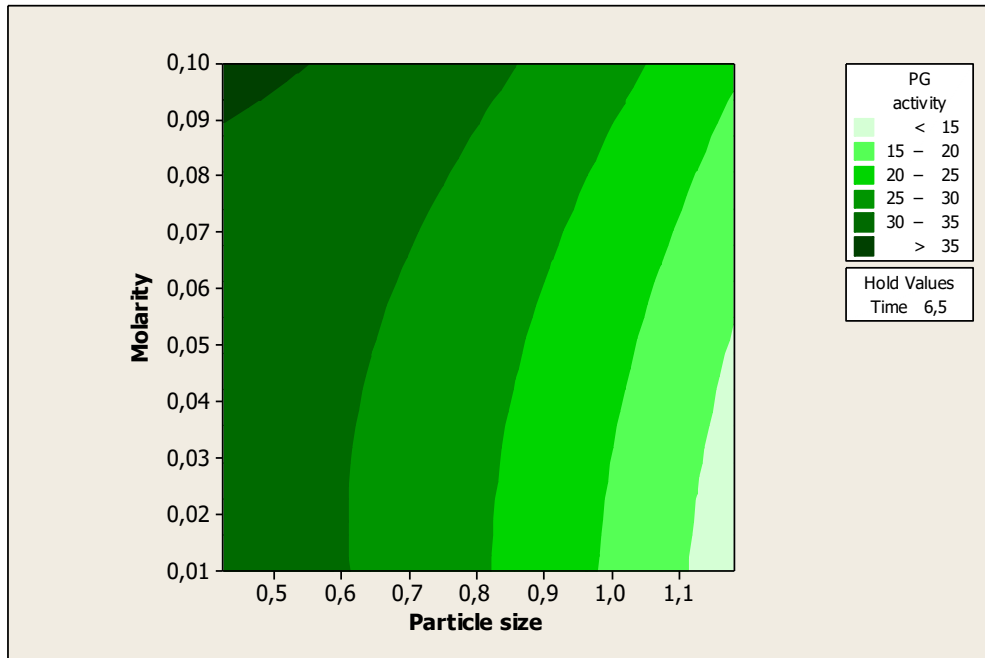
(a)
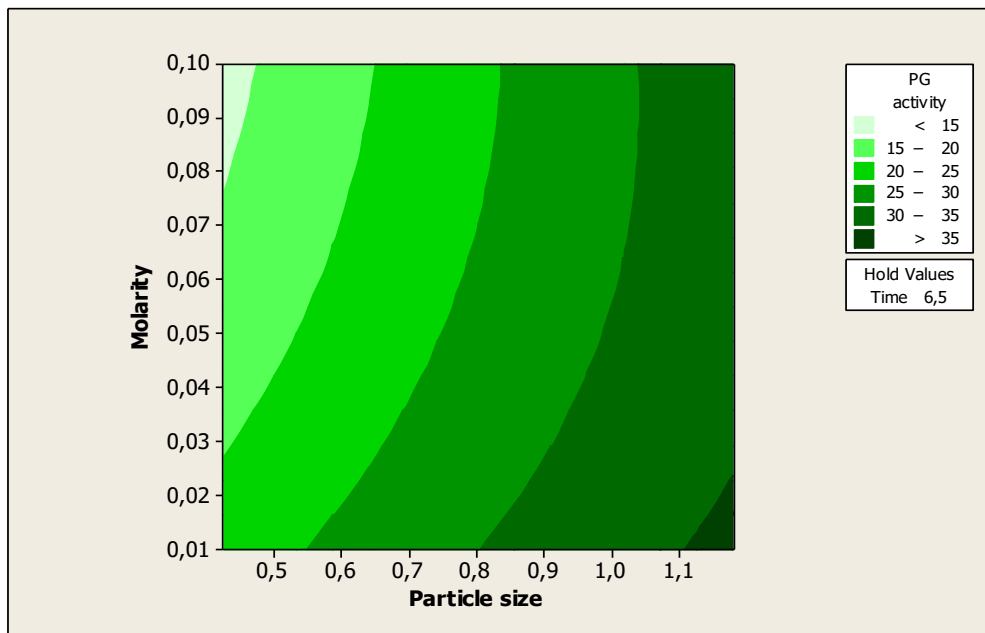
(b.)

Figure 4.1. Effect of Substrate Particle Size and NaOH Molarity on PG Activity Produced using Alkaline Pretreated Peel: (a) Orange Peel, (b) Plantain Peel
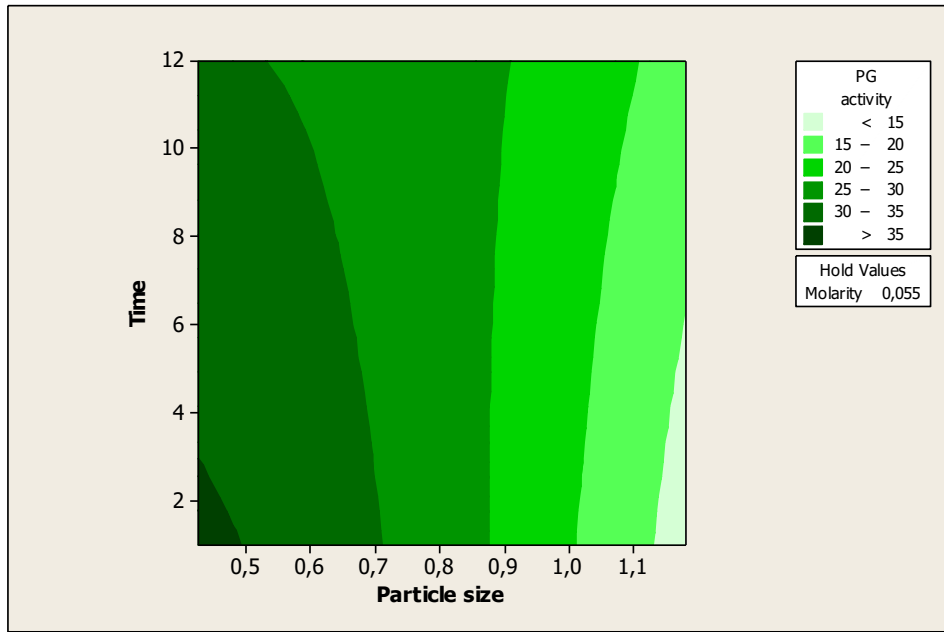
(a)
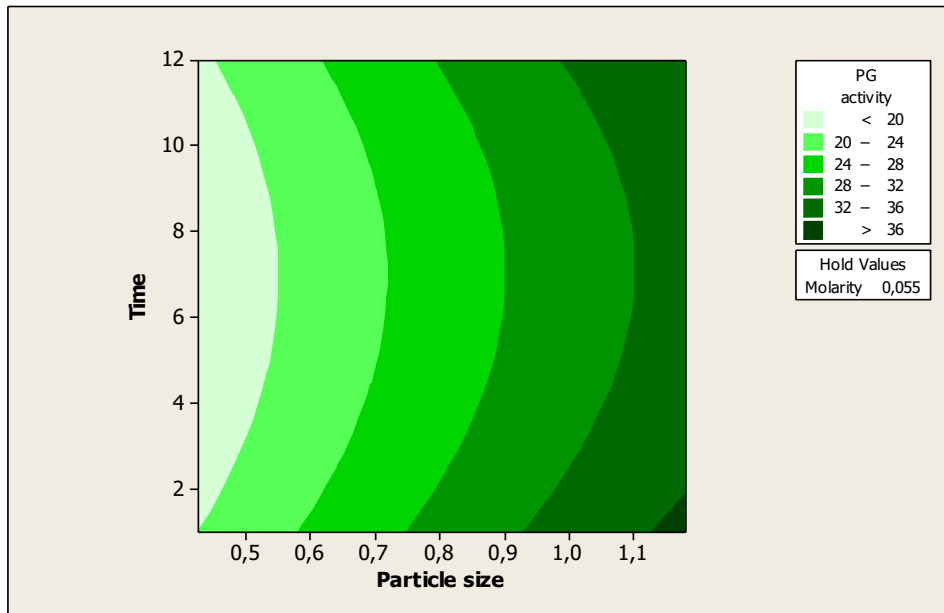
(b)

Figure 4.2. Effect of Substrate Particle Size and Pretreatment Time on PG Activity Produced using Alkaline Pretreated Peel: (a) Orange Peel, (b) Plantain Peel

consequence of surface area and time. Maximum PG activity was obtained from OPF with particle size of <0.425 mm pretreated for 1 h. In addition, PPF pretreated for 1 h gave the highest PG activity, however, at substrate particle size of 0.8025<x<1.18mm. Polygalacturonase activity reduced for both PGs produced from OPF and PPF as substrate pretreatment time increased.

Figure 4.3a shows the effect of NaOH molarity and pre-treatment time on the activity of PG produced fron orange peel. Pre-treatment time had no significant effect (p>0.05) on PG activity produced from OPF pretreated with 0.1 M NaOH. Maximum PG activity (>32 U/mL) was recorded at 0.1 M NaOH irrespective of pretreatment time. However, at lower NaOH molarity, high pretreatment time (> 4 h) resulted in reduction of PG activity. Reduction in enzymatic hydrolysis was also reported for wheat straw due to long (>1½ h) alkaline pretreatment time. Reduction in PG activity at prolonged pretreatment time may be due to destruction of inducer substrates e.g. pectin owing to increased saponification rate of their ester bonds (Santos *et al.,* 2011). For PG produced from PPF, maximum activity was obtained at low NaOH molarity and high pretreatment time (Figure 4.3b). Sahare *et al.* (2012) also reported maximum enzymatic hydrolysis of corn cob that was subjected to prolonged (>4 h) alkali pretreatment.

4.1.3   Model validation for alkaline pretreatment of orange and plantain peels

Adequacy of regression equation was validated by conducting the experiment using the terms of the optimized conditions. Percentage deviation (Table 4.3) was 0.94 and 3.40% for OPF and PPF, respectively. Since the values were less than 5.0% (Ezekiel and Aworh, 2018), it can be concluded therefore, that the model was suitable for the description of the experiment.
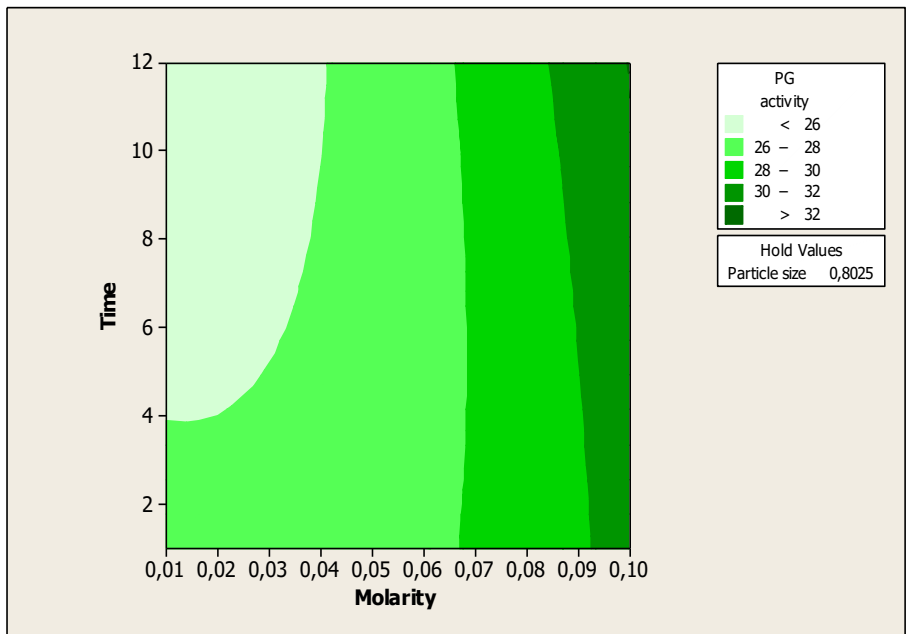
(a.)
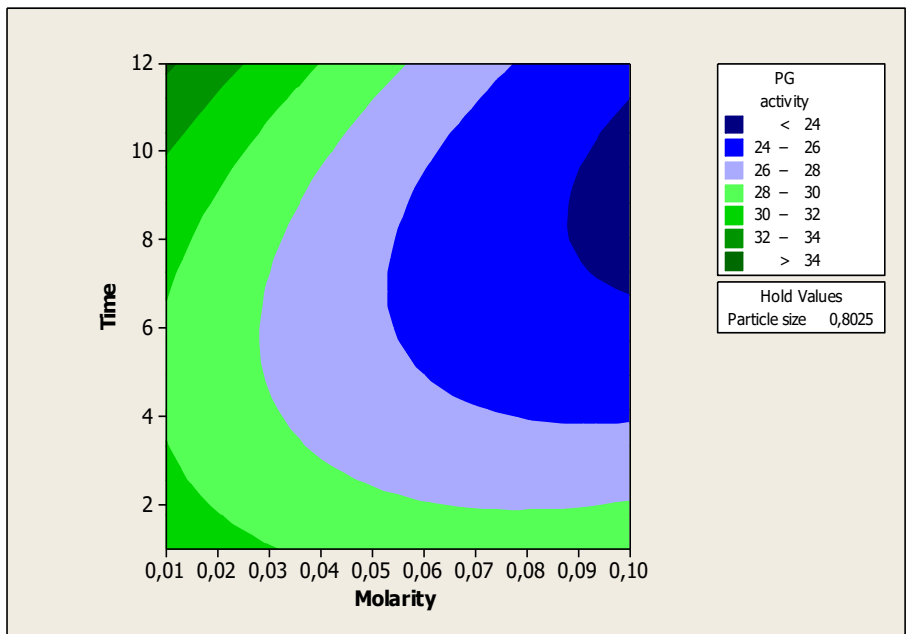
(b.)

Figure 4.3. Effect of NaOH Molarity and Pretreatment Time on PG Activity Produced using Alkaline Pretreated Peel: (a) Orange Peel, (b) Plantain Peel

Table 4.3. **Model Validation for Alkaline Pretreatment of Orange and Plantain Peels**

Substrate	Particle size (mm)	NaOH molarity (M)	Time (h)	PG activity (U/mL)	
Orange peel	<0.425	0.1	1	Experimental	38.46
				Predicted	38.10
				Deviation (%)	0.94
Plantain peel	0.8025<x<1.18	0.01	1	Experimental	38.82
				Predicted	37.50
				Deviation (%)	3.40

4.2 Optimisation of Microwave Pretreatment Condition of Orange and Plantain Peels

Table 4.4 shows PG activity produced from *Aspergillus awamori* produced from microwave (MW) pretreated orange and plantain peels. Polygalacturonase activity ranged from 6.97 (Run 3) to 26.21 U/mL (Run 8) and from 10.22 (Run 13) to 26.72 U/mL (Run 8) on OPF and PPF, respectively. Results obtained showed that MW pretreatment variables: substrate particle size, MW power and pretreatment time had significant effect on PG activity. For both OPF and PPF, maximum PG activity of 26.21 and 26.72 U/mL, respectively were obtained at Run 8 which corresponded to substrate particle size of 0.8025<x<1.18 mm, MW power of 720 W and pretreatment time of 10 min. Similarity observed may be due to high efficiency of microwave pretreatment which probably resulted in substrates with similar properties. MW treatment has been described as a technology with a high degree of heating efficiency and uniformity (Nomanbhay *et al*., 2013). Predicted PG activity of 24.70 and 26.03 U/mL for OPF and PPF, respectively showed that both the experimental and predicted values were highly correlated. Lowest PG activity of 6.97 U/mL was recorded for OPF with <0.425 mm particle size pretreated at 720W for 2.5 min (Run 3). However, Run 13 which corresponded to PG activity produced from PPF with particle size of 0.8025<x<1.18 mm, 480W MW power and pretreatment time of 2.5 min gave lowest value of 10.22 U/mL.

4.2.1 Significance of terms from microwave pretreatment variables

Analysis of variance (Table 4.5) showed the adequacy of the models in the characterization of the independent variables. F and p values obtained for OPF were 35.42 and 0.00, respectively while those obtained for PPF were 5.71 and 0.006, respectively. These values implied that the models were significant ($p<0.05$). R^2 and R^2 (adj) of 96.96 and 94.22%, respectively were obtained for PG activity produced from OPF while 90.71 and 79.04% were respectively recorded for PG activity produced form PPF. These suggested that 96.96% and 90.71% of the

Table 4.4.   Polygalacturonase Activity from *Aspergillus awamori* on Microwave Pre-treated Orange and Plantain Peels

| | | | | PG activity (U/mL) | | | |
| | | | | From orange peel | | From plantain peel | |
Run	Particle size (mm)	Power level (W)	Time (min)	Experimental	Predicted	Experimental	Predicted
1	<0.425	240	2.5	14.04	15.42	22.84	23.68
2	0.8025<x<1.18	240	2.5	25.43	24.27	11.29	10.52
3	<0.425	720	2.5	6.97	6.05	19.69	17.32
4	0.8025<x<1.18	720	2.5	17.12	18.15	13.27	16.19
5	<0.425	240	10	10.13	8.97	22.22	19.44
6	0.8025<x<1.18	240	10	15.04	15.83	14.27	16.79
7	<0.425	720	10	13.55	14.59	15.74	16.66
8	0.8025<x<1.18	720	10	26.21	24.70	26.72	26.03
9	<0.425	480	6.25	7.13	6.79	15.09	18.48
10	0.8025<x<1.18	480	6.25	15.43	16.27	20.56	16.58
11	0.425<x<0.8025	240	6.25	14.99	15.13	15.80	15.99
12	0.425<x<0.8025	720	6.25	14.53	14.88	18.22	17.44
13	0.425<x<0.8025	480	2.5	15.62	15.29	10.22	9.59
14	0.425<x<0.8025	480	10	14.51	15.34	12.36	12.39
15	0.425<x<0.8025	480	6.25	13.06	12.93	13.33	13.45
16	0.425<x<0.8025	480	6.25	13.11	12.93	13.06	13.45
17	0.425<x<0.8025	480	6.25	13.01	12.93	13.25	13.45
18	0.425<x<0.8025	480	6.25	13.16	12.93	13.12	13.45
19	0.425<x<0.8025	480	6.25	13.00	12.93	13.42	13.45
20	0.425<x<0.8025	480	6.25	13.21	12.93	13.35	13.45

Table 4.5. **Analysis of Variance of Fitted Models of Polygalacturonase from Microwave Pretreated Orange and Plantain Peels**

Source		Orange peel					Plantain peel			
	DF	Sum of square	Mean square	F-value	P-value	DF	Sum of square	Mean square	F-value	P-value
Model	9	396.79	44.09	35.42	0.00	9	305.55	33.95	5.71	0.006
X_1	1	224.71	12.98	10.43	0.09	1	8.97	109.25	18.37	0.002
X_2	1	0.16	39.52	31.75	0.00	1	5.21	59.74	10.05	0.01
X_3	1	0.01	39.67	31.87	0.00	1	19.60	0.96	0.16	0.70
X_1^2	1	8.26	5.37	4.31	0.07	1	103.79	45.68	7.68	0.02
X_2^2	1	28.38	11.93	9.58	0.01	1	17.49	29.34	4.92	0.051
X_3^2	1	15.68	15.68	12.59	0.005	1	16.64	16.36	2.80	0.125
X_1X_2	1	5.30	5.30	4.26	0.07	1	72.36	72.36	12.17	0.006
X_1X_3	1	1.97	1.97	1.58	0.24	1	55.13	55.13	9.27	0.012
X_2X_3	1	112.28	112.28	90.19	0.00	1	6.37	6.37	1.07	0.325
Residual error	10	12.45	12.45			10	59.47	5.95		
Lack of fit	5	12.41	2.48	353.84	0.227	5	59.37	11.86	604.92	0.183
Pure error	5	0.04	0.01			5	0.10			
Total	19	409.24				19	365.03			
R^2	96.96%					90.71%				
R^2 (adj)	94.22%					79.04%				

X_1- particle size; X_2- Microwave power; X_3- pretreatment time; DF- degree of freedom

variations in the predicted and experimental data of PG obtained from pretreated OPF and PPF, respectively. were covered by the models. This showed a significant (p<0.05) agreement between the experimental and predicted data. For PG activity produced from OPF, a total of five terms including: X_2 (MW power), X_3 (pretreatment time), X_3^2 (pretreatment time $\times$ pretreatment time) and X_2X_3 (MW power $\times$ pretreatment time) were significant (p<0.05) in the model. Quadratic equation after deleting terms that were not significant (p>0.05) is presented in Equation 4.3.

$$Y = 25.89 - 0.069X_2 - 3.83X_3 + 0.17X_3^2 + 0.004X_2X_3 \qquad (4.3)$$

The following terms were significant (p<0.05) in the regression model for PG activity produced from MW-pretreated PPF: X_1 (substrate particle size), X_2 (MW power), X_1^2 (particle size $\times$ particle size), X_1X_2 (particle size $\times$ MW power) and X_1X_3 (particle size $\times$ pretreatment time). Residual terms in the model are shown in Equation 4.4.

$$Y = 5.71 - 75.93X_1 - 0.08X_2 + 28.60X_1^2 + 0.03X_1X_2 + 1.85X_1X_3 \qquad (4.4)$$

4.2.2 Interactive effect of microwave pretreatment variables on polygalacturonase activity

Activity of PG as influenced by synergistic effect of substrate particle size and MW power on OPF and PPF are presented in Figures 4.4a and b, respectively. PG activity obtained from both OPF and PPF increased with increasing MW power and substrate particle size. Irrespective of the source of the substrate, maximum PG activity was obtained at the highest boundary of MW power and substrate particle size. Li *et al*. (2015) reported 11.8% increase in exo-pectinase activity from *Aspergillus japonicus* using OPF pre-treated at high MW power of 630W and substrate particle size of 0.850 mm. According to Woldesenbet *et al*. (2012), microwave radiation at high power level results in accelerated rupturing of substrates, which makes polysaccharides to be more susceptible to microbial proliferation.
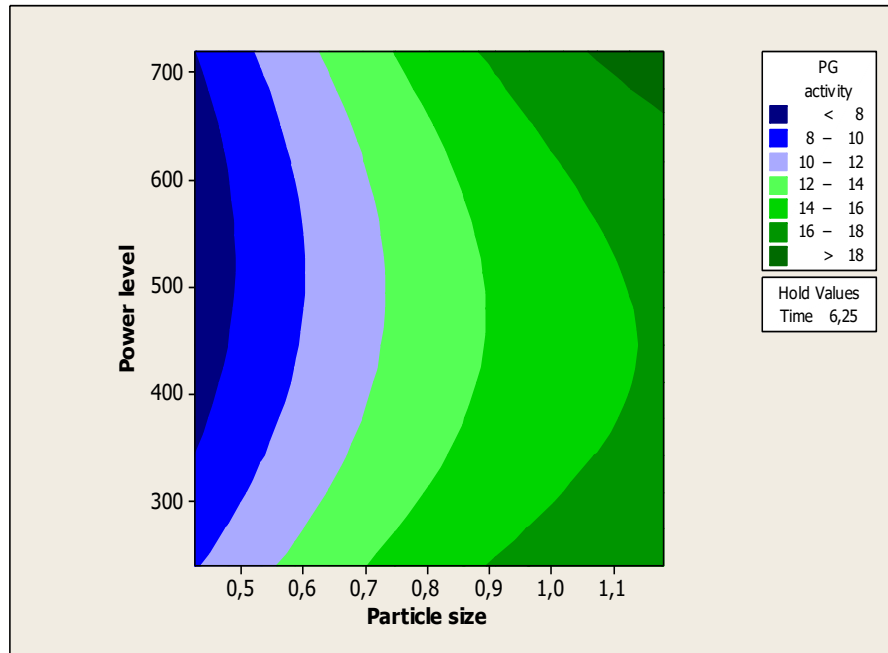
(a.)
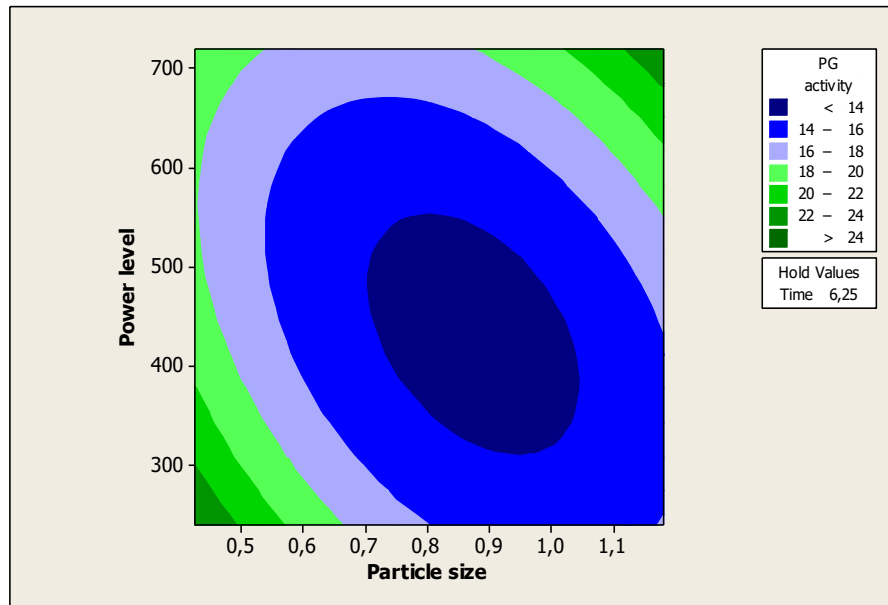
(b.)

Figure 4.4. Effect of Substrate Particle Size and Microwave Power on PG Activity Produced using Microwave Pretreated Peel: (a) Orange Peel, (b) Plantain Peel

Interactive effects between substrate particle size and pre-treatment time on PG activity produced from OPF and PPF are shown in Figures 4.5a and b, respectively. While holding MW power at 480 W, PG activity produced from OPF increased with increasing particle size irrespective of pretreatment time. Findings of Inan *et al*. (2016) also showed an increase in sugar concentration with increasing particle size of barley straw (x<1.0 mm) irrespective of pre-treatment time (2.5 – 10 min). Low PG activity recorded in OPF with small particle size could be due to crushing of inducer carbohydrate as a result of intensive size reduction. For PG produced from PPF, maximum activity was produced at substrate particle size of >1.0 mm and pre-treatment time of 6 – 10 min.

Figures 4.6a and b are contour plots showing the interaction between MW power and pretreatment time on the response. At a constant substrate particle size of 0.8025 mm, PG activity from OPF increased between MW power of 480 and 720 W and pretreatment time 7 to 10 min. In a similar pattern, PG activity from PPF increased with increasing MW power and pretreatment time. This result did not agree with the report of Tiwari *et al.* (2017) where maximum enzymatic hydrolysis of mango peel was obtained at MW power of 450 W and exposure time of 4 min. Differences in substrate composition may be responsible for the variation. However, the finding in this study is in agreement with the work of Inan *et al.* (2016) who observed a decrease in total sugar from barley pretreated at a microwave power level of 300 W and pretreatment time of 2.5 min.

4.2.3 Model validation for microwave pre-treatment of orange and plantain peels

Data presented in Table 4.6 show the percentage deviation for experimental and predicted data of OPF and PPF to be 4.81 and 2.58%, respectively. Suitability of the model in fitting the experimental data was thus validated since the values obtained were less than 5.0% (Ezekiel and Aworh, 2018).
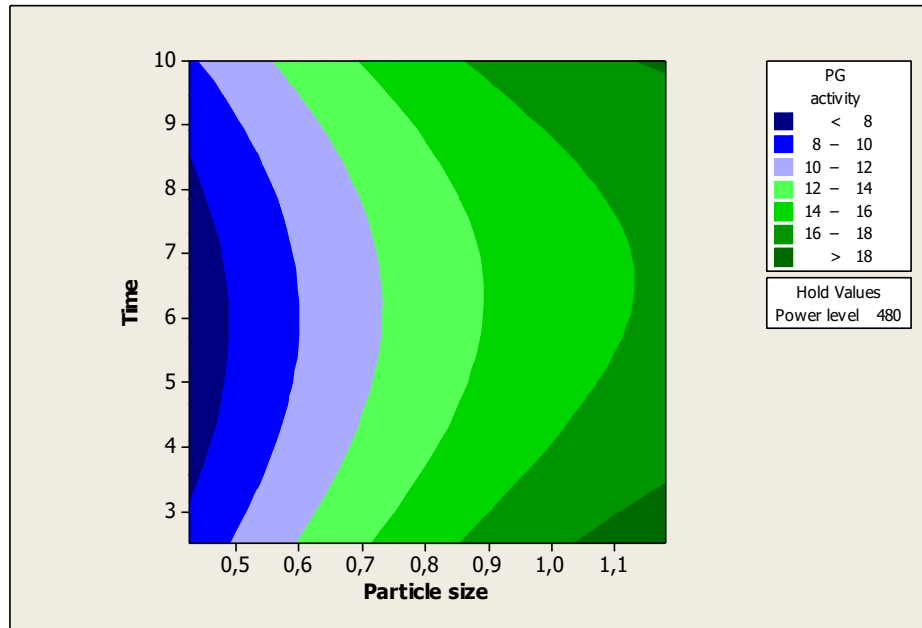
(a.)
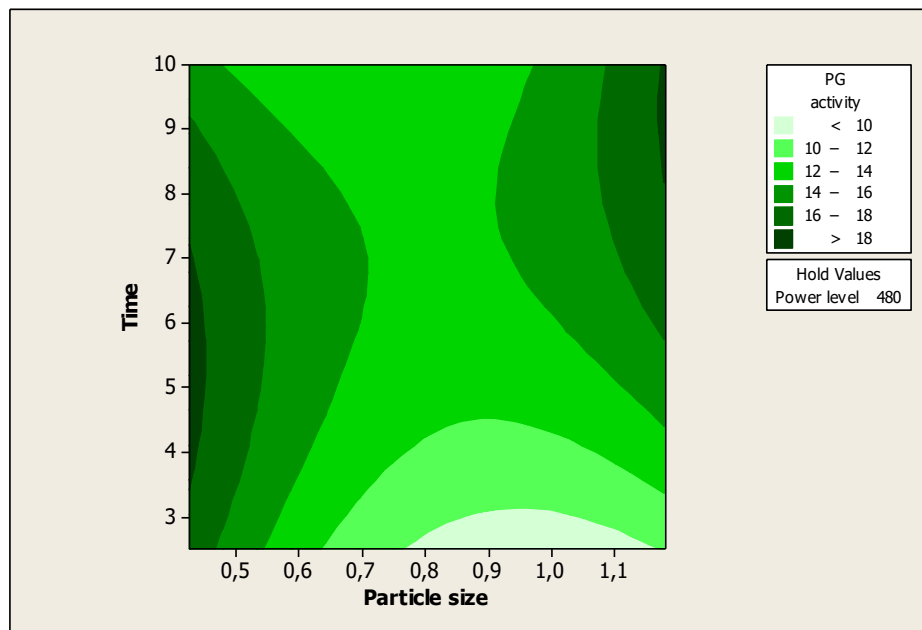
(b.)

Figure 4.5. Effect of Substrate's Particle Size and Pre-treatment Time on PG Activity Produced using Microwave Pretreated Peel: (a) Orange Peel, (b) Plantain Peel
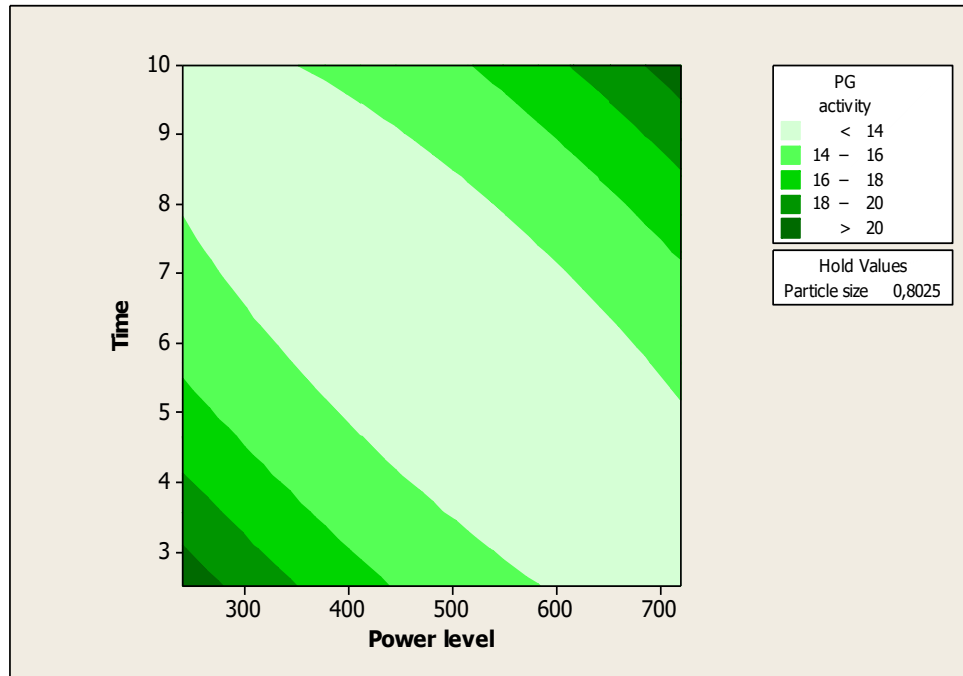
(a.)
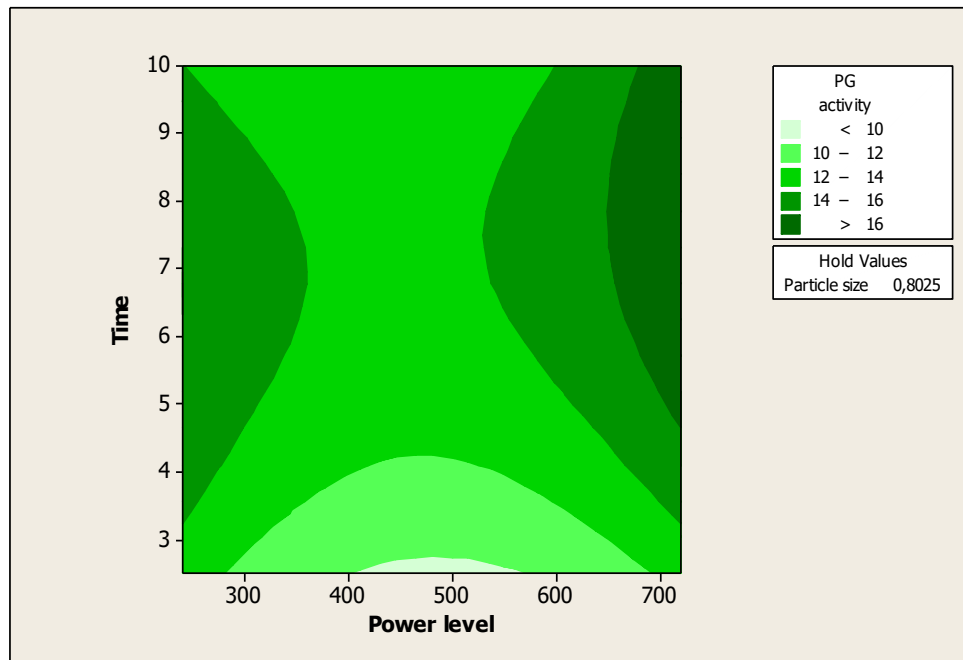
(b)

Figure 4.6. Effect of Microwave Power and Pretreatment Time on PG Activity Produced using Microwave Pretreated Peel: (a) Orange Peel, (b) Plantain Peel

Table 4.6. **Model Validation for Microwave Pretreatment of Orange and Plantain Peels**

Substrate	Particle size (mm)	Microwave power (W)	Time (min)	PG activity (U/mL)	
Orange peel	0.8025<x<1.18	720	10	Experimental	26.21
				Predicted	24.95
				Deviation (%)	4.81
Plantain peel	0.8025<x<1.18	720	10	Experimental	26.72
				Predicted	26.03
				Deviation (%)	2.58

4.3   Effect of Substrate Pretreatment Methods on Polygalacturonase Activity

Figure 4.7 shows comparison between PG activity produced from pretreated and un-treated OPF and PPF. Pretreatment of OPF and PPF with NaOH caused a 52.8 and 49.08% increase respectively in PG activity. This increase might be due to increase in lignin and hemicelluloses removal consequent to NaOH pre-treatment, thereby increasing pectin and cellulose concentrations. High pectin and cellulose are attributable to increase in microbial proliferation and hence, increased hydrolysis and activity of bio-products (Han *et al.,* 2012; Nomanbhay *et al.,* 2013). Elevated level of cellulase was also recorded from *Aspergillus niger* inoculated on NaOH pre-treated rice bran (Narasimha *et al.,* 2016). Treatment of substrates with NaOH results in swelling which in turn causes an increase in internal surface area of substrates. As a result of this, there is a decrease in degree of polymerization and disruption in lignin and carbohydrate linkages (Kashaninejad and Tabil, 2011).

Higher PG activity was recorded for NaOH pre-treated OPF and PPF compared to those pre-treated with microwave. Microwave assisted pretreatment caused an increase of 4.13 and 2.61% in PG activity produced from OPF and PPF, respectively. Li *et al.* (2015) also reported increase of 11.8% in exo-pectinase produced from MW pretreated OPF. This suggests inefficiency of MW method in the pretreatment of pectin-rich substrates. A combination of MW and NaOH (NaOH-MW) pretreatment resulted in a higher PG activity as increments of 67.06 and 72.73% were obtained for OPF and PPF, respectively. Earlier studies had reported improved yield and activity of bio-products from substrates as a result of synergistic effect of two pretreatment methods (Inan *et al*., 2016; Ethaib *et al.,* 2016; Chang *et al.,* 2017; Lai *et al*., 2017; Wang *et al*., 2017). A significant improvement in enzymatic saccharification of oil palm fibre consequent to microwave assisted NaOH pre-treatment was reported by Nomanbhay *et al.* (2013). Also, an increase in exo-glucanase and β-glucosidase activities as a result of synergistic effect of NaOH and ethylene glycol modification of corn stover was reported by Lai *et al.* (2017).
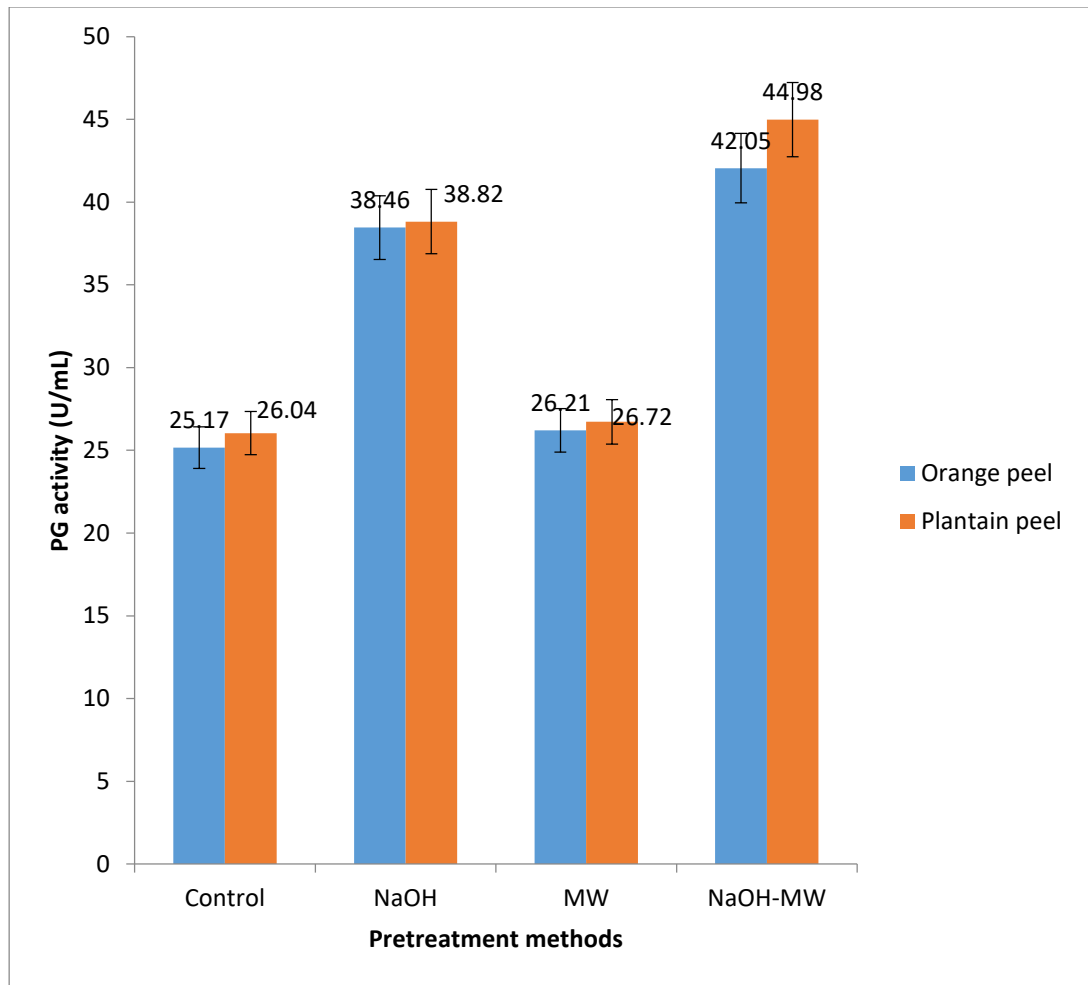
Figure 4.7. Effect of Substrate Pretreatment Methods on Polygalacturonase Activity. MW= microwave.

4.4. Effect of Substrate Pretreatment Methods on Percentage Solids Recovered from Orange and Plantain peels

Pretreatment methods influenced total percentage of solids recovered (Figure 4.8). Percentage solids recovered ranged from 56.2 to 85.4% and 60.8 to 79.4% in OPF and PPF, respectively. Highest percentage solids recovery of 85.4 and 79.4% were obtained for MW pre-treated OPF and PPF, respectively. This suggests low solids loss of substrate as a result of high salvage capability of MW pretreatment (Raj *et al*., 2016). This is in agreement with low solids loss of MW pre-treated dragon fruit foliage (Ethaib *et al.,* 2016). Lower percentage solids recovery of 60.8 and 56.2% were obtained for NaOH and NaOH+MW pre-treated OPF. In the same vein, NaOH and NaOH+MW pre-treated PPF yielded 71.4 and 56.6%, respectively. These were equivalent to 28.6 to 43.8% solids loss. According to Kataria and Gosh (2014), 15.6 to 47.5% solids were lost when Kans grass (*Saccharum spontaneum)* was subjected to NaOH pre-treatment. Low percentage solids recovery recorded for NaOH pretreated substrates could be due to dissolution of molecules such as lignin and sugars by the alkali (Cotana *et al*., 2015).

4.5 Effect of Pre-treatment Methods on Chemical Composition of Orange and Plantain Peel Flours

Chemical constituents of un-treated and pre-treated OPF and PPF are presented in Tables 4.7 and 4.8, respectively. Pectin, cellulose, hemicelluloses, lignin, extractives, ash, moisture and total sugar ranged from 15.52 to 31.91%, 18.68 to 51.69%, 16.41 to 27.63%, 4.02 to 8.10%, 14.11 to 26.26%, 2.78 to 8.29%, 10.11 to 11.86% and 158.34 to 217.08 mg/g in un-treated and pre-treated OPF. For PPF, pectin, cellulose, hemicelluloses, lignin, extractives, ash, moisture and total sugar ranged from 14.55 to 24.62%, 20.72 to 41.14%, 17.89 to 31.49%, 3.95 to 10.03%, 16.04 to 23.48%, 2.80 to 9.14%, 9.67 to 11.94% and 188.16 to 207.36 mg/g, respectively. Significant (p<0.05) increase in pectin content was recorded in OPF pre-treated with NaOH, MW and NaOH-MW. This increase could be due to reduction of lignin and hemicelluloses as a result of pretreatment.
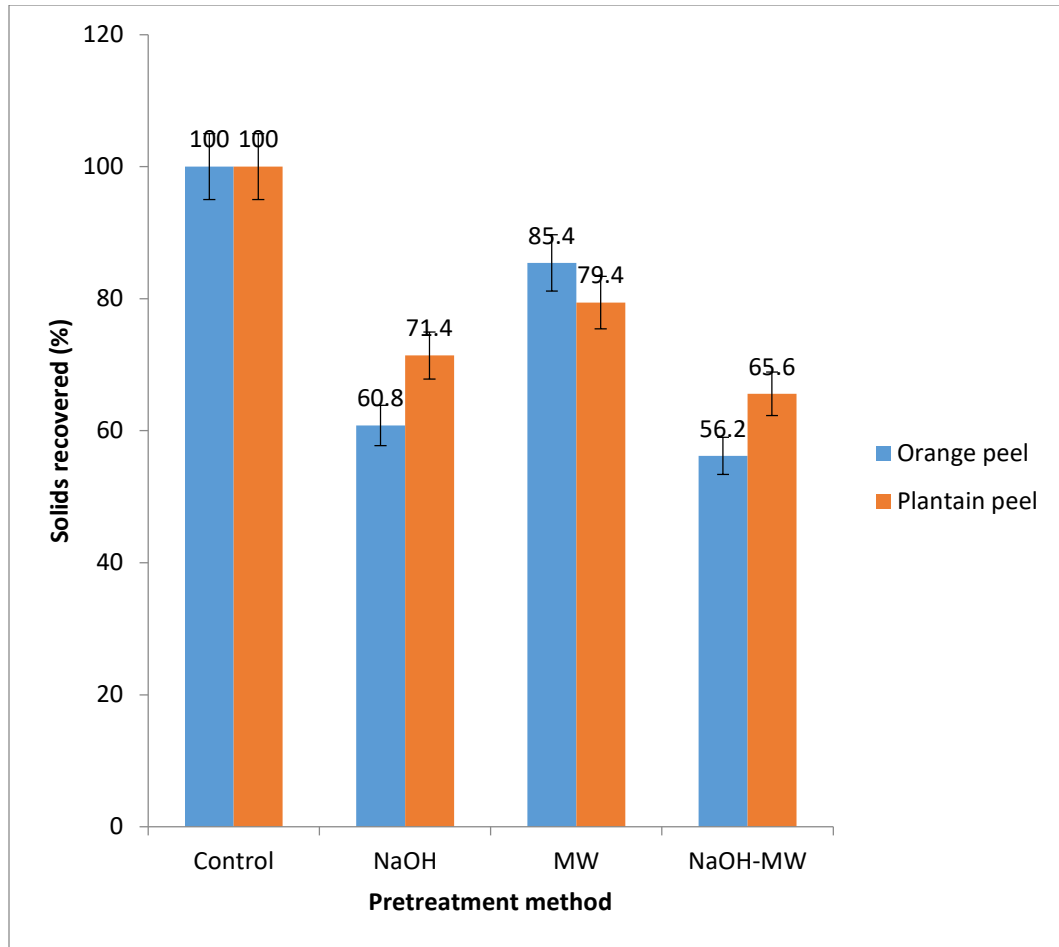
Figure 4.8. Percentage Solids Recovery of Orange and Plantain Peels as Influenced by Pre-treatment Methods. MW= microwave.

Table 4.7.   Chemical Composition of Un-treated and Pretreated Orange Peel

Pretreatment method	Pectin (%)	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Extractives (%)	Ash (%)	Moisture content (%)	Total sugar (mg/g)
Control sample (No pretreatment)	$15.52^{c}\pm1.29$	$18.68^{d}\pm1.11$	$27.63^{a}\pm0.01$	$8.10^{a}\pm1.17$	$26.26^{a}\pm0.20$	$8.29^{a}\pm0.06$	$11.04^{c}\pm0.06$	$158.34^{b}\pm7.20$
NaOH	$24.38^{b}\pm0.03$	$44.56^{c}\pm0.06$	$20.74^{b}\pm0.00$	$4.11^{c}\pm0.03$	$14.88^{c}\pm0.07$	$2.88^{c}\pm0.03$	$11.86^{a}\pm0.07$	$175.22^{b}\pm11.26$
MW	$26.01^{b}\pm0.10$	$47.43^{b}\pm0.01$	$16.41^{d}\pm0.28$	$5.77^{b}\pm0.10$	$16.80^{b}\pm0.10$	$3.48^{b}\pm0.06$	$10.11^{d}\pm0.03$	$217.08^{a}\pm21.21$
NaOH-MW	$31.91^{a}\pm0.04$	$51.69^{a}\pm0.03$	$18.83^{c}\pm0.18$	$4.02^{c}\pm0.03$	$14.11^{d}\pm0.08$	$2.78^{c}\pm0.08$	$11.56^{b}\pm1.17$	$161.42^{b}\pm6.92$

Values are means ± standard deviations of 3 replications. Means within a column with different superscripts were significantly ($p<0.05$) different. MW= microwave

Table 4.8.   **Chemical Composition of Un-treated and Pretreated Plantain Peel**

Pretreatment method	Pectin (%)	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Extractives (%)	Ash (%)	Moisture (%)	Total sugar (mg/g)
Control sample (No pretreatment)	$14.55^c \pm 1.17$	$20.72^d \pm 1.17$	$31.49^a \pm 0.04$	$10.03^a \pm 0.03$	$23.48^a \pm 0.01$	$9.14^a \pm 1.11$	$11.15^b \pm 0.08$	$193.46^a \pm 11.75$
NaOH	$20.17^b \pm 0.04$	$39.39^b \pm 0.07$	$25.23^c \pm 0.27$	$4.50^c \pm 0.08$	$16.04^d \pm 0.06$	$2.90^c \pm 1.13$	$11.94^a \pm 0.04$	$207.14^a \pm 1.68$
MW	$19.17^b \pm 0.08$	$41.14^a \pm 0.04$	$17.89^d \pm 0.38$	$5.26^b \pm 0.10$	$20.49^b \pm 0.10$	$4.95^b \pm 0.21$	$9.67^c \pm 1.14$	$207.36^a \pm 5.54$
NaOH-MW	$24.62^a \pm 1.13$	$35.23^c \pm 0.06$	$27.93^b \pm 0.04$	$3.95^d \pm 0.07$	$18.40^c \pm 0.04$	$2.80^c \pm 0.10$	$11.69^a \pm 0.10$	$188.16^a \pm 7.03$

Values are means ± standard deviations of 3 replications. Means within a column with different superscripts were significantly ($p<0.05$) different. MW- microwave

Significantly (p<0.05) higher pectin was obtained in OPF and PPF pretreated with NaOH+MW compared to other methods. Li *et al.* (2015) also reported higher pectin content in MW+surfactant pretreated orange peel compared to the use of MW only. Similar findings wre also obtained in pretreated jack fruit (Koh *et al.*, 2014).

There was a significant (p<0.05) increase in cellulose content following pre-treatment of OPF and PPF with NaOH and MW. Increase in cellulose of pre-treated agricultural residues and food wastes has been attributed to cellulose bulging, a phenomenon which occurs as a result of cellulose condensation owing to lignin and hemicellulose removal (Pandey and Negi, 2015).   OPF pre-treated with NaOH+MW gave the highest cellulose content of 51.69% while the MW pre-treated PPF gave the maximum cellulose content of 41.14%. The variation suggests differences in the composition of orange and plantain peels, hence their varying susceptibility to the pre-treatment methods considered. Cellulose comprises of crystalline and amorphous fractions (Brodeur *et al.*, 2011). While the former contributes to crystallinity of cellulose and is recalcitrant to breakdown, the latter is susceptible to breakdown through chemical, physical and biological means (Brodeur *et al.*, 2011). Therefore, one of the objectives of pre-treatment is to reduce the crysallinity of cellulose which will invariably increase the amorphous fraction. The degree at which this occurs is a function of pre-treatment method, structural properties and chemical composition of the substrate (Raj *et al.*, 2016).

Pre-treatment caused a significant (p<0.05) reduction of hemicellulose contents of both OPF and PPF. This agrees with the findings of Maeda *et al.* (2011) who also recorded marked reduction of hemicelluloses in banana pseudo-stem subjected to chemical pre-treatment. Substrates pre-treated with MW showed highest hemicellulose removal i.e. a reduction from 27.63 to 16.41% in OPF and 31.49 to 17.89% for PPF. Microwave pre-treatment is known to be very efficient in the disintegration of hemicelluloses (Kumar *et al.*, 2009). A remarkable decrease in hemicellulose of wheat straw pre-treated with microwave radiation was also reported by Kashaninejad *et al.* (2010).

Pre-treatment caused a reduction in lignin to the extent of 28.76 to 50.37% and 47.57 to 60.62% in OPF and PPF, respectively. The extent of loss of lignin recorded in this study was more than those reported in highly ligno-cellulosic materials such grass clipping (Cotana *et al.,* 2015) and wheat bran (Wang *et al.,* 2017). This is advantageous because low lignin contents of pre-treated OPF and PPF will increase their accessibility to microbial attack for subsequent hydrolysis (Yang *et al*., 2017). Also, there were significant ($p<0.05$) differences in the degree of lignin reduction by the pre-treatment methods. NaOH pre-treatment was more efficient than MW for the removal of lignin in both substrates. NaOH has high polarity which probably caused increased lignin solubilization (Ethaib *et al*., 2016). Lowest residual lignin contents of 4.02 and 3.95% were recorded in NaOH+MW pre-treated OPF and PPF, respectively. This implies that NaOH+MW pre-treatment was most efficient for lignin removal. Similar studies also showed that better lignin removal was achieved when MW and NaOH were combined for the pre-treatment of switch grass (Keshwani *et al*., 2007), wheat straw (Kashanenejad *et al*., 2010) and barley straw (Inan *et al*., 2016). In the presence of alkali, microwave increases degradation of lignin fibres through the dissolution of phenolic hydroxyl group and β-carbons that are adjacent to α-carbonyl groups, which are the major building blocks of lignin (Starr *et al*., 2015).

Extractives reduced significantly ($p<0.05$) as a result of microwave and NaOH pretreatment. This agreed with the loss of extractives in a similar pre-treatment of timber mill sawdust (Trevorah and Othman, 2015). Loss of extractives was more intense in NaOH pre-treated OPF and PPF than the MW pre-treated ones. This could be due to higher dissolution efficiency of NaOH. Similarly, reduction of extractives was found in bisulphite pre-treated sugarcane bagasse (Liu *et al*., 2017). Pretreatment also reduced ash content of OPF and PPF. The reduction was more pronounced in NaOH pre-treated samples.

Low ash recorded in NaOH and NaOH+MW pre-treated samples may be due to washing of the substrates to a neutral pH in order to remove NaOH. According to Yu and Chan (2010), loss of ash often accompanies substrate washing after pre-treatment. Since mineral elements contribute to instability of pH in solutions, low

ash contents in NaOH and NaOH+MW pre-treated samples will help to improve pH stability during downstream processing (Liu *et al*., 2017).

Moisture content of the un-treated and pre-treated substrates ranged between 9.67 and 11.94%. For both OPF and PPF, moisture content increased significantly ($p < 0.05$) in NaOH and NaOH+MW pretreated substrates, however, it decreased significantly ($p < 0.05$) in MW pre-treated samples probably due to drying effect from microwave radiation. Microwave radiation, a form of dielectric energy has been shown to be an efficient drying method and has been adopted for drying many valuable products (Bejar *et al*., 2011). Total sugar increased significantly ($p < 0.05$) consequent to MW and NaOH pre-treatments. In the same vein, Chang *et al.* (2017) reported an increase in total sugar of alkaline pre-treated rice straw. This could be due to increased concentration of cellulose in pre-treated substrates which breaks down to yield more sugar (El-Shishtawy *et al*., 2015). Highest sugar content was recorded in MW pretreated OPF (217.08 mg/g) and PPF (207.36 mg/g).. On the contrary, Inan *et al.* (2016) reported that MW pre-treated barley straw gave a lower total sugar content compared to NaOH+MW pre-treated ones.

4.6   Effect of Pre-treatment Methods on the Morphology of Orange and Plantain Peels

Morphology of un-treated and pre-treated samples as analyzed by scanning electron microscopy (SEM) are presented in Figures 4.9 to 4.12. Morphology of un-treated OPF (Figure 9a) and PPF (Figure 9b) show rigidity and proper alignment of structures of the substrates. This might have contributed to low growth rate of *Aspergillus awaamori* which probably culminated in poor activity of PG (Section 4.3). However, pre-treatment of OPF and PPF with MW, NaOH and NaOH+MW resulted in increased surface area and porosity of surface structures. This corroborated the findings of earlier studies that pre-treatment of
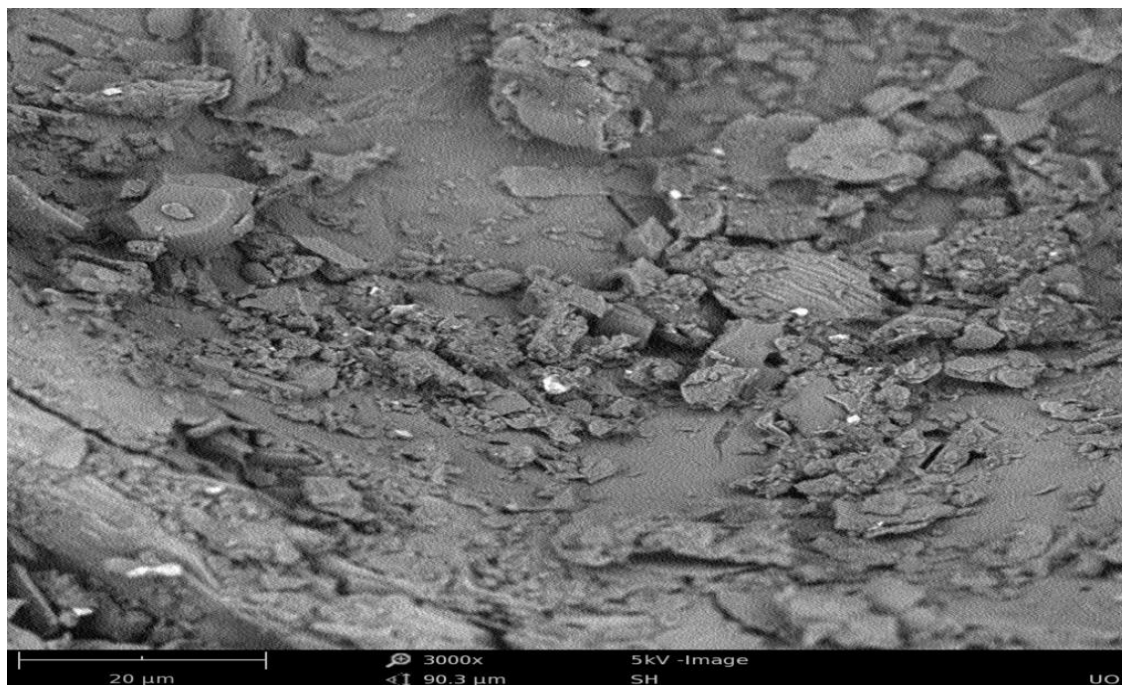
(a.)
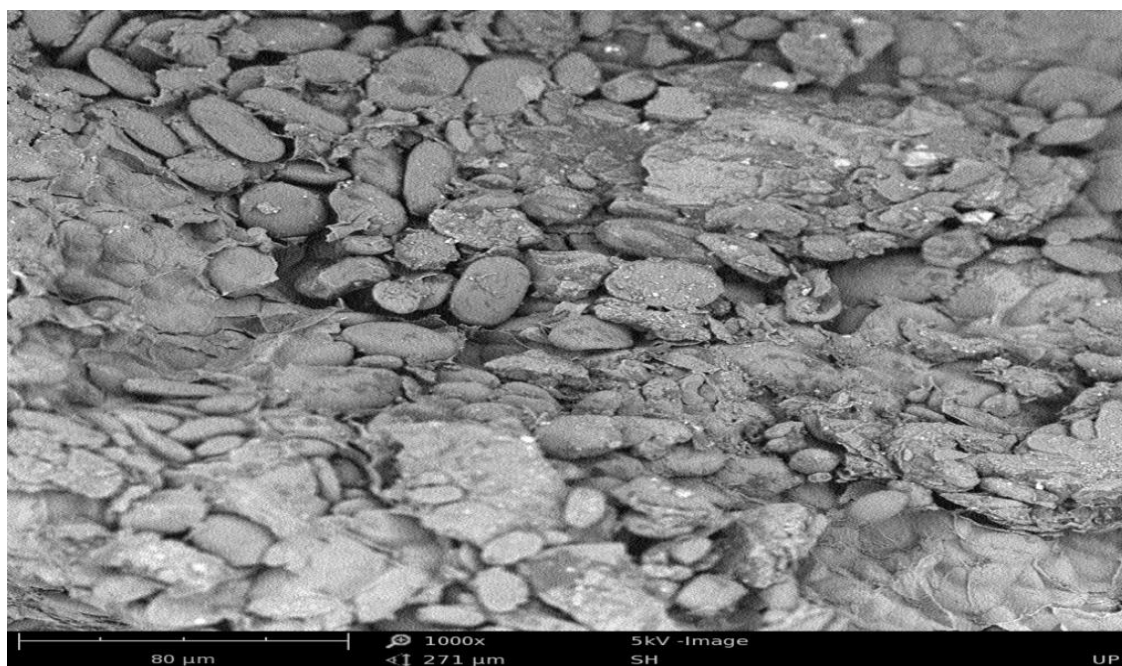
(b.)

Figure 4.9. Morphology of Untreated Peel: (a) Orange Peel, (b) Plantain Peel
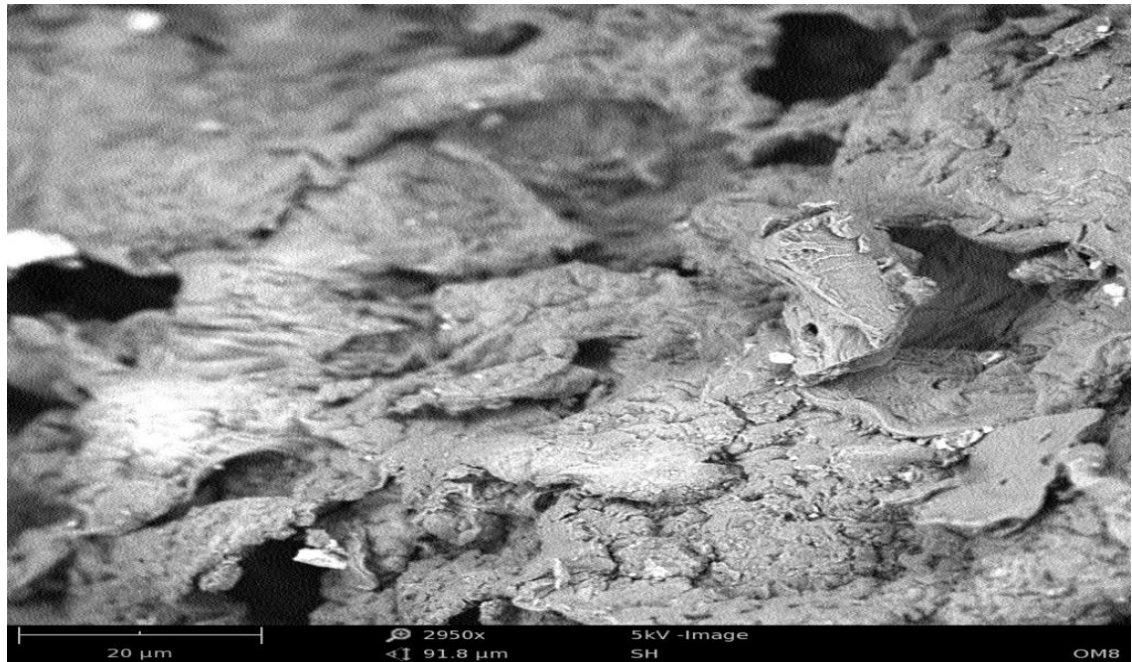
(a.)
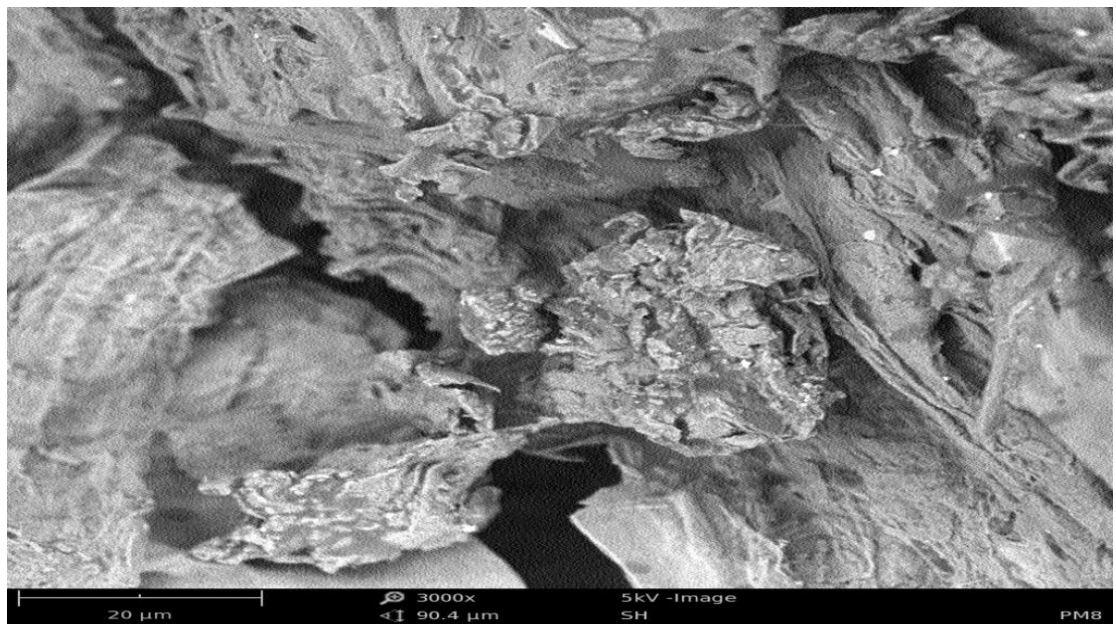
(b.)

Figure 4.10. Morphology of Microwave Pretreated Peel: (a) Orange Peel, (b) Plantain Peel
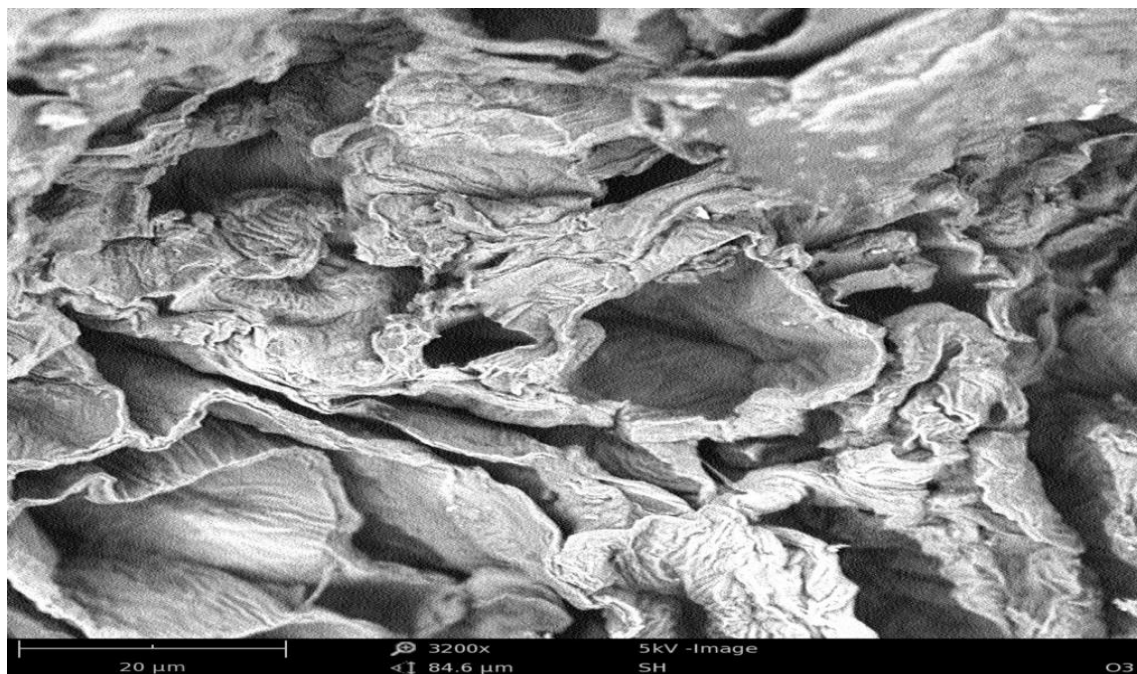
(a.)
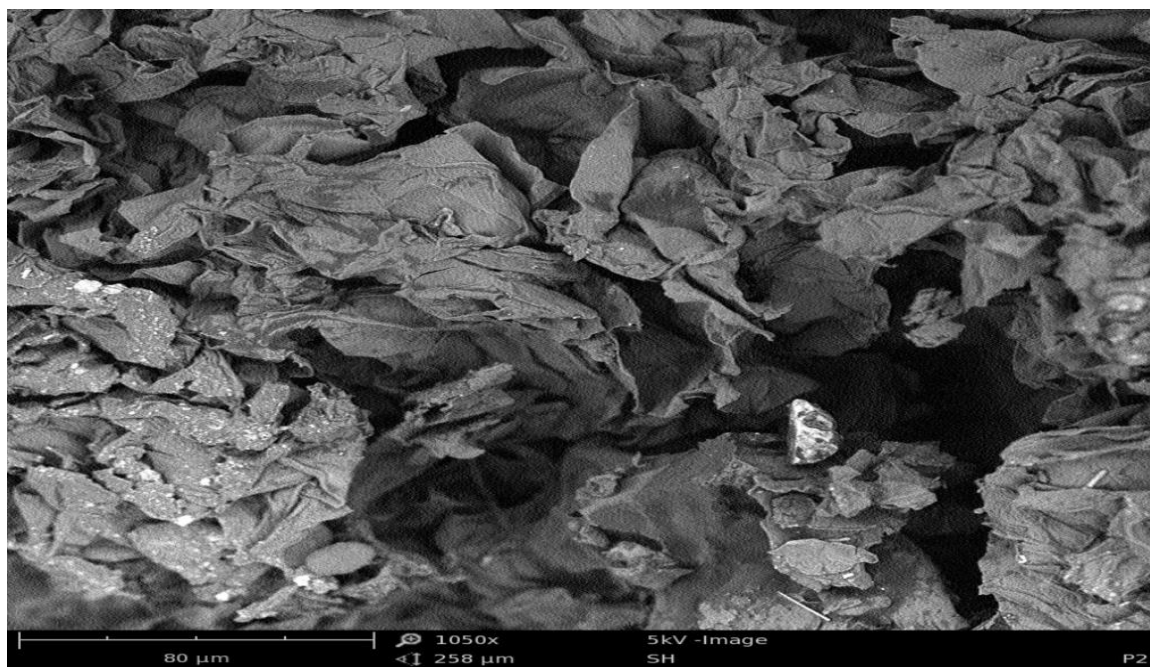
(b.)

Figure 4.11. Morphology of NaOH Pretreated Peel: (a) Orange Peel, (b) Plantain Peel
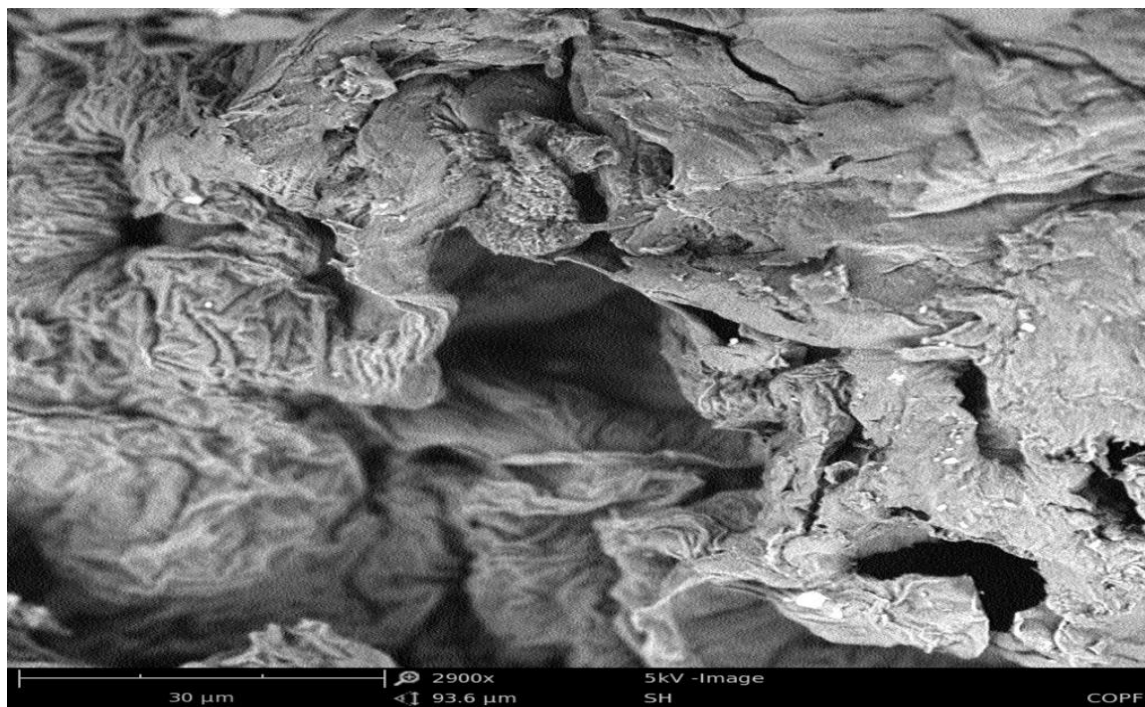
(a.)
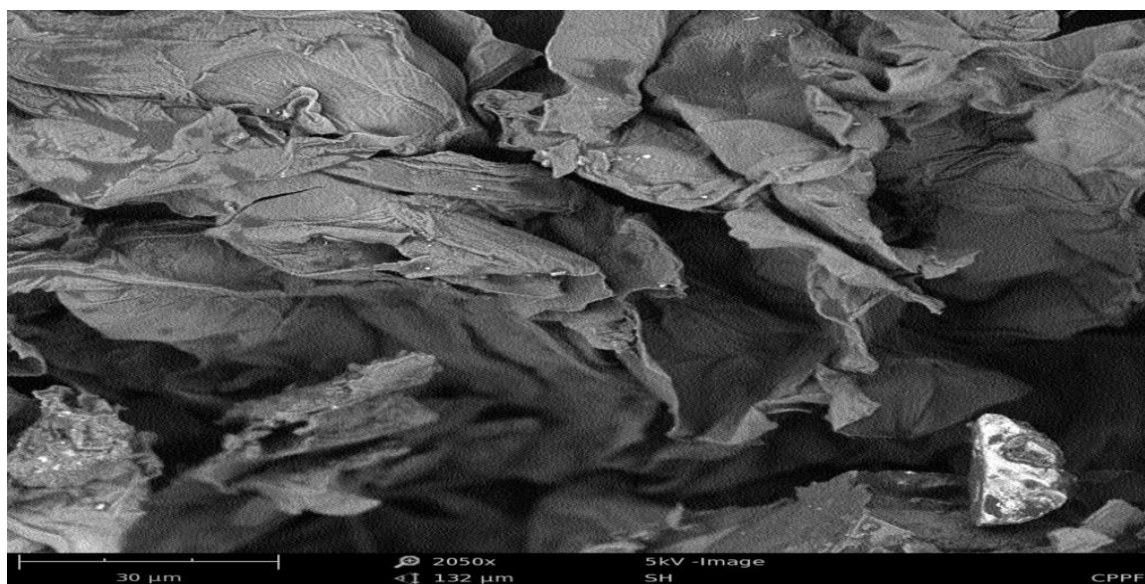
(b.)

Figure 4.12. Morphology of NaOH+microwave Pre-treated Peel: (a) Orange
Peel, (b) Plantain Peel

feedstock resulted in increased porosity (Han *et al.* 2012; Ethaib *et al.,* 2016; Yang *et al*., 2017). According to Raj *et al.* (2016), one of the objectives of pre-treatment is to reduce the rigid nature of substrates thereby giving room for increased activity of microorganisms and/or enzymes for improved production of bio-products.

It was observed from this study that severity of substrates' surface deformation varied with the methods of pre-treatment. During the pre-treatment of rice straw for improved delignification, Chang *et al.* (2017) also found out that the structural properties of pre-treated rice straw varied depending on the pre-treatment type. This suggests that different degrees of pre-treatment of feedstock can be achieved by different methods and no two pre-treatment methods will give the same substrates' structural properties. Microwave pre-treated substrates (Figures 10a and b) were characterized by pronounced porosity while NaOH pre-treated ones (Figures 11a and b) had severe surface roughness. Han *et al*. (2012) also observed that the surface of wheat straw became rough, soft and loose consequent to NaOH pre-treatment. The most severe disruption of microstructure was observed in OPF and PPF subjected to NaOH+MW pre-treatment (Figures 12a and b). This might have contributed to increased microbial proliferation which probably led to the improvement in PG activity (Section 4.3). Sahare *et al.* (2012) had attributed increased saccharification of pre-treated corn cob to rough external surface and expansion of lignin fibre.

4.7   Effect of Substrate Pre-treatment Methods on the Structures of Orange and Plantain peels

Figures 4.13 (a-h) show FTIR spectra of both untreated and pre-treated OPF and PPF. According to these spectra, there were noticeable changes in the bands of the selected wave numbers as a result of different pre-treatment conditions. Table 4.9 shows percentage relative change in intensity of different bands consequent to OPF pre-treatment.
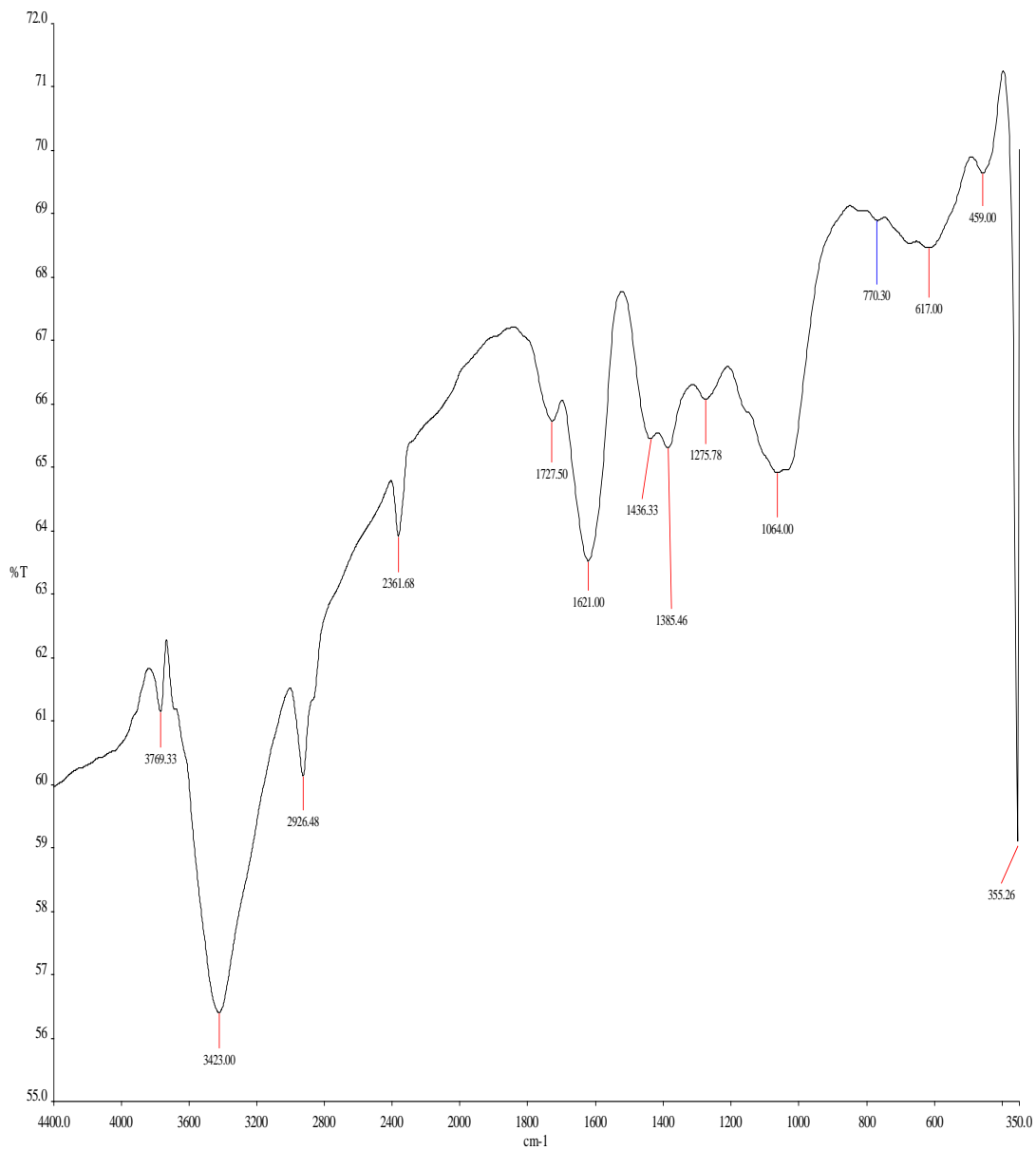
Figure 4.13a. FTIR Spectrum of Untreated Orange Peel
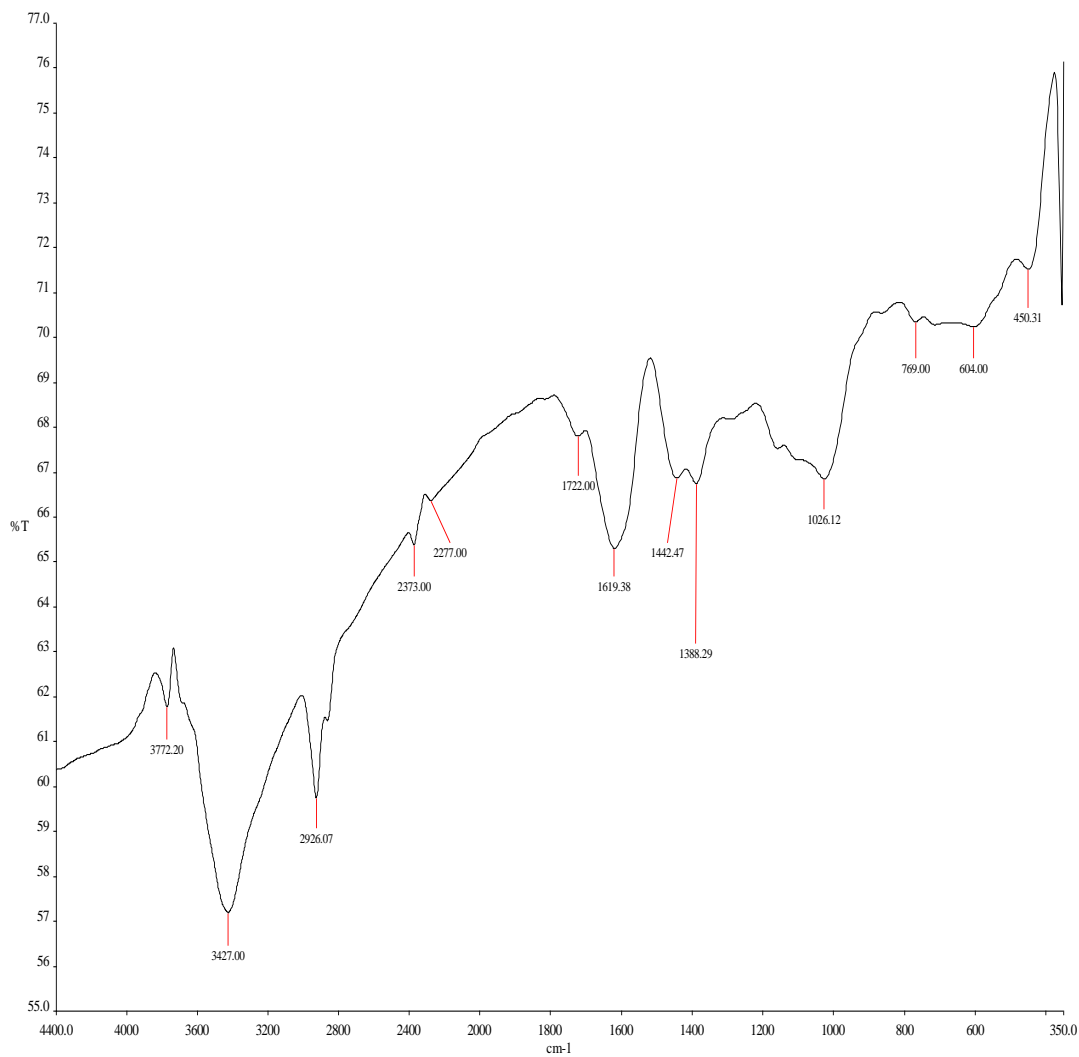
Figure 4.13b. FTIR Spectrum of Untreated Plantain Peel
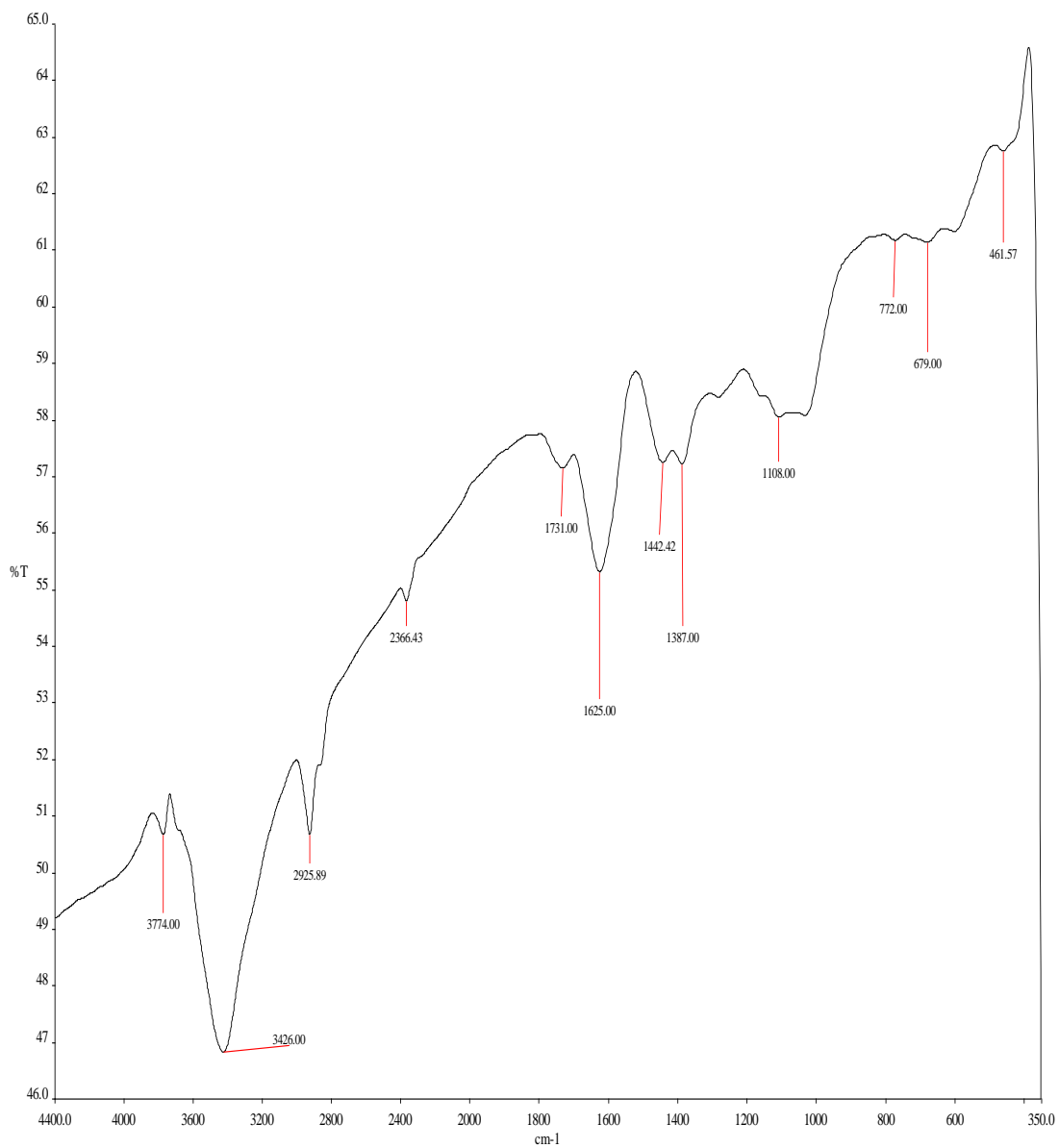
4.13c. FTIR Spectrum of Microwave Pretreated Orange Peel
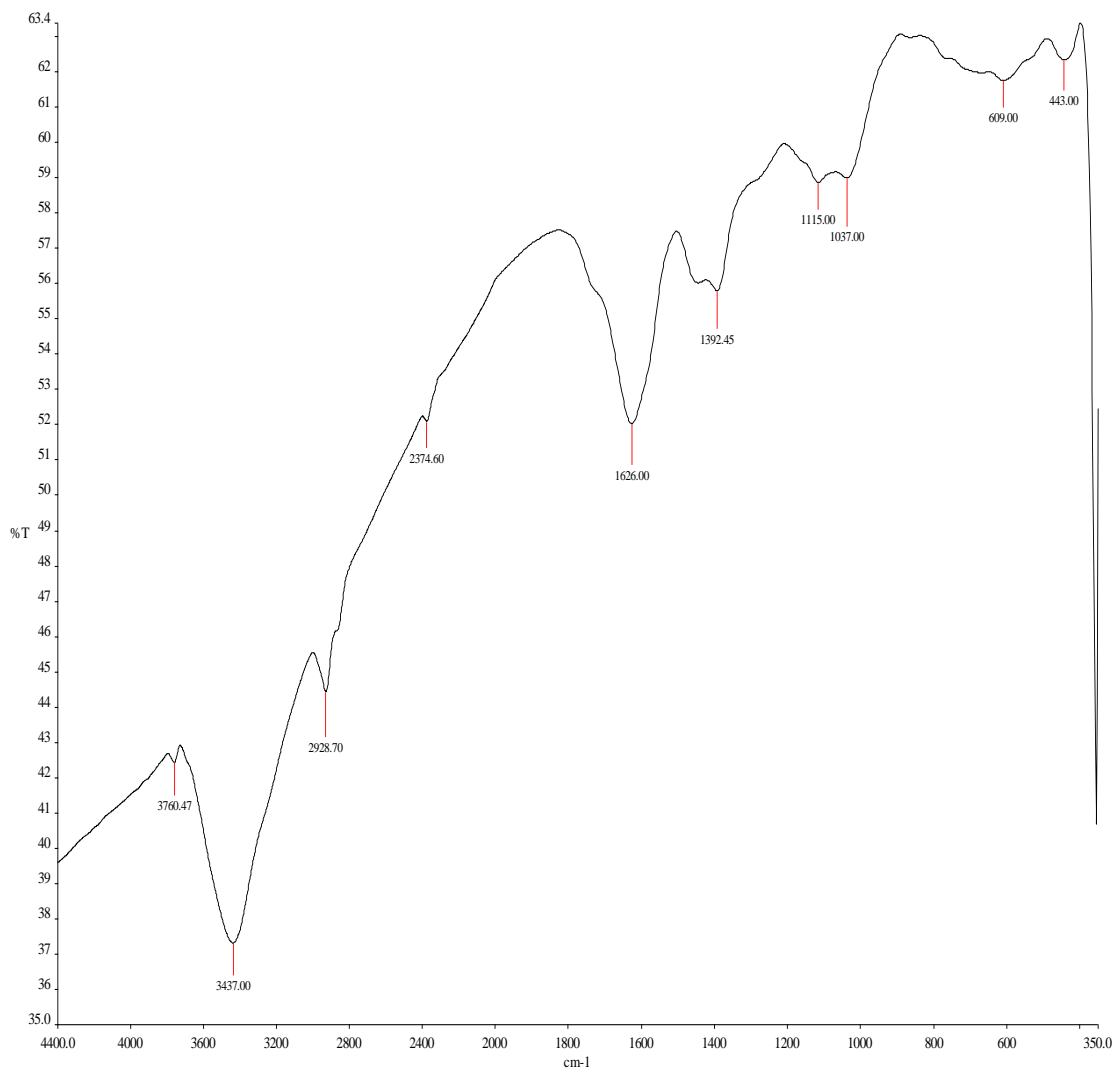
4.13d. FTIR Spectrum of Microwave Pretreated Plantain Peel
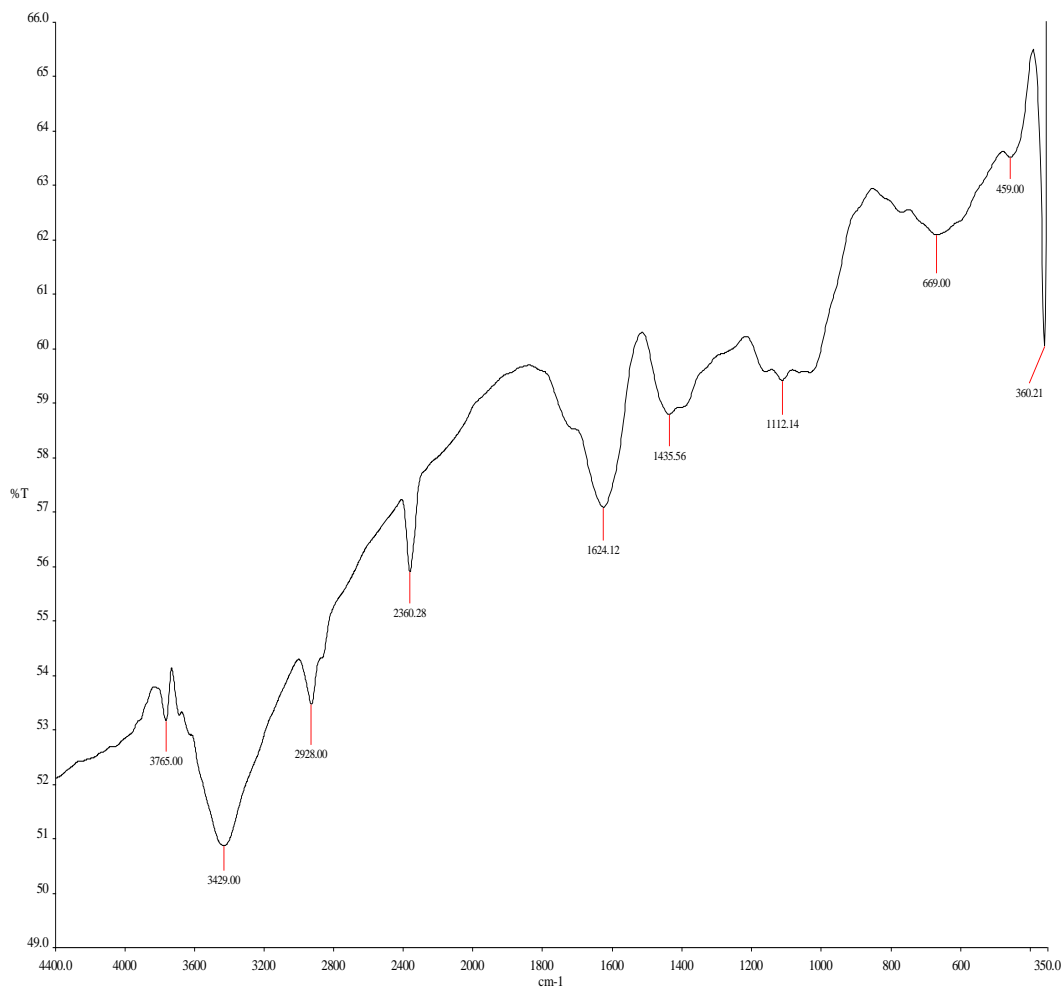
4.13e. FTIR Spectrum of NaOH Pretreated Orange Peel
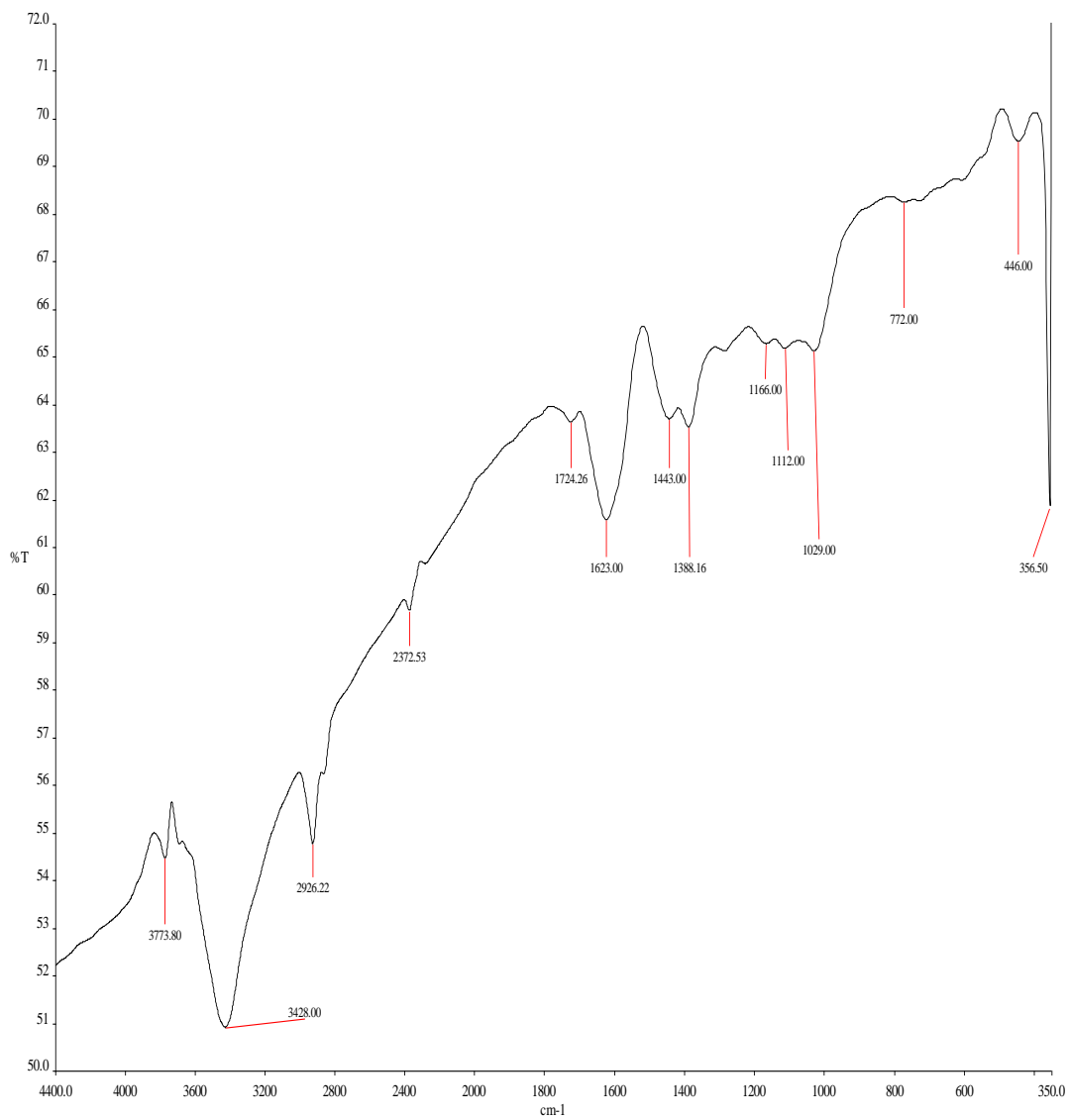
4.13f. FTIR Spectrum of NaOH Pretreated Plantain Peel
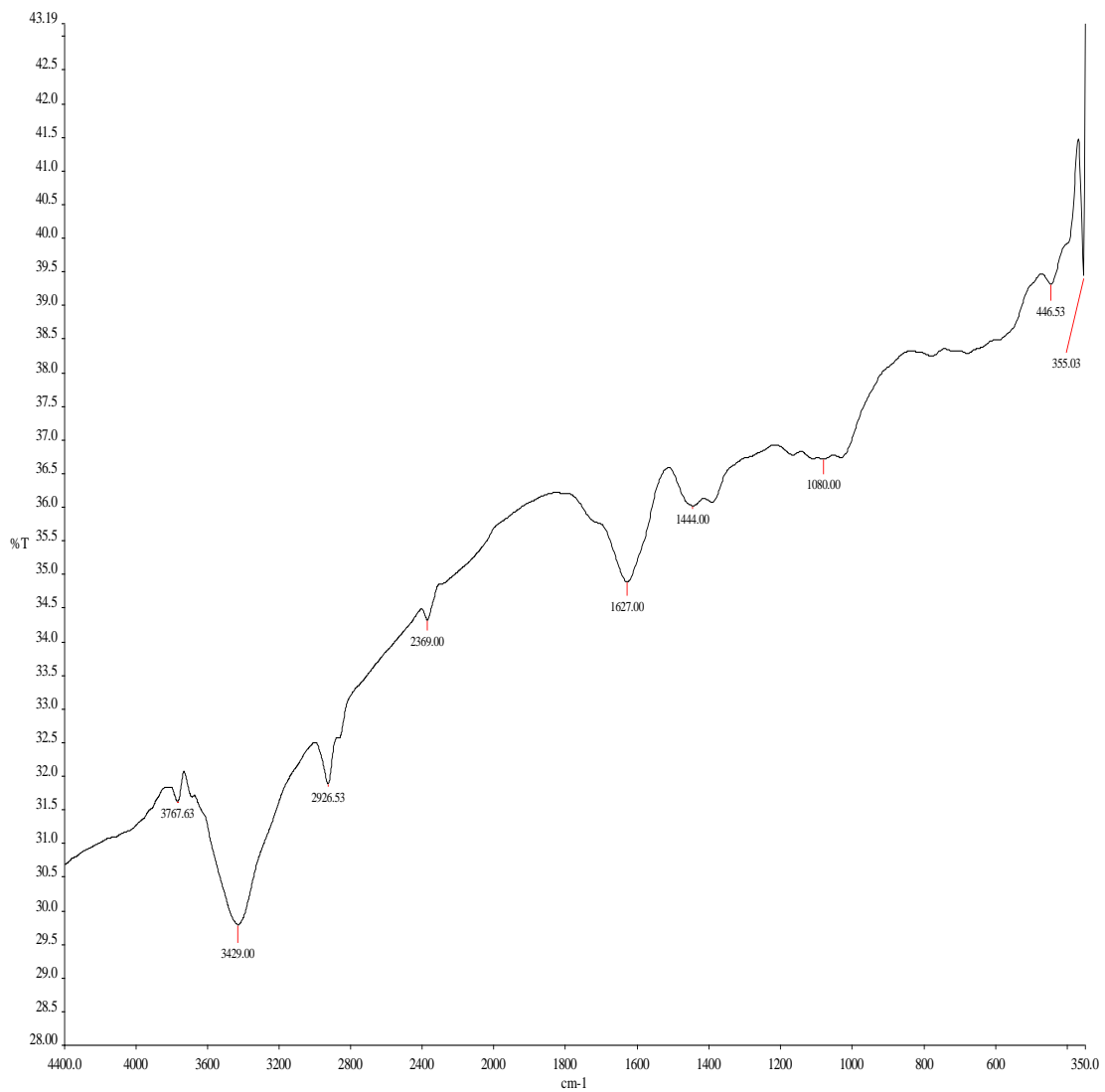
4.13g. FTIR Spectrum of Combined NaOH and Microwave Pretreated Orange Peel
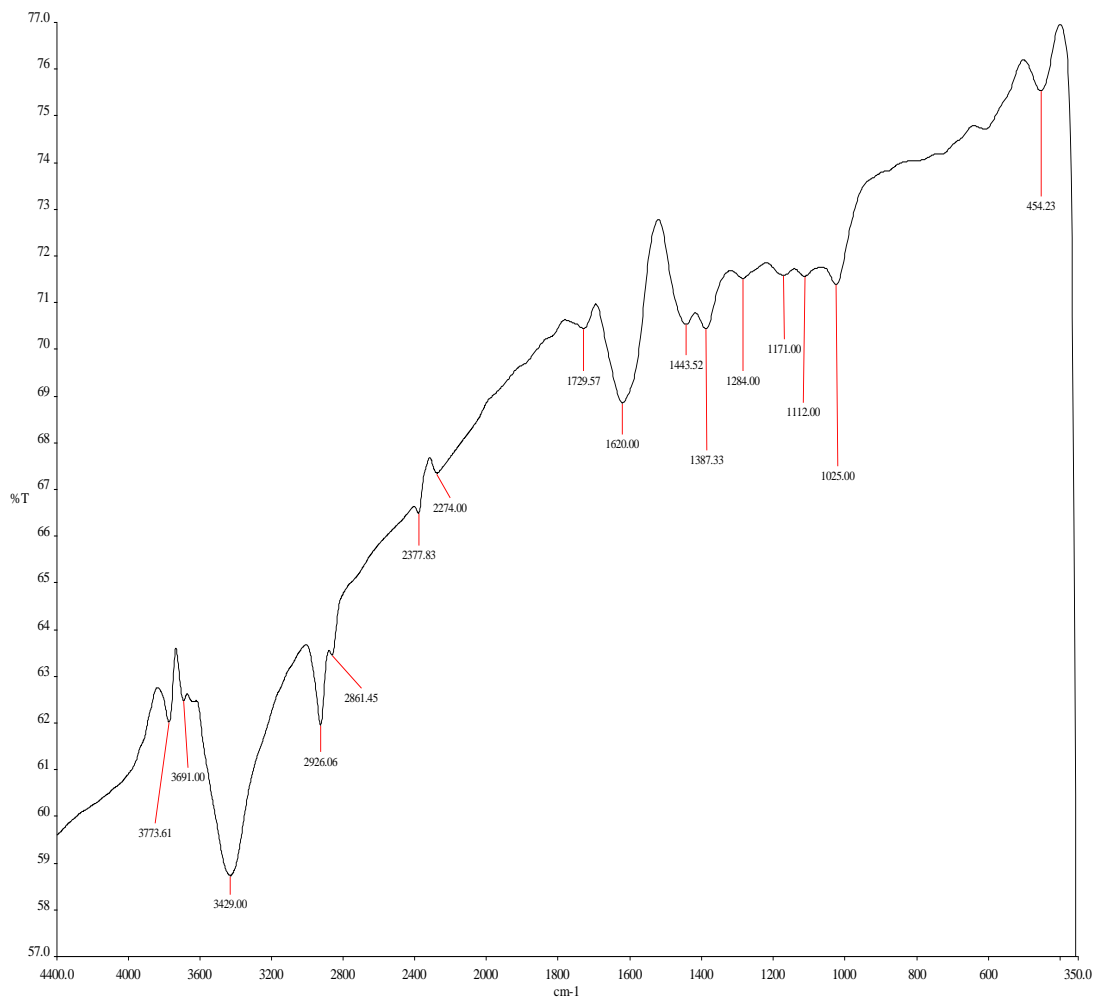
4.13h. FTIR Spectrum of Combined NaOH and Microwave Pretreated Plantain Peel

Table 4.9. FTIR Characterization of Pre-treated Orange Peel in Terms of Percentage Relative Change in Intensity

Wave number (cm^{-1})	Assignment/functional group	Probable polymer	Percentage relative change*		
			MW	NaOH	NaOH+MW
3423	O-H stretching/bending of alcohol group (Xu *et al.,* 2013)	Cellulose	-0.09	-0.18	-0.18
2926	Stretching of C=H in alkane (Santale and Oh, 2015)	Lignin/cellulose	0	-0.03	-0.07
2362	C-H stretching/deformation (Santale and Oh, 2015)	Cellulose	-0.17	-0.30	0.08
1728	C=O stretching of aldehyde/ketone (Singh *et al.,* 2014b)	Hemicellulose	-0.17	5.84	6.02
1621	C=C stretching of alkene (Xu *et al.*, 2013)	Lignin	-0.25	10.92	11.41
1436	-C-H bending of alkane (He *et al.,* 2008)	Lignin	-0.42	24.79	22.56
1385	-C-H bending of alkane (He *et al.,* 2008)	Hemicellulose	-0.14	-	-
1276	C-F stretching of alky halide (Trevorah and Othman, 2015)	Lignin/Hemicellulose	13.76	-	-
1064	C-O stretching (Pandey and Negi, 2015)	Cellulose/lignin	-	-	-
770	=C-H bending of alkene (Pandey and Negi, 2015)	Cellulose/hemicellulose	-0.26	-	-

*Percentage relative change = (difference in intensities of un-treated and pre-treated solids/intensity of untreated solid)×100. Positive percentage relative change indicates reduction in intensity. MW – microwave

Positive percentage relative change implies reduction in the wave number of a band in relation to the control band (band of un-treated biomass). No visible band reduction was observed at wave numbers 2362, 2926 and 3423 cm^{-1} which was distinguished peaks for cellulose (Liu *et al.,* 2017). This suggested reduction in asymmetric stretching of CH$_2$/CH of alkane functional group and O-H bending of alcohol group of cellulose (Singh *et* al., 2014b). This agreed with the results of chemical composition of pre-treated OPF (Table 4.7) where cellulose increased consequent to microwave and NaOH pre-treatment. MW pre-treatment of OPF did not result in reduction in the peak with wave number 1728 cm^{-1} which represented hemicellulose band, however, significant reduction to the tune of 5.84 and 6.02% in this peak was observed in samples subjected to NaOH and NaOH+MW pre-treatment, respectively. The reduction was probably as a result of C=O stretching of aldehyde/ketone functional group (He *et al*., 2008; Singh *et al.,* 2014b). Significant reductions in the magnitude of 10.92 and 11.41% for NaOH and NaOH+MW pre-treated OPF, respectively were recorded in wave number 1621 cm^{-1} owing to C=C stretching of alkene of lignin side chains which probably resulted in disruption of linkages between carbohydrate and lignin (Xu *et al*., 2013). Higher percentage relative change in intensity in NaOH+MW pre-treated OPF is indicative of higher capacity of lignocellulosic-carbohydrate disruption (Yang *et al.,* 2017), hence increased proliferation of *Aspergillus awamori* and subsequent improvement in PG activity. Also in MW and NaOH+MW pre-treated OPF, intermolecular -C-H bond of alkane in lignin side chains appeared to be disrupted (Liu *et al*., 2017) as a result of reduction in the absorption at 1436 cm^{-1} whereas, no significant change was observed in OPF pre-treated by MW only. There were disappearance of peaks with wave numbers 1385, 1276, 1064 and 770 cm^{-1} in OPF pre-treated with NaOH and NaOH-MW. These peaks were related to -C-H bending of alkane, C-F stretching of alky halide, C-O stretching and =C-H bending of alkene, respectively (Pandey and Negi, 2015; Trevorah and Othman, 2015) and their disappearance could be connected to structural disruption of hemicelluloses, lignin and cellulose as a result of the pre-treatment methods (Garmakhany *et al.,* 2013).

Table 4.10 shows the percentage relative change of selected bands after PPF pre-treatment. Increase in absorption was recorded for pre-treated PPF at wave numbers 3427, 2926 and 2373 cm^{-1}. This suggested reduction in asymmetric stretching of CH$_2$/CH of alkane functional group and O-H bending of alcohol group of cellulose (Singh *et* al., 2014b). On the other hand, significant reduction in absorption peaks was observed at wave numbers 2277 and 1722. These bands represent OH, C-O combination bands (Santale and Oh, 2015) and C=O stretching of aldehyde/ketone (Singh *et al.,* 2014b) and are representative bands for cellulose and hemicelluloses respectively. Reduction in these absorption peaks with attendant increase in percentage relative change in intensity implies significant reduction in hemicelluloses by all the pre-treatment methods. The degree of reduction in absorption peak of pre-treated PPF is of the order: NaOH-MW<NaOH<MW and NaOH<MW< NaOH+MW at 2277 and 1722 cm^{-1}, respectively. Among the pre-treatment methods considered in this study, NaOH+MW was the most efficient for effective lignin and hemicelluloses removal. This is so because highest percentage relative change in intensity at absorption peaks 1619, 1442 and 1026 cm^{-1} which represent C=C stretching of alkene (Xu *et al*., 2013) and -C-H bending of alkane of lignin-hemicellulose linkages (He *et al.,* 2008) was recorded for PPF pre-treated with NaOH-MW.

4.8   Purification of Polygalacturonase

Purification efficiency of PG by activated charcoal is discussed in this Section.

4.8.1   Degree of polygalacturonase purification

Protein content of crude and purified PG produced from OPF and PPF are presented in Tables 4.11 and 4.12, respectively. Protein content of crude and purified PG from OPF were 145.65-168.84 mg/mL and 8.84-10.36 mg/mL, respectively. Protein content of crude and purified PG produced

Table 4.10. FTIR Characterization of Pre-treated Plantain Peel in Terms of Percentage Relative Change in Intensity

Wave number (cm^{-1})	Assignment/functional group	Probable polymer	Percentage relative change		
			MW	NaOH	NaOH+MW
3427	O-H stretching/bending of alcohol group (Xu *et al.,* 2013)	Cellulose	-0.29	-0.03	-0.06
2926	Stretching of C=H in alkane (Santale and Oh, 2015)	Lignin/cellulose	-0.10	0	0
2373	C-H stretching/deformation (Santale and Oh, 2015)	Cellulose	-0.08	0	-0.21
2277	OH, C-O combination bands (Santale and Oh, 2015)	Cellulose	28.59	24.28	4.52
1722	C=O stretching of aldehyde/ketone (Singh *et al.,* 2014b)	Hemicellulose	19.16	5.75	22.76
1619	C=C stretching of alkene (Xu *et al.*, 2013)	Lignin	-	10.87	18.47
1442	-C-H bending of alkane (He *et al.,* 2008)	Lignin	-	3.74	13.80
1388	-C-H bending of alkane (He *et al.,* 2008)	Hemicellulose	-	15.99	16.48
1026	C-O stretching (Pandey and Negi, 2015)	Cellulose/lignin	-8.67	-0.29	0.10

*Percentage relative change in intensity = (difference in intensities of un-treated and pre-treated solids/intensity of untreated solid)×100. Positive percentage relative change indicates reduction in intensity. MW= microwave

Table 4.11.   Activity of Crude and Purified Polygalacturonase from *Aspergillus awamori* on Orange Peel

PG	PGc (U/mL)	PCc (mg/mL)	PCp (mg/mL)	PGp (U/mL)	SUc (U/mL)	SUp (U/mL)	LCI (%)	FP	PR (%)
Uo	$25.17^{d}\pm0.03$	$168.84^{a}\pm0.00$	$10.36^{a}\pm1.56$	$14.33^{c}\pm0.04$	$0.15^{c}\pm0.00$	$1.38^{c}\pm0.03$	$56.46^{a}\pm1.56$	$9.20^{a}\pm0.03$	$56.93^{a}\pm1.44$
O3	$38.46^{b}\pm0.28$	$163.77^{b}\pm0.03$	$9.57^{a}\pm0.10$	$19.66^{b}\pm0.06$	$0.23^{ab}\pm0.03$	$2.05^{b}\pm0.04$	$50.71^{bc}\pm1.41$	$8.91^{a}\pm1.44$	$51.11^{ab}\pm2.84$
OM8	$26.21^{c}\pm0.01$	$145.65^{d}\pm1.14$	$8.91^{a}\pm1.41$	$12.54^{d}\pm0.07$	$0.18^{bc}\pm0.01$	$1.41^{c}\pm0.06$	$47.19^{c}\pm1.42$	$7.83^{a}\pm1.43$	$47.84^{b}\pm1.47$
CPOF	$42.05^{a}\pm0.07$	$150.72^{c}\pm0.28$	$8.84^{a}\pm0.08$	$22.27^{a}\pm0.08$	$0.28^{a}\pm0.04$	$2.42^{a}\pm0.07$	$52.60^{b}\pm0.28$	$8.64^{a}\pm0.01$	$52.96^{ab}\pm2.87$

Values are means ± standard deviations of 3 replications. Means within a column with different superscripts were significantly ($p<0.05$) different. Uo= PG produced from un-treated orange peel; O3= PG produced from alkaline pre-treated orange peel; OM8= PG produced from microwave pre-treated orange peel; CPOF= PG produced from combined alkaline and microwave pre-treated orange peel; PGc=Activity of crude PG; PCc=protein content of crude enzyme; PCp=protein content of purified enzyme; PGp=Activity of purified PG; SUc=specific activity of crude enzyme; Sup=specific activity of purified enzyme; LCI=rate of purification; FP=fold purification; PR=PG recovery.

Table 4.12.   Activity of Crude and Purified Polygalacturonase from *Aspergillus awamori* on Plantain Peel

PG	PGc (U/mL)	PCc (mg/mL)	PCp (mg/mL)	PGp (U/mL)	SUc (U/mL)	SUp (U/mL)	LCI (%)	FP	PR (%)
Up	$26.04^{d}\pm0.06$	$168.12^{a}\pm1.41$	$10.29^{b}\pm0.03$	$14.66^{c}\pm0.10$	$0.15^{a}\pm0.04$	$1.42^{b}\pm0.08$	$55.81^{a}\pm4.24$	$9.47^{a}\pm0.07$	$56.30^{a}\pm1.44$
P2	$38.82^{b}\pm0.14$	$157.97^{b}\pm1.17$	$9.49^{b}\pm1.40$	$21.78^{b}\pm1.11$	$0.25^{a}\pm0.07$	$2.30^{a}\pm0.10$	$55.71^{a}\pm1.39$	$9.20^{a}\pm0.34$	$56.11^{a}\pm1.14$
OM8	$26.72^{c}\pm0.03$	$151.45^{c}\pm0.07$	$13.04^{a}\pm0.06$	$14.55^{c}\pm1.13$	$0.18^{a}\pm0.00$	$1.12^{b}\pm0.11$	$53.91^{a}\pm1.11$	$6.22^{c}\pm0.16$	$54.45^{ab}\pm1.48$
CPPF	$44.98^{a}\pm0.03$	$162.32^{b}\pm2.86$	$9.49^{b}\pm0.04$	$22.97^{a}\pm1.14$	$0.28^{a}\pm0.06$	$2.35^{a}\pm0.14$	$50.73^{a}\pm0.10$	$8.39^{c}\pm0.04$	$52.07^{c}\pm0.10$

Values are means $\pm$ standard deviations of 3 replications. Means within a column with different superscripts were significantly ($p<0.05$) different. Up= PG produced from un-treated plantain; P2= PG produced from alkaline pre-treated plantain peel; PM8= PG produced from microwave pre-treated plantain peel; CPPF= PG produced from combined alkaline and microwave pre-treated plantain peel; PCc=protein content of crude enzyme; PCp=protein content of purified enzyme; PGp=PG activity of purified enzyme; SUc=specific activity of crude enzyme; Sup=specific activity of purified enzyme; LCI=rate of purification; FP=fold purification; PR=PG recovery.

from PPF were 151.45-168.12mg/mL and 9.49-13.04 mg/mL, respectively. Purification of PG caused a significant ($p < 0.05$) reduction in protein content. This suggests that other components in the crude enzyme contributed to its total protein content. In a similar manner, a reduction in protein content of PG from *Aspergillus niger* was recorded after purification (Kant *et al*., 2013). Also, Silva *et al.* (2007) reported a reduction in the protein content of exo-PG from *Penicillium viridicatum* after purification. Protein content varied significantly ($p < 0.05$) among the crude enzymes. Lower protein content was recorded for crude enzymes produced from pre-treated substrates compared to those produced from un-treated substrates. On the other hand, there was no significant ($p > 0.05$) difference in protein content among the purified enzymes. This could be due to the removal of impurities that had contributed to high protein content of the crude PG by partial purification. Activated charcoal has been shown to be a cost-effective method for efficient purification and recovery of enzymes (Dey and Banerjee, 2014; Poletto *et al.,* 2015).

Activity of purified PG ranged from 14.33 to 22.27U/mL in OPF, and from 14.66 to 22.97 U/mL in PPF. These values were significantly ($p < 0.05$) lower compared to those recorded for crude PG (25.17 to 42.05 U/mL for OPF and 26.04 to 44.98 U/mL for PPF). This might be due to reduction in protein content as a result of purification. This agreed with the finding of Joshi *et al.* (2011) who reported reduction in the PG activity of pectinase produced from *Aspergillus niger* after purification. Specific activity of both the crude and partially purified enzymes varied significantly ($p < 0.05$). For crude PG, specific activity ranged from 0.15 to 0.28 U/mL. It increased significantly ($p < 0.05$) in purified PG (1.12 to 2.42 U/mL). Higher specific activity of purified PGs implies higher catalytic potential (Dogan and Tari, 2008). For both crude and purified PG, higher specific activity was recorded in PG produced from pre-treated OPF and PPF. PG produced from OPF and PPF pretreated with NaOH+MW gave the highest results. There was also variation in rate of purification. Results obtained ranged from 47.19 to 56.46%. Variation in the chemical and structural properties of substrates consequent to pretreatment might have been responsible for differences in properties of PG

produced from them. This probably resulted in variations in dielectric constants of the enzymes, hence, different interactive patterns of enzymes' protein molecules (Kumar and Parrack, 2003). According to Jaswail and Ravindra (2016), purification of enzymes increases their specific activity due to the removal of unwanted impurities. Fold purification varied from 6.22 to 9.47 and corresponded to PG recovery that ranged between 51.11 and 56.93%. Fold purification and PG recovery obtained for partially purified PG in this study were higher than 3.13 and 4.33%, respectively reported for purified PG obtained from *Aspergillus fumigatus* (Anand *et al*., 2016). However, Dey and Banerjee (2014) reported higher fold purification (8.6 – 34.8) and PG recovery (65.1 – 69.8) in activated carbon purified PG from *Aspergillus awamori* Nakazawa MTCC 6652. There was no significant ($p > 0.05$) difference in fold purification among the samples except PG from MW pre-treated OPF and PPF. Pre-treatment of OPF and PPF also caused significant ($p < 0.05$) reduction in PG recovery of their enzymes. This could be attributed to reduction in dielectric constant of enzyme which probably increased the interaction between protein molecules (Ngo *et al*., 2008).

4.8.2 Protein purity and molecular weight of crude and purified polygalacturonase

Figure 4.14 shows the bands of standard marker, crude and purified PG from *Aspergillus awamori.* Crude PGs produced from *Aspergillus awamori* using OPF (Lane 3) and PPF (Lane 5) showed many protein bands that ranged from 20 to 80 kDa and from 20 to 96.2 kDa, respectively. Different protein bands that ranged between 36 and 72 kDa were also found in alkaline PG produced from *Bacillus* spp. (Paudel *et al.,* 2015). Numerous protein bands on crude PG could be an indication of presence of other proteins and impurities (Kogo *et al.,* 2017). Purification of PG resulted in a single protein band in PG produced using OPF and PPF. Molecular weight for purified PG were 32 and 31 kDa for OPF (Lane 2) and PPF (Lane 4), respectively.
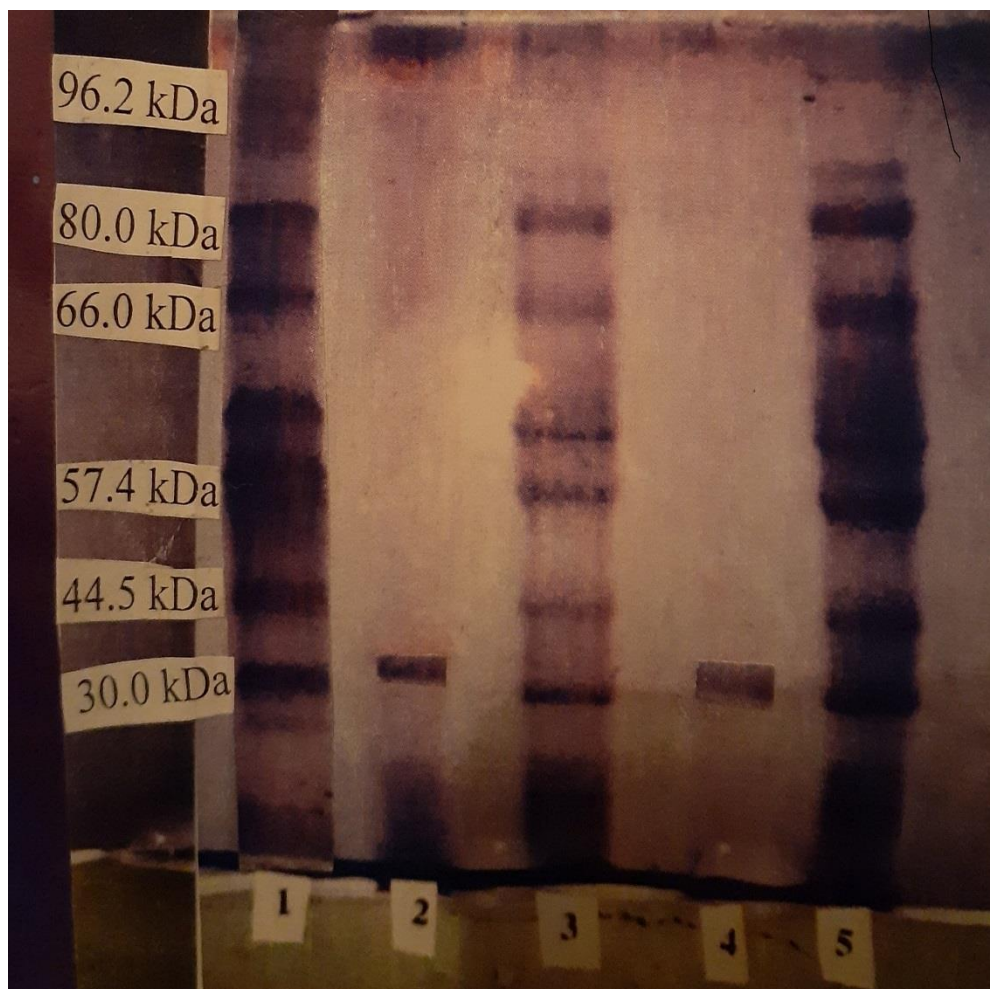
Figure 4.14.   Protein Bands of Crude and Purified Polygalacturonases.

Lane 1- standard protein marker; Lane 2- purified polygalacturonase produced by *Aspergillus awamori* using pre-treated orange peel; Lane 3- crude polygalacturonase produced by *Aspergillus awamori* using pre-treated orange peel; Lane 4- purified polygalacturonase produced by *Aspergillus awamori* using pre-treated plantain peel; Lane 5- crude polygalacturonase produced by *Aspergillus awamori* using pre-treated plantain peel.

These results suggested probable presence of PG. These findings are similar to those reported by earlier workers. Ahmed *et al.* (2015) reported molecular weight of 30 kDa for purified pectinase produced from *Aspergillus niger.* Also, Kant *et al.* (2013) reported a molecular weight of 34 kDa for a purified PG produced from *Aspergillus niger* MTCC 3323. Differences in the molecular weight of PG produced from OPF and PPF could be due to differences in their chemical constituents. Ahmed *et al.* (2015) had reported that the properties of enzyme's proteins are based on factors such as substrate type, nature of microorganisms and analytical methods.

4.8.3 Colour order properties of purified polygalacturonase as influenced by substrate pre-treatment

Degree of lightness (L) of PGs produced from OPF and PPF are presented in Figures 4.15a and b, respectively. Higher L value was recorded in PG produced from pre-treated substrates. This is probably due to removal of colours and pigments during pre-treatment operation. According to Adiguzel *et al.* (2016), extracts contain impurities and melanine-like colouring matters that have strong affinity for enzymes. Among the PGs produced from pre-treated OPF and PPF, highest L value was recorded for the NaOH pre-treated ones while the least was recorded for MW pre-treated ones. Purification of PG also caused an increase in L values probably due to decolorization during the operation. PGs produced from both pre-treated OPF and PPF had higher L values than those from un-treated ones. Purification of PG produced from OPF caused a deviation from redness to greenness. This is evident in the negative values obtained for a (Figure 4.16a). Highest deviation was observed in PG produced from MW pre-treated OPF. PG produced from PPF showed the same pattern (Figure 4.16b) except the PG produced from NaOH+MW pre-treated that showed deviation from greenness to redness. For both PG produced from OPF and PPF, purification caused a reduction in b (Figures 4.17a and b) value. This implies reduction in yellowness. Figure 18a and b show that colour intensity increased consequent to purification of PG with activated charcoal. Higher colour intensity was recorded for purified PG. Increased colour intensity could be as a result of loss of colour compounds
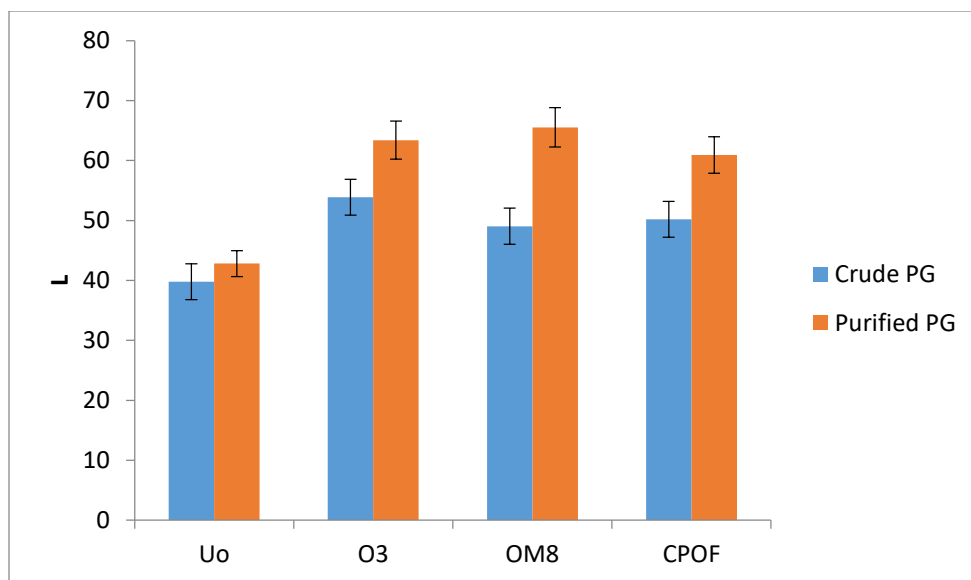
(a.)
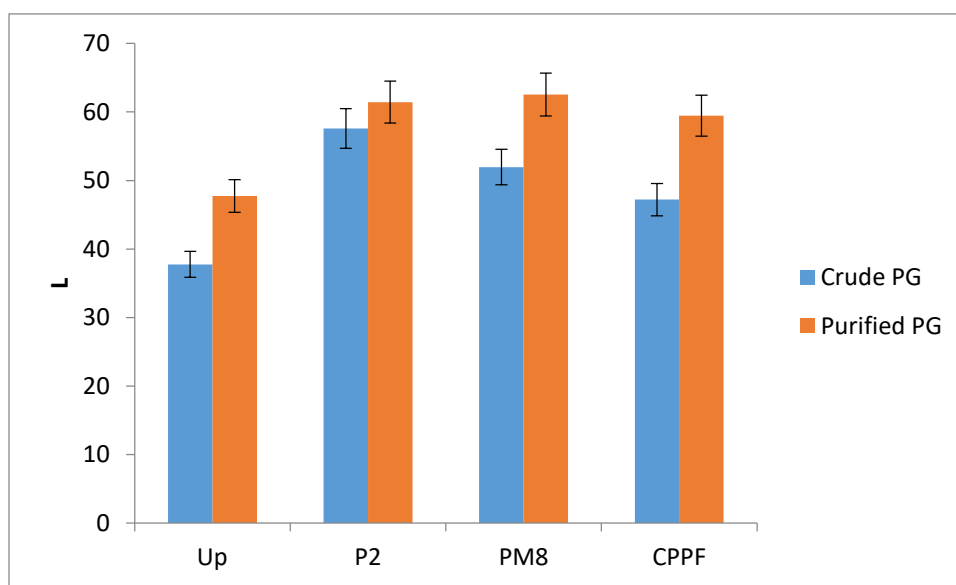
(b.)

Figure 4.15. L Values of Crude and Purified Polygalacturonases: (a) using orange peel, (b) using plantain peel. Uo- PG produced from un-treated orange peel; O3- PG produced from alkaline pre-treated orange peel; OM8- PG produced from microwave pre-treated orange peel; CPOF- PG produced from combined alkaline and microwave pre-treated orange peel, Up- PG produced from un-treated plantain; P2- PG produced from alkaline pre-treated plantain peel; PM8- PG produced from microwave pre-treated plantain peel; CPPF- PG produced from combined alkaline and microwave pre-treated plantain peel.
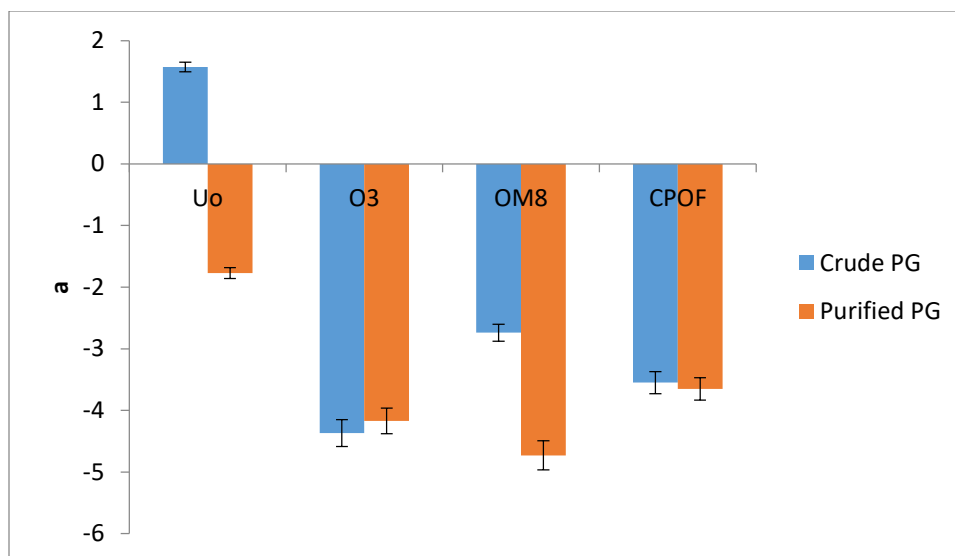
(a.)
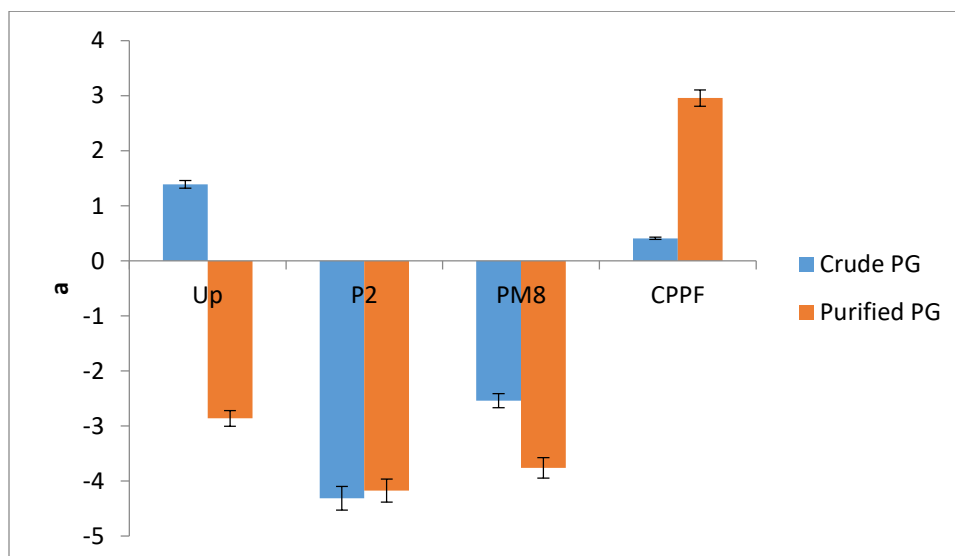
(b.)

Figure 4.16. a Values of Crude and Purified Polygalacturonase: (a) using orange peel, (b) using plantain peel. Uo- PG produced from un-treated orange peel; O3- PG produced from alkaline pre-treated orange peel; OM8- PG produced from microwave pre-treated orange peel; CPOF- PG produced from combined alkaline and microwave pre-treated orange peel, Up- PG produced from un-treated plantain; P2- PG produced from alkaline pre-treated plantain peel; PM8- PG produced from microwave pre-treated plantain peel; CPPF- PG produced from combined alkaline and microwave pre-treated plantain peel.
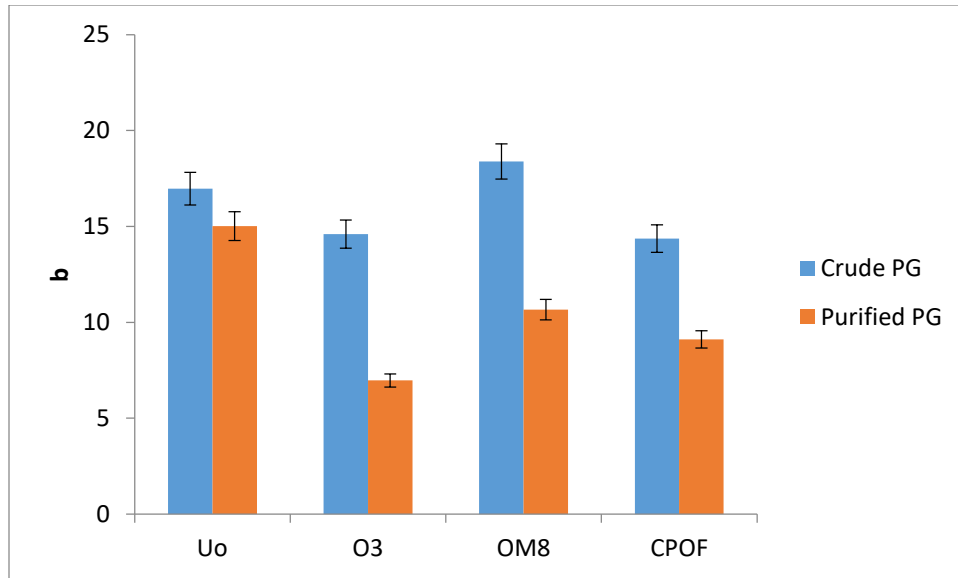
(a.)
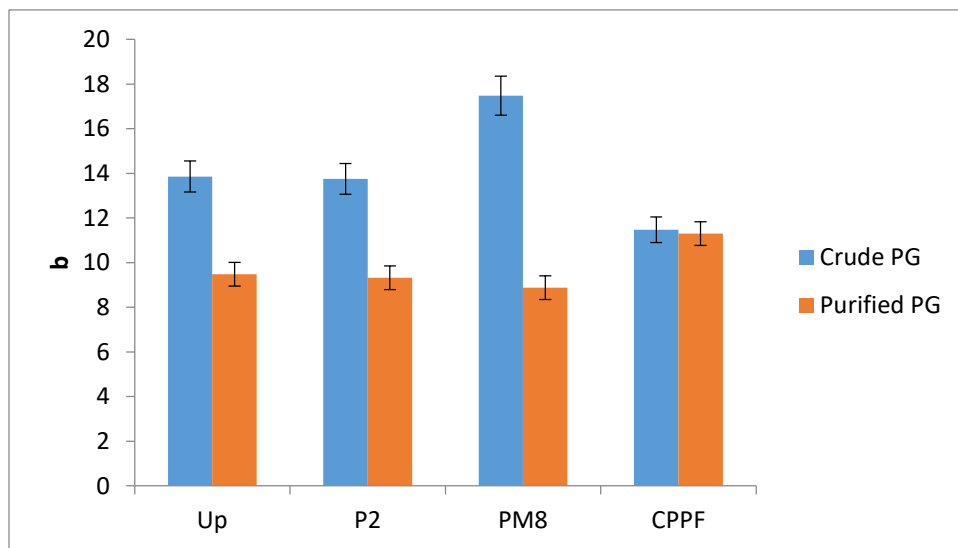
(b.)

Figure 4.17. b Values of Crude and Purified Polygalacturonase: (a) using orange peel, (b) using plantain peel. Uo- PG produced from un-treated orange peel; O3- PG produced from alkaline pre-treated orange peel; OM8- PG produced from microwave pre-treated orange peel; CPOF- PG produced from combined alkaline and microwave pre-treated orange peel, Up- PG produced from un-treated plantain; P2- PG produced from alkaline pre-treated plantain peel; PM8- PG produced from microwave pre-treated plantain peel; CPPF- PG produced from combined alkaline and microwave pre-treated plantain peel.
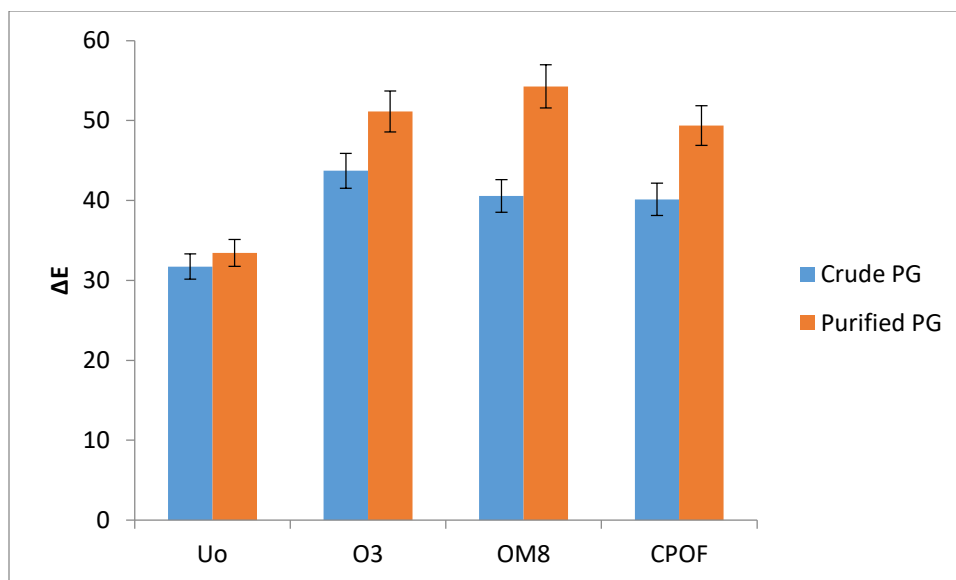
(a.)
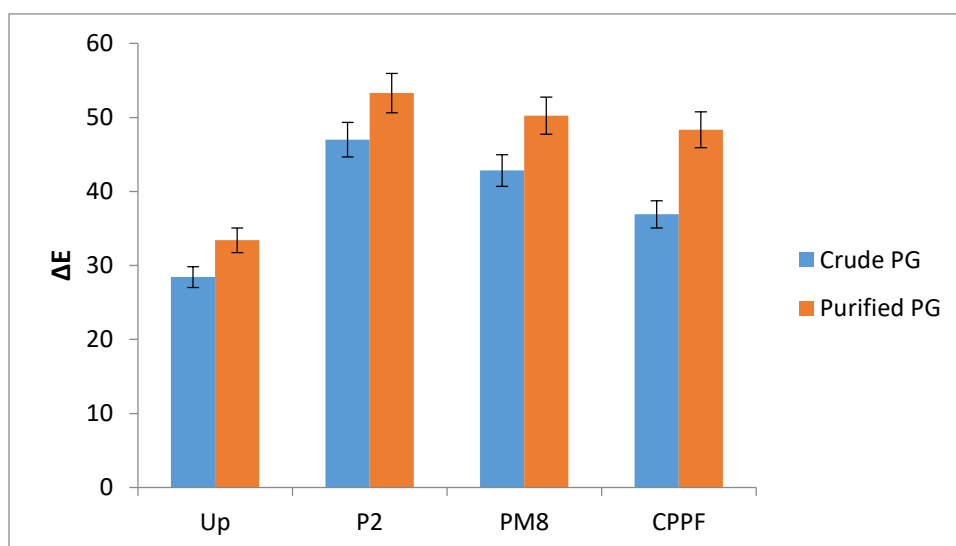
(b.)

Figure 4.18. Colour Intensity (ΔE) of Crude and Purified Polygalacturonase: (a) using orange peel, (b) using plantain peel. Uo- PG produced from un-treated orange peel; O3- PG produced from alkaline pre-treated orange peel; OM8- PG produced from microwave pre-treated orange peel; CPOF- PG produced from combined alkaline and microwave pre-treated orange peel, Up- PG produced from un-treated plantain; P2- PG produced from alkaline pre-treated plantain peel; PM8- PG produced from microwave pre-treated plantain peel; CPPF- PG produced from combined alkaline and microwave pre-treated plantain peel.

in the pre-treated substrates. There were variations in the degree of colour intensity based on substrate pre-treatment methods. For OPF (Figure 18a), highest colour

intensity was recorded in purified PG produced from OPF pre-treated with MW while highest value was recorded in purified PG produced from alkaline pre-treated PPF.

4.9 Characteristics of Polygalacturonase

Stability and thermodynamic properties of PG are discussed in this Section.

4.9.1 Polygalacturonase activity at different pH values

Figures 4.19 (a and b) and 20 (a and b) respectively show the activity of crude and purified PG produced from un-treated and pre-treated substrates at different pH. Maximum activity was obtained at pH 4.0 for Uo (crude PG produced from un-treated OPF), O3 (crude PG produced from NaOH pre-treated OPF) and CPOF (crude PG produced from NaOH+MW pre-treated OPF). Also, maximum activity was obtained for PUo, (purified PG produced from un-treated OPF), PO3 (purified PG produced from NaOH pre-treated OPF) and PCPOF (purified PG produced from NaOH+MW pre-treated OPF) at pH 4.0. Earlier studies had shown optimum activity of fungal PG to range between 4.0 and 6.0 (Suresh and Viruthagiri, 2010; Kant *et al.,* 2013; Ahmed *et al.*, 2015). However, Joshi *et al.* (2011) reported a lower optimum pH 3.5 for PG produced from *Aspergillus niger.* Optimum pH varied among the PGs produced from PPF i.e.7.0 for Up (crude PG produced from un-treated plantain peel), 4.0 for P2 (crude PG produced from NaOH pre-treated PPF), PUp (purified PG produced from un-treated plantain peel) and 5.0 for CPPF (crude PG produced from NaOH+MW pre-treated PPF), PP2 (purified PG produced from NaOH pre-treated PPF) and PCPPF (purified PG produced from NaOH+MW pre-treated PPF). pH optima varies among PGs depending on the source, fermentation type, post-fermentation extraction methods etc. (Zaslona and Trusek-Holownia, 2015). Optimum pH 7.0 obtained for Up showed a slight deviation from other PG produced from this study. Neutral or
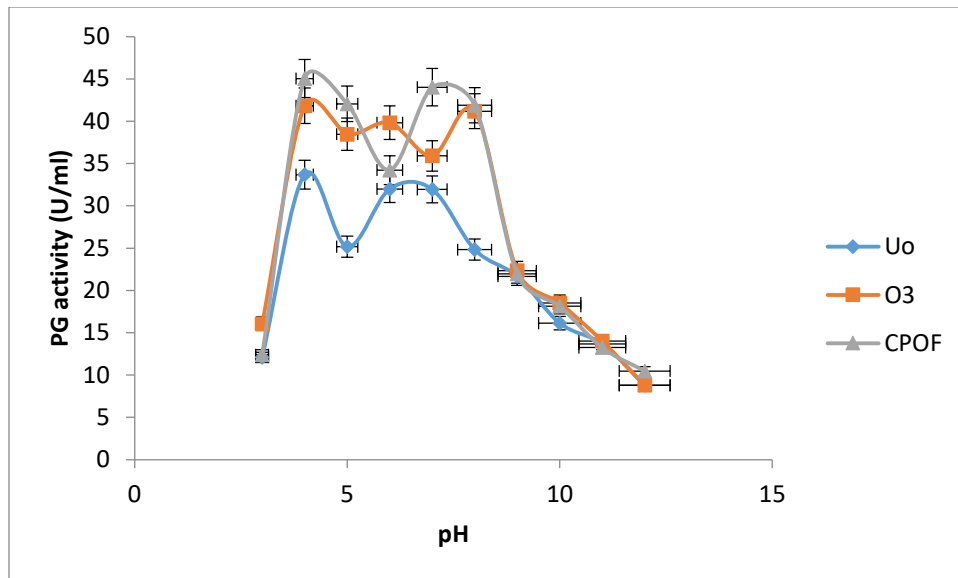
(a.)
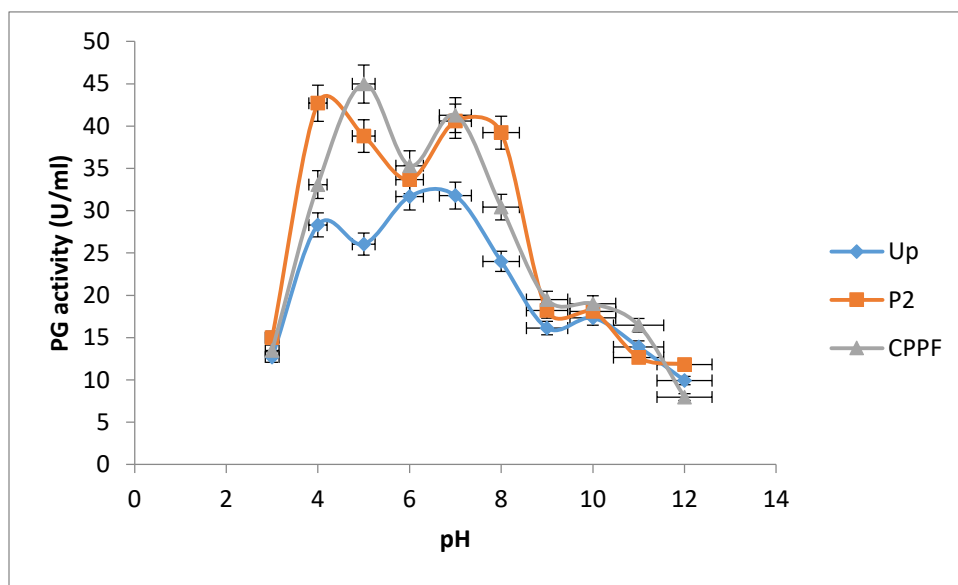
(b)

Figure 4.19. Activity of Crude Polygalacturonase at Different pH Values: (a) using orange peel, (b) using plantain peel. Uo= Crude PG produced from untreated orange peel; O3= Crude PG produced from alkali pretreated orange peel; CPOF= Crude PG produced from combined alkaline and microwave pretreated orange peel Up= Crude PG produced from untreated plantain peel; P2= Crude PG produced from alkali pretreated plantain peel; CPPF= Crude PG produced from combined alkali and microwave pretreated plantain peel.
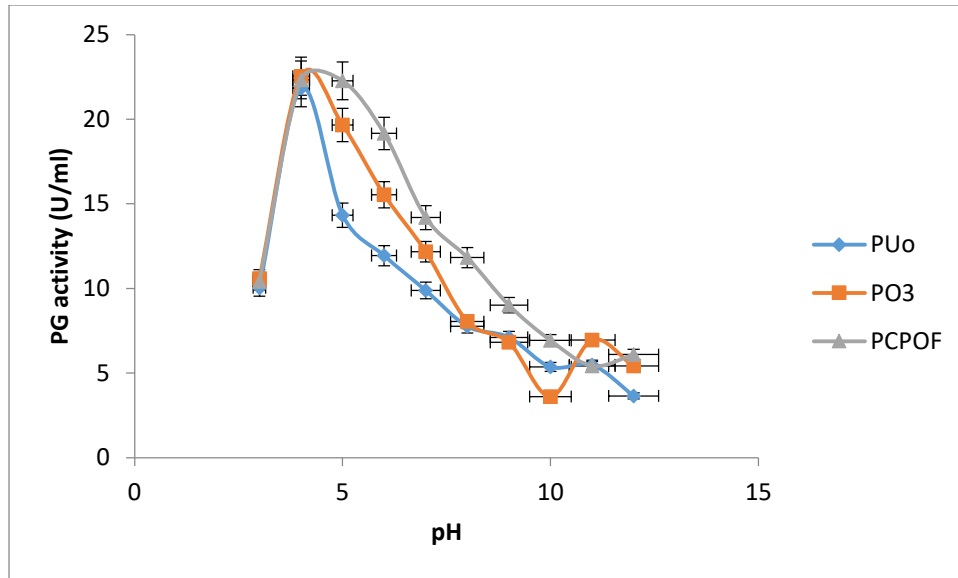
(a.)
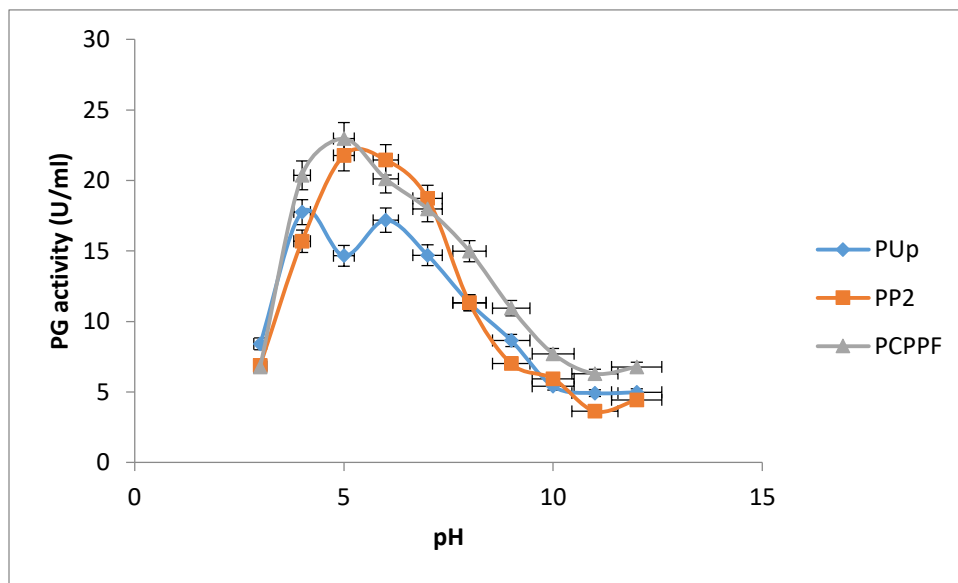
(b.)

Figure 4.20. Activity of Purified Polygalacturonase Produced at Different pH Values: (a) using orange peel, (b) using plantain peel. PUo=Purified PG produced from untreated orange peel; PO3= Purified PG produced from alkali pretreated orange peel; PCPOF= Purified PG produced from alkali and microwave pretreated orange peel Pup= Purified PG produced from untreated plantain peel; PP2= Purified PG produced from alkali pretreated plantain peel; PCPPF= Purified PG produced from combined alkali and microwave pretreated plantain peel.

alkaline pectinase is often produced by bacteria especially *Bacillus* spp. (Qureshi *et al.,* 2012; Paudel *et al.,* 2015), however, a recent study by Khatri *et al.* (2015) showed the pH optimum of PG produced from *Aspergillus niger* MCAS2 to be 8.2. Relative activity (Tables 4.13 and 4.14) of the PGs varied at different pH. It can be seen that crude PGs produced from pre-treated OPF retained at least 90% of their activities at pH 8.0 while Uo retained 73.69% of its activity at the same pH. Also PGs produced from PPF showed tolerance at alkaline pH 8.0 as PG activity obtained at the pH ranged from 67.63 to 91.85%. Beyond pH 8.0, relative activity of the PGs decreased with increasing pH. PUo had poor stability at pH above 5.0 while other purified PGs maintained an appreciable percentage of their pH up to pH 6.0. Ahmed *et al.* (2015) had reported stability of PG produced from *Aspergillus niger* at pH 9.2.

4.9.2 Polygalacturonase activity at different temperatures

Maximum activity was produced by all the crude PGs at 55 ^{o}C (Figures 4.21a and b). This implies that substrate pre-treatment had little or no effect on thermostability of crude PG. However, maximum activity was obtained at lower temperatures (35 and 45°C) for all the purified PGs (Figures 4.22a and b). This is advantageous because less energy barrier will be needed to be overcome during application of purified PG. Higher temperature for optimum activity of crude PGs could be due to the presence of interfering contaminant. Maximum PG activity obtained in this study agrees with the findings of Ahmed *et al.* (2015) who also reported maximum PG activity at 55 ^{o}C. However, this value contradicted 30°C maximum activity reported for PG produced from *Aspergillus niger* (Joshi *et al.,* 2011). Also, Gomes *et al.* (2011) reported maximum activity at 37 ^{o}C for PG produced from *Aspergillus niger* ATCC 9642. These variations could be due to the fact that fungal strains produce proteins with different biochemical properties (Zaslona and Trusek-Holownia, 2015). Relative activity (Tables 4.15 and 4.16) of the PGs differed at different temperature considered. For example, Up retained 73.72% of its activity at 35°C while O3, CPOF, P2, CPPF and Uo retained smaller

Table 4.13. Percentage Relative Activity of Crude Polygalacturonase at Different pH Values

PG	pH 3	4	5	6	7	8	9	10	11	12
Uo	39.93	100.00	74.73	94.98	93.08	73.69	64.34	47.89	13.68	8.73
O3	38.42	100.00	91.94	95.19	85.85	98.45	53.36	44.27	33.49	21.09
CPOF	27.49	100.00	93.36	75.98	97.74	93.00	48.71	40.28	29.40	23.25
Up	39.96	89.08	81.94	99.65	100.00	75.55	50.76	54.50	43.74	31.25
P2	35.08	100.00	84.95	78.88	95.04	91.85	42.60	42.34	29.63	27.70
CPPF	29.92	73.54	100.00	78.50	91.80	67.63	43.35	42.26	36.57	17.72

Uo= Crude PG produced from un-pretreated orange peel; O3= Crude PG produced from alkali pretreated orange peel; CPOF= Crude PG produced from alkali and microwave pretreated orange peel; Up= Crude PG produced from un-pretreated plantain peel; P2= Crude PG produced from alkali pretreated plantain peel; CPPF= Crude PG produced from alkali and microwave pretreated plantain peel.

Table 4.14. Percentage Relative Activity of Purified Polygalacturonase at Different pH Values

	pH									
	3	4	5	6	7	8	9	10	11	12
PG										
PUo	70.06	100	65.64	54.70	45.26	35.55	32.57	24.55	25.10	16.72
PO3	46.94	100	87.12	68.94	53.99	35.67	30.21	16.02	27.36	24.00
PCPOF	46.73	100	99.78	85.84	63.58	52.96	40.37	31.05	24.28	27.28
PUp	47.35	100	82.55	86.73	82.77	63.73	48.70	30.41	27.7	28.04
PP2	31.63	72.04	100	98.53	86.00	52.07	32.19	27.27	16.67	20.39
PCPPF	29.52	88.64	100	87.55	88.31	65.26	47.63	32.57	27.43	29.52

PUo-Purified PG produced from un-pretreated orange peel; PO3- Purified PG produced from alkali pretreated orange peel; PCPOF- Purified PG produced from alkali and microwave pretreated orange peel; PUp- Purified PG produced from un-pretreated plantain peel; PP2- Purified PG produced from alkali pretreated plantain peel; PCPPF- Purified PG produced from alkali and microwave pretreated plantain peel.
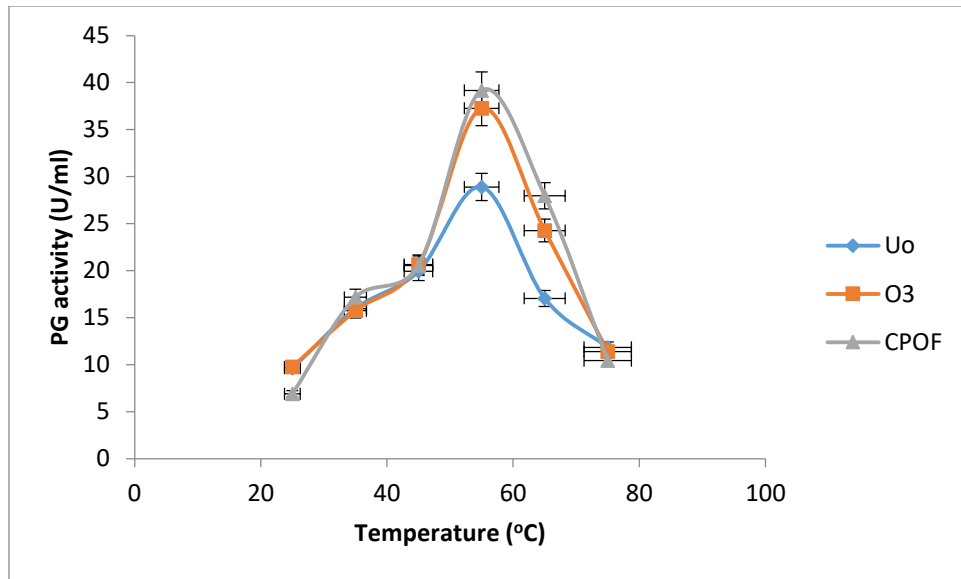
(a.)
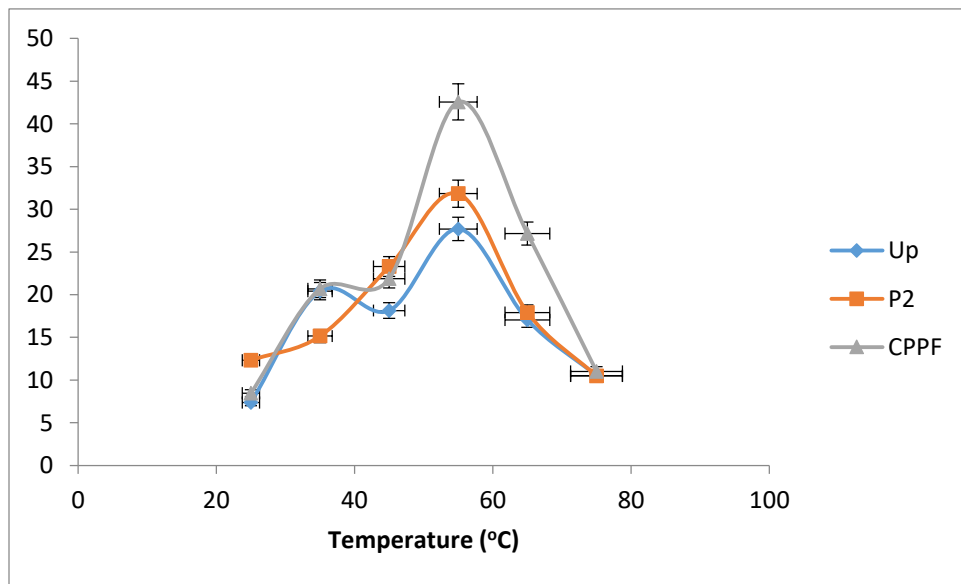
(b.)

Figure 4.21. Activity of Crude Polygalacturonase at Different Temperatures: (a) using orange peel, (b) using plantain peel. Uo= Crude PG produced from untreated orange peel; O3= Crude PG produced from alkali pretreated orange peel; CPOF= Crude PG produced from combined alkaline and microwave pretreated orange peel Up= Crude PG produced from untreated plantain peel; P2= Crude PG produced from alkali pretreated plantain peel; CPPF= Crude PG produced from combined alkaline and microwave pretreated plantain peel.
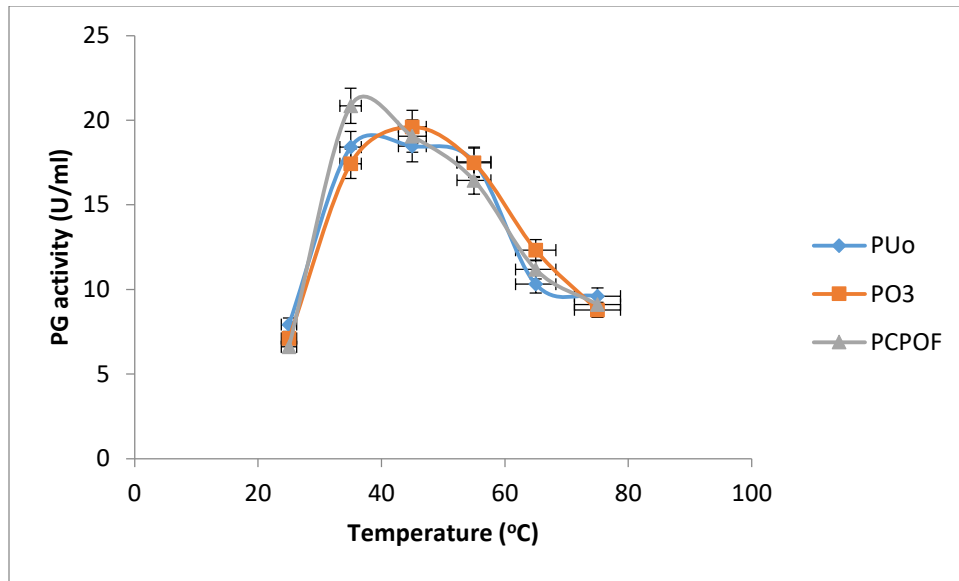
(a.)
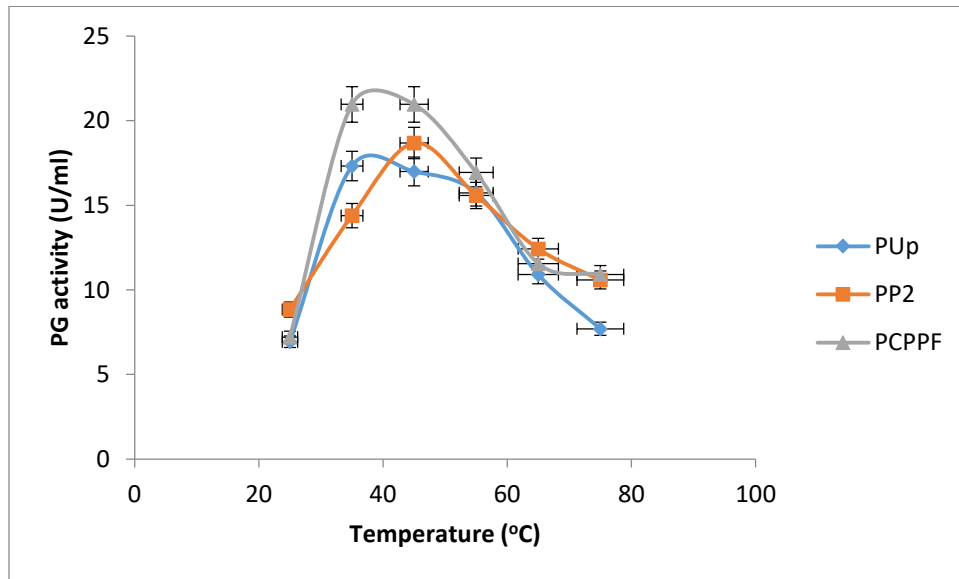
(b.)

Figure 4.22. Activity of Purified Polygalacturonase Produced at Different Temperatures: (a) using orange peel, (b) using plantain peel. PUo=Purified PG produced from untreated orange peel; PO3= Purified PG produced from alkali pretreated orange peel; PCPOF= Purified PG produced from alkali and microwave pretreated orange peel; Pup= Purified PG produced from untreated plantain peel; PP2= Purified PG produced from alkaline pretreated plantain peel; PCPPF= Purified PG produced from combined alkali and microwave pretreated plantain peel.

Table 4.15. **Percentage Relative Activity of Crude Polygalacturonase at Different Temperatures**

PG	_Temperature (°C)_					
	25	35	45	55	65	75
Uo	33.22	55.22	68.96	100.00	59.00	40.93
O3	26.21	42.23	55.38	100.00	65.12	30.59
CPOF	17.59	43.80	52.40	100.00	71.39	26.60
Up	26.64	73.72	65.63	100.00	61.55	38.01
P2	38.71	47.60	73.17	100.00	56.30	32.89
CPPF	19.87	48.60	51.40	100.00	63.80	25.89

Uo= Crude PG produced from un-pretreated orange peel; O3= Crude PG produced from alkali pretreated orange peel; CPOF= Crude PG produced from alkaline and microwave pretreated orange peel; Up= Crude PG produced from un-pretreated plantain peel; P2= Crude PG produced from alkali pretreated plantain peel; CPPF= Crude PG produced from alkaline and microwave pretreated plantain peel.

Table 4.16. **Percentage Relative Activity of Purified Polygalacturonase at Different Temperatures**

PG	25	35	45	55	65	75
			Temperature (°C)			
PUo	42.90	99.73	100	95.02	55.85	52.00
PO3	36.72	88.93	100	89.18	77.00	44.85
PCPOF	31.70	100	91.41	78.90	53.62	43.69
PUp	40.07	100	98.09	90.00	62.93	44.46
PP2	47.32	77.03	100	83.40	66.54	56.64
PCPPF	34.40	100	100	80.82	55.15	52.00

PUo= Purified PG produced from un-pretreated orange peel; PO3= Purified PG produced from alkali pretreated orange peel; PCPOF= Purified PG produced from alkali and microwave pretreated orange peel; Pup= Purified PG produced from un-pretreated plantain peel; PP2= Purified PG produced from alkali pretreated plantain peel; PCPPF= Purified PG produced from alkali and microwave pretreated plantain peel.

percentage of their activities i.e. 42.23, 43.80, 47.60, 48.60 and 55.22% respectively at the same temperature. Overall, the PGs showed low activity at 65 $^{\circ}$C and above. This suggests that PG from *Aspergillus awamori* CICC 2040 had low thermal stability beyond 65 $^{\circ}$C. This could be due to denaturation of the PG's protein. According to Zohdi and Amid (2013), denaturation of enzymes' proteins occurs at 50°C and above. Joshi *et al.* (2011) also reported reduction in the activity of PG produced from *Aspergillus niger* at temperature above 50 $^{\circ}$C. Nevertheless, certain PGs showed thermostability at 65 $^{\circ}$C and above. Khatri *et al.* (2015) reported that 82% activity of pectinase produced by *Aspergillus niger* MCAS2 was retained when subjected to a temperature of 100 $^{\circ}$C. Microbial strains produce enzymes with different cysteine moiety in their amino acid sequences (You *et al.,* 2010). Cysteine promotes formation of disulphide bonds which have strong hydrophobic effect that results to high stability of bio-products (Singh *et al.,* 2012a).

4.9.3 Percentage relative activity of polygalacturonase in the presence of selected chloride ions

Tables 4.17 and 4.18 present the percentage relative activity of crude and purified PGs, respectively as influenced by different metal ions. The rate of PG stimulation or inhibition varied significantly ($p<0.05$) among the samples. For crude PGs, Uo and Up were stimulated in the presence of Na$^+$, Cu^{2+} and Mg^{2+} while other PGs were inhibited. Also, all the PGs were inhibited in the presence of Ca^{2+}. An increase in Uo activity was recorded in the presence of NH$_4$$^+$ and K$^+$. For the purified PGs, PUo, PO3 and PUp were enhanced by Na$^+$ and K$^+$; PUo, PUp and PCPPF by Ca^{2+} while Cu^{2+} enhanced all the purified PGs except PCPOF. Overall, higher stability was recorded for purified PGs probably due to reduction in concentration of contaminating proteins as a result of the purification operation. Differences in the stability of PGs in the presence of metal ions could have been influenced by their sources (Dogan and Tari, 2008). Findings from this study clearly showed that the nature of substrate and purification influence the properties of PG. Review of earlier works showed variations of PG properties in

Table 4.17.   Percentage Relative Activity of Crude Polygalacturonase in the Presence of Selected Chloride Ions

| PG	Na^+	Ca^{2+}	NH_4^+	Cu^{2+}	K^+	Mg^{2+}
Uo	$121.49^a \pm 1.41$	$77.23^a \pm 0.28$	$137.27^a \pm 2.83$	$138.14^b \pm 2.86$	$102.26^a \pm 1.47$	$123.68^a \pm 2.86$
O3	$65.53^e \pm 0.04$	$57.75^b \pm 1.42$	$93.94^b \pm 0.03$	$94.36^c \pm 0.51$	$53.22^c \pm 0.31$	$81.77^d \pm 0.28$
CPOF	$79.19^c \pm 0.06$	$51.80^c \pm 0.11$	$86.04^d \pm 0.06$	$91.20^d \pm 0.28$	$65.49^b \pm 1.44$	$101.00^b \pm 2.83$
Up	$104.92^b \pm 2.84$	$76.54^a \pm 4.24$	$75.97^f \pm 0.04$	$157.30^a \pm 0.42$	$99.50^a \pm 0.71$	$125.38^a \pm 1.43$
P2	$65.48^e \pm 0.28$	$75.09^a \pm 0.13$	$90.39^c \pm 0.01$	$80.89^e \pm 0.14$	$64.63^b \pm 0.14$	$84.80^d \pm 0.14$
CPPF	$74.77^d \pm 0.31$	$47.69^c \pm 1.43$	$84.79^e \pm 0.14$	$65.34^f \pm 0.28$	$50.36^c \pm 4.24$	$95.29^c \pm 1.56$

Data are means± standard deviations of 2 scores. Values in column with different superscripts were significantly different ($p<0.05$). Uo=PG produced from un-pretreated orange peel; O3= Crude PG produced from alkaline pretreated orange peel; Crude CPOF= PG produced from alkali and microwave pretreated orange peel; Up= Crude PG produced from un-pretreated plantain peel; P2= Crude PG produced from alkali pretreated plantain peel; CPPF= Crude PG produced from alkaline and microwave pretreated plantain peel.

**Table 4.18.   Percentage Relative Activity of Purified Polygalacturonase in the Presence of Selected Chloride Ions**

PG	Na$^+$	Ca^{2+}	NH$_4^+$	Cu^{2+}	K$^+$	Mg^{2+}
PUo	123.17^b±0.03	142.08^a±2.86	207.75^a±1.41	115.91^a±0.00	107.61^c±1.70	140.20^a±0.28
PO3	108.85^c±1.41	98.63^c±0.00	137.03^b±0.03	116.02^a±2.84	131.23^a±3.11	77.31^e±2.82
PCPOF	85.59^f±2.86	98.74^c±0.04	79.48^e±4.24	88.28^e±0.28	79.48^e±0.57	84.87^d±1.41
PUp	143.38^a±1.44	114.62^b±5.66	138.88^b±2.84	111.11^b±0.16	117.05^b±0.07	130.76^b±1.56
PP2	90.50^e±1.70	83.53^d±0.10	88.52^d±0.42	100.73^d±1.44	75.05^e±2.05	91.28^c±0.00
PCPPF	99.78^d±1.43	109.49^b±0.03	123.20^c±0.85	105.44^c±0.57	85.81^d±2.84	94.56^c±2.84

Data are means± standard deviations of 2 scores. Values in column with different superscripts were significantly different ($p<0.05$). PUo= Purified PG produced from un-pretreated orange peel; PO3= Purified PG produced from alkaline pretreated orange peel; PCPOF= Purified PG produced from alkali and microwave pretreated orange peel; Pup= Purified PG produced from un-pretreated plantain peel; PP2= Purified PG produced from alkali pretreated plantain peel; PCPPF= Purified PG produced from alkaline and microwave pretreated plantain peel.

the presence of metal ions. Khatri *et al.* (2015) reported inhibition of PG produced from *Aspergillus niger* MCAS2 in the presence of sodium and calcium ions. Also, Kant *et al.* (2013) observed an inhibition of PG in the presence of K^+, Ca^+ while Zn^{2+} while Mg^{2+} and Cu^{2+} stimulated it. Many enzymes, PG inclusive are inhibited by the presence of some metals. The inhibition could be due to denaturation of proteins (Zohdi and Ahmid, 2013) or protein polymerization which forms bridges between peptide chains, blockage of thiol group of proteins (Silva *et al.,* 2007) etc. These phenomena interfere with protein's geometry, hence reduction in its activity.

4.9.4 Percentage relative activity of polygalacturonase in the presence of selected compounds

Percentage relative activity of PGs as influenced by the presence of selected compounds are presented in Tables 4.19 and 4.20. Among the crude enzymes, only Uo and Up were stimulated in the presence of EDTA, citric acid, glycerol and Tween 20 while other PGs were inhibited by the compounds. On the other hand, all the purified PGs were enhanced by all the compounds. Enhancement of purified PGs, Uo and Up implies that the enzymes can be successfully applied in systems that contain the compounds. It can also be said that purification caused a reduction in concentration of contaminants which would otherwise cause the inhibition of the PGs in the presence of the compounds. Mechanisms of enzyme enhancement vary among compounds.

Surfactant such as Tween 20 might cause an increase in affinity of PG's active sites to the substrate which would culminate in reduction in surface tension (Paudel *et al.,* 2015). EDTA acts by chelating metal ions from enzymes (Juwon *et al.,* 2012). Published data have also shown variation in the stability of PGs in the presence of different compounds. Dogan and Tari (2008) reported improved PG activity in the presence of EDTA and glycerol while Tween 80 inhibited it. Paudel *et al.* (2015) reported an inhibition of pectinase in the presence of Triton-X-100.

Table 4.19.   Percentage Relative Activity of Crude Polygalacturonase in the Presence of Selected Compounds

PG	EDTA	Citric acid	Glycerol	Tween 20
Uo	$112.87^a\pm1.56$	$118.91^a\pm1.46$	$125.82^a\pm1.56$	$108.99^b\pm2.83$
O3	$90.54^c\pm0.28$	$76.13^e\pm0.04$	$99.01^c\pm1.43$	$78.81^c\pm0.16$
CPOF	$81.78^d\pm0.03$	$77.50^e\pm0.71$	$75.96^e\pm0.06$	$69.37^d\pm0.42$
Up	$108.87^b\pm2.83$	$109.10^b\pm0.14$	$106.60^b\pm0.57$	$114.94^a\pm1.47$
P2	$67.57^f\pm0.40$	$85.37^c\pm0.42$	$82.41^d\pm0.57$	$76.68^c\pm2.86$
CPPF	$72.21^e\pm1.43$	$81.77^d\pm0.16$	$69.43^f\pm1.56$	$61.09^e\pm1.54$

Data are means± standard deviations of 2 scores. Values in column with different superscripts were significantly different ($p<0.05$). Uo= Crude PG produced from un-pretreated orange peel; O3= Crude PG produced from alkaline pretreated orange peel; CPOF= Crude PG produced from alkali and microwave pretreated orange peel; Up= Crude PG produced from un-pretreated plantain peel; P2= Crude PG produced from alkaline pretreated plantain peel; CPPF= Crude PG produced from alkaline and microwave pretreated plantain peel.

Table 4.20. **Percentage Relative Activity of Purified Polygalacturonase in the Presence of Selected Compounds**

PG	EDTA	Citric acid	Glycerol	Tween 20
PUo	$119.79^a\pm1.41$	$208.09^a\pm2.84$	$203.91^b\pm2.86$	$157.29^b\pm1.41$
PO3	$149.19^c\pm1.43$	$161.65^c\pm1.56$	$116.63^d\pm2.83$	$148.93^c\pm0.03$
PCPOF	$115.58^e\pm2.86$	$130.00^d\pm4.24$	$172.20^c\pm3.11$	$130.00^e\pm0.71$
PUp	$182.26^b\pm2.89$	$187.86^b\pm4.24$	$212.19^a\pm1.44$	$216.37^a\pm4.26$
PP2	$136.91^d\pm5.71$	$154.41^c\pm4.26$	$163.64^d\pm0.42$	$139.39^d\pm2.86$
PCPPF	$122.73^e\pm4.29$	$130.78^d\pm0.14$	$176.93^c\pm1.46$	$122.25^f\pm3.18$

Data are means± standard deviations of 2 scores. Values in column with different superscripts were significantly different ($p<0.05$). PUo= Purified PG produced from un-pretreated orange peel; PO3= Purified PG produced from alkali pretreated orange peel; PCPOF= Purified PG produced from alkaline and microwave pretreated orange peel; Pup= Purified PG produced from un-pretreated plantain peel; PP2= Purified PG produced from alkali pretreated plantain peel; PCPPF= Purified PG produced from alkaline and microwave pretreated plantain peel.

Juwon *et al.* (2012) also reported that PG produced from *Aspergillus niger* CSTRF was inhibited by EDTA.

4.9.5 Substrate specificity of polygalacturonase

The affinity of PGs to different substrates is shown in Tables 4.21 and 4.22. For crude PG, activity varied from 25.17 to 44.48, 9.06 to 10.36, 15.27 to 20.26 and 21.23 to 38.90 on pectin, starch, malt extract and potato dextrose, respectively. For purified PG, activity varied from 14.33 to 22.97, 8.30 to 9.71, 11.72 to 15.04 and 12.61 to 20.73 on pectin, starch, malt extract and potato dextrose, respectively. For all the PGs, maximum activity was obtained when pectin was used as substrates. Overall, affinity of both crude and purified PGs to substrates was in the order: pectin>potato dextrose>malt extract>starch. This consolidated the findings of Kant *et al.* (2013) who recorded higher activity of PG on pectin compared to other non-pectic polysaccharides. High affinity of PG on pectin stems from its high hydrolytic capability on glycosidic bonds that link the galacturonic acid residues of pectin (Heerd *et al.,* 2012). Results obtained from this study showed variations ($p<0.05$) in the affinity of samples to the substrates considered. Activity of both crude and purified PG produced from pre-treated OPF and PPF was significantly ($p<0.05$) higher on pectin and potato dextrose than PG produced from un-treated OPF and PPF. Also for both crude and purified PGs, there was no significant ($p<0.05$) difference among the PGs on malt extract. Pre-treatment of PPF caused reduction of affinity of PG produced from it on malt extract. Conversely, pre-treatment of OPF caused a significant ($p<0.05$) increase of affinity of PG produced from it on malt extract. This implies differences in the properties of the substrates. This corroborated earlier claim that the properties of PG is dependent on the nature of substrates (Kant *et al.,* 2013).

Table 4.21. **Activity (U/mL) of Crude Polygalacturonase on Selected Substrates**

PG	Pectin	Starch	Substrates Malt extracts	Potato dextrose
Uo	$25.17^c \pm 1.41$	$9.28^a \pm 1.70$	$15.27^c \pm 0.33$	$21.23^d \pm 0.28$
O3	$38.46^b \pm 1.47$	$10.09^a \pm 0.13$	$18.35^{ab} \pm 0.21$	$32.38^b \pm 0.28$
CPOF	$42.05^a \pm 0.07$	$10.20^a \pm 0.28$	$17.21^{bc} \pm 1.41$	$35.72^{ab} \pm 1.44$
UP	$26.04^c \pm 0.06$	$9.06^a \pm 2.87$	$20.26^a \pm 0.37$	$25.27^c \pm 0.38$
P2	$38.82^b \pm 0.17$	$10.36^a \pm 0.28$	$17.54^b \pm 0.06$	$35.46^{ab} \pm 2.18$
CPPF	$44.48^a \pm 2.15$	$10.30^a \pm 0.71$	$17.64^b \pm 1.50$	$38.90^a \pm 2.84$

Data are means± standard deviations of 2 scores. Values in column with different superscripts were significantly different ($p<0.05$). Uo=PG produced from un-pretreated orange peel; O3= Crude PG produced from alkaline pretreated orange peel; CPOF= Crude PG produced from alkaline and microwave pretreated orange peel; Up= Crude PG produced from un-pretreated plantain peel; P2= Crude PG produced from alkaline pretreated plantain peel; CPPF= Crude PG produced from alkaline and microwave pretreated plantain peel.

Table 4.22. **Activity (U/mL) of Purified Polygalacturonase on Selected Substrates**

PG	Pectin	Starch	Malt extracts	Potato dextrose
		Substrates		
PUo	14.33[c]±1.46	8.30[a]±0.03	12.10[a]±2.97	13.41[c]±0.10
PO3	19.66[b]±0.28	9.71[a]±1.70	13.19[a]±0.00	16.73[b]±0.31
PCPOF	22.27[ab]±1.44	9.55[a]±0.71	11.72[a]±0.14	16.30[b]±0.42
PUP	14.66[c]±1.46	8.30[a]±0.42	12.32[a]±1.58	12.61[c]±1.41
PP2	21.78[ab]±0.03	8.79[a]±0.14	12.49[a]±0.49	19.38[a]±0.45
PCPPF	22.97[a]±0.04	9.33[a]±3.11	15.04[a]±0.06	20.73[a]±0.16

Data are means± standard deviations of 2 scores. Values in column with different superscripts were significantly different ($p < 0.05$). PUo= Purified PG produced from un-pretreated orange peel; PO3= Purified PG produced from alkaline pretreated orange peel; PCPOF= Purified PG produced from alkaline and microwave pretreated orange peel; Pup= Purified PG produced from un-pretreated plantain peel; PP2= Purified PG produced from alkaline pretreated plantain peel; PCPPF= Purified PG produced from alkali and microwave pretreated plantain peel.

4.9.6   Kinetics of polygalacturonase

Kinetic constants obtained for the PGs are presented in Tables 4.23 and 4.24. Vmax ranged between 29.41 and 40 U/mL for crude PGs and 55.55 to 90.91 U/mL for purified PGS. The range of values obtained in this study was smaller than 294.12U/mL reported by Suresh and Viruthagiri (2010). Variations could be due to differences in microbial strains (Zaslona and Trusek-Holownia, 2015).   Higher Vmax reported for purified enzymes implies better catalytic capability than the crude PGs. Higher Vmax were recorded for PGs produced from pre-treated OPF and PPF compared to those from un-treated ones. Since Vmax indicates catalytic activity of enzyme (Adiguezel *et al*., 2016), higher Vmax of PGs from pre-treated OPF and PPF implies higher catalysis of substrates, hence improved hydrolysis and yield/activity of bio-products. In spite of high catalytic properties of PGs produced from the pre-treated substrates, lower affinity for pectin compared to Uo and Up was recorded for them. This was evident in their higher Km compared to Uo and Up. Generally, the PGs under consideration in this study had better substrate affinity (low Km) than those reported by earlier workers (Dogan and Tari, 2008; Suresh and Viruthagiri, 2010). R^2 values obtained for the fitness of kinetic parameters ranged between 0.862 and 0.913 for crude PGs and 0.850 to 0.994 for the purified PGs. This suggests the suitability of Lineweaver-Burk model in fitting the data.

4.9.7   Inactivation   kinetics   and   thermodynamic   properties   of polygalacturonase

(a)   Inactivation kinetics of polygalacturonase

Inactivation kinetics of crude and purified PGs are presented in Tables 4.25 and 4.26. Inactivation rate constant (Kd) of all the polygalacturonases investigated in this study increased with increasing temperature. This suggests reduction in PG

Table 4.23.   Michaelis-Menten Kinetics of Crude Polygalacturonase

Sample	Vmax (U/mL)	Km (mg/mL)	R^2
Uo	30.30	0.212	0.862
CPOF	33.33	0.267	0.896
Up	29.41	0.206	0.877
CPPF	40.00	0.320	0.913

Uo= Crude PG produced from un-treated orange peel; CPOF= Crude PG produced from pre-treated orange peel; Up= Crude PG produced from un-treated plantain peel; CPPF= Crude PG produced from pre-treated plantain peel.

Table 4.24.   Michaelis-Menten Kinetics of Purified Polygalacturonase

Sample	Vmax (U/mL)	Km (mg/mL)	R^2
PUo	55.55	0.722	0.994
PCPOF	83.33	0.750	0.850
PUp	66.67	0.933	0.964
PCPPF	90.91	0.909	0.967

PUo- Purified PG produced from un-treated orange peel; PCPOF- Purified PG produced from pre-treated orange peel; PUp- Purified PG produced from un-treated plantain peel; PCPPF- Purified PG produced from pre-treated plantain peel.

Table 4.25.   Inactivation Kinetics of Crude Polygalacturonase

Temperature (^{o}C)	PG	Kd (min^{-1})	R^2	$t_{1/2}$ (min)	D (min)
60	Uo	0.0151	0.961	43.90	145.49
	CPOF	0.0152	0.973	45.60	151.49
	Up	0.0142	0.907	36.10	119.93
	CPPF	0.0154	0.905	45.01	149.52
70	Uo	0.0202	0.970	31.31	113.99
	CPOF	0.0232	0.977	34.31	119.99
	Up	0.0214	0.900	24.41	81.08
	CPPF	0.0222	0.905	31.22	103.72
80	Uo	0.0315	0.980	20.00	73.10
	CPOF	0.0335	0.994	22.00	78.10
	Up	0.0346	0.949	20.03	66.55
	CPPF	0.0394	0.914	23.58	78.32
90	Uo	0.0387	0.945	17.91	59.50
	CPOF	0.0425	0.937	21.33	70.85
	Up	0.0608	0.927	11.40	37.87
	CPPF	0.0681	0.943	14.41	47.87

Kd=inactivation rate constant; $t_{1/2}$=half life; D= decimal reduction time; Uo= Crude PG produced from un-treated orange peel; CPOF= Crude PG produced from pre-treated orange peel; Up= Crude PG produced from un-treated plantain peel; CPPF= Crude PG produced from pre-treated plantain peel.

Table 4.26.   Inactivation Kinetics of Purified Polygalacturonase

Temperature (°C)	PG	K_d (min^{-1})	R^2	$t_{1/2}$ (min)	D (min)
60	PUo	0.0326	0.911	13.26	70.63
	PCPOF	0.0380	0.949	18.24	80.59
	PUp	0.0336	0.955	20.63	68.53
	PCPPF	0.0496	0.961	23.97	76.42
70	PUo	0.0371	0.924	18.68	52.06
	PCPOF	0.0636	0.932	22.90	66.20
	PUp	0.0441	0.962	15.72	52.21
	PCPPF	0.0552	0.978	17.56	61.71
80	PUo	0.0571	0.974	7.14	40.33
	PCPOF	0.0703	0.994	9.86	32.75
	PUp	0.0481	0.987	8.41	47.87
	PCPPF	0.0648	0.994	10.70	53.53
90	PUo	0.0671	0.933	7.33	23.32
	PCPOF	0.0852	0.970	8.14	27.03
	PUp	0.0707	0.987	8.80	32.57
	PCPPF	0.0770	0.996	9.00	37.90

K_d=inactivation rate constant; $t_{1/2}$=half life; D= decimal reduction time; PUo= Purified PG produced from un-treated orange peel; PCPOF= Purified PG produced from pre-treated orange peel; Pup= Purified PG produced from un-treated plantain peel; PCPPF= Purified PG produced from pre-treated plantain peel.

thermostability with increasing temperature. Increase in Kd as temperature increased could be due to denaturation of protein consequent to the breakdown of primary, secondary, tertiary and quaternary structures of proteins without disruption of covalent bonds (Dogan and Tari, 2008). This follows earlier trends described for glucoamylase (Melikoglu *et al.,* 2013) and protease (Ortiz *et al*., 2016). At every temperature considered, Kd differed depending on the source of substrate used for PG production. Higher Kd was obtained for PG produced using pretreated OPF and PPF compared to PG produced using untreated peels. This implies higher thermostability of PG produced using pretreated OPF and PPF. This could be due to modification of the nutrient density and structures of the peels following their pretreatment. Han *et al.* (2015) had reported differences in Kd of glucoamylase produced from *Aspergillus awamori* on different wastes: cake, pastry and bread due to their different chemical constituents. According to Norouzian *et al.* (2006), variation in carbon and nitrogen sources and their ratio could have a crucial influence on enzyme properties. High R^2 (0.905-0.994) recorded for all the PGs at different temperature suggests good fitness of experimental data. Half life and decimal reduction time of PGs decreased with increasing temperature. This was consistent with increasing Kd and temperature. Han *et al.* (2015) also reported decreasing half life of glucoamylase with increasing temperature. Also, decimal reduction time of peroxidase reduced with increasing temperature (Deylami *et al.*, 2014). Variation in half life and decimal reduction time of the PGs considered in this study suggests differences in thermodynamic properties. According to Melikoglu *et al.* (2013), half life and decimal reduction time are good indicators of enzyme thermostability, therefore, Up which had lowest half life and decimal reduction time at all the temperatures considered could be said to be the least thermostable PG.

(b) Temperature dependency of polygalacturonase inactivation

Temperature dependency of studied PGs as described in terms of activation energy of denaturation is presented in Tables 4.27 and 4.28. Positive activation energy was obtained for all the PGs. This indicated catalytic reaction of enzyme

Table 4.27. Temperature Dependency of Crude Polygalacturonase

PG	k_o (min^{-1})	E_a (kJ/mol)	R^2
Uo	0.011	3.71	0.982
CPOF	0.010	3.07	0.987
Up	0.013	3.03	0.966
CPPF	0.012	2.26	0.919

k_o- Arrhenius constant; E_a- activation energy; Uo= Crude PG produced from un-treated orange peel; CPOF= Crude PG produced from pre-treated orange peel; Up= Crude PG produced from un-treated plantain peel; CPPF= Crude PG produced from pre-treated plantain peel.

Table 4.28. Temperature Dependency of Purified Polygalacturonase

PG	ko (min^{-1})	Ea (kJ/mol)	R^2
PUo	0.024	2.15	0.953
PCPOF	0.033	2.10	0. 891
PUp	0.027	1.92	0.947
PCPPF	0.042	1.23	0.989

Ko= Arrhenius constant; Ea= activation energy; PUo= Purified PG produced from un-treated orange peel; PCPOF= Purified PG produced from pre-treated orange peel; Pup= PG produced from un-treated plantain peel; PCPPF= PG produced from pre-treated plantain peel.

deactivation below the reflection point (Melikoglu *et al*., 2013). Dogan and Tari (2008) also reported positive activation energy for fungal PG. Activation energy ranged from 2.26 to 3.07kJ/mol for crude PGs and 1.23 to 2.15 kJ/mol for purified PGs. This implies higher reaction rates of purified PGs owing to reduction of energy barrier. PGs produced from pre-treated OPF had lower activation energy than the ones produced from un-treated ones. However, activation energies of PGs produced from PPF were close. Also, activation energy of PG from PPF was higher than those from PPF. According to Lam *et al.* (2013), activation energy connotes the energy required to form activation complex for protein hydrolysis, therefore, low activation energy of PG produced from pre-treated OPF probably required less energy to form the complex. Arrhenius model used for temperature dependency of inactivation rate constant showed linear conformations with high R^2 which ranged from 0.891 to 0.989. This suggests single conformation up to the transition temperature (Melikoglu *et al.,* 2013).

(c) Thermodynamic properties of polygalacturonase

Thermodynamic properties of PGs as described by enthalpy, free energy and entropy at different temperatures (60 to 90°C) are presented in Tables 4.29 and 4.30. The enthalpies of the PGs were quite similar and this suggests little or no difference in degree of disruption of non-covalent linkages of proteins (Melikoglu *et al*., 2013) as a result of substrate pre-treatment and PG purification. Also, negative enthalpies were recorded for all the PGs. Irrespective of substrate pre-treatment and enzyme purification, ΔG increased with increasing temperature. This suggests reduction in energy barrier for inactivation and increasing degree of spontaneity as temperature increased (Deylami *et al*., 2014). Negative entropy was obtained for all the PGs at all temperatures and this, according to Riaz *et al.* (2007) could be attributed to low disorganization of enzyme structure as a result of enzyme deactivation. Negative entropy and enthalpy recorded for the PGs depicts spontaneity of the inactivation process (Melikoglu *et al*., 2013). Similar result was also reported for glucoamylase (Han *et al.,* 2015).

Table 4.29. **Thermodynamic Properties for Thermal Inactivation of Crude Polygalacturonase**

Temperature (ºC)	PG	ΔH (J/mol)	ΔG (kJ/mol)	ΔS (kJ/molK)
60	Uo	-2,768,559.29	93,347.42	-288.64
	CPOF	-2,768,558.93	93,453.98	-272.34
	Up	-2,768,558.97	92,807.20	-287.01
	CPPF	-2,768,559.97	92,417.79	-285.84
70	Uo	-2,851,699.29	95,529.75	-286.83
	CPOF	-2,851,698.93	95,529.75	-286.83
	Up	-2,851,698.97	94,558.16	-283.99
	CPPF	-2,851,699.74	95,260.53	-286.04
80	Uo	-2,934,839.29	97,095.99	-283.37
	CPOF	-2,934,838.93	97,095.99	-283.37
	Up	-2,934,838.97	96,820.51	-282.74
	CPPF	-2,934,839.74	97,298.47	-283.95
90	Uo	-3,017,979.29	99,315.42	-281.91
	CPOF	-3,017,978.93	98,842.36	-280.61
	Up	-3,017,978.97	97,952.05	-278.15
	CPPF	-3,017,979.74	98,659.18	-280.10

Uo= Crude PG produced from un-treated orange peel; CPOF= Crude PG produced from pre-treated orange peel; Up= Crude PG produced from un-treated plantain peel; CPPF= Crude PG produced from pre-treated plantain peel.

Table 4.30. Thermodynamic Properties for Thermal Inactivation of Purified Polygalacturonase

Temperature (ºC)	PG	ΔH (J/mol)	ΔG (kJ/mol)	ΔS (kJ/molK)
60	PUo	-2,768,559.85	91,341.52	-282.61
	PCPOF	-2,768,559.90	90,917.17	-281.34
	PUp	-2,768,568.08	91,257.87	-282.36
	PCPPF	-2,768,560.77	90.179.62	-279.12
70	PUo	-2,851,699.85	93,796.11	-281.77
	PCPOF	-2,851,699.90	92,259.05	-277.29
	PUp	-2,851,700.08	93,303.21	-280.34
	PCPPF	-2,851,700.77	92,662.99	-278.45
80	PUo	-2,934,839.85	95,350.30	-278.43
	PCPOF	-2,934,839.90	94,739.94	-278.43
	PUp	-2,934,840.08	95,853.68	-279.85
	PCPPF	-2,934,840.77	94,979.03	-277.38
90	PUo	-3,017,979.85	97,654.49	-277.33
	PCPOF	-3,017,979.90	96,837.39	-275.08
	PUp	-3,017,980.08	97,496.77	-276.90
	PCPPF	-3,017,980.77	97,239.15	-276.19

PUo= Purified PG produced from un-treated orange peel; PCPOF= Purified PG produced from pre-treated orange peel; Pup= Purified PG produced from un-treated plantain peel; PCPPF= Purified PG produced from pre-treated plantain peel.

4.10   Quality of Mango Juice as Influenced by Enzymatic Extraction

Chemical composition, rheological and sensory properties of mango juice are discussed in this Section.

4.10.1 Effect of enzymatic extraction on yield of mango juice

Figure 4.23 shows that enzymatic maceration with pectinase enzyme increased the juice of mango. Mango yield increased from 52.8% in CTT (mango juice extracted without enzyme) to 79.2, 84.4 and 87.6% in CRE (mango juice extracted with crude polygalacturonase), PUE (mango juice extracted with purified polygalacturonase) and CME (mango juice extracted with commercial pectinase enzyme), respectively. Earlier works also showed increase in juice yield as a result of enzymatic extraction: 17.5% for bael (*Aegle marmelos*) (Singh *et al.,* 2012b); 26% in plum fruit (Joshi *et al.,* 2011); 11.46% in carrot juice (Kaur and Sharma, 2013) and 13.5% in apple juice (Oszmianski *et al.,* 2009). The increase in yield in enzymatically extracted mango juice could be attributed to reduction in water holding capacity of pectin consequent to its degradation by pectinase. Therefore, more water containing less complex materials such as sugars is released into the system which increases yield (Lee *et al.,* 2006). Findings obtained from this study showed that higher juice was obtained in PUE (84.4%) than CRE (79.2%). Yield in CME (87.6%) was higher than PUE. The variation could be due to differences in activity of the enzymes (Padma *et al.,* 2017).

4.10.2 Effect of enzymatic extraction on chemical composition of mango juice
Table 4.31 shows the chemical composition of mango as influenced by enzymatic extraction. There was a significant ($p < 0.05$) increase in the percentage transmittance (clarity) of mango juice extracted with polygalacturonase. This is in agreement with earlier studies on apple and orange (Tribess and Tadini, 2006; Joshi *et al.,* 2011). There was no significant ($p > 0.05$) difference in clarity between
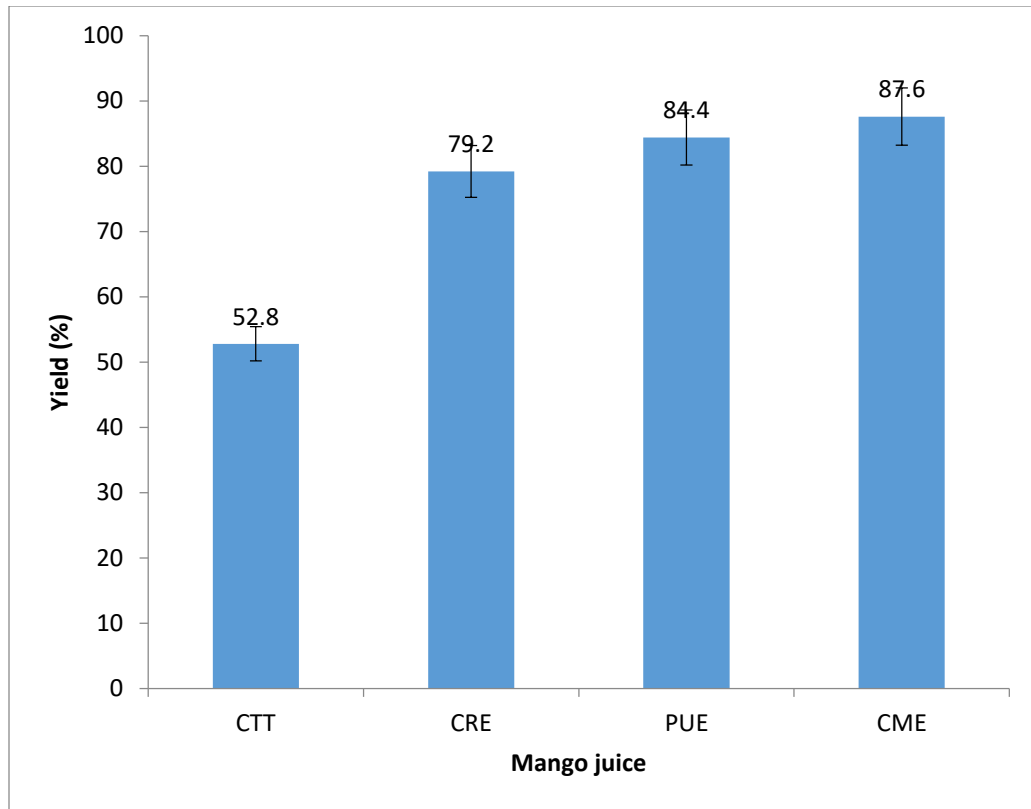
Figure 4.23. Percentage Yield of Mango Juice as Influenced by Polygalacturonase Extraction. CTT= mango juice extracted without enzyme (control); CRE= mango juice extracted with crude polygalacturonase; PUE= mango juice extracted with purified polygalacturonase; CME= mango juice extracted with commercial pectinase enzyme.

Table 4.31.   Chemical Composition of Mango Juice as Influenced by Enzymatic Extraction

Mango juice	$\%T_{650}$	Soluble solids (^{o}Brix)	pH	Total sugar (mg/100mL)	Reducing sugar (mg/100mL)	TTA (%)	Ascorbic acid (mg/100mL)	Specific gravity
CTT	$13.80^c\pm0.04$	$16.60^a\pm0.42$	$3.76^a\pm0.01$	$11.94^c\pm1.13$	$6.89^d\pm0.08$	$0.12^a\pm0.03$	$1.60^a\pm0.28$	$1.10^a\pm0.14$
CRE	$19.05^b\pm0.28$	$15.60^{ab}\pm0.28$	$3.71^b\pm0.01$	$20.47^a\pm0.16$	$10.09^b\pm0.31$	$0.09^a\pm0.04$	$1.30^{ab}\pm0.00$	$1.07^a\pm0.01$
PUE	$21.43^a\pm0.07$	$15.40^b\pm0.57$	$3.58^c\pm0.01$	$12.79^c\pm0.31$	$8.90^c\pm0.20$	$0.12^a\pm0.01$	$1.10^b\pm0.14$	$1.07^a\pm0.00$
CME	$21.12^a\pm1.44$	$16.20^{ab}\pm0.14$	$3.51^d\pm0.00$	$17.70^b\pm0.04$	$14.79^a\pm0.23$	$0.11^a\pm0.02$	$1.15^{ab}\pm0.07$	$1.08^a\pm0.06$

Data are means± standard deviations of triplicate scores. Values in column with different superscripts were significantly different ($p<0.05$). CTT= mango juice without enzyme (control); CRE= mango juice extracted with crude polygalacturonase; PUE= mango juice extracted with purified polygalacturonase; CME= mango juice extracted with commercial pectinase enzyme; $\%T_{650}$= percentage transmittance at 650nm; TTA= titratable acidity.

PUE and and CME. This implies that the purified polygalacturonase compared very well with the commercial pectinase enzyme. There was a significant ($p<0.05$) reduction in soluble solids as a result of enzymatic extraction. Also, significantly ($p<0.05$) lower soluble solids was recorded for PUE compared to CRE and CME. This might be due to increased hydrolysis of sugar and phenol conjugant leading to the production of aglycone moieties (Cerreti *et al.,* 2016).

There was significant ($p<0.05$) variation in pH of samples with CTT having the highest pH of 3.76. Enzymatic extraction caused a significant ($p<0.05$) reduction in pH. This is advantageous because reduction in pH could result to better shelf stability for the juice. Abbes *et al.* (2011) also reported reduction in pH in enzymatically extracted date syrup. Lowest pH value was recorded for CME followed by PUE. There was a significant ($p<0.05$) increase in reducing and total sugar content of mango juice as a result of enzymatic clarification. This agreed with increase in sugar content in enzymatically extracted date juice (Bahramian *et al.,* 2011). Incease was in the order: PUE<CME<CRE for reducing sugar and PUE<CRE<CME for total sugar. Variation in sugar contents among the sample extracted with enzyme could be due to difference in enzyme properties. According to Cerreti *et al.* (2017), properties that are related to enzyme such as concentration, types, purity are important factors that influence juice extraction and clarification.

There was no significant ($p>0.05$) difference in titratable acidity (TTA). This contradicted the report of Joshi *et al.* (2011) who reported increased TTA in pear juice extracted with pectinase enzyme. Result obtained from this study could imply that enzymatic extraction does not always cause increase in TTA. There was a significant ($p<0.05$) reduction in ascorbic acid due to enzymatic activity during extraction operation. Vijayanand *et al.* (2010) had attributed reduction in vitamin C in enzymatically extracted umbu (*Spondias tuberose*) juice to oxidation of ascorbic acid during extraction operation.

There was no significant ($p>0.05$) difference in specific gravity of the samples, however, lower values were recorded for the enzymatically extracted samples. Reduction in specific gravity could be due to reduction in density as a result of the

breakdown of denser polysaccharides such as pectin, cellulose etc. to simple ones such as sugars (Sharma *et al.,* 2014).

4.10.3 Effect of enzymatic extraction on the rheological properties of mango juice

Shear stress versus shear rate patterns of mango juices at different temperatures are presented in Figure 4.24 (a-e). At all temperatures considered, shear stress increased with an increasing shear rates. This pattern suggested a pseudoplastic or shear-thinning non-Newtonian behaviour. Gouvea *et al.* (2017) also reported pseeudoplastic behaviour for umbu juice extracted with pectinase enzymes rapidase and pectinex. Enzymatic extraction caused a marked reduction in shear stress at all the corresponding shear rates. This resulted in the reduction of pseudoplasticity as the enzymatically extracted juices tended toward Newtonian behaviour. This corroborated the finding of Belibagli and Dalgic (2007) for sour cherry (*Prunus cerasus*) juice.

Figures 4.25 (a-e) shows decreasing apparent viscosity as shear rate increased. This validated the claim that mango juice exhibits pseudoplastic behaviour. Also, there was reduction in viscosity as a result of extraction with PG. Gouvea *et al.* (2017) also reported reduction in viscosity of umbu juice extracted with pectinase enzyme. Reduction in viscosity could be attributed to the breakdown of polysaccharides such as pectin which has high jelly-forming capability (Sharma *et al.,* 2014). According to Singh *et al.* (2012b), one of the objectives of enzymatic juice extraction is to disintegrate the jelly nature of fruit pulp for easy juice recovery.
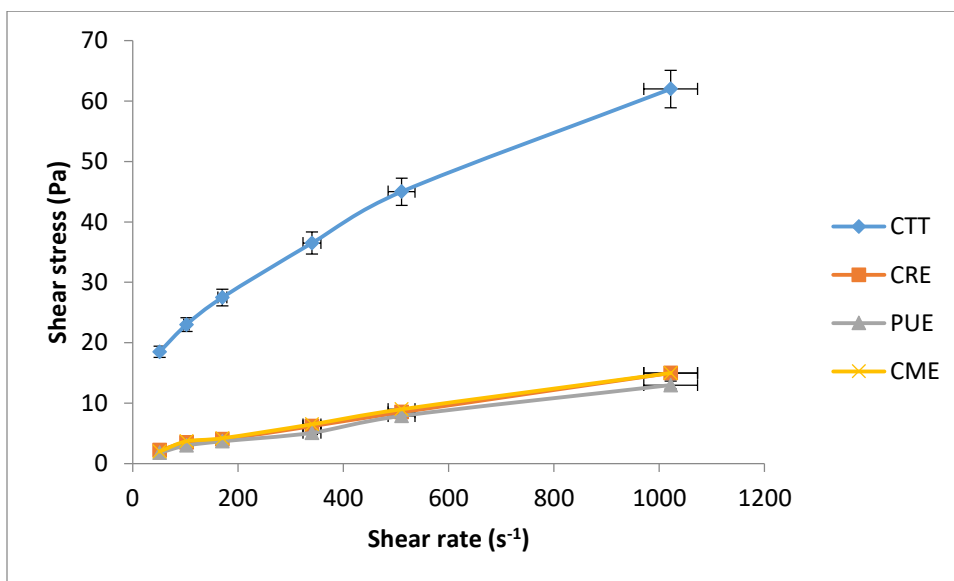
(a.)
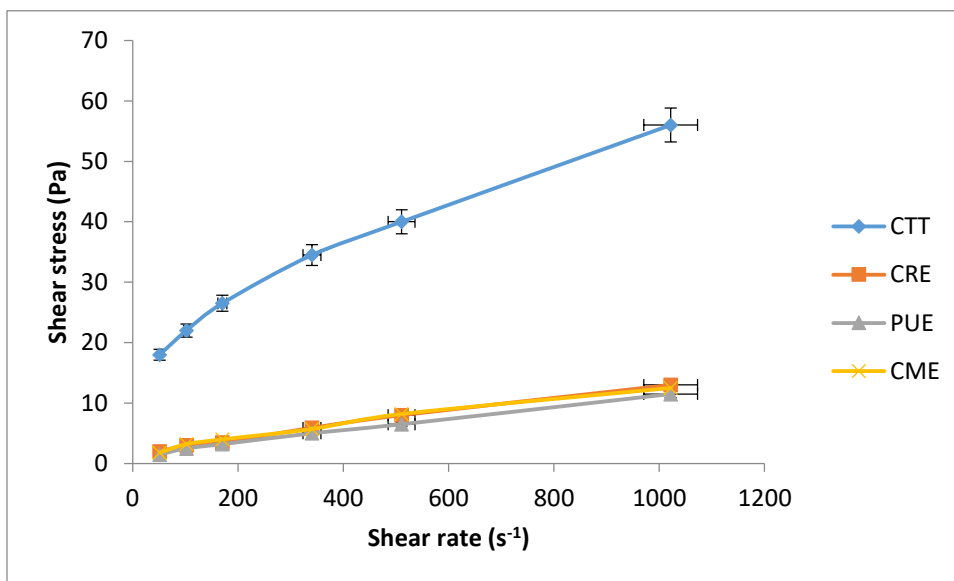
(b.)

Figure 4.24. Effect of Enzymatic Extraction on Shear Stress versus Shear Rate Patterns of Mango Juice: (a) at 10°C, (b) at 20°C. CTT= mango juice without enzyme (control); CRE= mango juice extracted with crude polygalacturonase; PUE= mango juice extracted with purified polygalacturonase; CME= mango juice extracted with commercial pectinase enzyme.
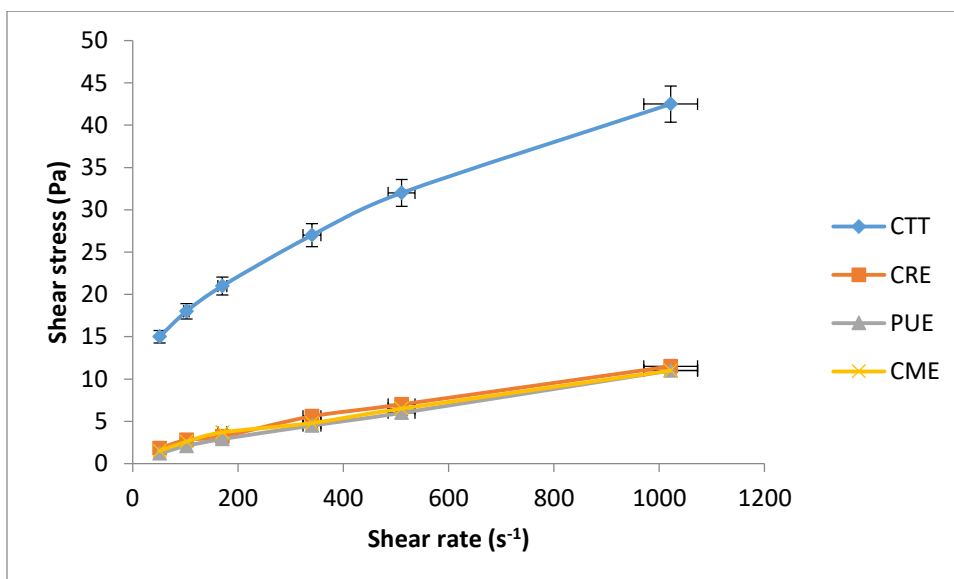
(c.)
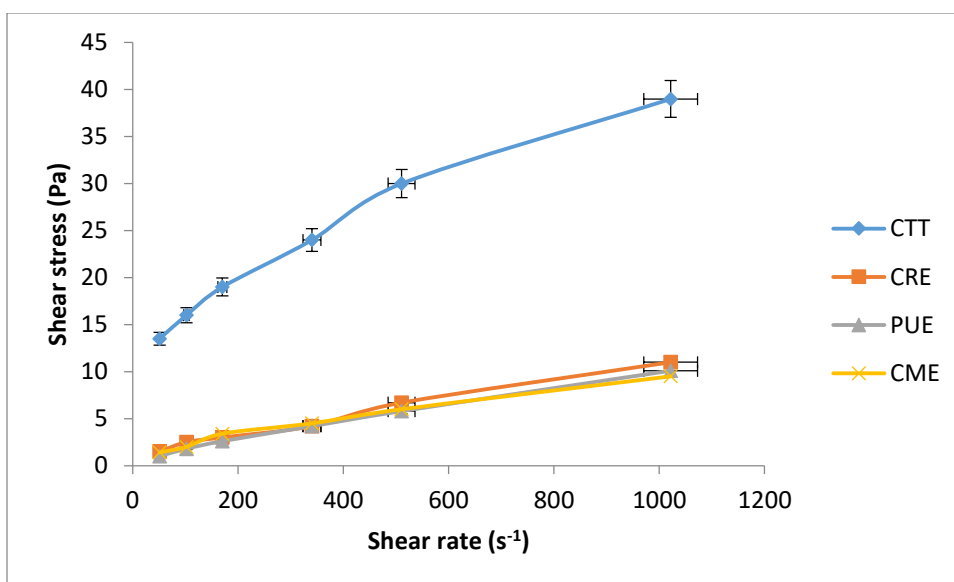
(d.)

Figure 4.24. Effect of Enzymatic Extraction on Shear Stress versus Shear Rate Patterns of Mango Juice: (c) at 30°C, (d) at 40°C. CTT= mango juice without enzyme (control); CRE= mango juice extracted with crude polygalacturonase; PUE= mango juice extracted with purified polygalacturonase; CME= mango juice extracted with commercial pectinase enzyme.
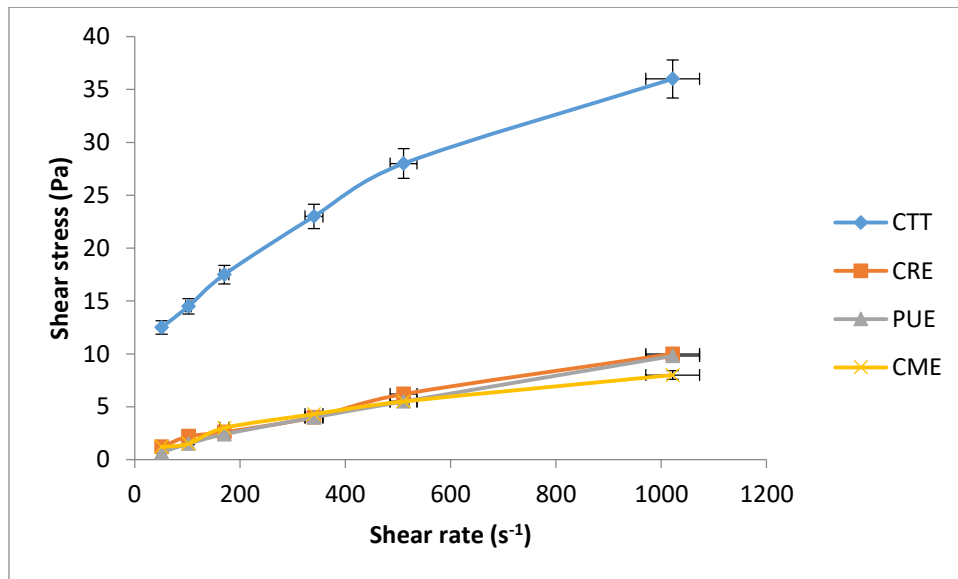
(e.)

Figure 4.24. Effect of Enzymatic Extraction on Shear Stress versus Shear Rate Patterns of Mango Juice: (e) at 50°C. CTT= mango juice without enzyme (control); CRE= mango juice extracted with crude polygalacturonase; PUE= mango juice extracted with purified polygalacturonase; CME= mango juice extracted with commercial pectinase enzyme.
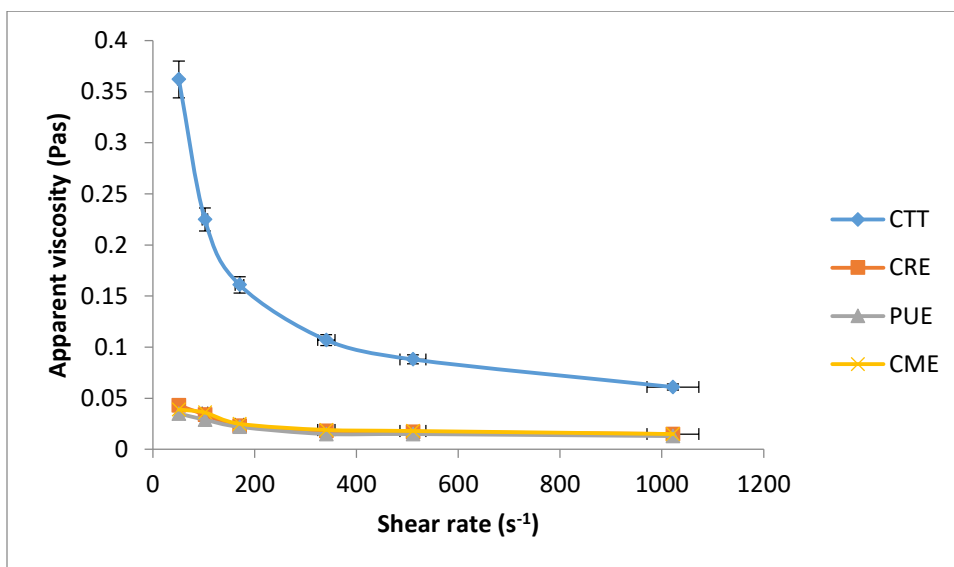
(a.)
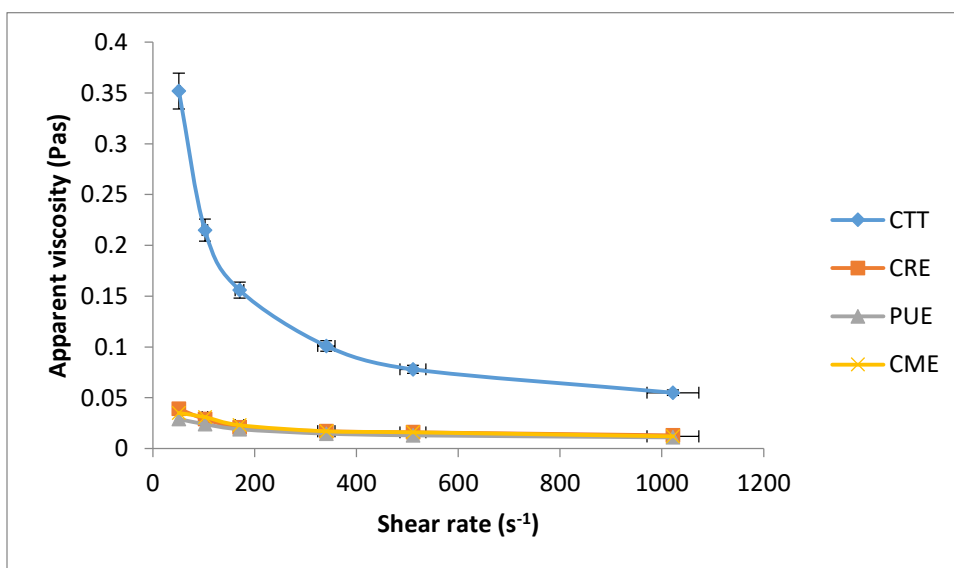
(b.)

Figure 4.25. Effect of Enzymatic Extraction on Apparent Viscosity versus Shear Rate Patterns of Mango Juice: (a) at 10ºC, (b) at 20ºC. CTT= mango juice without enzyme (control); CRE= mango juice extracted with crude polygalacturonase; PUE= mango juice extracted with purified polygalacturonase; CME= mango juice extracted with commercial pectinase enzyme.
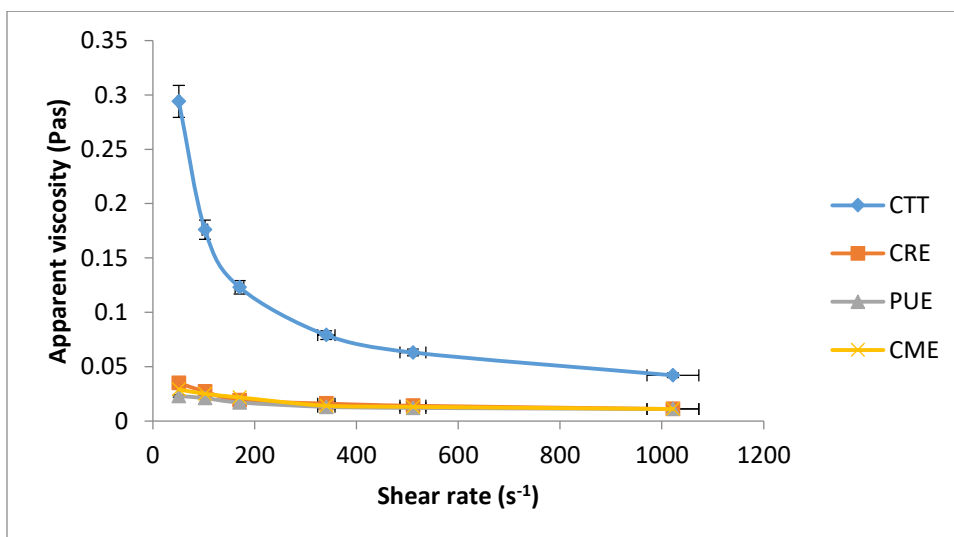
(c.)
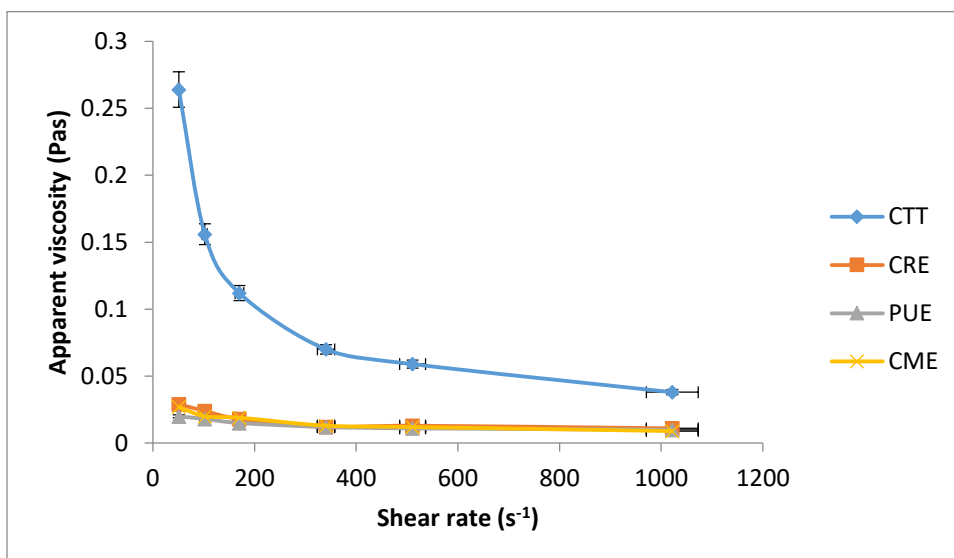
(d.)

Figure 4.25d. Effect of Enzymatic Extraction on Apparent Viscosity versus Shear Rate Patterns of Mango Juice: (c) at 30°C, (d) at 40°C. CTT= mango juice without enzyme (control); CRE= mango juice extracted with crude polygalacturonase; PUE= mango juice extracted with purified polygalacturonase; CME= mango juice extracted with commercial pectinase enzyme.

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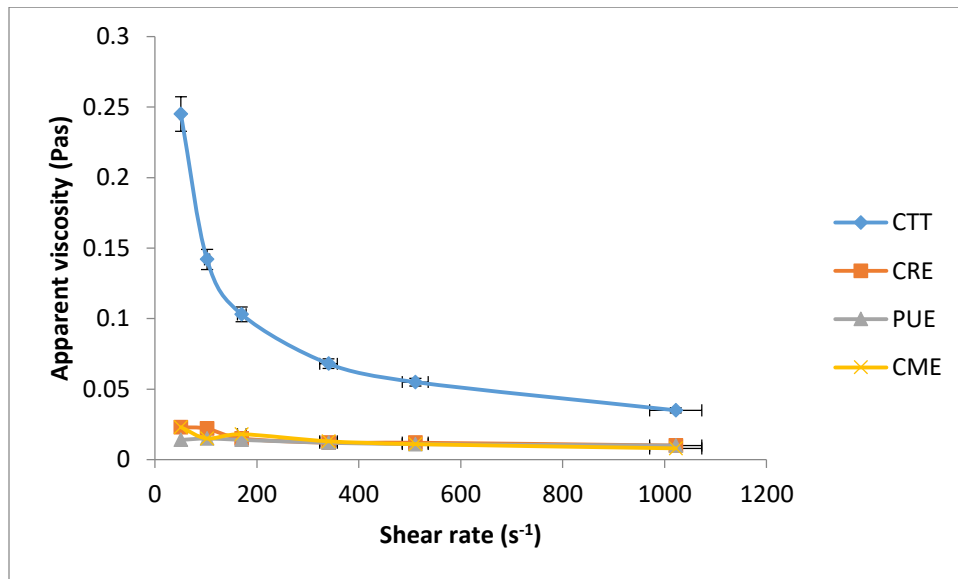
(e.)

Figure 4.25e. Effect of Enzymatic Extraction on Apparent Viscosity versus Shear Rate Patterns of Mango Juice: (e) at $50^{\circ}C$. CTT= mango juice without enzyme (control); CRE= mango juice extracted with crude polygalacturonase; PUE= mango juice extracted with purified polygalacturonase; CME= mango juice extracted with commercial pectinase enzyme.

Rheological parameters of mango juice as characterized by Power Law model are presented in Table 4.32. At all the temperatures considered, higher flow behaviour index (n) and lower consistency index (k) were recorded for juice extracted with PG. This was probably due to reduced viscous nature of enzymatically extracted juice. There were variations in n and k of mango juice extracted with PG. At all the temperatures considered, PUE had higher 'n' than CRE. Also, CRE compared with CME as 'n' obtained for both were close at each temperature. Lower 'k' was obtained for PUE and CME compared to CRE. High 'n' and low 'k' obtained for PUE suggest that it had good flow characteristics that were comparable with CME. Variations in 'n' and 'k' of the un-treated and enzymatically extracted juices could be due to differences in their soluble solids content. According to Deshmukh *et al.* (2015), flow behaviour properties are dependent on solids/solute (mainly sugars and organic acids) content. Solids and solute contents determine their level of interaction and this interaction (i.e. water-solute interaction) is based on the nature of solute in terms of its shape, size and molecular weight; and its degree of hydration in solvent (Diamante and Umemoto, 2015). High soluble solids content increases water and solute interaction which results in increased molecular hydration (Deshmukh *et al.,* 2015; Sakhale *et al.,* 2016). Generally, the flow characteristics of the mango juice increased with increasing temperature. This is probably due to reduction in viscosity due to enhanced mobility of molecules which led to increased inter-molecular collision as temperature increased (Rao, 2007). Deshmukh *et al.* (2015) also reported higher flow characteristics of sapota (*Achras sapota*) juice with increasing temperature. R^2 ranged between 0.973 and 0.999. This implies the suitability of Power Law for rheological characterization of mango juice. Flow properties ('n' and 'k') and occurrence of yield stress obtained from Herschel Bulkley model are presented in Table 4.33. Lower yield stress was recorded for mango juice extracted with PG at all the temperatures considered. Sharoba and Ramadan (2011) also reported reduction of yield stress in golden berry (*Physalis peruviana*) juice. Reduction in yield stress in mango juice

Table 4.32. **Rheological Characterisation of Enzymatically Extracted Mango Juice by Power Law Model**

Mango juice	Temperature (°C)	n	k (Pas)	R^2
CTT	10	0.406	3.557	0.991
	20	0.377	3.912	0.992
	30	0.350	3.615	0.991
	40	0.361	3.083	0.987
	50	0.366	2.782	0.988
CRE	10	0.619	0.184	0.982
	20	0.625	0.161	0.988
	30	0.614	0.154	0.989
	40	0.644	0.116	0.980
	50	0.689	0.081	0.987
PUE	10	0.638	0.145	0.984
	20	0.659	0.112	0.995
	30	0.716	0.073	0.996
	40	0.759	0.052	0.999
	50	0.860	0.026	0.994
CME	10	0.644	0.164	0.981
	20	0.627	0.160	0.992
	30	0.633	0.131	0.989
	40	0.640	0.112	0.990
	50	0.668	0.083	0.973

CTT= mango juice without enzyme (control); CRE= mango juice extracted with crude polygalacturonase; PUE= mango juice extracted with purified polygalacturonase; CME= mango juice extracted with commercial pectinase enzyme; n= flow behaviou index; k- consistency index.

Table 4.33.   Rheological Characterisation of Enzymatically Extracted Mango Juice by Herschel-Bulkley Model

Mango juice	Temperature (°C)	τ_o (Pa)	n	k (Pas)	R^2
CTT	10	19.32	1.671	0.000757	0.858
	20	18.96	0.003	2.565	0.807
	30	15.79	0.003	1.218	0.888
	40	14.07	0.003	1.455	0.867
	50	13.09	0.003	1.271	0.872
CRE	10	1.838	1.100	0.00725	0.963
	20	1.774	0.003	0.699	0.804
	30	1.716	0.004	0.303	0.867
	40	1.284	0.003	0.655	0.808
	50	1.096	0.003	0.482	0.827
PUE	10	1.607	1.247	0.00254	0.924
	20	1.368	0.003	0.565	0.839
	30	1.023	0.003	0.623	0.865
	40	0.884	0.003	0.497	0.840
	50	0.630	0.003	0.416	0.889
CME	10	1.985	1.100	0.00725	0.963
	20	1.986	0.004	0.316	0.874
	30	1.600	0.003	0.383	0.812
	40	1.494	0.004	0.155	0.851
	50	1.376	0.004	0.155	0.862

CTT= mango juice without enzyme (control); CRE= mango juice extracted with crude polygalacturonase; PUE= mango juice extracted with purified polygalacturonase; CME= mango juice extracted with commercial pectinase enzyme; τ_o= yield stress; n= flow behaviou index; k= consistency index.

extracted with PG could be due to reduction in viscosity. Yield stress is a form of potential energy which must be overcome before flow of liquid can commence, therefore, its reduction is advantageous because less energy will be required for equipment design (Rao, 2007). Yield stress recorded for PUE was lower than those of CRE and CME at all temperatures considered. There were no marked differences among the samples in terms of 'n', however, 'k' reduced as a result of enzymatic extraction. Sharoba and Ramadan (2011) attributed reduction in 'k' in golden berry juice extracted with pectinase to reduction in pulp content owing to enzymatic hydrolysis.In comparison to Power Law model, Herschel-Bulkley model was less suitable for the rheological characterization of mango juice due to lower R^2 values (0.804-0.963) obtained.

Casson model (Table 4.34) also showed that yield stress and 'k' reduced consequent to mango juice extraction with PG. Also, these parameters varied among the samples with PUE having better flow properties than CRE and CME. The variation could be due to differences in enzyme properties (Diamante and Umemoto, 2015). Also, variation in 'k' has been attributed to differences in soluble solids content (Sogi *et al.,* 2010). Casson model with R^2 that ranged between 0.948 and 0.997, gave more consistent flow characteristics for mango juice than the Herschel Bulkley model.

Temperature dependency study of mango juice as influenced by enzymatic extraction is presented in Table 4.35. Activation energy (Ea) increased consequent to enzymatic extraction of mango juice. This agreed with the report of Sharoba and Ramadan (2011) who reported significant increase in Ea of golden berry juice extracted with pectinase enzyme. Ea for the juice extracted by enzyme followed the order: CME>PUE>CRE. The higher the Ea, the higher the rate of viscosity reduction i.e. increased rate of reduction of activation energy complex (Manjunatha *et al.,* 2012).

Table 4.34. **Rheological Characterisation of Enzymatically Extracted Mango Juice by Casson Model**

Sample	Temperature (^{o}C)	τ_o (Pa)	k (Pas)	R^2
CTT	10	3.338	0.144	0.997
	20	3.397	0.129	0.997
	30	3.169	0.107	0.996
	40	2.962	0.105	0.994
	50	2.840	0.102	0.990
CRE	10	0.865	0.095	0.972
	20	0.786	0.088	0.997
	30	0.809	0.077	0.948
	40	0.656	0.082	0.989
	50	0.563	0.081	0.991
PUE	10	0.743	0.089	0.991
	20	0.666	0.084	0.997
	30	0.526	0.086	0.997
	40	0.446	0.086	0.997
	50	0.295	0.090	0.993
CME	10	0.821	0.095	0.992
	20	0.838	0.086	0.990
	30	0.754	0.080	0.988
	40	0.712	0.075	0.986
	50	0.649	0.071	0.965

CTT= mango juice without enzyme (control); CRE= mango juice extracted with crude polygalacturonase; PUE= mango juice extracted with purified polygalacturonase; CME= mango juice extracted with commercial pectinase enzyme; τ_o= yield stress; k= consistency index.

Table 4.35. **Temperature Dependency of Mango Juice as Influenced by Enzymatic Extraction**

Mango juice	Shear rate (s^{-1})	Uo (Pa)	Ea (kJ/mol)	R^2
CTT	51.09	0.908	7.08	0.900
	102.2	0.687	8.24	0.981
	170.3	0.825	8.22	0.900
	340.6	0.911	8.61	0.903
	510.9	1.174	8.46	0.948
	1021.8	0.921	9.79	0.931
CRE	51.09	0.033	9.87	0.901
	102.2	0.134	7.57	0.981
	170.3	0.205	6.90	0.976
	340.6	0.214	7.94	0.817
	510.9	0.824	5.44	0.951
	1021.8	0.809	6.75	0.989
PUE	51.09	0.00264	15.27	0.931
	102.2	0.021	11.60	0.977
	170.3	0.156	7.35	0.993
	340.6	0.795	4.36	0.905
	510.9	0.576	5.99	0.962
	1021.8	1.60	4.83	0.989
CME	51.09	0.0507	8.57	0.956
	102.2	0.0061	15.05	0.921
	170.3	0.405	5.50	0.906
	340.6	0.273	7.33	0.953
	510.9	0.210	8.75	0.962
	1021.8	0.173	10.38	0.979

CTT= mango juice without enzyme (control); CRE= mango juice extracted with crude polygalacturonase; PUE= mango juice extracted with purified polygalacturonase; CME= mango juice extracted with commercial pectinase enzyme; Uo= Arrhenius constant; Ea= activation energy.

For all the juice samples, there was a general reduction in activation energy with increasing shear rate. This could be due to increased mobility and collision of molecules at high shear which probably led to reduction in activation energy complex (Manjunatha *et al.,* 2012).

4.10.4 Effect of enzymatic extraction on the sensory properties of mango juice

Table 4.36 shows the sensory properties of mango juice as influenced by enzymatic extraction. There were significant ($p < 0.05$) differences among the samples in terms of colour, consistency, mouthfeel and overall acceptability. Higher score (7.12) was recorded for PUE in terms of colour. This was followed by CME (6.56), CTT (6.04) and CRE (5.80). High scores recorded for PUE and CME could not be unconnected to higher colour degradation efficacy of the purified PG and commercial pectinase (Kaur and Sharma, 2013). Higher scores (6.94 – 6.96) were recorded for the enzymatically extracted mango juice in terms of taste. This could be due to increased sugar content of the juice consequent to degradation of complex polysaccharides (Abdullah *et al.,* 2007). Improved taste could also be attributed to bio-availability of flavour compounds as a result of enzymatic juice extraction (Kumar*,* 2015).

CME had highest score for mouthfeel (6.96) followed by PUE (6.28). Both samples had significantly ($p < 0.05$) higher scores in the parameter than CRE (5.72) and CTT (6.06). Also, both were more preferred in terms of consistency; however, PUE enjoyed better preference than CME. High preference for PUE and CME in terms of consistency and mouthfeel could be due to increased rate of depectinization during juice extraction operation. According to Abdullah *et al.* (2007), increased depectinization results in viscosity reduction which lead to improved flavour and consistency. Overall, samples extracted enzymatically enjoyed better acceptability than the control sample.

Table 4.36.   Sensory Properties of Mango Juice as Influenced by Enzymatic Extraction

Mango juice	Colour	Taste	Consistency	Mouthfeel	Overall acceptability
CTT	$6.04^c\pm1.12$	$6.72^a\pm0.93$	$6.06^{bc}\pm1.10$	$6.08^{bc}\pm1.07$	$5.78^c\pm1.30$
CRE	$5.80^c\pm1.11$	$6.94^a\pm0.84$	$5.72^c\pm0.99$	$5.70^c\pm1.02$	$6.92^a\pm0.85$
PUE	$7.12^a\pm1.12$	$6.96^a\pm1.09$	$6.68^a\pm1.32$	$6.28^b\pm1.01$	$6.34^b\pm0.87$
CME	$6.56^b\pm0.99$	$6.90^a\pm0.81$	$6.34^{ab}\pm1.04$	$6.96^a\pm0.92$	$6.70^{ab}\pm0.86$

Data are means±standard deviations of 50 scores. Values in column with different superscripts were significantly different ($p<0.05$). CTT= mango juice without enzyme (control); CRE= mango juice extracted with crude polygalacturonase; PUE= mango juice extracted with purified polygalacturonase; CME= mango juice extracted with commercial pectinase enzyme.

4.11   Quality of Apple Juice as Influenced by Enzymatic Clarification

Chemical composition, colour order, rheological and sensory properties of apple juice are discussed in this Section.

4.11.1 Effect of enzymatic clarification on chemical composition of apple juice

Table 4.37 shows the chemical composition of un-clarified (CTTA) and clarified apple juice samples. Percentage transmittance ranged from 13.89 to 52.41%. Enzymatic clarification caused a significant ($p<0.05$) increase in percentage transmittance (clarity). This implies reduction in turbidity and removal of materials that would have contributed to cloudiness. According to Tribess and Tadini (2006), apple juice is cloudy due to the presence of pectin, cellulose and other complex polysaccharides, proteins, tannins, metals etc. Success has earlier been reported on the use of PG for the clarification of apple (Joshi *et al.,* 2011; Dey *et al.,* 2014; Dey and Banerjee, 2014) and guava (Kant *et al.,* 2013) juices. Enzymes such as PG degrade complex polysaccharides especially pectin which leads to crystal formation. The crystals are easily removed via centrifugation, decantation, and filtration or by any other mechanical separation method (Sharma *et al.,* 2014). Clarified samples varied significantly ($p<0.05$) from one another in terms of clarity with the sample clarified with crude PG (CREA) having the lowest value. Highest performance was recorded for the sample clarified with commercial pectinase enzyme (CMEA), followed by sample clarified with purified PG (PUEA). Soluble solids ranged from 8.00 to 9.62 °Brix. There was no significant difference ($p>0.05$) among the samples except PUEA which had significantly ($p<0.05$) lower value. Lower soluble solids exhibited by PUEA could be attributed to its higher degradation ability. Performance of enzymes in juice clarification is dependent on enzyme related properties such as activity, purity and catalytic activity (Levaj *et al.,* 2012). Clarification of juice caused a significant ($p<0.05$) reduction in pH from 3.57 in CTTA to 3.48, 3.49 and 3.51 in CREA, PUEA and CMEA, respectively. This could be due to liberation of organic acid which lowered the pH of the juice. Earlier reports had shown reduction in pH of juices consequent to enzymatic clarification

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Table 4.37. **Chemical Composition of Apple Juice as Influenced by Enzymatic Clarification**

Apple juice	%T_{650}	Soluble solids (°Brix)	pH	Reducing sugar (mg/100mL)	Total sugar (mg/100mL)	TTA (%)	Ascorbic acid(mg/100mL)	Specific gravity
CTTA	$13.89^c \pm 0.57$	$9.62^a \pm 0.14$	$3.57^a \pm 0.00$	$18.26^d \pm 0.78$	$36.12^c \pm 0.74$	$0.06^a \pm 0.01$	$0.84^d \pm 0.04$	$1.14^a \pm 0.01$
CREA	$35.23^b \pm 0.12$	$8.8^a \pm 0.28$	$3.48^c \pm 0.06$	$45.18^a \pm 1.44$	$65.36^a \pm 5.66$	$0.07^a \pm 0.01$	$2.08^a \pm 0.00$	$1.08^b \pm 0.00$
PUEA	$45.67^{ab} \pm 0.51$	$8.00^b \pm 0.00$	$3.49^{ab} \pm 0.00$	$28.88^c \pm 2.83$	$50.31^b \pm 0.04$	$0.08^a \pm 0.03$	$1.87^b \pm 0.06$	$1.07^b \pm 0.03$
CMEA	$52.41^a \pm 0.27$	$9.10^a \pm 0.42$	$3.51^{ab} \pm 0.01$	$34.17^b \pm 1.46$	$43.52^{ab} \pm 11.72$	$0.09^a \pm 0.01$	$1.65^c \pm 0.03$	$1.06^b \pm 0.01$

Data are means± standard deviations of triplicate scores. Values in column with different superscripts were significantly different (p<0.05). CTTA= un-clarified apple juice (control); CREA= apple juice clarified with crude polygalacturonase; PUEA= apple juice clarified with purified polygalacturonase; CMEA= apple juice clarified with commercial pectinase enzyme; %T_{650}= percentage transmittance at 650nm; TTA= titratable acidity.

(Joshi *et al.,* 2011; Kumar, 2015). While enzymatic clarification caused a significant (p<0.05) reduction in pH, there was a corresponding non-significant (p>0.05) increase in titratable acidity which might have also been as a result of organic acid production. There was no significant (p<0.05) difference among the clarified samples in terms of pH and titratable acidity. This implies the suitability of both the crude and purified PGs in these regards.

Reducing and total sugars ranged from 18.26 to 45.18 mg/100mL and from 36.12 to 65.36 mg/100mL, respectively. There were significant (p<0.05) increases in reducing and total sugars of apple juice as a result of enzymatic clarification. This agreed with earlier reports where sugars also increased after juice clarification (Joshi *et al.,* 2011; Sharoba and Ramadan, 2011; Bahramian *et al.*, 2011). Increase in sugars content could be due to their increased bioavailability consequent to degradation of complex organic materials such as pectins, cellulose, hemicelluloses etc. Among the clarified samples, CREA had highest reducing and total sugar contents. Variation could be due to enzyme-related factors (Levaj *et al.,* 2012; Diamante and Umemoto, 2015).

Ascorbic acid content ranged between 0.84 and 2.08mg/100mL. It increased significantly (p<0.05) as a result of enzymatic clarification probably due to its bio-availability during the operation. Khandare *et al.* (2011) had reported increased bioavailability of organic materials and phytochemicals during enzymatic clarification of black carrot (*Daucus carota*). Both CREA and PUEA had significantly (p<0.05) higher ascorbic acid than CME. Specific gravity ranged from 1.06 to 1.14. Significantly (p<0.05) higher specific gravity was recorded for CTTA. This implies that clarification caused significant reduction in density of apple juice. This reduction could be caused by degradation of heavier polysaccharides to sugars which are lighter (Diamante and Umemoto, 2015).

4.11.2 Physical properties of apple juice as influenced by enzymatic clarification

Colour order properties of apple juice as influenced by enzymatic clarification by polygalacturonase enzyme are presented in Table 4.38. L, a degree of lightness varied from 35.48 to 50.96. It increased significantly ($p < 0.05$) in clarified samples. The extent of increase varied significantly ($p < 0.05$) among the clarified samples with CREA having the highest value. This agreed with the result reported for sapota juice (Kaur and Sharma, 2013). Increase in L could be an indication of increased clarity (Kaur and Sharma, 2013) or colour degradation as a result of auto-oxidation of phenols (Deshmukh *et al.,* 2015). a and b ranged from -2.39 to 3.31 and 13.91 to 19.17, respectively. There was a significant ($p < 0.05$) reduction in a as a result of enzymatic clarification. Reduction in a was most pronounced in CREA followed by CMEA. Reduction in a is an indication of reduction in redness. Enzymatic clarification resulted in significant ($p < 0.05$) increase in b. Also, highest b was recorded in CREA. Increased b implies increased yellowness which might have occurred probably as a result of bio-availability of colour compounds such as pro-vitamin A. Similar finding was reported by Kaur and Sharma (2013). Hue angle, deltachrome and colour intensity also increased as a result of enzymatic clarification. They ranged from 76.63 to 89.29. 13.68 to 18.33 and 26.66 to 42.61, respectively. For all the values, CREA and PUEA had higher values than CMEA.

Table 4.39 shows the relative viscosity of apple juice as influenced by enzymatic clarification. At lower shear rates i.e. 51.09 and 102.2 s^{-1}, enzymatic clarification significantly ($p < 0.05$) reduced viscosity. Reduction in viscosity could be attributed to the breakdown of polysaccharides such as pectin which has high jelly-forming capability that makes juices highly viscous (Sharma *et al.,* 2014). Sharoba and Ramadan (2011) also reported reduction in viscosity of golden berry juice as a result of enzymatic clarification. Among the clarified samples,

Table 4.38.   Colour Order Parameters of Apple Juice as Influenced by Enzymatic Clarification

Apple juice	L	a	b	Hue angle	Chroma	Colour intensity
CTTA	$35.48^d \pm 0.04$	$3.31^a \pm 0.03$	$13.91^d \pm 0.03$	$76.63^d \pm 0.08$	$13.68^c \pm 0.03$	$26.66^d \pm 0.05$
CREA	$50.96^a \pm 0.02$	$-2.39^d \pm 0.01$	$19.17^a \pm 0.01$	$82.88^c \pm 0.04$	$18.33^a \pm 0.01$	$42.61^a \pm 0.01$
PUEA	$45.73^c \pm 0.04$	$0.24^b \pm 0.02$	$19.02^b \pm 0.02$	$89.29^a \pm 0.07$	$18.30^a \pm 0.02$	$37.89^b \pm 0.04$
CMEA	$46.15^b \pm 0.21$	$-1.37^c \pm 0.01$	$17.76^c \pm 0.14$	$85.57^b \pm 0.03$	$17.00^b \pm 0.15$	$37.64^c \pm 0.25$

Data are means± standard deviations of triplicate scores. Values in column with different superscripts were significantly different ($p<0.05$). CTTA= un-clarified apple juice (control); CREA= apple juice clarified with crude polygalacturonase; PUEA= apple juice clarified with purified polygalacturonase; CMEA= apple juice clarified with commercial pectinase enzyme.

Table 4.39. **Relative Viscosity of Apple Juice as Influenced by Enzymatic Clarification**

| Apple juice | Shear rate (s^{-1}) |
	51.09	102.2	170.3	340.6	510.9	1021.8
CTTA	$2.00^a\pm0.28$	$1.50^a\pm0.28$	$2.50^a\pm0.42$	$2.00^a\pm0.14$	$1.60^a\pm0.28$	$2.00^a\pm0.23$
CREA	$1.60^{ab}\pm0.57$	$1.00^b\pm0.00$	$1.50^a\pm0.42$	$1.67^a\pm0.28$	$1.40^a\pm0.14$	$1.67^a\pm0.42$
PUEA	$1.00^b\pm0.28$	$1.00^b\pm0.14$	$2.00^a\pm0.71$	$1.87^a\pm0.00$	$1.20^a\pm0.28$	$1.67^a\pm0.14$
CMEA	$1.00^b\pm0.00$	$1.00^b\pm0.14$	$1.50^a\pm0.71$	$1.67^a\pm0.14$	$1.20^a\pm0.28$	$1.67^a\pm0.31$

Data are means±standard deviations of triplicate scores. Values in column with different superscripts were significantly different ($p<0.05$). CTTA= un-clarified apple juice (control); CREA= apple juice clarified with crude polygalacturonase; PUEA= apple juice clarified with purified polygalacturonase; CMEA= apple juice clarified with commercial pectinase enzyme.

Significantly (p<0.05) lower relative viscosity was recorded for PUEA and CMEA compared to CREA. However, there was no significant (p>0.05) among the clarified samples at 102.2 s^{-1}. At higher shear rate i.e. 170.3 s^{-1} and above, clarification did not cause viscosity reduction. This could be due to low viscosity of apple juice and could not be shared at higher shear rate.

4.11.3 Sensory properties of apple juice as influenced by enzymatic clarification

Effect of enzymatic clarification on the sensory properties of clarified juice is presented in Table 4.40. Colour, taste, consistency, clarity, mouthfeel and overall acceptability ranged from 5.50 to 6.86, 6.56 to 7.02, 5.76 to 6.86, 4.70 to 7.00, 5.86 to 6.94 and 5.72 to 6.76, respectively. There was no significant (p>0.05) difference between PUEA and CMEA in terms of colour and taste. Highest scores were recorded for CREA in terms of taste and overall acceptability; CMEA enjoyed best preference in terms of clarity and mouthfeel while PUEA performed best in consistency. Variation in performances of the clarified juices could be probably due to differences in the properties of the enzymes. Clarification of apple juice with PG resulted in increased acceptability in terms of listed sensory parameters. This corroborated earlier findings of improved sensory properties as a result of enzymatic clarification (Joshi *et al.,* 2011). Several principles could be attributed to improved sensory properties as a result of enzymatic clarification. Increase in preference for colour, consistency, clarity and mouthfeel could be due to degradation and subsequent removal of colloidal pigments especially polysaccharides (Abdullah *et al*., 2007). Increase in taste could be as a result of increased bioavailability of sugars consequent to breakdown of complex polysaccharides through the actions of PG.

Table 4.40.   Sensory Properties of Apple Juice as Influenced by Enzymatic Clarification

Apple juice	Colour	Taste	Consistency	Clarity	Mouthfeel	Overall acceptability
CTTA	$5.50^b\pm0.88$	$6.56^b\pm0.86$	$6.12^{bc}\pm1.02$	$4.70^c\pm0.97$	$5.92^c\pm0.88$	$5.72^c\pm1.18$
CREA	$5.86^b\pm1.03$	$7.02^a\pm1.00$	$5.76^c\pm1.25$	$6.18^b\pm1.14$	$5.86^c\pm1.16$	$6.76^a\pm1.06$
PUEA	$6.82^a\pm1.14$	$6.92^{ab}\pm1.03$	$6.86^a\pm1.20$	$6.24^b\pm0.87$	$6.30^b\pm0.86$	$6.28^b\pm0.99$
CMEA	$6.86^a\pm0.88$	$6.82^{ab}\pm0.86$	$6.26^b\pm1.02$	$7.00^a\pm0.97$	$6.94^a\pm0.88$	$6.36^{ab}\pm1.18$

Data are means±standard deviations of triplicate scores. Values in column with different superscripts were significantly different ($p<0.05$). CTTA= un-clarified apple juice (control); CREA= apple juice clarified with crude polygalacturonase; PUEA= apple juice clarified with purified polygalacturonase; CMEA= apple juice clarified with commercial pectinase enzyme.

CHAPTER FIVE

5.0 CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

This study showed that alkaline and microwave pre-treatments were effective for compositional and morphological improvement of orange and plantain peels for increased proliferation of *Aspergillus awamori* CICC 2040 and subsequent enhancement of PG production. Polygalacturonase activity was favoured by pre-treatment of OPF and PPF with 0.1M and 0.01M concentration of NaOH, respectively. Short pre-treatment time of 1 h resulted in maximum PG activity for both substrates. Also, the extent of substrate improvement depended on pre-treatment methods.

Pretreatment of OPF and PPF with NaOH, NaOH+MW and MW caused significant ($p<0.05$) reduction in lignin, hemicelluloses, extractives and ash contents while cellulose and total sugar increased. NaOH and NaOH+MW pre-treatment of OPF and PPF resulted in improved structural and morphological properties, hence, increased proliferation of *Aspergillus awamori* for increased PG production. In addition, there were reductions in representative bands of FTIR spectra for lignin and hemicelluloses consequent to NaOH, NaOH+MW and MW pretreatment of OPF and PPF.

Pre-treatment of substrates reduced impurities and enhanced enzyme purification operation. Purification of PG improved its efficiency in terms of stability in various conditions such as pH and temperature. Purified PGs showed better stability in the presence of metal ions and other compounds. Higher stability in the presence of metal ions and other compounds was exhibited by crude PGs produced from un-treated OPF and PPF in comparison to those produced from pre-treated ones. Both crude and purified PGs showed highest specificity in the presence of pectin. Purified PG showed better conformity with Michaelis-Menten equations than crude PG. Polygalacturonase produced from pre-treated substrates had higher inactivation rate, thermostability and

reaction rate than PG produced from un-treated substrates. Irrespective of substrate pre-treatment methods and enzyme purification, all the PGs produced in this work gave negative enthalpy and entropy which imply spontaneity of the inactivation process.

Extraction of mango juice with PG resulted in higher yield. Purified PG compared with commercial pectinase enzyme in this regard. Enzymatic extraction of mango juice also caused improvement in flow characteristics and sensory properties of the juice. Enzymatic clarification caused a significant increase in clarity, L* value, sugars and ascorbic acid of apple juice. Enzymatic clarification resulted in reduction in pH, specific gravity and relative viscosity of the juice.

5.2   Recommendations

- Alkaline pretreatment condition of <0.425 mm particle size, 0.1 M NaOH and pre-treatment time of 1 h is recommended for orange peel to obtain optimum PG activity.

- Alkaline pretreatment condition of 0.8025<x<1.18 mm particle size, 0.01 M NaOH and pre-treatment time of 1 h is recommended for plantain peel to obtain optimum activity of PG.

- Orange and plantain peels should be subjected to 0.8025<x<1.18 mm, 700 W microwave power and pre-treatment time of 10 min for optimum activity of PG.

- Crude polygalacturonase should be purified for improved efficiency: stability at various conditions such as temperature, pH and in metal ions, different compounds and substrates, thermostability and thermodynamic properties. Further works should be conducted in the following areas:

- Investigation on the effects of other pretreatment methods such as the use of surfactant, ionic liquids and ultrasound for compositional improvement of pectin rich fruit wastes for improved activity of pectinase enzymes.

- Investigation on the effect of substrate pretreatment on the growth of *Bacillus* spp. for increased activity of alkaline pectinases and proteases.

5.3   Contributions to Knowledge

- Alkaline and microwave pretreatment conditions of orange and plantain peels for maximum PG production were established.

- Kinetic and thermodynamic properties of PG as influenced by substrate pretreatment methods were generated.

- Chemical and thermal stability of PG as influenced by substrate pretreatment methods were established.

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APPENDICES

Appendix I

Preparation of Reagents and pH Buffers

(a) Preparation of 3, 5 di-nitro salicylic acid

One gramme of 3, 5 di-nitro salicylic acid was dissolved in 20 mL NaOH in 100 mL volumetric flask. Thereafter, 30 g of sodium potassium tartarate was added to the solution, and made to mark with distilled water. The solution was stored in the dark until required.

(b) Preparation of reagents for protein analysis

A: 2% Na_2CO_3 in 0.1 M NaOH

B: 1% of sodium potassium tartarate in distilled water

C: 0.5% $CUSO_4.5H_2O$ in distilled water

Reagent I: 48 mL of A + 1 mL of B + 1 mL of C

Reagent II: 5 mL each of 2 M Folin-phenol and distilled water

(c) Preparation of malt extract agar

Fifty gramme of malt extract was dissolved in 1 L of distilled water and boiled for few minutes. The mixture was sterilized by autoclaving at 115 °C for 10 min.. it was cooled to 55 °C and 1 mL of 10% lactic acid was added. The agar was stored in a sterile refrigerator until required.

(d) Preparation of pH buffers

Buffers were prepared as follows: pH 3.0 to 5.0 were prepared by mixing varying volumes of acetic acid (0.1 M) and sodium acetate (0.1 M); For pH 6.0, 6.81 g of KH_2PO_4 was added to 56 mL of 0.1 M NaOH and was made up to 1 L with distilled

water; For pH 7.0, 6.81 g of KH_2PO_4 was added to 291 mL of 0.1M NaOH and was made up to 1 L with distilled water; For pH 8.0, 6.81 g of KH_2PO_4 was added to 467 mL of 0.1 M NaOH and was made up to 1 L with distilled water; For pH 9, 150 mL of 0.238 M $NaHCO_3$ was mixed with 26 mL of 0.05 M Na_2CO_3; For pH 10, 100 mL of 0.05M $NaHCO_3$ was added to 21.4 mL 0.1M NaOH; For pH 11, 100 mL of 0.05 M Na_2HPO_4 was mixed with 8.2 mL of 0.1 M NaOH; For pH 12, 100 mL of 0.05 M Na_2HPO_4 and 53.8 mL of 0.1 M NaOH were combined.

Calibration curves

(a) Standard glucose
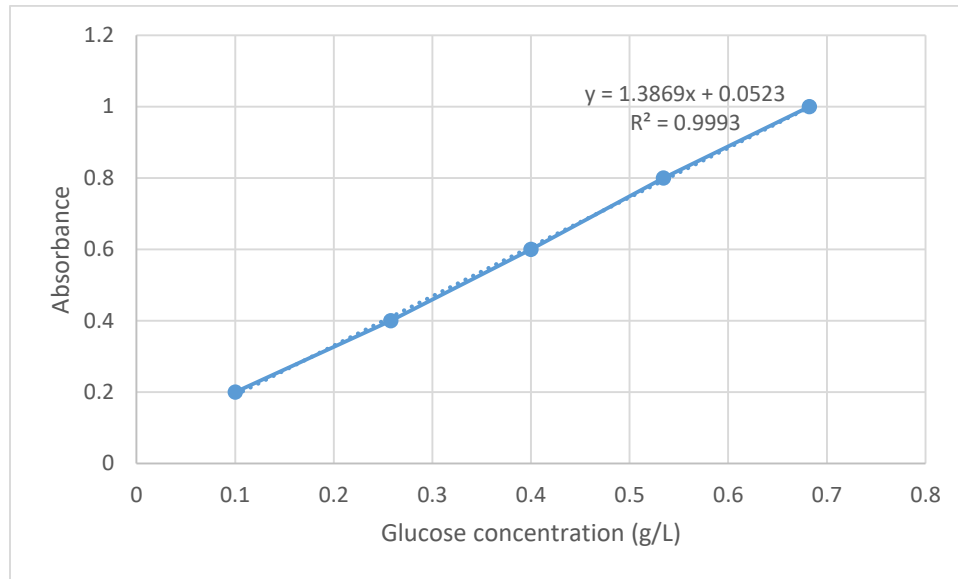
y = 1.3869x + 0.0523
R² = 0.9993

(b) D-galacturonic acid
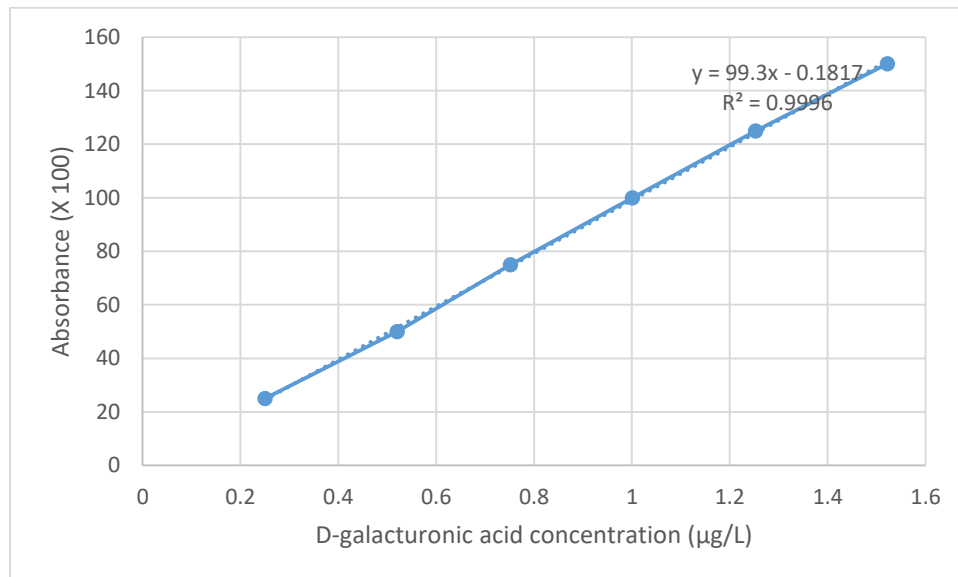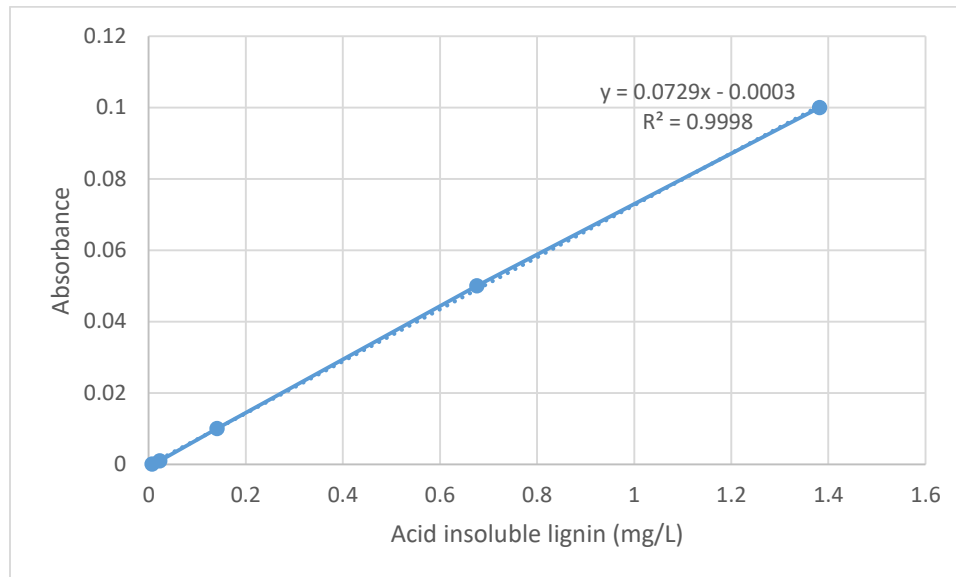
y = 99.3x - 0.1817
R² = 0.9996

(c) Acid insoluble lignin

y = 0.0729x - 0.0003
R² = 0.9998

Absorbance (y-axis)

Acid insoluble lignin (mg/L) (x-axis)

(d) Bovine serum albumin

y = 7894.2x - 16.93
R² = 0.998

Absorbance (y-axis)

Bovine serum albumin (µg/mL) (x-axis)
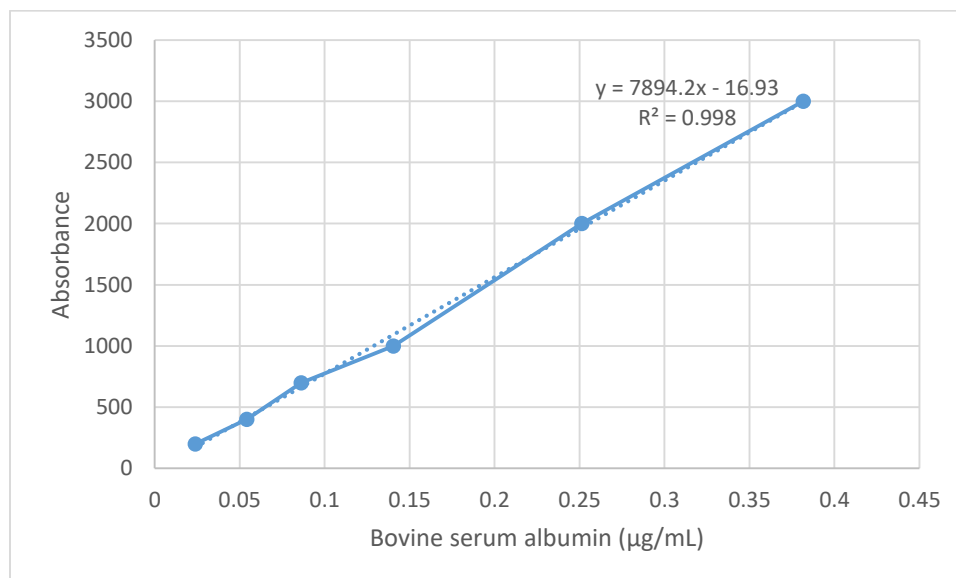
Michaelis-Menten Kinetics of Crude and Purified Polygalacturonase
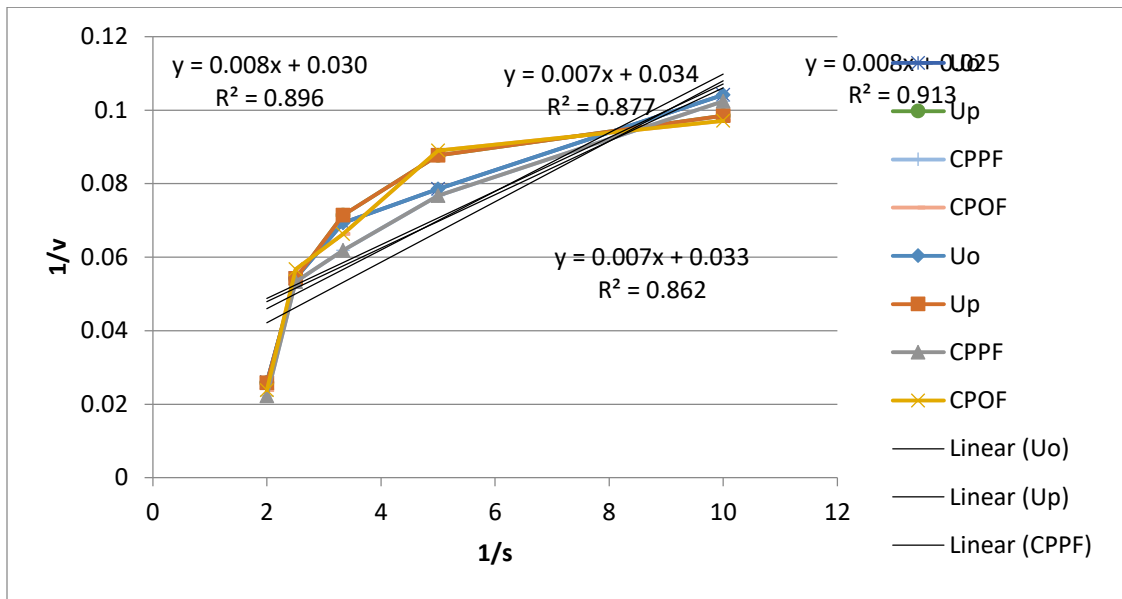
a.
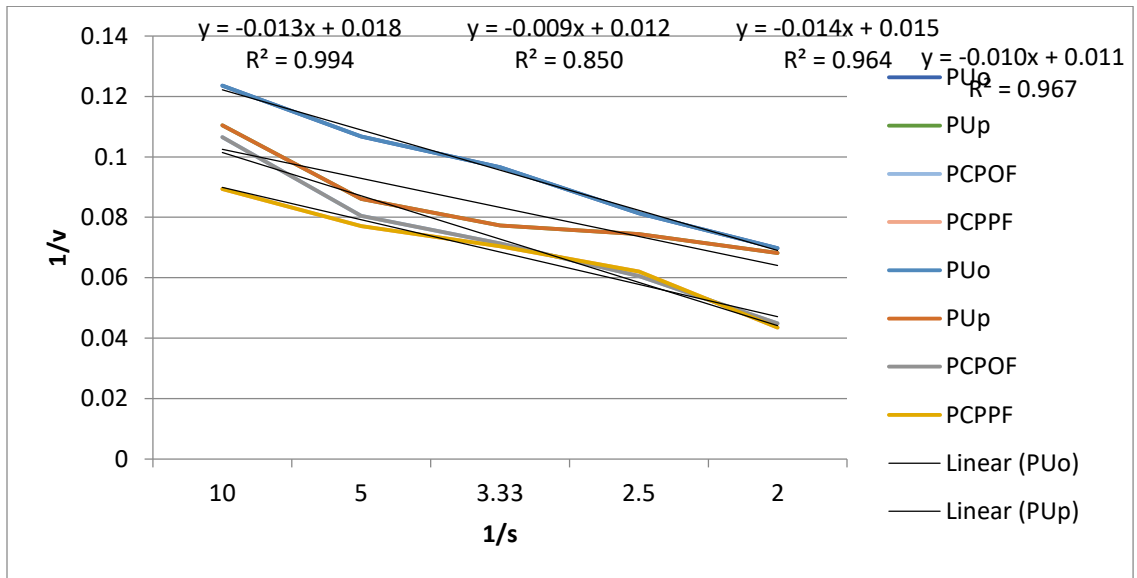
b.

Michaelis-Menten kinetics of polygalacturonase: (a) crude polygalacturonase, (b) purified polygalacturonase

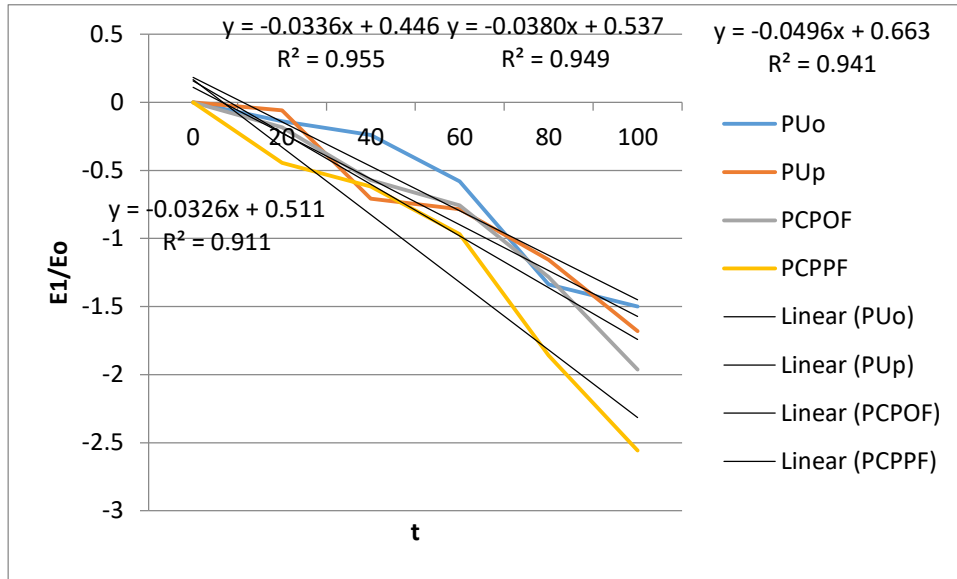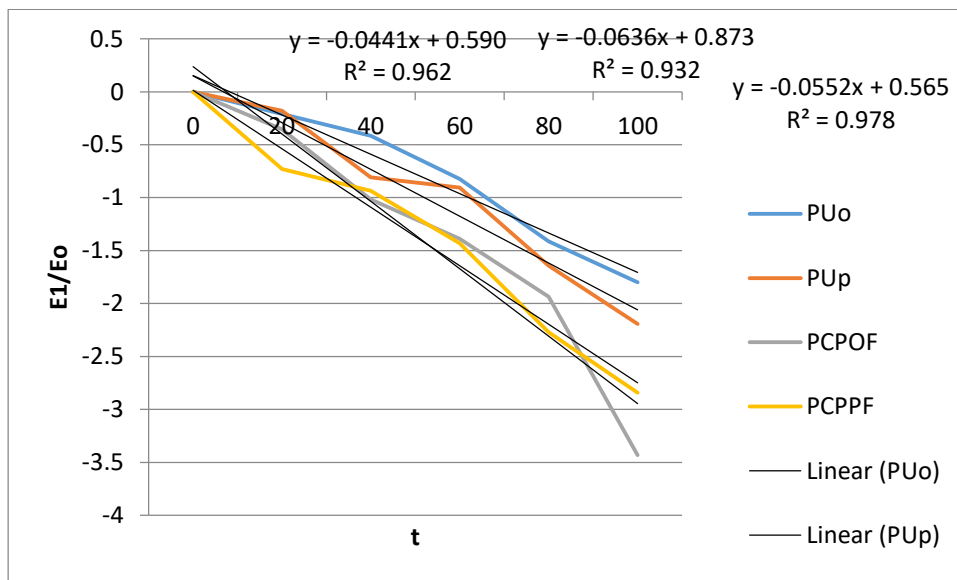**Inactivation kinetics of crude polygalacturonase at different temperatures**

a.

b.

c.

d.

Inactivation kinetics of crude polygalacturonase at different temperatures: (a) 60ºC, (b) 70ºC, (c) 80ºC and (d) 90 ºC

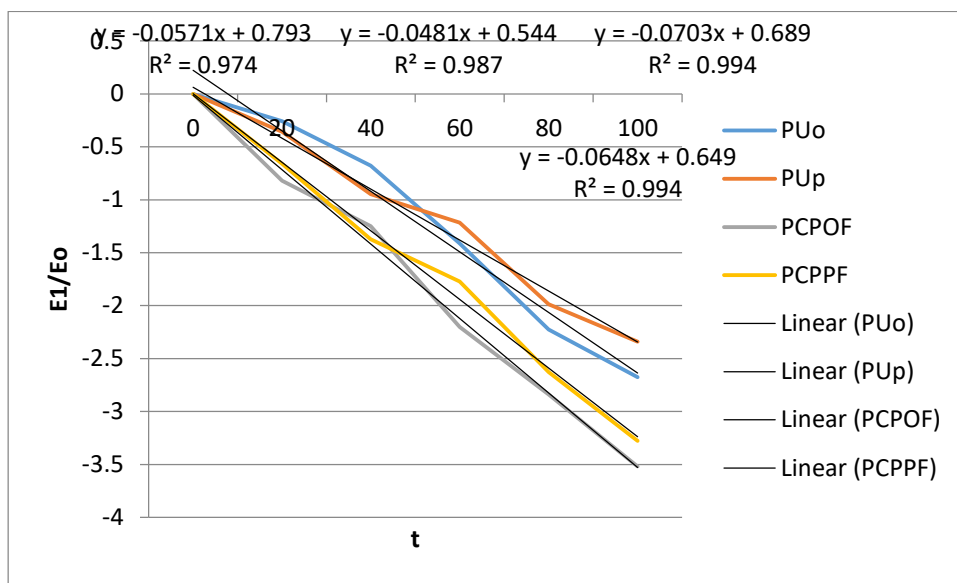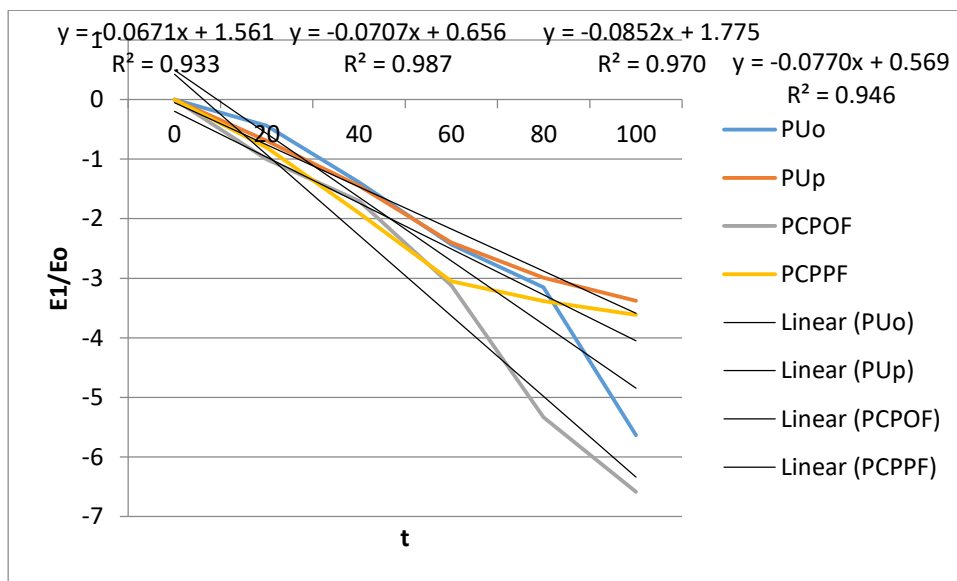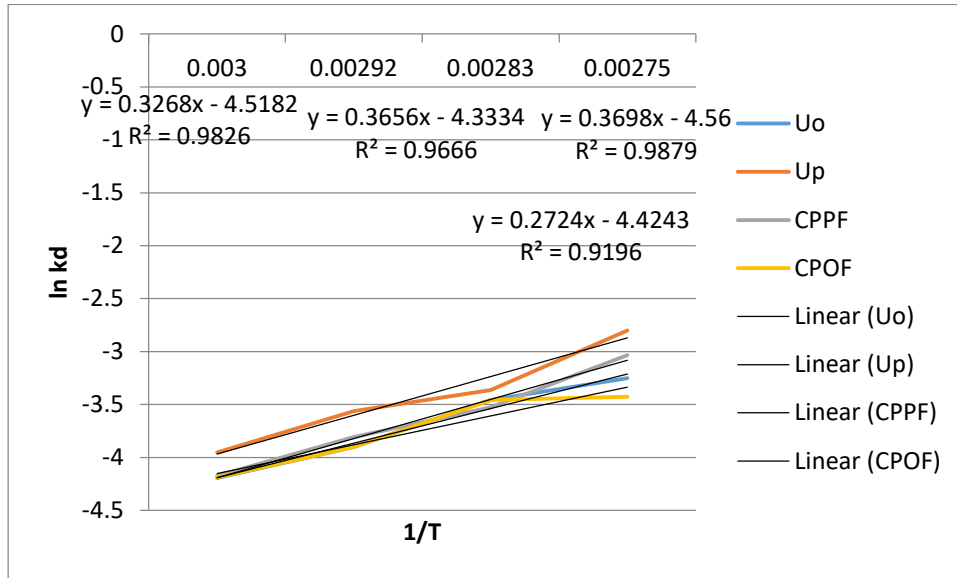**Inactivation kinetics of purified polygalacturonase at different temperatures**

a.

b.

c.

d.
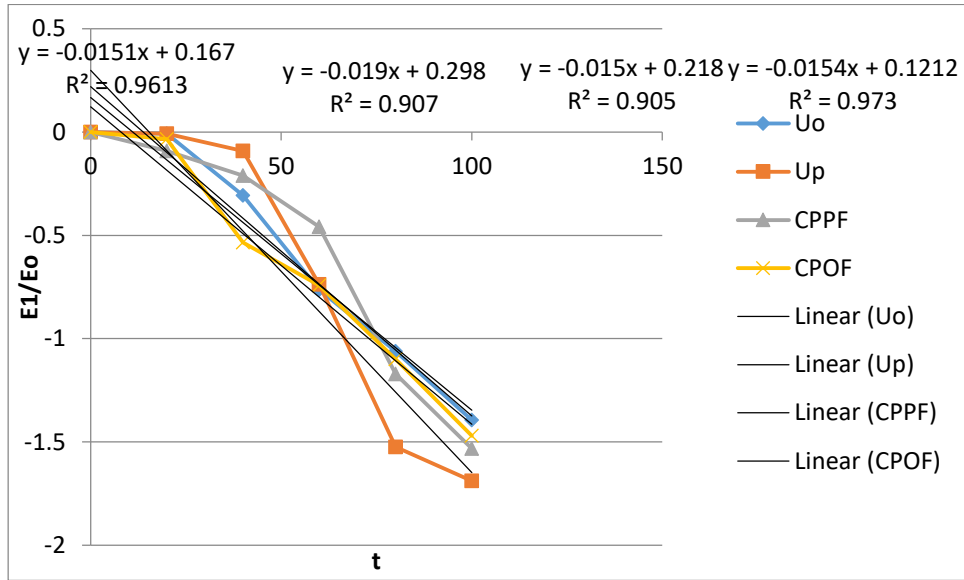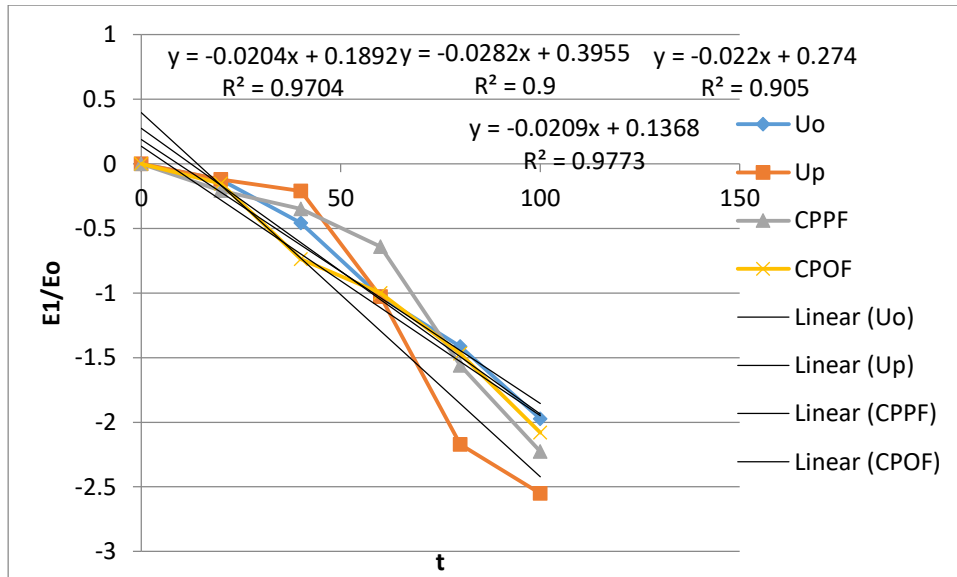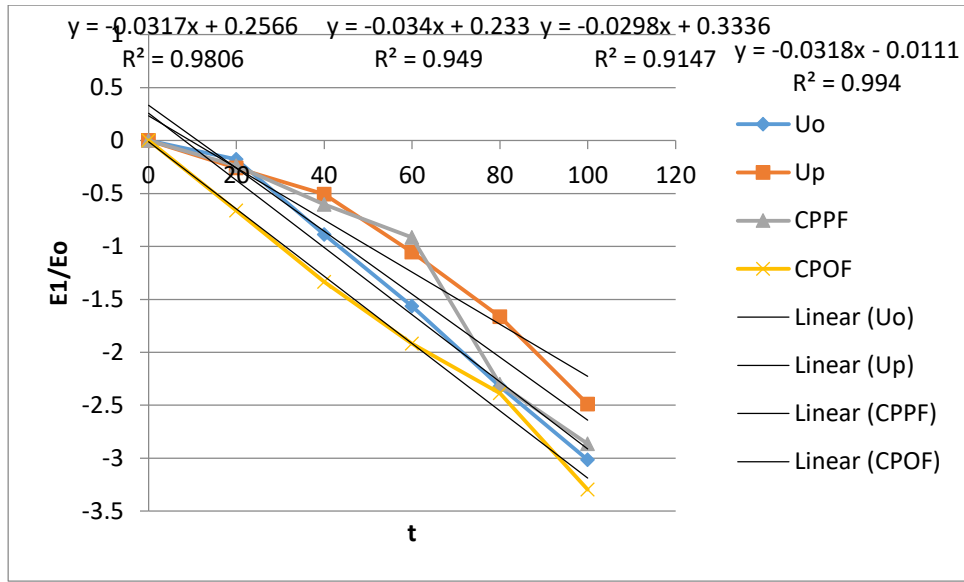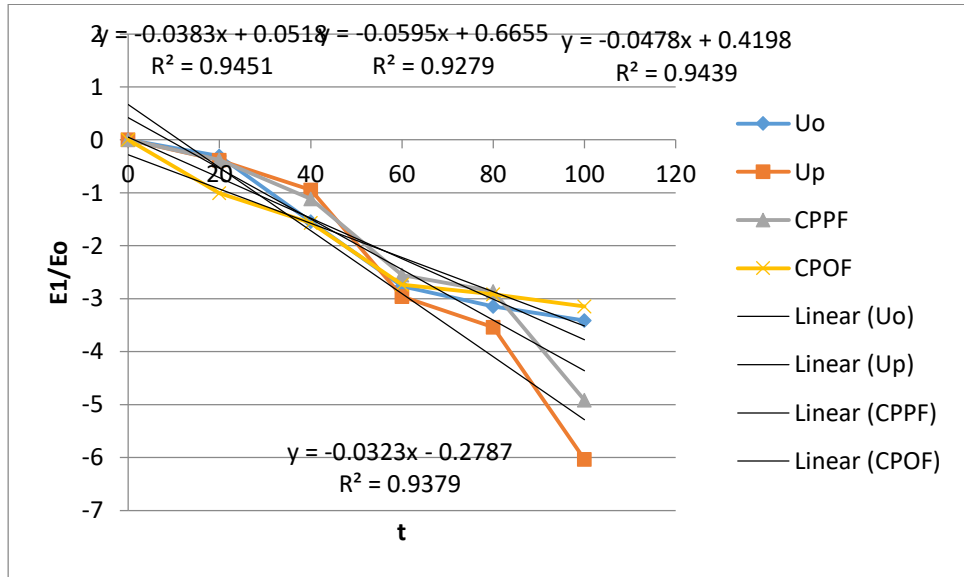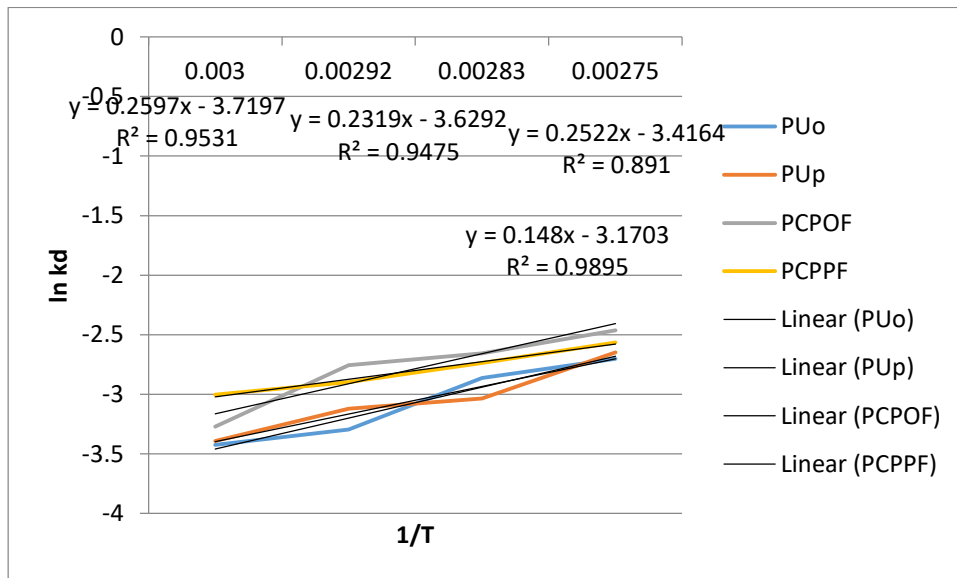
Inactivation kinetics of purified polygalacturonase at different temperatures: (a) 60°C, (b) 70°C, (c) 80°C and (d) 90 °C

Temperature dependency of polygalacturonase

a.

b.

Temperature dependency of: (a) crude polygalacturonase, (b) purified polygalacturonase

Questionnaire for sensory evaluation of mango juice samples

Presented before you are four coded samples of mango juice, carefully assess and score each based on the scale provided. Rinse your mouth with water in between samples.

Sample	Colour	Taste	Consistency	Mouthfeel	Overall acceptability
CME					
CRE					
PUE					
CTT					

9.	Like extremely	8.	Like very much
7.	Like moderately	6.	Like slightly
5.	Neither like nor dislike	4.	Dislike slightly
3.	Dislike moderately	2.	Dislike very much
1.	Dislike extremely		

Comment:

………………………………………………………………………………………

…………..……………………………………………………………………………

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Thanks for your time.

Appendix VIII

Questionnaire for sensory evaluation of clarified apple juice samples

Presented before you are four coded samples of clarified apple juice, carefully assess and score each based on the scale provided. Rinse your mouth with water in between samples.

Sample	Colour	Taste	Consistency	Clarity	Mouthfeel	Overall acceptability
CME						
CRE						
PUE						
CTT						

9.	Like extremely		8.	Like very much
7.	Like moderately		6.	Like slightly
5.	Neither like nor dislike		4.	Dislike slightly
3.	Dislike moderately		2.	Dislike very much
1.	Dislike extremely			

Comment:

……………………………………………………………………………………………
…………..

Thanks for your time.