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Optimization of conditions for pectinase production by *Saccharomyces cerevisiae* ATTC 52712 in solid state fermentation (SSF) and evaluation of its efficacy in orange juice extraction

By

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Thesis submitted to the Department of Biochemistry and Biotechnology, College of Science in partial fulfilment of the requirements for the degree of

MASTER OF SCIENCE IN BIOTECHNOLOGY

APRIL, 2016

DECLARATION

I, George Dzorgbenya Ametefe, declare that I undertook the study reported herein under the supervision of Prof. (Mrs.) V.P. Dzogbefia, and that except for those references which have been duly acknowledged in the text, this dissertation is the outcome of my research.

Ametefe George Dzorgbenya..... .....

(PG 8011712) Signature Date

Certified by

Prof. (Mrs.) V.P. Dzogbefia..... .....

(Supervisor) Signature Date

Head of Department

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Signature Date

DEDICATION

In humility, I dedicate this project to the Almighty God for making it possible to be educated in this great institution.

ABSTRACT

Pectinases are the group of enzymes which degrade pectin and are one of the most used enzymes in food industry. In fruit juice industry, pectinases are often used to enhance juice extraction. This study sought to optimize conditions for pectinase production from *Saccharomyces cerevisiae* ATCC 52712 and evaluate its efficacy in orange juice extraction. The study was undertaken in two phases, that is, optimization of conditions during fermentation for the enzyme production and its extraction from the solid medium, and assessing the efficacy of the enzyme produced in juice extraction. The optimal conditions investigated in first stage were fermentation time, proportion of corn cobs and orange peels, pH, temperature and inoculum size during the fermentation period for enzyme production, agitation time during enzyme extraction and the best extraction solvent for enzyme recovery. In the second stage, the crude enzyme was concentrated by ammonium sulphate precipitation and used to study the best reaction time and enzyme dosage required for optimal juice extraction. The optimal conditions obtained in the first stage were 6 days of fermentation, 80:20 % for corn cobs to orange peels, pH of 4 at 30 ^{o}C, 10.46 x 10^{6} cells/ml, 30 minutes agitation during enzyme extraction and 0.1 M NaCl as best extraction solvent. The optimal conditions obtained with respect to assessment of the efficacy of the enzyme produced with 60 % $(NH_4)_2SO_4$ saturation of the crude enzyme solution resulted in 45 minutes reaction/holding time and an enzyme dosage of 40 mg total protein per 200 g of orange mash (0.02 %). Significant increase ($p<0.05$) in activity was obtained when the crude enzyme produced was saturated to 60 % $(NH_4)_2SO_4$ concentration. During juice extraction, an enzyme dosage of 10 mg total protein per 200 g of orange mash resulted in 15 % increase in free-run juice while 40 mg total protein per 200 g of orange mash enzyme dosage gave an increase of 123.4 % juice extracted over control. This study showed that the activity of pectolytic enzymes from *S. cerevisiae* produced under optimal conditions enhanced orange juice extraction significantly thereby making the technology useful in improving orange juice production.

ACKNOWLEDGEMENT

All praises to the Almighty God, my director, protector and sustainer for granting me this golden opportunity and enablement to undertake this program.

My heartfelt gratitude goes to my supervisor Prof. (Mrs.) V.P. Dzogbefia for her guidance, understanding and the many constructive criticisms that have greatly enlightened me. Above all for her patience in the course of this work. You are indeed a scholar and a mother. May God continually release His blessings on you and your household.

I also thank Mr. Eric Acheampong of the Biological Science Department laboratory, Mr. Blutse (Biochemistry and Biotechnology laboratory) and the Technicians of the Chemistry laboratory whose pieces of advice made this work a success. May God continually bless you.

At this juncture, I thank my parents and sponsors Dr. and Mrs. Gilbert Tory Doe Ametefe for the support, encouragement and prayers that their children advance in their educational pursuit. Not forgetting my siblings Jerry, Faith, Divine and Marvelous for their cooperation in the course of this work. May God richly bless you.

To my lecturers, I sincerely thank **you all** for your impartation of knowledge, discipline and dedication. You all will reap your rewards right here on earth.

To my colleagues, it has been interesting, and a blessing knowing you. God bless you all.

My spiritual fathers Bishop Fred Addo, Bishop Dag Heward-Mills, Rev. Dr (Mrs.) Joy Bruce, I am very grateful for your moral and spiritual support. And finally to my seniors in the field, you as pioneers left a fertile ground and I have harvested bountifully. God bless you all. AMEN.

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CHAPTER ONE

1.0 INTODUCTION 1.1 BACKGROUND

A large quantity of waste is derived from industries that use agricultural raw materials in their production processes. This leads to increase in clean-up problems from the environment, which in turn become breeding ground for pathogens, causing diseases such as cholera, malaria, and a host of other diseases. Martin *et al.,* (2004) also emphasized the increasing energy demand in the use of renewable agricultural and industrial wastes, as their disposal poses environmental problems.

At present, through controlled biological degradation of food wastes by microbes, valuable compounds such as enzymes, citric acids and other raw materials for medical and industrial uses are obtained from these wastes (Magdy, 2011).

Pectic substances are a group of polysaccharides made up of sugar acids containing polymers of α (1, 4) D-galacturopyranosyl units and are found in the middle lamella between adjacent cell walls (Rastogi, 1998). Pectin is one of the vital components of plant cell wall and has been reported (Debra, 2008) to have important functions in plant development, growth, morphology, plant defense; serves as a gelling/stabilizing polymer in diverse foods, specialty products and also shown to have positive effects on human health and multiple biomedical uses. Plant pathogens attack target cells by producing a number of cell degrading enzymes (including pectin enzymes) which facilitate the entry and expansion of the pathogen in the host tissue (Jayani *et al*., 2005).

Pedrolli *et al.,* (2009), defines pectinases as a large class of enzymes that split pectic polysaccharides of plant tissues into simpler molecules like galacturonic acids. Of the overall manufacturing of enzyme preparations, pectinase production accounts for 10%, with its application in juice and wine production (Semenova *et al*., 2006). In the processing of fruit juice, it is employed

mainly in the extraction and clarification of juices, that is; the reduction in viscosity leading to the formation of clear juice. Pectinases are predominantly used in the wine industry for reducing sourness by making anthocyanins soluble without leaching out procyadinpolyphenols while extraction of more anthocyanins could also be obtained using pectinases with increase in pigmentation of the extracted juice (Tucker and Woods, 1991). Commercial pectin enzymes have also been employed in starch extraction from sweet potato (Rahman and Rakshit, 2003), yam (Daiuto *et al.,* 2005) and cassava (Dzogbefia *et al.,* 2008).

Several conventional industrial processes over the years have used pectinases for plant fiber processing, treatment of industrial wastewater containing pectinaceous material, textile, tea and coffee industries, oil extraction, purification of viruses and paper manufacturing (Jayani *et al.,* 2005). The two fermentation techniques used for enzyme production from filamentous fungi are: submerged fermentation (SmF) and solid state fermentation (SSF) (Elander and Lowe, 1992).

Orange is a widely cultivated fruit. The abundance of antioxidants (e.g. beta-carotene), vitamins A and C, fiber and phytonutrients in orange foods make this fruit good for sight (eyes), skin and heart (Yvelette, 2010). Corn cobs are also agro-wastes obtained after harvesting and processing the corn kernel. Corn cobs make up about 8 to 9% of the above ground dry matter (grain plus residues) at grain physiological maturity (Hanway, 1963; Pordesimo *et al*., 2005).

These agro wastes, including orange peels and corn cobs, contain pectin-the substrate required for pectinase production in addition to other components. The importance of these pectin containing agro waste substrates indicates the use of agro wastes for the production of pectinases from yeasts (Reid and Ricard, 2000) and the growing commercial production of pectinase from fungal sources due to their GRAS (generally regarded as safe) status as opposed to bacteria, due to their pathogenicity (Huang and Mahoney, 1999; Singh *et al.,* 1999; Pandey *et al.,* 2009). Among fungi,

Aspergillus niger, *Penicillum* and *Rhizopus* have many advantages aside their recognition as GRAS strains; they yield extracellular products which can easily be recovered from fermented medium (Blanco *et al.,* 1999).

Fermentation has been used extensively in enzyme production. The concept for using microorganisms in enzyme production is that, as microorganisms degrade the substrates, desired enzymes are released into solution (Renge *et al.,* 2012).

Subramaniyam and Vimala (2012), considers fermentation as a technique employed in the conversion of biological substrates (complex in nature) into simple compounds by microorganisms such as bacteria and fungi and in the process of this breakdown, secondary metabolites such as alcohol and carbon dioxide are released. A major advantage of fermentation is that medium compositions and culture conditions such as temperature, pH, dissolved oxygen and build-up of waste metabolites that influence cell growth and product synthesis can be examined and controlled to enhance product synthesis (Rosso *et al.*, 2002; Danquah and Forde, 2007). The main methods used for enzyme production are the submerged and solid state fermentations.

Microorganism cultivation in liquid broth requiring high volume of water, continuous agitation and generation of lot of effluents in the process is referred to as submerged fermentation; while the growth of microorganisms on/or within solid substrates leading to the formation of products under aerobic condition in the absence, or near absence of free water and which generally does not require aseptic conditions for enzyme production is known as solid state fermentation (Mudgett, 1986).

Some advantages of solid state fermentation (SSF) over submerged fermentation (SmF) include; high concentration of product, high volumetric productivity, cheaper product recovery, lower capital investment, less effluent generation and simple fermentation equipment (Cook, 1994).

The biotechnological exploitation of solid state fermentation has, however, been limited despite the many advantages it offers both economically and environmentally (Aidoo *et al*, 1982; Pandey, 1992). Below are some of the reasons:

- Limitation on microorganism
- Medium heterogeneity and
- Scale up problems.

The production of pectinase in developing countries like Ghana using agro-wastes would go a long way in promoting the food, leather, feed and textiles industries. This is because breaking of the pectin content of the raw materials involved in these industrial processes would be enhanced, thereby leading to increase in productivity and also reduction in the cost of the overall product(s) from these industries.

1.2 STATEMENT OF PROBLEM

Despite the wide range use of enzymes including pectinases in pulp and paper industries (Ricard *et al.,* 2005), bio-bleaching of kraft pulp (Amanjot *et al.,* 2010), preparation/clarification of fruit and vegetable juices in order to increase juice yield (Alkorta *et al.,* 1998; Kashyap *et al.,* 2000), degumming of plant fiber and in waste water treatment (Hoondal *et al.,* 2002), their application in Ghana is "relatively" non-existent.

The main reasons are:

- Pectinase production locally (by SSF) has not been exploited despite the abundance of pectin containing substrates.
- Imported enzymes add to cost of production

- Instability in power supply leading to enzyme instability on storage

Previous trials show pectinase influence in increasing the energy and amino acid availability from vegetable proteins to broiler chickens, thereby reducing the need for feed formulation with lysine and sulphur amino acids (Cowan *et al.,* 1999). It is encouraging to know that, pectinase production by submerged fermentation (SmF) has been investigated (Acuna-Arguelles *et al*., 1995; Joshi *et al*., 2013; Ezugwu *et al*., 2014), but its production by SSF has not been exploited to any large extent, though reports indicate enzyme activity is higher in SSF than SmF (AcunaArguelles *et al*., 1995). Additionally, substrates for SSF for enzyme production are readily abundant (Sarvamangala and Agasar, 2006); however, no comprehensive study on pectinase production in SSF by *Saccharomyces cerevisiae* ATCC 52712 has been carried out.

1.3 PURPOSE OF STUDY

- To investigate optimal conditions needed for pectinase production on corn cobs supplemented with orange peels and study the efficacy of the use of the enzyme produced in the extraction of orange juice.

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Specific objectives

- To assess the effect of fermentation time and substrate supplementation with orange peels on optimal activity during pectinase production by SSF.
- To study the optimal conditions of pH, temperature and inoculum concentration on pectinase production and activity by SSF.
- To assess the effect of period of agitation during extraction on enzyme activity.
- To investigate the best solvent for enzyme extraction from the fermentation medium.

- To produce pectinase under the established optimal conditions and study its efficacy in orange juice extraction process.

1.4 JUSTIFICATION

Both orange peels and corn cobs are agro wastes in Ghana which could be put to alternative use as substrates for enzyme production locally through SSF. Additionally, *Saccharomyces cerevisiae* ATCC 52712, a common yeast strain, can be capitalized on for use in simple cultivation methods to produce pectinases, since most pectinase production uses *A. niger* or bacteria which are not as easy to handle compared to *Saccharomyces cerevisiae*. The success of the study can pave way for pectinase production locally for industrial applications, when the fermentation process has been optimized for best enzyme production.

CHAPTER TWO

2.0 LITERATURE REVIEW

The increasing energy demands have focused worldwide attention on the utilization of renewable resources, particularly agriculture and forest residues; the major components of which are cellulose, starch, lignin, xylan and pectin (Pedrolli *et al*., 2009). These materials have attracted considerable attention as alternative feedstock and energy source, since they are abundantly available. Many microbes are capable of utilizing these substances as carbon and energy sources by producing a vast array of enzymes in different environmental niches (Antranikian, 1992; Kaur *et al*., 2004).

2.1 PECTIC SUBSTANCES

The term “pectin” was introduced by Henri Braconnot because of the gelling properties of these substances (Braconnot, 1825). Compounds acted upon by pectinolytic enzymes are generally termed “pectic substances” (Reddy and Sreeramulu, 2012). Complex high molecular mass glycosidic macromolecules found in higher plants are often also referred to as pectic substances (Khodjaeva *et al.,* 2013). They are found in the primary cell wall and are the main components of the middle lamellae, a thin extracellular adhesive layer formed between the walls of adjacent young cells in the form of calcium pectate and magnesium pectate (Rastogi, 1998). In short, the structural integrity and cohesion of plant tissues is largely due to the presence of pectic substances (Rombouts and Pilnik, 1980; Semenova *et al*., 2006).

Pectins constitute a large part of some algal biomass (up to 30%) and occur in low concentration in forestry or agricultural residues and mainly found in fruits and vegetables (Arunachalam and Asha, 2010; Horikoshi, 1990). Polysaccharides from cell walls of ripe pears have been reported to contain 11.5 % pectic substances, 21.4 % glucosan, 3.5 % galactan, 1.1 % mannan, 16.1 % lignin, 21 % xylan and 10 % arabinan (Horikoshi, 1990). It has been observed that, the relative molecular mass of most pectic substances ranges from 25 to 360 KDa, as shown for some pectic substances in Table 2.1.

Table 2.1. **Molecular weight of pectic substances in some fruits**

Source	**Molecular weight (KDa)**
Apple and lemon	200-360
Pear and prune	25-35

Orange	40-50
Sugar beet pulp	40-50

Source: Sakai *et al.* (1993).

The classifications of pectic substances into 4 main types by the American Chemical Society are explained below:

i. Protopectin: refers to pectic substance present in intact tissue and not soluble in water. Pectin or pectic acids are the products obtained on restricted hydrolysis of protopectin.

ii. Pectic acid: is the soluble polymer of galacturonans that contains negligible amount of methoxyl groups. Normal or acid salts of pectic acid are called pectates.

iii. Pectinic acid: is the polygalacturonan chain that contains greater than zero "0" and less than 75 % methylated galacturonate units. Normal or acid salts of pectinic acids are referred to as pectinates.

iv. Pectin (polymethyl galacturonate): in this class of pectic substances, at least 75 % of the carboxyl groups of the galacturonate units are esterified with methanol on the polymeric material. This accounts for rigidity on the cell wall when bound to cellulose (Alkorta *et al*., 1998).

It is important to note that pectin is present in all plants, but the content and composition varies depending on the species, variety, maturity, plant part and growing conditions (Holloway *et al*., 1983).

Pectin is naturally found as protopectin (water insoluble pectic substance) in unripe fruits, bound to cellulose microfibrils thus accounting for rigidity on cell walls (Pedrolli *et al.,* 2009). What occurs during ripening is that, the pectin structure is altered by fruit enzymes leading to breaking of the pectin backbone or side chains giving rise to a more soluble molecule (Kashyap *et al*., 2000).

It is necessary to point out that, water and solutes are trapped due to the three dimensional crystalline network formed when portions of homogalacturonan (linear polymer of D-galcturonic acid) are cross-linked, leading to pectic substances forming a gel structure (Jayani *et al*., 2005; Pedrolli *et al.,* 2009).

Pectic polysaccharides have been reportedly used as detoxifying agents and bioactive food ingredients and also found to be adequate infant food supplements (Gummadi and Panda, 2003).

2.1.1 Industrial Uses and Application of Pectins

Owing to the colloidal properties of pectins, they can be applied as jelling agents in the production of jams and thickening foods. Examples of such foods include cake, ketchups, yoghurt and fruit jelly (Holloway *et al*., 1983). Pectin can also be used to increase the foaming power of gases in water (Madhav and Pushpalatha, 2002); for medicinal uses such as defecation improving effect (due to its strong activity as dietary fiber), repression effect of the level of cholesterol content of blood and hypertensive repression effects (Uchida and Watebe, 1998).

2.2 PECTINASES

For many decades, microbes have been used for pectinases production (Janani *et al*., 2011; Reddy and Sreeramulu, 2012). As reported by Jayani *et al.* (2005), microbial pectinases account for 25 % of the global food enzyme sales. Many microorganisms such as bacteria, yeasts and moulds have been found to be capable of producing pectinases (Saranjaj and Naidu, 2014). Aside the definition of pectinases by Pedrolli *et al.* (2009), pectinase can also be simply defined as a group of enzymes that act on pectin and depolymerize it by hydrolysis and trans-elimination as well as de-esterification reactions, thereby hydrolyzing the ester bond between methyl and carboxyl groups of pectin (Ceci and Loranzo, 2008).

2.3 CLASSIFICATION OF PECTINASES

i. Protopectinases

Protopectinases act on protopectin by solubilizing the substrate to soluble pectin that is highly polymerized (Alkorta *et al.,* 1998; Kashyap *et al.,* 2000). They are categorized into two: one reacts with the polygalacturonic acid region of protopectin (A type) while the other reacts with the polysaccharide chains that may connect the polygalacturonic acid chain and cell wall constituents (B type) (Sakai *et al.,* 1993).

ii. Pectin Methyl Esterases (PME)

This class of enzyme gives rise to pectic acid and methanol by de-esterifying the methoxyl group of pectin. This enzyme acts preferentially when a methyl ester group of galacturonate unit is next to a non-esterified galacturonate unit. Since both polygalacturonase and pectate lyases need nonesterified substrates to function, PME acts before these enzymes (Kashyap *et al.,* 2000).

iii. Polymethylgalacturonases (PMG)

Polymethylgalacturonase catalyzes the hydrolytic cleavage of α-1, 4-glycosidic bonds in pectin backbone, preferentially highly esterified pectin, forming 6-methyl-D-galacturonate (Jayani *et al.,* 2005). The yeast *Saccharomyces cerevisiae* (ATCC 52712) has been reported to produce this type of pectin enzyme (Dzogbefia *et al.,* 1999; Dzogbefia *et al.,* 2001; Dzogbefia *et al.,* 2008). **iv. Polygalacturonases (PG)**

Polygalacturonase produces D-galacturonate by catalyzing the hydrolysis of α-1, 4-glycosidic linkages in polygalacturonic acid. Both groups of these hydrolase enzymes (PMG and PG) have been found to act in endo- or exo- modes. Endo-PG and endo-PMG catalyze random cleavage of substrate; exo-PG and exo-PMG produce monogalacturonate or digalacturonate (in some cases) by catalyzing the hydrolytic cleavage at the non-reducing end of the substrate (Rombouts and Pilnik, 1980; Kashyap *et al.,* 2000).

v. Pectate Lyases (PGL)

Pectate lyase gives rise to unsaturated product by cleaving glycosidic linkages preferentially on polygalacturonic acid (Pedrolli *et al.,* 2009).

vi. Pectin Lyases (PL)

The random cleavage of pectin is catalyzed by pectin lyase, preferentially highly esterified pectin producing unsaturated methyl-oligogalacturonates through trans-elimination of glycosidic linkages (Van-Alebeek *et al*., 2002; Jayani *et al*., 2005; Sinitsyna *et al*., 2007).

2.4 MODE OF ACTION OF PECTINASES

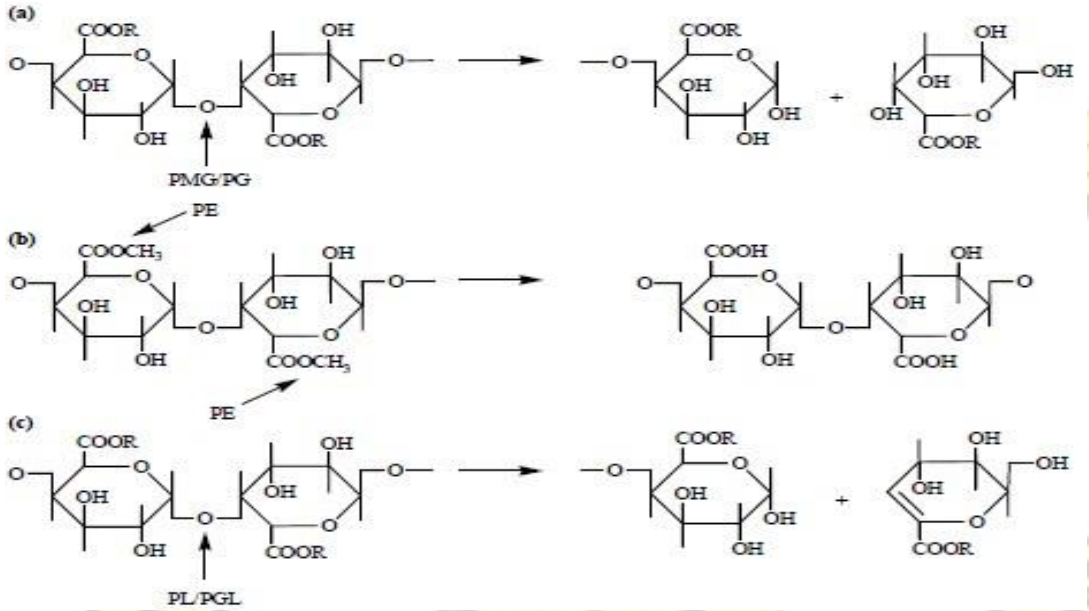

Figure 2.1. Some pectinases and mode of action

a) R=H for PG and CH_3 for PMG; b). PE cleaves the bond between COO^- and CH_3; and c) R=H for PGL and CH_3 for PL.

NB: The arrow indicates the place(s) where the pectinase reacts with the pectic substances.

PMG, polymethylgalacturonases; PG, polygalacturonases; PE, pectinesterase; PL, pectin lyase (Lang and Dörnenburg, 2000).

The above mode of action of pectinases shows that various forms of pectic substances occur in plants, and this could probably account for the different forms of pectinases produced.

On the other hand, Alkorta *et al*. (1998) classified the pectinases as being of three types: depolymerizing enzymes (pectinases; hydrolases and lyases); de-esterifying enzymes (pectinesterases) and protopectinases. The depolymerases split the ß-(1, 4) glycosidic bonds between galacturonic monomers in pectic substances by either hydrolysis (hydrolases) or ßelimination (lyases). The hydrolases are also divided into two groups: those preferring pectate are called polygalacturonases and those preferentially degrading pectin are called polymethylgalacturonases.

The solubilization of protopectin is catalyzed by protopectinase, with polygalacturonase the most common type among all pectinolytic enzymes. The trans-eliminative cleavage of the galacturonic acid polymer is catalyzed by lyases (Pastore, 2001).

2.5 SOURCES OF PECTIN ENZYMES

Microorganisms have been identified as the most common source of enzymes for industrial use: 50 % from moulds and yeasts, 35 % from bacteria and 15 % are either of plants or animal origin (Smith and Aidoo, 1999). In pectinase production, the most studied microorganisms are the moulds with *Aspergillus niger* being the most prominently used in pectinase production (Blanco *et al.,* 1999).

2.5.1 Yeasts as a sources of pectinase

Moulds and bacteria have been known to secret pectinases, but they also occur in some yeasts

(Fernández-González *et al.,* 2004; Jayani *et al.,* 2005; Oliveira *et al.,* 2006). Certain strains of *Saccharomyces cerevisiae* have been found to break down polygalacturonic acid, which could be important for the fermentation of plant-derived substrates (McKay, 1990; Fernández-González *et al.,* 2004). It has been clearly shown that when the enzyme extract from *Saccharomyces bayanus* is added to fresh mash, the effects on turbidity are the same as when a commercial enzyme preparation is added (Gainvors *et al.,* 1994).

In another study by Blanco *et al.* (1999), they also viewed yeasts as good sources of pectinases and more advantageous than *A. niger,* because in the process of producing pectinases from *A. niger*, many other non-pectin enzymes are produced which are not of interest. However, a fairly recent report indicates that effective technology was used to produce pectinase and cellulase with *Saccharomyces cerevisiae* for citric acid production (Magdy, 2011). *Saccharomyces cerevisiae* (ATCC 52712) has also been reported to produce polymethylgalacturonase (Dzogbefia *et al.,* 2001; Dzogbefia *et al.,* 2008).

2.6. METHODS OF PECTIN ENZYME PRODUCTION

Renge *et al.* (2012) stated that fermentation is a method of generating enzymes for industrial purposes. Ward (1992) supported the fact that the mode of pectin enzyme production has been fermentation, in which the microorganism degrades the complex substrate to produce energy.

Many of the useful enzymes (including pectinases) have been produced with *Aspergillus niger* (Abe *et al*., 1988; Tjamos *et al*., 2004; Perrone *et al*., 2006). Nowadays pectinase is one of the most sought after enzymes in food processing industries (Khan *et al*., 2012). The two major types of fermentation methods used in pectinase production are the solid state fermentation (SSF) (Soccol and Larroche, 2008) and the submerged fermentation (SmF) (Rangarajan *et al*., 2010).

The term solid state fermentation (SSF) is used to depict the processes by which insoluble materials in water are used to aid the growth of microorganisms for the production of useful products (Moo-Young *et al.,* 1983). This technique simulates the natural microbiological processes like composting and ensiling (Toca-Herrera *et al.,* 2007). In this type of fermentation, the solid bed in which the microorganisms grow though water is needed, it is not expected to exceed saturated point (Laukevics *et al.,* 1984). It is important to emphasize that water is vital for microbial growth and in SSF it is present in thin layers and in some instances absorbed inside the substrates (Mudgett, 1986). In SSF, it is also important to know that the produced metabolites remain in a solid matrix, and must therefore be extracted by solid-liquid extraction or leaching. This is the first step in any recovery and purification process that is intended for any desirable metabolite produced by SSF (Castilho *et al.,* 1999; Gupta *et al.,* 2008).

For SmF, the nutrients and microorganisms are both submerged in water (Grigelmo-Miguel and Martin-Belloso, 1998). SmF utilizes free flowing liquid substrates, such as molasses and broths. The bioactive compounds are secreted into the fermentation broth. The substrates are utilized quite rapidly hence, the need for constant supplementation/replacement with nutrients. This fermentation technique is best suited for microorganisms such as bacteria that require high moisture content (Subramaniyam and Vimala, 2012).

SSF presents some advantages over SmF, viz;

- There is high growth rate of organism with large quantity of enzyme produced
- Low water content of SSF substrates is a selection factor that prevents undesired bacterial contaminations

- The substrates used are simple and inexpensive
- Less water requirement
- The volume of effluents generated is low
- SSF system (with regards to the substrates used) is closer to the natural habitat of microbes, thus aiding the production of certain enzymes and metabolites from the fermented solids (Archana and Satyanarayona, 1998).
- Another important advantage of SSF over SmF is that, metabolites from the fermented solid materials are easier to recover from the medium (Praveen *et al*., 2008).

Despite the above advantages however, SSF also has some disadvantages, namely:

- It favours only microorganisms that grow in low moisture levels (Babu and Satyanarayana, 1996)
- The substrate/substrates usually require pre-treatment
- Frequent need of high inoculum concentrations
- Difficulty encountered in the removal of metabolic heat as compared to SmF
- Aeration can be difficult due to the high solid concentration in some SSF processes with longer cultivation than in SmF (Pérez-Guerra *et al*., 2003).

In summary, almost all industrial enzymes can be produced in SSF using wild type microorganisms (Filer, 2001; Pandey *et al.,* 2001).

2.7 SUBSTRATES FOR ENZYME PRODUCTION IN SOLID STATE FERMENTATION

The best substrates for SSF processes are generally agro-industrial residues. Evidence available showed that, though enzymes such as pectinases can be produced from different carbon sources, they are inducible (Janani *et al*., 2011). Some of the substrates that have been used for the

production of enzymes include; wheat bran for polygalacturonase production (Hours *et al.,* 1994), rice bran for protease (Ikasari and Mitchell, 1998) and citrus waste in polygalacturonase production (Garzon and Hours, 1991), among many other substrates used for the production of many other enzymes as reported by Ashok *et al.* (2004). In addition, some other substrates used for pectinase production include; sugarcane bagasse, wheat straw, corn cobs, tea waste rice straw, coconut coir pith, banana waste, saw dust, and orange peels (Pilar *et al.,* 1999).

2.7.1 Corn cobs

Corn cobs (*Zea mays*) are regarded as one of the potential agricultural biomass feedstock for renewable energy industries in the U.S. to abate energy and the greenhouse gas problems (Christiansen, 2009). About 15 to 20 % of aboveground corn residues (non-grain) are corn cobs (Sokhansanj *et al.,* 2002; Pordesimo *et al.,* 2004; Pordesimo *et al.,* 2005). Cobs account for about 8 to 9 % of the above ground dry matter (grain plus residues) at grain physiological maturity (Hanway, 1963; Pordesimo *et al.,* 2005). The average yield of corn cobs may range from 1.42 to 1.53 tones per hectare (Shinners *et al.,* 2006). For every 100 kg of corn grain processed, approximately 18kg of corn cobs is generated (Knob *et al*., 2014). Large quantities of corn cobs produced have negligible value and are mostly discarded, with small quantities sold at very low prices for supplementing animal feed or used as fertilizer (Topakas *et al*., 2004; Ashour *et al*., 2013). Though the time at which corn cobs are harvested determines the moisture content, corn cobs have been generally reported to have moisture content of about 20 to 55 % (Johnson and Lamp, 1966; Morey and Thimsen, 1980).

2.7.2 Orange (peel)

Most fruits are known to be highly perishable; their wastes are a problem to processing industries and pollution monitoring agencies (Apsara and Pushpalatha, 2002; Chikku, 2014). Suitable

methods have to be adopted to utilize them for conversion into value-added products (Konno *et al.,* 1981). By-product recovery from fruit wastes can improve the overall economics of processing units. Beside this, the problem of environmental pollution can also be minimized. A valuable by-product that can be obtained from fruit "waste" such as orange peel is pectin, since it exists in varying amounts in fruit cell walls. While the percentage of pectin in fresh orange pulp is between 3.5-5.5 %, the percentage of pectin on dry weight basis is 30-40 % (Pilnik and Voragen, 1993; Girdhari *et al*, 1998).

Pectic substance in fruits was discovered by French chemist Louis Nicolas Vauquelin in 1790 in tamarin fruit (Vauquelin, 1790). The pectin content in orange and lemon peels were previously exploited by treating citrus peels with 96 % ethanol and the obtained alcohol-insoluble solids (AIS) subjected to sequential extraction with hot distilled water and 0.5 % HCl (Georgiev *et al*. 2012).

Table 2.2.Comparison of pectin content in some fruits.

Fruit	**% pectin**	**References**
Apple	0.63-1.15 (CaP)	Gautam *et al.* (1986)
Banana	0.58-0.89 (CaP)	Kertesz (1951)
Blackberry	0.40-1.19 (CaP)	Money and Christian (1950)
Grape	0.70-0.80 (AGA)	Silacci and Morrison (1990)
Lemon	0.63 (AGA)	Vollendorf and Marlett (1993)
Orange	0.25-0.76 (CaP) 0.57 (AGA)	Ross *et al.* (1985); Money and Christian (1950)

(CaP= calcium pectate; AGA= anhydrogalacturonic acid)

2.8. PRETREATMENT OF SUBSTRATES FOR SSF

Ward (1992) described some pretreatment methods for substrate(s) presentation in a suitable form for fermentation processes:

- Reduction of substrate size to increase availability by chopping, grinding, chemical and enzymatic hydrolysis.
- Supplementation of the medium with nutrients such as phosphorus, nitrogen and salt.
- Setting the pH through addition of mineral solution (and/or adjusting the moisture content using distilled water).
- Vapour treatment of the macromolecules to degrade and eliminate some of the major contaminants.

2.9 Some major factors affecting the production of pectinases

The use of surfactants such as Tween-20 has been found to increase the production of pectinase due to favorable effect on cell membrane permeability, thereby enhancing enzyme secretion (Apoorvi and Vuppu, 2012). The microbial sources used, for instance; wild type, recombinant, mutagenized along with various parameters considered during enzyme production, such as pH, metal ions and temperature affect pectinase production. The extent to which an enzyme degrades its substrates is, however also enhanced by agitation (Sonia *et al.,* 2009; Apoorvi and Vuppu, 2012). It is therefore important to consider these factors in detail as outlined below:

i. The type of substrate used for fermentation

The culture medium used for a particular organism may not be appropriate for another, hence the need to understand the type of medium used for different organisms; grape pomace is nutrient

medium for *Aspergillus awamori* (Botella *et al.,* 2005; Suneetha and Zaved, 2010), potato dextrose agar media for *Mucor flavus* (Ramchandra *et. al.,* 2003), yeast extract and wheat bran for *Bacillus subtilis* (Sonia *et al.,* 2009), and sabouraud dextrose agar for *Penicillium viridicatum* RFC3 (Denis *et al.,* 2005).

Carbon sources observed in a study (Des *et. al.*, 2003) showed that PGA (polygalacturonic acid), lactose and pectin increased pectinase production. Glycine and ammonium nitrate were, however, reported to inhibit pectinase production, while wheat bran, peptone, ammonium chloride and yeast extract enhanced its production (Des *et al.,* 2003). The use of pectin has been found to greatly induce pectate lyase as compared to polygalacturonic acid (Sunneetha and Zaved, 2010). **ii. Effect of pH during pectinase production**

Secretion of a particular pectic enzyme by test organism in culture and amount of activity of the crude enzyme extract are largely influenced by various components in the culture media including the pH of the broth and the incubation periods (Mehta, 1973).

Except for exoPGase secreted by *Fusarium oxysporum* and endoPGase from *Bacillus licheniformis,* all PGases have pH between 3.3 to 7 (Apoorvi and Vuppu, 2012). The biobleaching industry uses xylano-pectinolytic enzymes in their production and the enzymes were optimally reported to have been produced at pH 8.5 (Amanjot *et al.,* 2010).

The three PME isoforms have optimum pH between 6.5-9.0 and this optimum pH range has been found to be dependent on salt concentration, thus affecting the PME activity due to masking of the carboxylic charged groups by the salt thereby inhibiting enzyme from acting on substrates (blockage of the substrate recognition site on the enzyme) (Bruna *et al.,* 2008). Enzymes such as pectate lyases have best activity at pH of 8.5; moulds and yeasts produce PGase with acidic pH and for pectinase production by *Bacillus subtilis*, the production was observed at pH 9.5 (Suneetha

and Zaved, 2010). Most bacteria grow and produce pectinase optimally at pH between 7-10 (Sonia *et al.,* 2009), while some strains of bacteria such as *Streptomyces* sp and *Aspergillus aculeatus* produce PGase actively at pH of 3.0 (Ernesto *et al.,* 2006). This therefore shows the effect of pH on the growth and production of pectinases.

iii. Effect of temperature during pectinase production

The effect of temperature on pectinase production cannot be overlooked during its production since enzymes are proteins. In fruit juice industries (Saad *et al.,* 2007; Suneetha and Zaved, 2010) and wine processing, the sensitivity of *M. rouxii* which is sensitive at 30 ºC and effective at 20 ºC is used (Saad *et al.,*2007). *Bacillus subtilis* produces pectinases optimally at 37 °C (Sonia *et al.,* 2009), while the temperature at which pectate lyase was found to be optimally produced was at 70 °C (Suneetha and Zaved, 2010). Pectinase has been produced from *Streptomyces* sp having best activity at 60 °C; with PGase from moulds having optimum activity at 50 °C and between 40 °C to 60 °C for yeasts (Ernesto *et al.,* 2006), though Magdy (2011) reportedly produced pectinase and cellulase optimally at 25 °C.

iv. Effect of metal ions on pectinase production

As the activity of endo PGase is reduced by Cu^{2+} and Hg^{2+}, metal ions such as Zn^{2+} and Mg^{2+} also inhibit enzyme production due to the involvement of the thiol group in the active site of the enzyme (Sonia *et al.,* 2009; Suneetha and Zaved, 2010). Mn^{2+} increases the PGase activity; however, Li^{2+}, Fe^{2+}, Rb^{2+} have no effect on the activity (Sonia *et al.,* 2009). When the metal ions are in high concentration, enzyme production usually reduces due to blockage in protein secretion; though bacteria need Ca^{2+}in appropriate amount for growth and production of pectate lyases (Suneetha and Zaved, 2010), fungi do not need $Ca^{2+}$for growth (Apoorvi and Vuppu,

2012). This shows that the type of metal ion and concentration for a particular organism's growth and enzyme production should be carefully taken into consideration.

v. Effect of fermentation time and substrate composition on pectinase production

Penicillium atrovenetum, *Aspergillus flavus* and *Aspergillus oryzae* produced polygalacturonase optimally on the 5th day and endoglucanase on the 7th day of fermentation from orange peels according to Adebare *et al.* (2012).

In a study by Nazneen *et al.* (2011), solid state fermentation was undertaken with seven different strains of fungi to determine the best strain with maximum pectinase activity and was obtained with *Aspergillus niger* IM-6 after incubation for 7 days at 40 ˚C in 750 ml conical flask, maximum enzymatic activity was obtained after 7 days of incubation at 30 °C in 250 ml Erlenmeyer conical flask with *Aspergillus niger* (ATCC 16404); though one percent dextrose was used as carbon source, citric acid as carbon source showed better results, but starch was not productive in relation to cost.

However, substrate combination in the ratio of 9:1:1:1 of wheat bran, fresh mosambi, orange and lemon peels showed good result in solid state fermentation (Ashfaq *et al.,* 2012).

In another study, optimal pectinase production was achieved in 72 hours of SSF by *Aspergillus awamori* using substrate combination of 85 % rice bran and 15 % sugarcane bagasse (Suresh and Viruthagiri, 2011).

Pectinase production studied in solid-state fermentation process with wheat bran and sugarcane bagasse as substrates utilizing *Aspergillus niger,* showed that mixed substrates of 90 % of wheat bran and 10 % of sugarcane bagasse gave maximum pectinase yield during 96 hours fermentation period (Baladhandayutham and Thangavelu, 2010).

vi. Effect of some solvents used for pectinase extraction

Various solvents have been used for pectinase extraction. For instance distilled water, acetate buffer (0.05 M), Tween 80 and 40, glycerol among many others (Rezende *et al*., 2002; Silva *et al*., 2005; Giese *et al*., 2008; Patil and Chaudhari, 2010; Ahmed and Mostafa, 2013). Comparing distilled water, acetate buffer (0.05M), Tween 80 (0.05 %), Tween 40 (0.05 %) and glycerol (0.05 %) as solvents for pectinase extraction, acetate buffer was reported the best for pectinase extraction (Ahmed and Mostafa, 2013). Some enzymes produced under SSF have been recovered from the solid phase by treatment with distilled water (Silva *et al*., 2005; Patil and Chaudhari, 2010), de-ionized water (Giese *et al*., 2008), surfactant Tween 80 (Rezende *et al*., 2002) and buffer (Rezende *et al*., 2002; Phutela *et al*., 2005; Linde *et al*., 2007). While determining the best solvent for polygalacturonase extraction, Castilho *et al*. (2000) obtained highest activity with acetate buffer. Singh *et al*. (1999) on the other hand obtained the best activity using Na_2SO_4 (0.1 M). From the above studies, the solvents used helped to break the bond between carbohydrates and proteins (Solarito *et al.*, 2010).

However, more buffer/solvent for extraction has been shown to release more enzymes from fermented material (Ahmed and Mostafa, 2013). Therefore, solvent volume must be sufficient for complete enzyme extraction. Excessively large volume of buffer used for extraction would also yield an enzyme solution too dilute to be profitably utilized (Aikat and Bhattacharyya, 2000). Decrease in total activity when a lower volume of buffer was used for extraction has also been found (Ahmed and Mostafa, 2013); this might be due to insufficient solvent volume to penetrate the solid fermented mass.

vii. Effect of Inoculum size on enzyme production

Inoculum concentration has been shown to affect the production of pectinases by *A. niger* (Kiro, 2010). Inoculum concentration of 1 x 10^7 spores/ml contributed to the maximum xylanase activity relative to other concentrations, with least activity observed using 1 x 10^6 spores/ml (Maria *et. al*., 2006). Therefore, to optimize for enzyme activity, the need for spore concentration to be high enough to colonize the substrate particles is of importance for optimum enzyme production (Sikyta, 1983).

Many studies, however, indicated that there can be a decline in enzyme activity above an optimal spore concentration. For instance, Kuhad *et al*. (1998) obtained maximum xylanase activity by *Fusarium oxysporium* using 1 x10^7 spores/ml, but 2 x10^7 spores/ml led to decrease in activity.

2.9.1 Some factors worth considering for effective enzyme application

After production of the crude enzyme, it is equally important to enhance enzyme activity by concentrating the crude enzyme filtrate and examine the appropriate dosage for optimum reaction. Therefore, it is important to consider these factors in detail as outlined below;

i. Effect of ammonium sulphate precipitation on enzyme activity

The solubility of proteins is dependent on the amount of salt in the solution. The various charged groups on protein molecules are stabilized at low salt concentration thereby enhancing protein solubility, commonly referred to as *salting in*. A point is, however, reached where as more and more salt is added, insufficient water is available to make the protein soluble making the protein precipitate, commonly referred to as *salting-out* due to the presence of the excess salt (Jakoby, 1971; http://www.eng.umd.edu/~nsw/ench485/lab6a.htm accessed 4/07/2014).

According to Ezugwu *et al*. (2012), increasing ammonium sulphate concentration from 20 to 80

% led to increase in pectinase activity from 7.10 to 46.04 µmole/min, making 80 % ammonium sulphate saturation of the crude protein suitable for pectinase precipitation. In another study involving the purification of pectin methyl esterase (PME) with 20 to 80 % ammonium sulphate, as the percentage of ammonium sulphate saturation increased, the activity of PME (units/g) also increased from 8.25 for 0 % to 21.50 for 80 % (160.6 % increase) (Joshi *et al.,* 2011). However, an observable decrease in soluble protein content from 62mg/ml to 21mg/ml in the fraction of 80 % ammonium sulphate saturation of the crude protein was obtained (Joshi *et al.,* 2011). A 30 % yield of purified pectinase using $(NH_4)_2SO_4$ fractionation has also been reported (Singh *et. al*., 1999).

Crude protopectinase preparation obtained from *Kluyveromyces marxianus* cultures was also partially purified by fractional precipitation with ammonium sulphate and at 65 % ammonium sulphate saturation, the most active fraction was obtained (Kabli, 2007). In the production of pectinase from orange peels that is, with respect to the crude enzyme produced, ammonium sulphate saturation of the crude and dialysis of the enzyme showed specific activities of 26.47, 28.78 and 46.02 units/mg protein respectively; dialysis showed further increase in purification fold to 1.74 as against 1.08 for ammonium sulphate saturation of the crude enzyme (Ezugwu *et. al*., 2014).

Aside ammonium sulphate which is used for precipitation of crude protein/enzyme, ethanol (Englard and Seifter, 1990) as well as ethylene glycol (Walker, 2002) could also be used. **ii.**

Effect of enzyme dosage for effective juice extraction

In a study by Joshi *et al*. (2011), pectin enzyme dosage of 2.5 % was the best for apple juice extraction. Clarifications of apple and pear juice, however, were optimized at 1.0 and 0.5 % respectively (Joshi *et al*., 2011). Since plants cells are united together through the middle lamella consisting of xylan and pectin, treating fruits such as apples and pears with the appropriate pectin

enzyme dosage leads to optimum pectin hydrolysis or destruction of the middle lamella (leading to separation of cells) when observed with the scanning electron microscope resulting in the extraction of more juice (Jacob *et al.,* 2008; Azzaz *et. al.,* 2013).

2.10. SOME ASSAY METHODS FOR PECTIN ENZYMES

After producing pectin enzymes, the need for assay in order to characterize the activity of the enzyme cannot be overlooked (Apoorvi and Vuppu, 2012). Some of the assay methods are listed below:

I. Polymethylgalacturonase (PMG):-

The activity of PMG can be assayed by measuring reducing groups released by dinitrosalicylic acid method (Miller, 1959). In the dinitrosalicylic acid (DNSA) method, half milliliter of 1 % pectin in 0.1 M citrate buffer (pH5.8) is placed in a test tube and 0.5 ml of culture filtrate added. The culture filtrate is then incubated at 50 ^{o}C for 30 minutes and termination of the reaction by adding 1.5 ml DNSA reagent, followed by heating the tubes at 100 ^{o}C in boiling water bath for 15minutes plus cooling at room temperature and reading absorbance at 575 nm (Oyeleke *et al*., 2012).

A standard calibration curve is plotted by using galacturonic acid (GA Sigma). One unit of PMG is equivalent to the amount of enzyme which releases 1 µmol of GA per minute (Maria *et al*., 2013).

II. Pectate lyase (PL).

Pectate lyase activity can be assayed by the spectrophotometric method of Macmillan and Phaff, (1966).

In this assay type, a stock solution of substrate is prepared by mixing 150 ml sodium polygalacturonate, 90 ml 0.1 M Tris buffer and 30 ml 0.005 M $CaCl_2$. To 2.7 ml of substrate solution, a volume of 0.3 ml diluted enzyme is added and the increase in absorbance measured at 235 nm at 25 °C (Maria *et al*., 2013). One unit of pectate lyase (PL) activity is the amount of enzyme which produces1 µM of unsaturated product per minute at pH 8.0.

III. Pectin Esterase (PE)

Assay for pectin esterase can be carried out using colour change of a pH indicator (bromocresol green) added to the reaction mixture since carboxyl groups are being released during the reaction and using 0.5 % (w/v) citrus pectin (Sigma) in water at pH 5.0 as the substrate (Vilarino *et al*., 1993).

2.11. SOME INDUSTRIAL APPLICATIONS OF PECTIN ENZYMES

Enzymatic reactions are generally carried out under mild conditions as they are highly specific. They work under mild conditions of temperature and pH and are readily denatured under extreme pHs and temperatures with small amounts of energy needed to carry out reactions even on industrial scale (Aehle, 2007). Some applications of pectin enzymes are outlined below: **i. Fruit juice industries**

Acidic pectic enzymes used in fruit juice industries and wine making often come from fungal sources, especially from *Aspergillus niger* (Sunil *et al.*, 2015). In sparkling clear juice (such as apple, pear and grape juices), suspended matter is usually removed to give sparkling clear juices (free of haze) and this can be easily achieved using pectin enzyme, having also an added advantage in that the enzyme increases the yield of juice during pressing (Kashyap *et al.*, 2000).

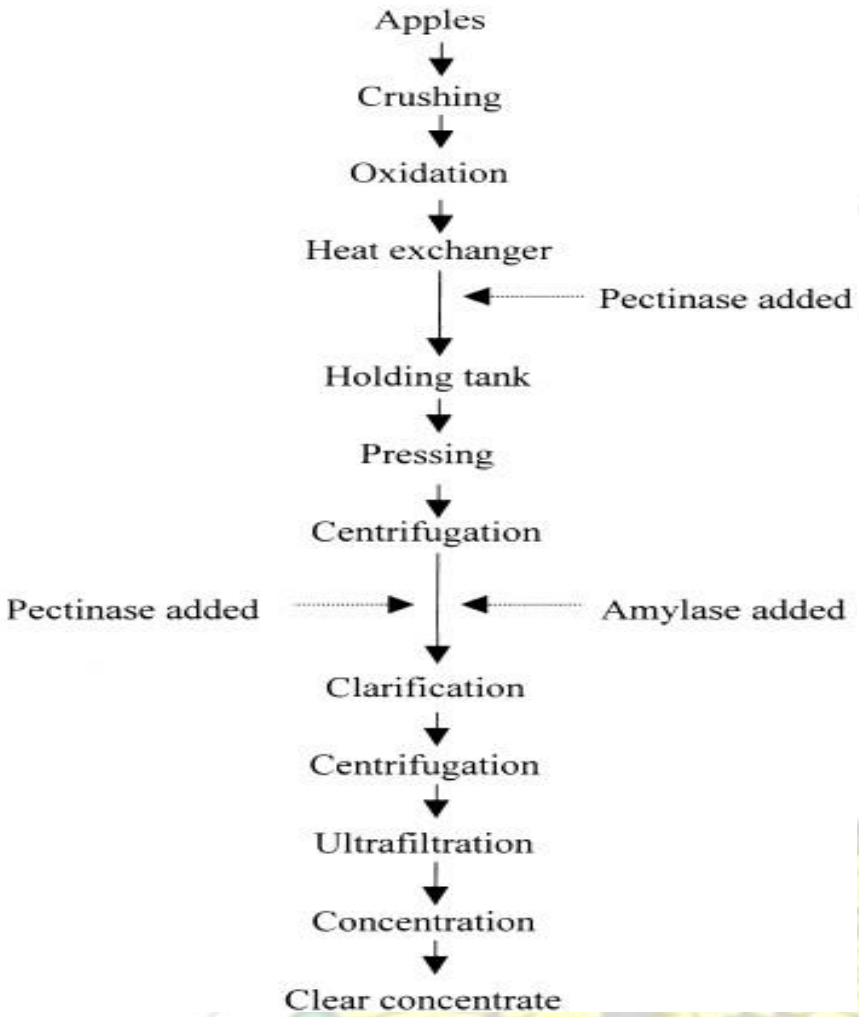

Figure 2.2. Stages of incorporation of pectinase in apple juice production (Grassin and Fauquembergue, 1996).

ii. Alkaline pectinases mainly from *Bacillus* spp have been used in the industrial sector for the following purposes:

A. Degumming and retting of fiber crops–in retting process, fermentation using certain bacteria (e.g. *Clostridium* or *Bacillus*) and some fungi (e.g. *Aspergillus* or *Penicillium*) degrade pectin of the bark and release fiber, of which pectinolytic enzymes secreted by these microbes are actively involved in the process (Sharma and Robinson, 1983). Commercially, dew retting (an aerobic process) can be used, where plant straw is exposed

to the action of fungi and aerobic bacteria for a few weeks (usually 2-10 weeks). Many species of microbes such as *Cladosporium*, *Penicillium* and *Aspergillus* have been isolated from dew-retted plants (Fogarty and Ward, 1972).

B. **Treatments of pectic wastewater-** The wastewater from the fruit processing industries contain pectinaceous materials that are barely decomposed by microbes during the activated-sludge treatment (Tanabe *et al.,* 1986). The usefulness of pectic substance removal from waste water containing pectinaceous substances has been proven through treatment in alkaline medium with extracellular endo-pectate lyase from *Bacillus* species. (GIR 621) (Tanabe *et al.,* 1987).

C. ***Coffee and tea fermentation-*** Pectinolytic microorganisms are often used for coffee fermentation to remove the pulpy layer of the bean (Kashyap *et al.,* 2000). Fungal pectinases are also used in the manufacture of tea owing to its ability to accelerate tea fermentation, although the dosage of enzyme used must be carefully monitored to avoid damage to the tea leaf. Improvement in the foam-forming property of instant tea powders by destroying tea pectins can also be achieved by addition of pectinase (Carr, 1985).

iii. Textile industry

Natural fiber treatment employed in the textile industry has been obtained with pectinases (Baracet *et al.,* 1991).

2.12. POTENTIAL MEDICAL APPLICATIONS OF PECTINASE IN HUMANS

It has been shown that, high number of negative charges and association with ions is enhanced when pectins are demethylated. Thus, it can behave as a weak cation exchange resin and depending on the pH conditions, chelate toxic ions or make available minerals in the gut (Khan *et al.,* 2013).

The splitting of the methyl group from pectins by pectinases to short chain fatty acids such as acetate, propionate and butyrate are more easily fermented by intestinal bacteria. Apart from the protection it offers the bowel against inflammatory diseases, it also modulates the release of gut hormones that control insulin release and appetite (Tolhurst *et al.,* 2012). The primary short chain fatty acid to reach the systematic circulation from the liver is acetate and thus, pectinases added to pectins find application in different food products for probiotic purpose (Khan *et al.,* 2013).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Chemicals

Some of the chemicals used for the study include; peptone (Biotechnica, USA), yeast extract (Andy Biotech-Xian Co. Ltd., China), dextrose agar (Biomark, China), Sodium hydroxide

(Qixian Country Dongfang Chemical Co. Ltd., China), copper sulphate (Jinzhou King Changsheng Chemical Co. Ltd., China), potassium sodium (+) – tartrate (Hubei Yuancheng Saichuang Technology, Co. Ltd., China), sodium chloride (Weifan Xinjiawei Co. Ltd., China), pectin and bovine serum albumin (BSA) from SIGMA-Aldrich, USA.

3.1.2 Microorganism

The pure yeast strain, *Saccharomyces cerevisiae* (ATCC 52712) was stored on agar slants in a freezer in the laboratory of the Biochemistry Department. It was originally purchased from the American Type Culture Collection, Maryland, USA; and has been maintained on agar slants at the Department of Biochemistry and Biotechnology, KNUST, Kumasi.

3.1.3 Substrates

Corn cobs and orange peels used for SSF were obtained from Tamale and KNUST campus respectively. However, the uniformly ripened but firm oranges for juice extraction were obtained from Kumasi Central Market.

3.1.4 Preparation of corn cobs and orange peels

They were separately washed, dried, milled and sieved to average particle size of 0.3 mm.

3.2 METHODS

3.2.1 Preparation of media

A yeast extract/peptone/dextrose agar medium was prepared by adding 5 g of yeast extract, 10 g of peptone, 10 g of dextrose plus 10 g of agar in 500 ml of distilled water and mixed properly for homogeneity by heating. The mixture was then autoclaved at 121 ºC for 15 minutes. The agar

slants were obtained by transferring 10 ml of the autoclaved liquid media into sterile tests tubes before allowing to cool overnight in a laminar hood.

3.2.2 Culturing of Sa*ccharomyces cerevisiae* (ATCC 52712)

Culturing of the yeast cells was undertaken in an inoculation chamber. A sterilized inoculation loop was used to scoop out a little of the microorganism from the frozen stock tube and then streaked on the prepared slants of yeast extract/peptone/dextrose agar. The tubes were incubated at 28 ºC in an incubator for the growth of the organism and then stored at 4 ºC for subsequent inoculum preparation and inoculation. Sub culturing was regularly done during the study.

3.2.3 Inoculum Preparation

The prepared agar slants were inoculated aseptically with yeasts cells. Marked growth observed on the agar slants after three days was further kept at 4ºC for later use.

3.2.4 Estimation of Yeast Cell Number

Colonies of yeast cells were picked from the cultured yeast slants using a sterilised inoculating pin and diluted with distilled water and the absorbance read with colorimeter (WPA Colourwave CO7500) at 660 nm against distilled water as blank. The absorbance determined was indicative of the cell concentration of the yeast cells measured as cells/ml. The turbidity of the yeast culture measured as the absorbance was correlated to the total number of yeast cells (www.pangloss.com/seidel/Protocols?ODvsCells.html accessed 20/09/2013).

This work was carried out in two stages, namely, production of the pectin enzyme under optimized conditions and its subsequent use in stage two for juice extraction.

3.3 PRODUCTION OF PECTIN ENZYME UNDER OPTIMIZED CONDITIONS

3.1 Solid state fermentation (SSF)

Five grams of corn cobs only and corn cobs supplemented with orange peels were separately measured into 100 ml Erlenmeyer flasks. To each parameter considered, four ml nutrient solution which consisted of NaCl (2.25 g), NH_4SO_4 (10.5 g), Na_3PO_4 (15.0 g) and Urea (2.25 g) dissolved in distilled water to the 100 ml mark was added to each of the measured substrates in the Erlenmeyer flasks, and the pH adjusted. Sterilization of substrates was carried out in a Gallenkamp autoclave at 121 ^oC for 15 minutes after which the flasks were allowed to cool. Inoculation of substrates was carried out with 2 ml of *S. cerevisiae* (ATCC 52712) cell suspension (1.52 x 10^7 cells/ml) followed by adjustment of the moisture content to 60 % in a laminar hood. The flasks were then incubated using the Gallenkamp incubator at 30 ^oC for different time intervals. At the end of each fermentation, the crude enzyme was extracted and pectinase activity and protein concentration determined. All determinations were carried out in triplicates. The details of how each parameter was investigated are described as follows:

3.3.1.1 Determination of optimal fermentation time

Fermentation was carried out for 15 days and the process monitored at 3 day intervals of 3, 6, 9, 12 and 15 days to determine the best time for solid state fermentation with pH of 4.5 and temperature of 30 ^oC using corn cobs as substrate. The Erlenmeyer flasks containing the fermented substrates were each harvested by diluting in 100 ml acetate buffer and the filtrates assayed for pectinase activity and protein concentration.

3.3.1.2 Ratio of corn cobs to orange peels determination

With total substrate weight of five grams in Erlenmeyer flasks, the ratio of corn cobs to orange peels was calculated in percentages viz; 90:10, 80:20, 70:30, 60:40 and 50:50 respectively and incubated for 6 days as determined for optimum fermentation time, pH 4.5 at 30 ºC.

3.3.1.3 Effect of pH on pectinase production

The effect of pH was studied at seven different pH levels: 3.0, 3.5. 4.0, 4.5, 5.0, 5.5, and 6.0. Solutions of 1 M HCl and 1 M NaOH were used for pH adjustments (with HANNA HI 83141 pH-meter) of the substrates after adding 4 ml nutrient solution which consisted of NaCl (2.25 g), NH_4SO_4 (10.5 g), Na_3PO_4 (15.0 g) and Urea-(2.25 g), all dissolved in distilled water to the 100 ml mark and incubated for 6 days at 30 ºC.

3.3.1.4 Effect of temperature on pectinase production

The Gallenkamp incubator was used for incubation at different temperatures (20 ºC – 50 ºC). The effect of temperature on pectinase production was determined by using substrate combination of 80 % corn cobs and 20 % orange peels, the pH was adjusted to 4 with 1 M HCl and 1 M NaOH and sterilization of the content in Gallenkamp autoclave at 121 ºC for 15 minutes and incubating at the temperature range of 20 ºC to 50 ºC for 6 days.

3.3.1.5 Determination of optimal inoculum size on pectinase production

Inoculum sizes of 1.28 x 10^6 to 18.50 x 10^6 cells/ml using the method described for estimation of yeast cell number were used for optimum inoculum size determination for pectinase production.

3.3.1.6 Preparation of crude extract

Generally, crude enzyme extracts were prepared from the solid fermented media by adding 100ml of acetate buffer (pH 5.0) to each flask, agitated for 5 minutes using the Fischer Scientific mini vortexer and filtered with F1001 grade qualitative filter paper prior to buffer optimization.

From each crude filtrate, total protein and pectinase assays were carried out.

3.3.1.6.1 Effect of agitation time on enzyme recovery

At the end of each fermentation, 100 ml acetate buffer, pH 5.0 was added to each flask, mixed and agitated for different time periods (15, 30 and 60 minutes) using the Fischer Scientific mini vortexer.

3.3.1.6.2 Effect of some extraction solvents on enzyme recovery

Some extraction solvents viz; acetate buffer (pH 5.0), citrate buffer (pH 5.0) and 0.1 M NaCl were used for extraction. These solvents were selected because they were previously used by other researchers with optimal effect on pectinase extraction from different fermented solid media (Singh *et al*., 1999; Castilho *et al*., 2000; Linde *et al*., 2007).

Acetate buffer was prepared by adding 0.1 M solution of acetic acid to 0.1 M sodium acetate while citrate buffer was prepared by adding 0.1 M citric acid solution to 0.1 M sodium citrate solution and monitoring the pH during addition with HANNA HI 83141 pH-meter. One hundred ml of each extraction solvent was added to each of the fermented substrates and same volume of the extraction solvent added to the residue to obtain the second crude filtrate. Prior to investigating the best solvent for pectinase extraction, only acetate buffer pH 5.0 was used as extraction solvent for all of the earlier parameters investigated.

3.3.1.7 Combination of the established optimized conditions for pectinase production All the established optimal conditions were used simultaneously for bulk pectinase production; that is, 80:20 % of corn cobs to orange peels, pH of 4, at 30 ^{o}C, inoculum concentration of 10.46 x 10^{6} cells/ml and solid state fermentation for 6 days. Crude enzyme recovery was achieved by addition of 100 ml of 0.1M NaCl solution to the fermented media, mixed and agitated for 30 minutes.

The protein concentration and pectinase activity of the crude extract obtained from each parameter investigated were determined using the standard procedures.

3.4 DETERMINATION OF TOTAL PROTEIN CONTENT BY BIURET METHOD

Biuret method for protein content measurement was one of the earliest colorimetric protein assay methods developed (Gornall *et al.,* 1949). The Biuret protein assay works under the principle that in alkaline conditions, substances containing two or more peptide bonds form a purple complex with copper salts in the reagent (Gornall *et al.,* 1949; Torten and Whitaker, 1964). This method was used for determining the total protein content in the present work from a standard curve prepared using egg albumin.

3.4.1 Preparation of Biuret reagent

Biuret reagent was prepared by weighing 1.50 g of cupric sulfate pentahydrate ($CuSO_4$. 5 H_2O) and 6.0 g sodium potassium tartrate-tetrahydrate ($NaKC_4H_4O_6$ 4 H_2O). The mixture was then dissolved in 500 ml of distilled water with 300 ml of 10 % NaOH added and then topped up to a total volume of 1 liter. This solution was stored in a plastic bottle protected from light at about 8 ^oC (Gornall *et al*., 1949).

3.4.2 Preparation of protein standard curve using egg albumin

Different concentrations of egg albumin viz; 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 mg/ml were prepared. To each test tube, 4 ml of Biuret reagent was added and mixed. The tubes were then incubated at 37 ^oC for 10 minutes and finally cooled and absorbance measured at 540 nm using the UVmini-1240 spectrophotometer (SHIMADZU). The absorbance values obtained were then plotted as the standard protein curve.

3.4.3 Determination of protein concentration of the crude enzyme solution

Total protein content of each enzyme solution was determined by using the Biuret protein assay method as described by Torten and Whitaker (1964), where 4 ml of Biuret reagent from prepared

stock solution was added to 1 ml of test solution in a sterile test tube with the mixture properly mixed. The tubes were then incubated at 37 ºC for 10 minutes and finally cooled and absorbance measured at 540 nm using the UVmini-1240 spectrophotometer (SHIMADZU). The concentrations were then extrapolated from the standard protein curve of egg albumin.

3.5 DETERMINATION OF PECTINASE ACTIVITY

Pectinase activity determination was carried out using a method for pectinase activity assay prepared at the 55th Joint Expert Committee for Food Additives- JECFA, (2000) with little modification. This assay is based on the hydrolysis of pectin and the resulting galacturonic acid determined spectrophotometrically at 235 nm. One unit of pectinase activity causes an increase of 0.010 of absorbance per minute under the conditions of the assay (pectin 0.5 %, pH 5.0 at 30 ºC).

3.5.1 Procedure

In a properly washed test tube, 0.1 ml of the enzyme solution was added to 3.0 ml of 0.5 % pectin solution, mixed and pre-warmed to 30 ºC for 5 minutes. One ml from the resultant mixture of pectin solution and crude enzyme extract was added to 10 ml of acetate buffer solution (pH 5.0) and mixed by shaking the tubes gently. After short mixing, the absorbance at 235 nm was read over 8 minutes at one minute intervals using acetate buffer as blank, except in the case of solvent optimization where the respective extraction solvents were used as blanks. Determination was done in triplicate.

3.5.2 Calculation of pectinase activity

The absorbance (sample-extraction solvent) was read over 8 minutes. A graph was plotted of time versus absorbance on the y-axis. The slope (change in Absorbance/ Change in time) was determined in the linear section of the function.

Pectinase activity in units was calculated as:

$$\text{Pectinase activity (U/ml)} = \frac{\text{Change in absorbance at 235 nm /change in time}}{0.01 \times V}$$

Where;

V= final reaction volume (0.1ml of enzyme solution plus 3.0 ml of the pectin solution).

3.5.3 Calculation of specific activity of pectinase

The specific activities of the pectinase after fermentation were calculated using the formula;

Specific activity of pectinase (U/mg) = Pectinase activity (U/ml)/ Protein concentration (mg/ml)

3.5.4 Concentration of the crude pectinase by salt precipitation

The reason for carrying out this step was to further enhance the activity of pectinase in the crude solution and assess its effect on orange juice extraction.

3.5.5 Effect of ammonium sulphate saturation on protein concentration

Ammonium sulphate precipitation of protein was performed (Englard and Seifter, 1990) to concentrate the crude extract solution. Various percentages of $(NH_4)_2SO_4$ were added to saturate the extracts (20 %, 30 %, 40 %, 50 %, 60 %, 70 % and 80 %). The amount of solid ammonium sulphate ($NH_4(SO_4)_2$) added to saturate the crude extract was calculated by using an online calculator for percentage ammonium sulphate saturation (http://www.encorbio.com/protocol/AM-SO4.htm accessed 21/03/2015) at room temperature. Each saturated solution was gently stirred and the extract left on ice for 30 minutes, followed by centrifuging at a speed of 3,600 g for 15 minutes using an MISTRAL 3000 E centrifuge to get the precipitates. The resulting precipitates were then resolubilised in 0.1 M NaCl solution with

continuous stirring, after which the protein concentrations and pectinase activities were determined.

3.6. DETERMINATION OF EFFICACY OF CRUDE PECTINASE IN ORANGE JUICE EXTRACTION

3.6.1 Effect of enzyme reaction/holding time on volume of orange juice extracted

Various times (15, 30, 45, 60, 75 and 90 minutes) were used to determine the optimum reaction time for extraction of juice (measured in ml). In this process, oranges (*Citrus sinensis*) with yellow coloration were washed under running tap water, peeled, cut and mashed, to increase the surface area while making more areas accessible to the enzyme to break down pectin in plant cell walls thereby releasing more juice (Shefali and Sudhir, 2013). Two-hundred grams of the mashed oranges were separately weighed into labeled beakers and one ml of the concentrated protein (10 mg/ml) with the best pectinase activity was added, stirred and then covered with plastic wraps and left to stand for the various reaction times. At the end of each reaction time, the contents were filtered over 6 minutes period using a funnel and Whatmann No. 1 filter paper and volumes of juice obtained compared to the control (orange mash with no enzyme).

3.6.2 Enzyme dosage effect on volume of juice produced

For this determination, different volumes (1-5 ml) of the concentrated enzyme extract containing approximately 10 mg/ml protein were added to 200 g orange mash to obtain different dosages ranging from 10 – 50 mg total protein per 200 g mash. The control used was orange mash with equal volume of buffer in place of enzyme.

3.7 STATISTICAL ANALYSES

The data were analyzed on the average of three replicates obtained from independent determinations. Statistical analyses of these averages were carried out with Minitab software at 95 % significance level. Graphs were drawn using the Microsoft Office Excel 2010 version.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

This study was undertaken in two stages. The first stage focused on optimizing conditions necessary for pectinase production by *Saccharomyces cerevisiae* ATCC 52712 in solid state fermentation process (Figures 4.1 to 4.14). The second stage aimed at using the established optimal conditions for pectinase production in stage one to study the efficacy of the pectin enzyme produced in orange juice extraction (Figures 4.15 to 4.18).

4.1. STAGE ONE

Enzymes function best at optimal conditions making optimization of conditions for effective pectinase activity important. It is, therefore, important to consider the effect of these conditions in detail.

4.1.1. Effect of fermentation time on pectinase activity on corn cob substrate only

The effect of duration of fermentation on pectinase activity with corn cobs as substrate is illustrated in Figure 4.1. Optimum total enzyme activity was obtained on the 6th day of fermentation for both filtrates when extraction was repeated. The difference between the first and second extractions with regard to pectinase activities and protein concentrations on the 6th day of fermentation was not statistically significant ($p>0.05$). Optimum specific activity of pectinase was also seen on the 6th day of fermentation (Figure 4.2), while there was an increase in protein concentration from day 3 to 15 in both first and second filtrates.

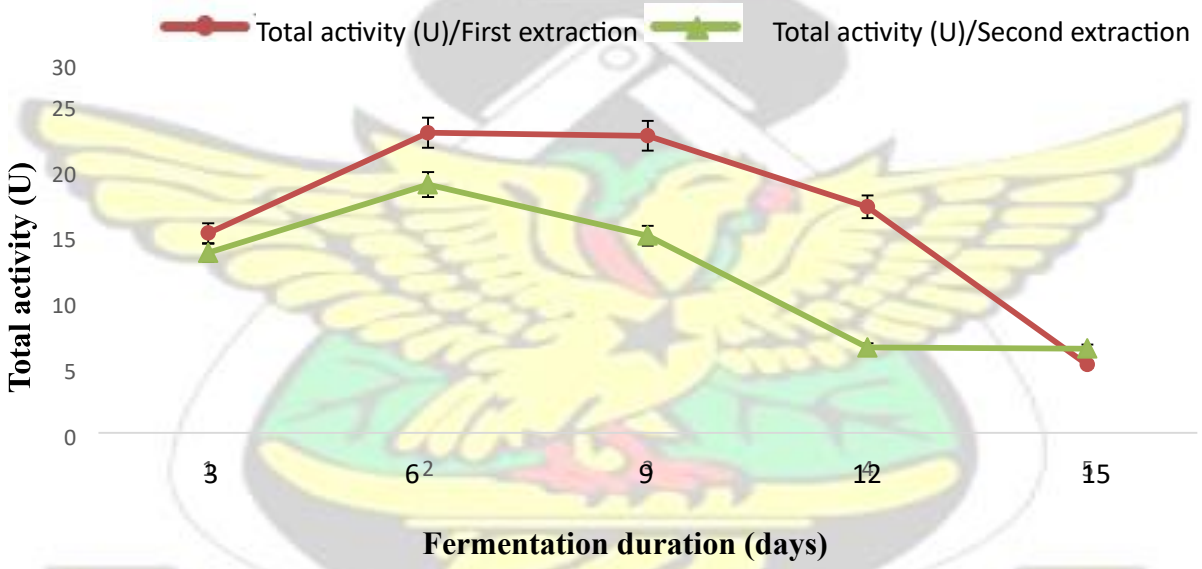

Figure 4.1. Changes in total pectinase activity produced by *Saccharomyces cerevisiae* with fermentation time.

***Fermentation conditions –100 % corn cobs, pH 4.5 at 30 °C with acetate buffer (pH 5.0) as extraction solvent.**

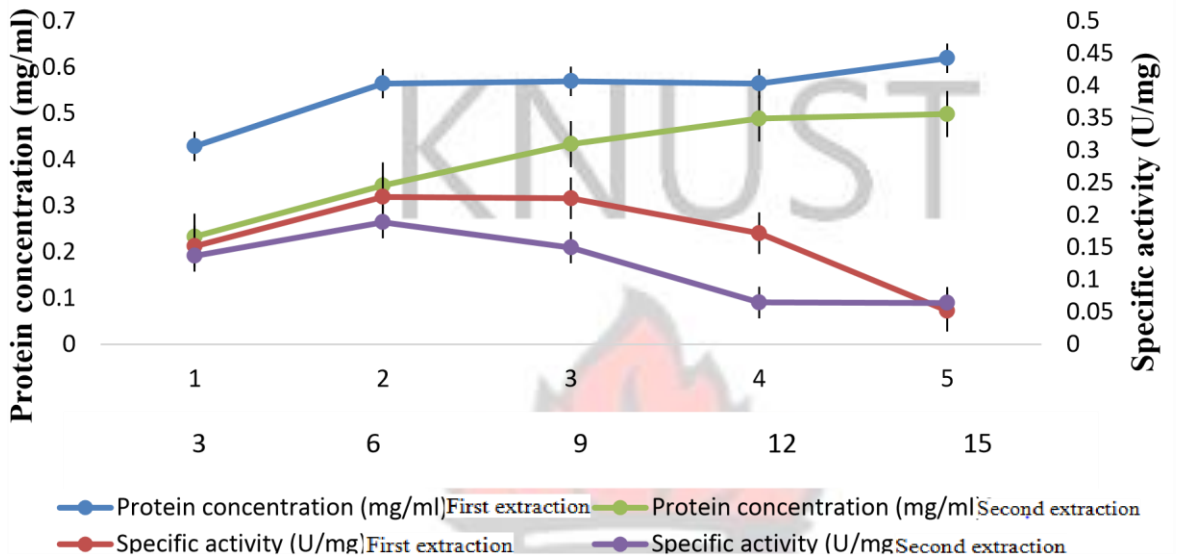

Figure 4.2: Changes in concentration of protein and specific activity of pectinase produced by *Saccharomyces cerevisiae* with fermentation time.

***Fermentation conditions -100% corn cobs, pH 4.5 at 30 °C with acetate buffer (pH 5.0) as extraction solvent.**

The decrease in activity of pectinase beyond the 6th day of fermentation could be attributed to catabolite repression (Richardson and Hyslop, 1992) and lower content of pectin in the medium for pectinase production (Dhilion *et al*., 2004).

Although the protein concentration was highest on the 15th day of fermentation (Figure 4.2), the optimum specific activity of pectinase was achieved on the 6th day. This therefore indicates that other proteins aside pectinases such as xylanases (La-Grange *et al*., 2001; Polizeli *et al*., 2005; Tain *et al*., 2013; Knob *et al*., 2014) and cellulases (Shahera *et al*., 2002; Omajasola and Jilani, 2008; Oyeleke *et al*., 2012) could be present in the crude extract, since the cell wall of plants is composed primarily of polysaccharides (such as cellulose, hemi cellulose and pectin substances) (Herron *et al*., 2000).

Namita *et al.,* (2011), used NS-2 strain of *Aspergillus niger* for fermentation and found out that it was capable of producing cellulolytic, hemicellulolytic, amylolytic and pectinolytic enzymes in appreciable titers on wheat bran, making these mixtures of enzymes to efficiently hydrolyze various domestic waste residues.

In a similar work, the production of a notable and highly effective pectinase and cellulase by the commercial baker's yeast *Saccharomyces cerevisiae* utilizing potato processing wastes, was achieved in 5-day solid state fermentation (SSF) (Magdy, 2011).

In other works, the optimum fermentation time using *Aspergillus niger* was obtained on the 7th (Khan *et al*., 2012) and the 5th day (Oyeleke *et al.,* 2012) on different substrates. Using *Aspergillus oryzae* and orange peels, the optimum pectinase activity was obtained on the 5th day of fermentation by Adebare *et al.* (2012). Thus, the result obtained in this study with regards to optimum fermentation time is consistent with others.

4.1.2. Effect of supplementation of corn cobs with different proportions of orange peels on pectinase production.

Various formulations of corn cobs supplemented with orange peels on pectinase production showed that, a ratio of 80 % **:** 20 % gave optimum results (Figure 4.3). Whereas protein concentration increased with increase in proportion of orange peels, enzyme activity peaked at 80:20 %. The combination of 80:20% of corn cobs to orange peels might have favoured adequate oxygen supply by increasing inter-particle spacing thereby enhancing growth of the yeasts and pectinase yield and easier solvent penetration during enzyme extraction leading to significant differences ($p<0.05$) obtained in pectinase activities and protein concentrations between first and second extractions (Mitchell *et al*., 1991; Bakri *et al*., 2003; Ikram-ul-Haq *et al*., 2006).

The trend observed in relation to increase in total protein concentrations as opposed to decrease in specific activity of pectinase (Figure 4.4) after the optimum was due to the effect of other proteins present as earlier discussed.

Similar result was observed with respect to the production of polygalcturonase (PG) whose activity was enhanced to 2.12 times when one part of orange bagasse (Ob) and three parts of molohkia stalks (Ms) were used as carbon sources as compared to the control (1 Ob :1 Ms) (Ahmed and Mostafa, 2013). Therefore, increasing the Ms quantity stimulated fungal growth and PG production. In another work (Gargade *et al.,* 2013), maximum amount of pectinase was produced by *Aspergillus spp*. at 6 % citrus peels substrate concentration. In addition to the increase in inter-particle spacing provided by the citrus peels, it also served as an inducer for pectinase production (Mitchell *et al*., 1991).

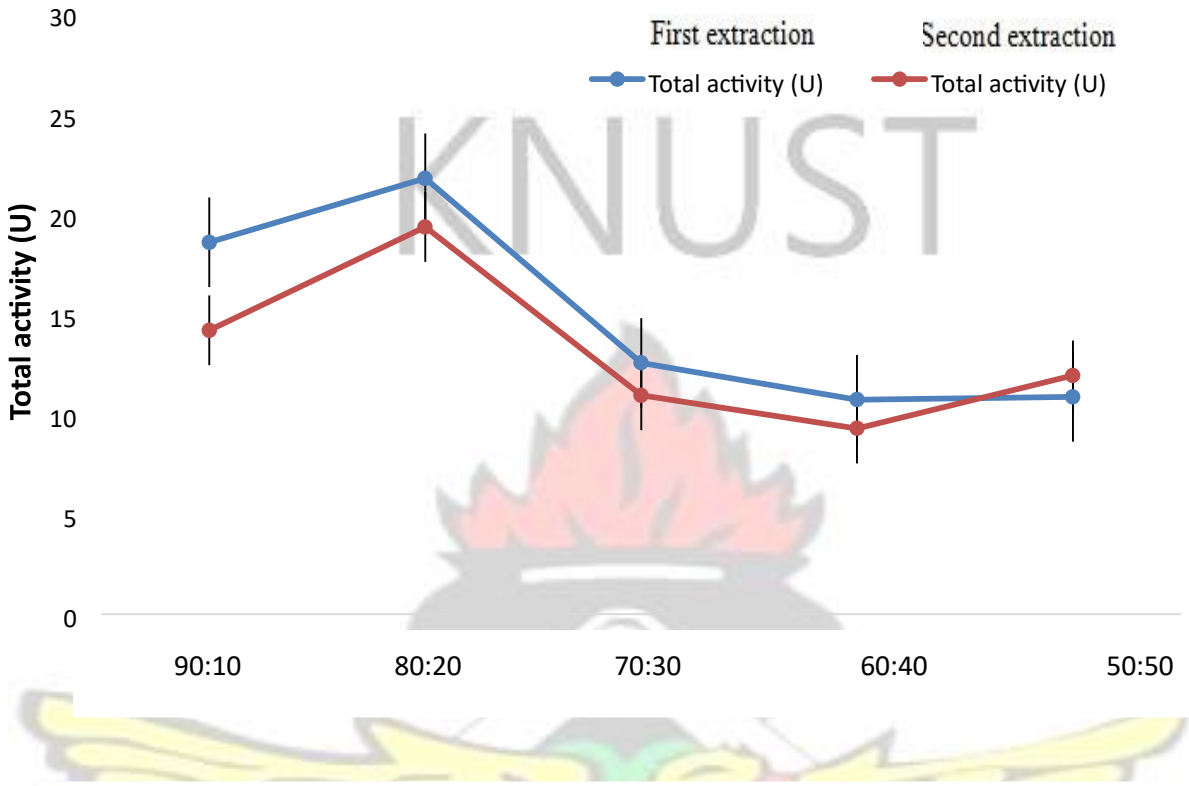

Figure 4.3. Effect of different proportions of corn cobs and orange peels on pectinase production during fermentation by *S. cerevisiae*.

***Fermentation conditions -6 days of fermentation, pH 4.5 at 30 °C with acetate buffer (pH 5.0) as extraction solvent.**

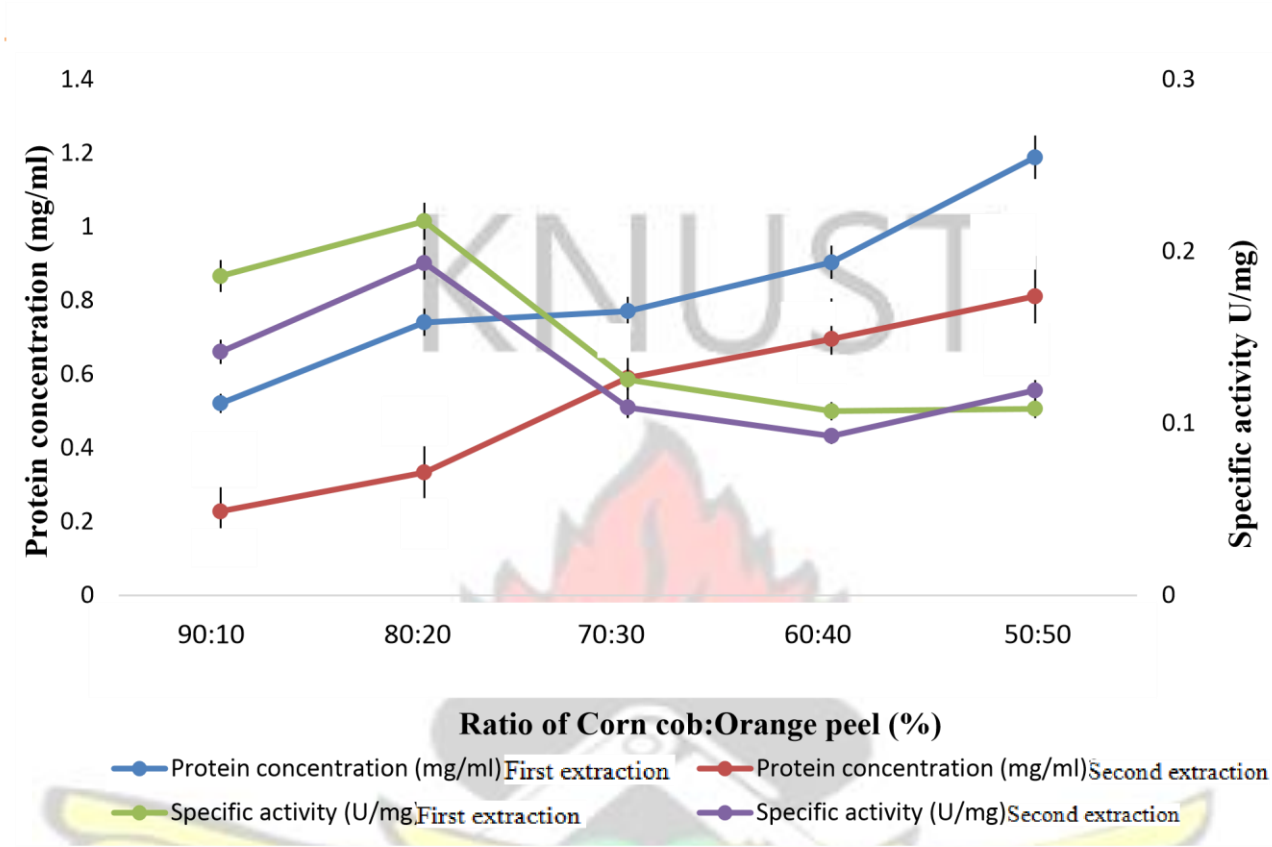

Figure 4.4. Changes in protein concentration and specific activity of pectinase during fermentation of different proportions of corn cobs and orange peels by *S. cerevisiae*.

***Fermentation conditions -6 days of fermentation, pH 4.5 at 30 °C with acetate buffer (pH 5.0) as extraction solvent.**

4.1.3. Effect of pH on pectinase production and activity pH alters enzyme conformation, recognition site, active site and substrate conformation (Palmer,

1995); hence determining the best pH for maximum pectinase activity is of importance. Optimum pectinase activity was observed at pH 4.0 (Figures 4.5 and 4.6). The decline in activity beyond pH of 4 may be due to denaturation of the enzyme which is a common phenomenon during fermentation due to the release of various by-products in the media (Dhilhion *et al*., 2004) or instability of the enzyme at extreme pH values since they are proteins that are generally denatured at such extreme pH values (Amaeze *et al*., 2015).

The production of a notable effective pectinase and cellulase by commercial baker's yeast

Saccharomyces cerevisiae utilizing potato processing wastes was obtained in the pH range of 4.0-5.0 (Magdy, 2011). Similarly, pectolytic enzymes from *Saccharomyces cerevisiae* (ATTC 52712) had been found to have broad activity at pH range of 3.5-5.0 (Ameko, 1998). The difference between the percentage reduction in viscosity (which can be used as a measure of pectolytic activity) at pH of 3.5 and 5.0 was only 5.31 %; and the enzyme was also found to be stable within this pH range (Ameko, 1998).

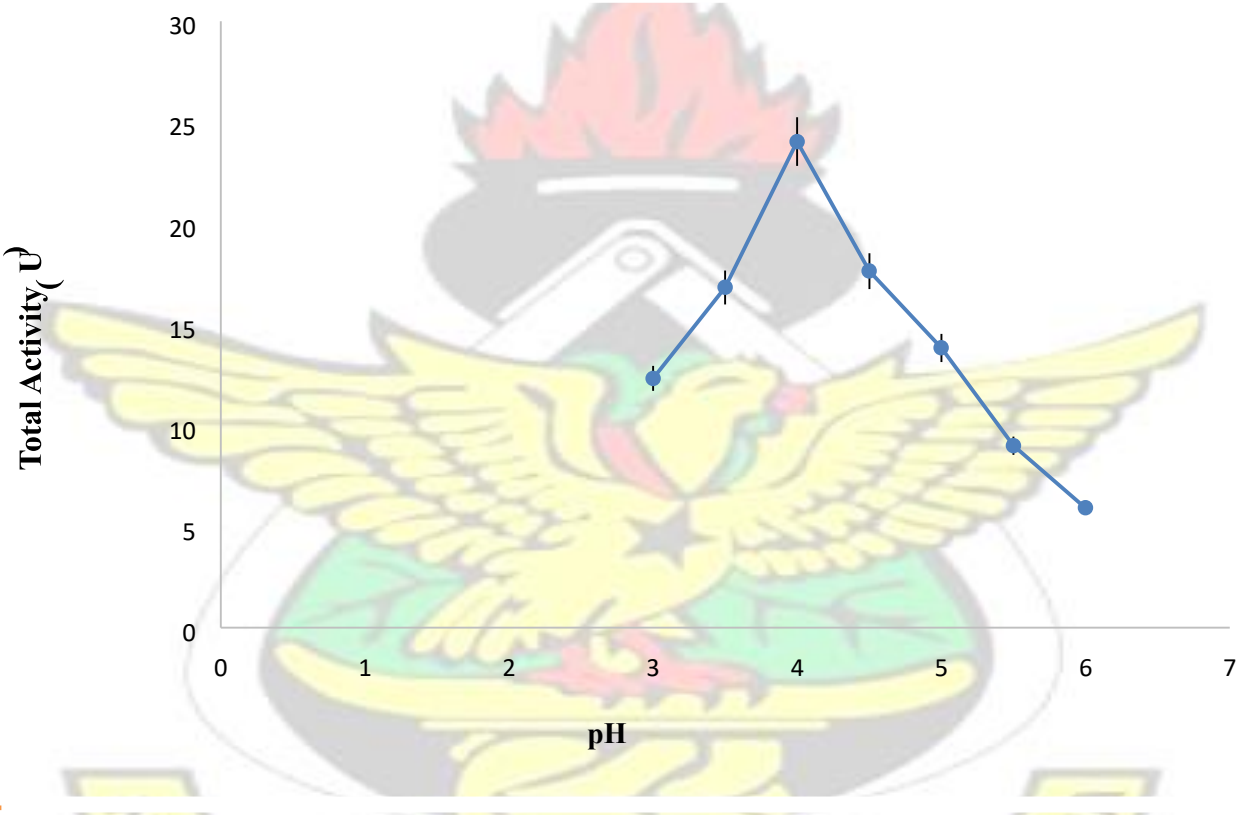

Figure 4.5. Effect of pH on total pectinase activity during fermentation with *S. cerevisiae*.

***Fermentation conditions -6 days of fermentation, 80 % corn cob: 20 % orange peel, pH 4.5 at 30 °C with acetate buffer (pH 5.0) as extraction solvent.**

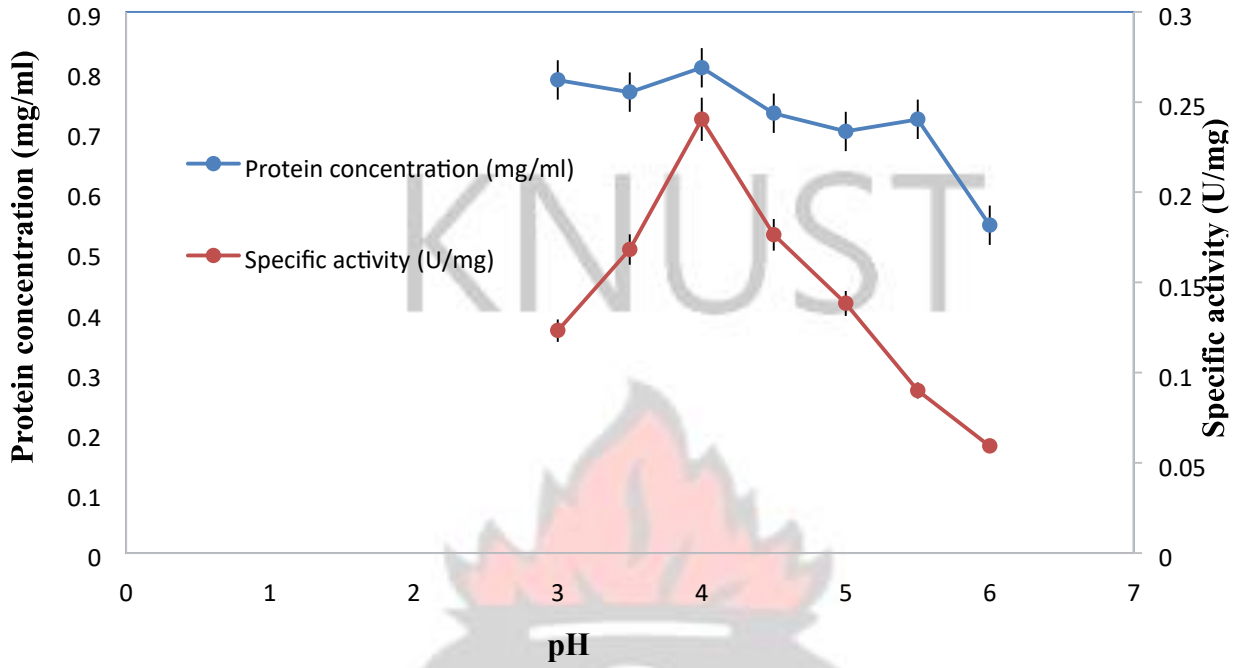

Figure 4.6. Effect of pH on protein concentration and specific activity of pectinase during fermentation with *S. cerevisiae.*

***Fermentation conditions -6 days of fermentation, 80% corn cob: 20% orange peel, pH 4.5 at 30 ºC with acetate buffer (pH 5.0) as extraction solvent.**

4.1.4. Effect of temperature on pectinase activity

Based on the results shown in Figures 4.7 and 4.8, the optimum pectinase activity was found to be at 30 ºC after which there was a decline. Best protein concentration was also obtained at 30 ºC and declined afterwards. The reduction in enzyme activity and protein concentration (Figure 4.8) beyond 30 ºC during fermentation at elevated temperatures, may be due to unfavourable heat stress encountered by the yeast cells as reported in a study on the metabolic response of *Saccharomyces cerevisiae* to continuous heat stress (http://www.rmsb.ubordeaux2.fr/BTK/abstracts/21-Mensonides.pdf) thereby discouraging the growth of S*accharomyces cerevisiae* (ATTC 52712) (Melo *et al*. 2007; Amir *et al*. 2011). Extreme

temperatures or excess heat effect possibly changed the physical properties of the organism's cell membrane thereby affecting protein secretion and uncoiling of some of the secreted proteins into random configurations due to heat stress, leading to decline in protein concentrations and pectinase activities (Sonia *et al*., 2013).

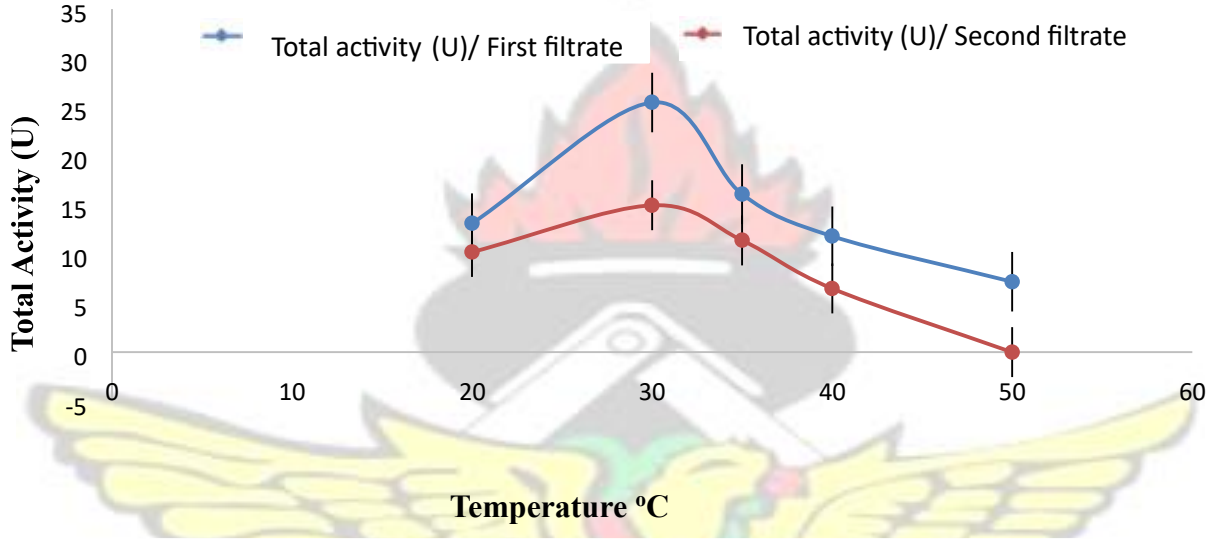

Figure 4.7. Effect of temperature on total pectinase activity during fermentation with *S. cerevisiae*.

***Fermentation conditions -6 days of fermentation, 80 % corn cob: 20 % orange peel, pH 4.0 at 30 ºC with acetate buffer (pH 5.0) as extraction solvent.**

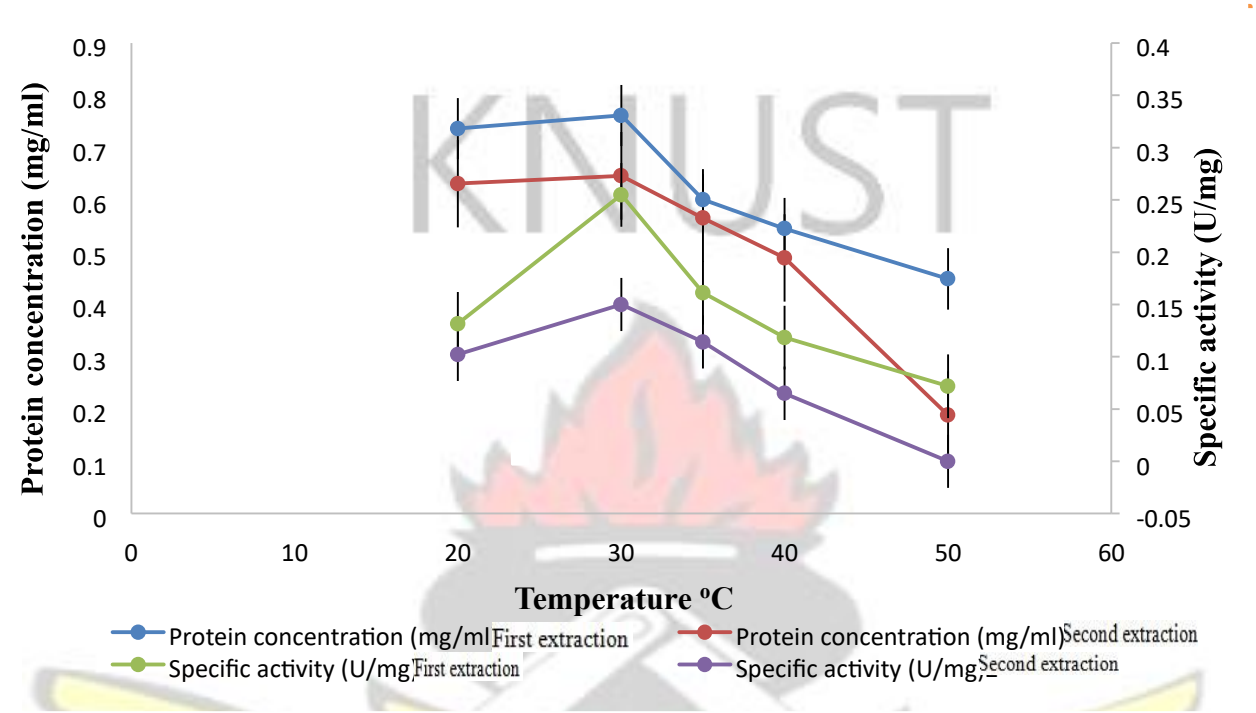

Figure 4.8. Effect of temperature on protein concentration and specific activity of pectinase during fermentation with *S. cerevisiae*.

***Fermentation conditions -6 days of fermentation, 80% corn cob: 20 % orange peel, pH 4.0 at 30 °C with acetate buffer (pH 5.0) as extraction solvent.**

4.1.5. Effect of inoculum size on pectinase production and activity

With respect to cell density of *Saccharomyces cerevisiae* on enzyme activity, 10.46 x 10^6 cells/ml was the optimum (Figures 4.9 and 4.10) indicating that this cell concentration was sufficient in colonizing the substrate particles for optimum enzyme activity to be obtained (Sikyta, 1983).

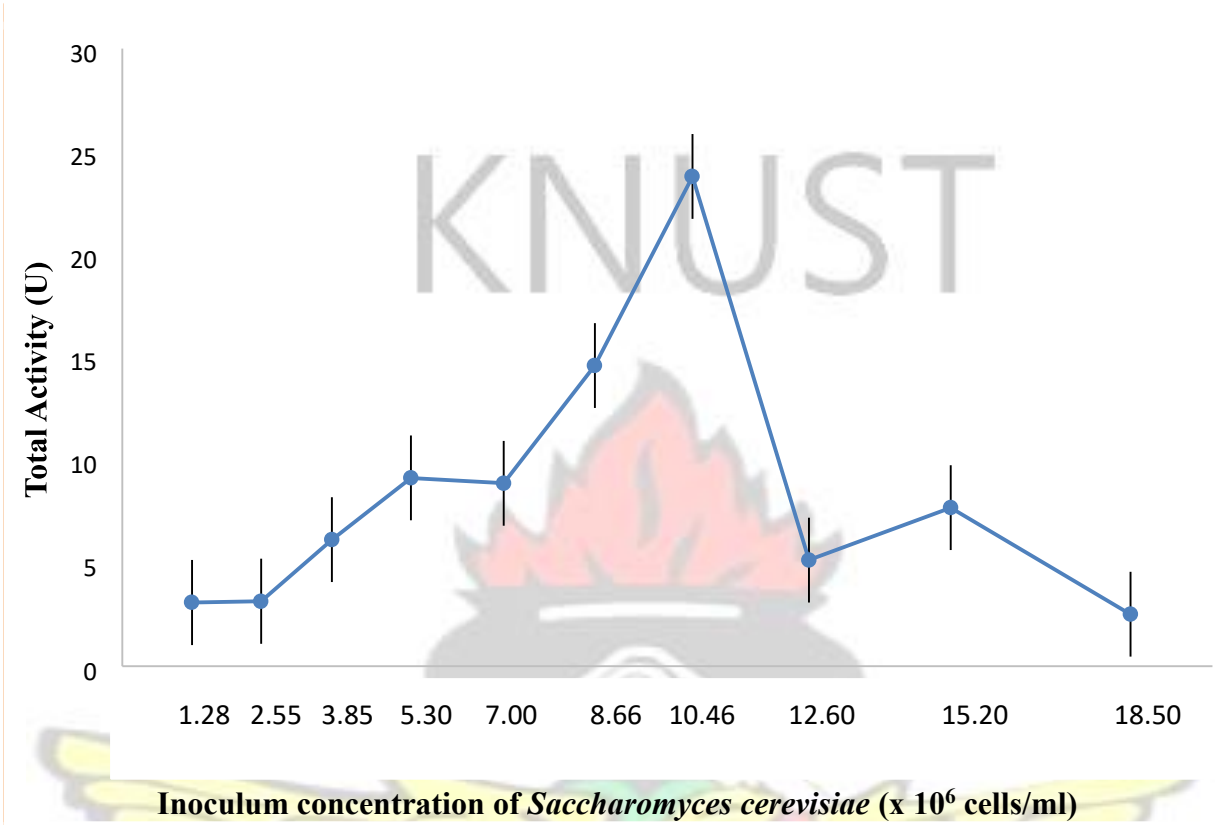

Figure 4.9. Effect of inoculum concentration on total pectinase activity during fermentation with *S. cerevisiae*.

*** Fermentation conditions -6 days of fermentation, 80 % corn cob: 20 % orange peel, pH 4.0 at 30 ºC with acetate buffer (pH 5.0) as extraction solvent.**

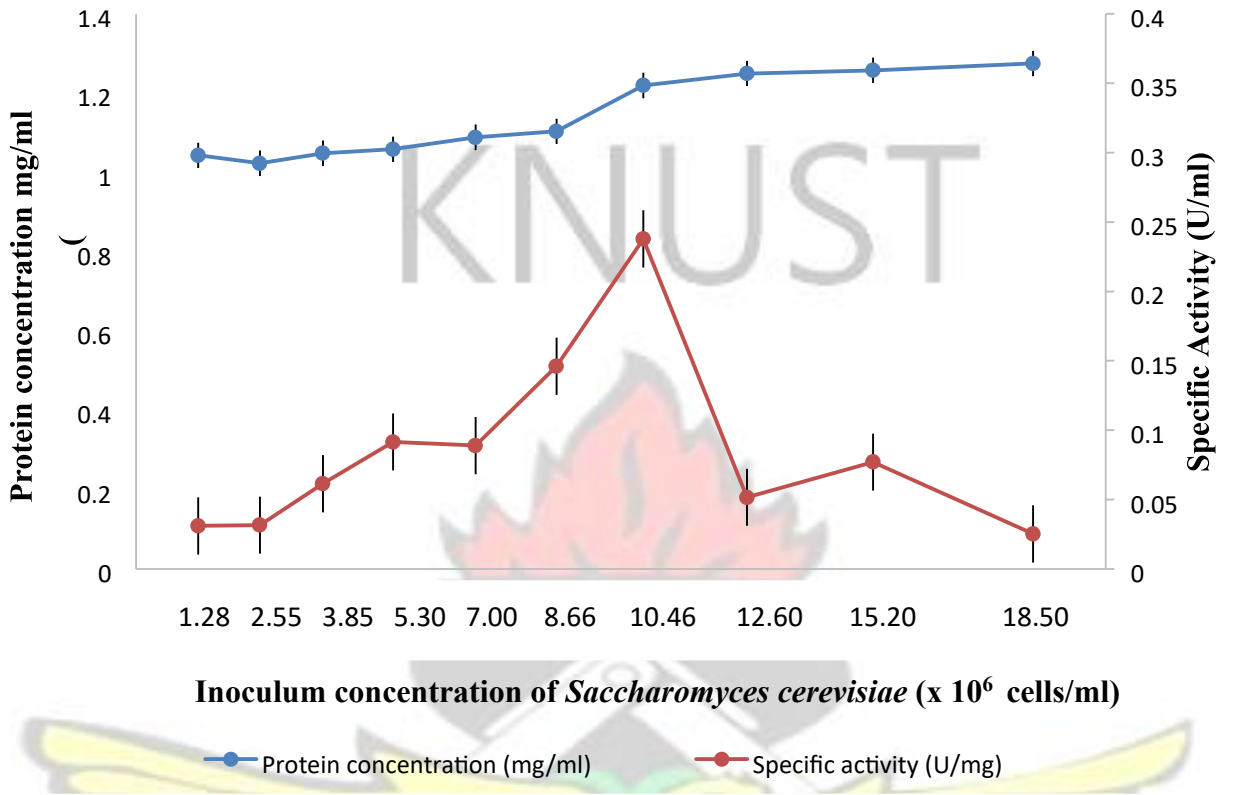

Figure 4.10. Effect of inoculum concentration on protein concentration and specific activity of pectinase during fermentation with *S. cerevisiae*.

***Fermentation conditions -6 days of fermentation, 80 % corn cob: 20 % orange peel, pH 4.0 at 30 ºC with acetate buffer (pH 5.0) as extraction solvent.**

Reduction in activity with further increase in cell concentration beyond the optimum might be due to clumping of cells, thereby reducing carbon and oxygen uptake leading to reduction in pectin enzyme release (Folakemi *et al*., 2008). Similar work reported that inoculum size of 2 x 10^6 cells/ml was favourable for enzyme production by *B. licheniformis* (Sen and Satyanarayana, 1993; Gajju *et al*. 1996; Prakasham *et al*. 2005). The inoculum variation with present findings might be due to difference in microbe and substrates used.

4.1.6 Effect of agitation time during enzyme extraction on pectinase activity

The effect of duration of agitation during enzyme extraction from the solid medium after fermentation is shown in Figures 4.11 and 4.12. Thirty minutes of agitation was the best to obtain maximum activity of pectinase. Though reports indicated that the ability of an enzyme to degrade a substrate is enhanced by agitation (Sonia *et al.*, 2009; Apoorvi and Vuppu, 2012), the results obtained in this study showed a decline after 30 minutes of agitation. This loss of activity beyond 30 minutes could be attributed to the fact that when a higher level of mechanical agitation was introduced into the system, the level of surface modification of the enzyme increased thereby decreasing its activity (Silva *et al.*, 2006).

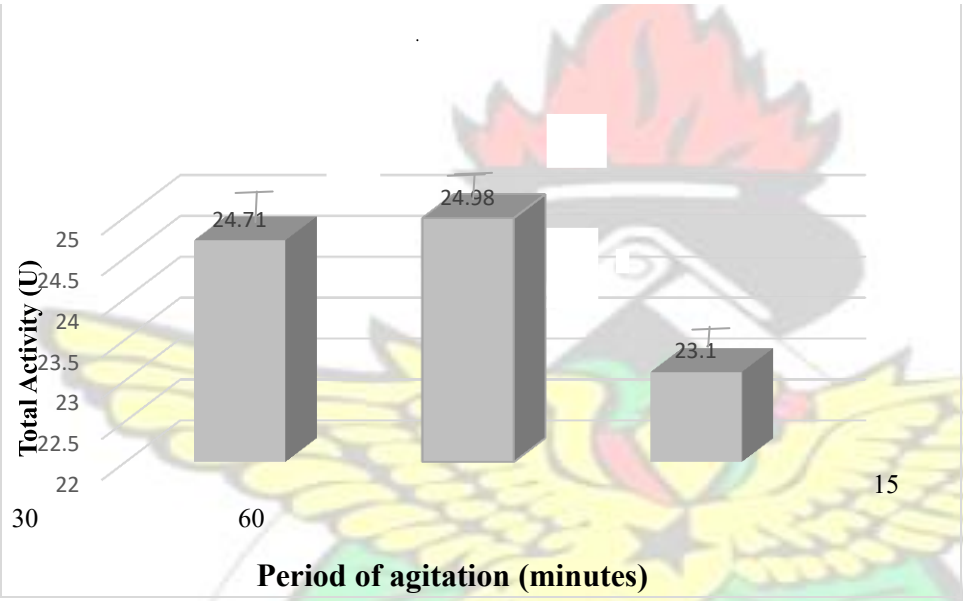

Figure 4.11. Effect of agitation time on total activity of pectinase released during extraction from the solid medium.

***Fermentation conditions -6 days of fermentation, 80 % corn cob: 20 % orange peel, pH 4.0 at 30 ºC, 10.46 x 10^6 cells/ml with acetate buffer (pH 5.0) as extraction solvent.**

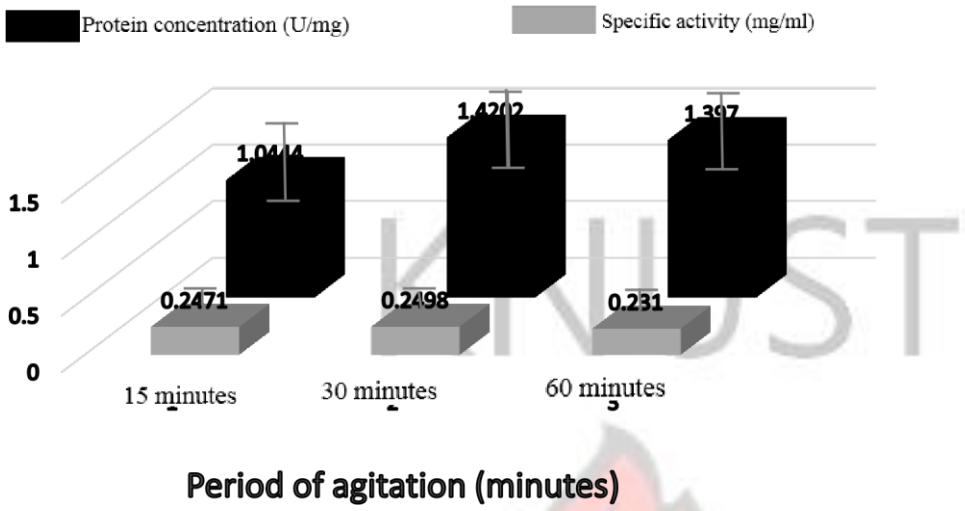

Figure 4.12. Effect of agitation time on protein concentration and pectinase specific activity during enzyme extraction after SSF.

***Fermentation conditions -6 days of fermentation, 80 % corn cob: 20 % orange peel, pH 4.0 at 30 ^{o}C, 10.46 x 10^{6} cells/ml with acetate buffer (pH 5.0) as extraction solvent.**

Other studies reported that the time required to remove enzyme from a solid substrate, ranged from 30 minutes (Castilho *et al*., 2000) to 48 hours under shaking conditions (Shata, 2005). In cultures of *Aspergillus niger*, the optimum time of agitation for polygalacturonase (PG) extraction was 30 minutes (Castilho *et al*., 2000), while in *Aspergillus carbonarius,* the optimum time was 15 minutes (Singh *et al*., 1999). This therefore showed microbe strain effect on period of agitation during extraction of enzymes from fermented substrates. Therefore, the substrate composition and the yeast used in this study had an effect on the optimum period of agitation for effective pectinase extraction.

4.1.7. Effect of extraction solvent on pectinase activity

Using three extraction solvents as shown in Figures 4.13 and 4.14, NaCl (0.1 M) was the best solvent with the highest pectinase activity. These solvents were selected because they were previously used by other researchers with optimal effect on pectinase extraction from different fermented solid media (Singh *et al*., 1999; Castilho *et al*., 2000; Linde *et al*., 2007).

NaCl (0.1 M) was significantly different when compared to acetate buffer ($p<0.05$). Adsorption of enzymes to cells or solid substrates has been attributed to ionic bond, hydrogen bond and Van *der* Waal's forces (Agrawal *et al*., 2005). This therefore makes extraction an area worth considering in the recovery of enzyme from fermented biomass; hence, selection of a suitable solvent is necessary.

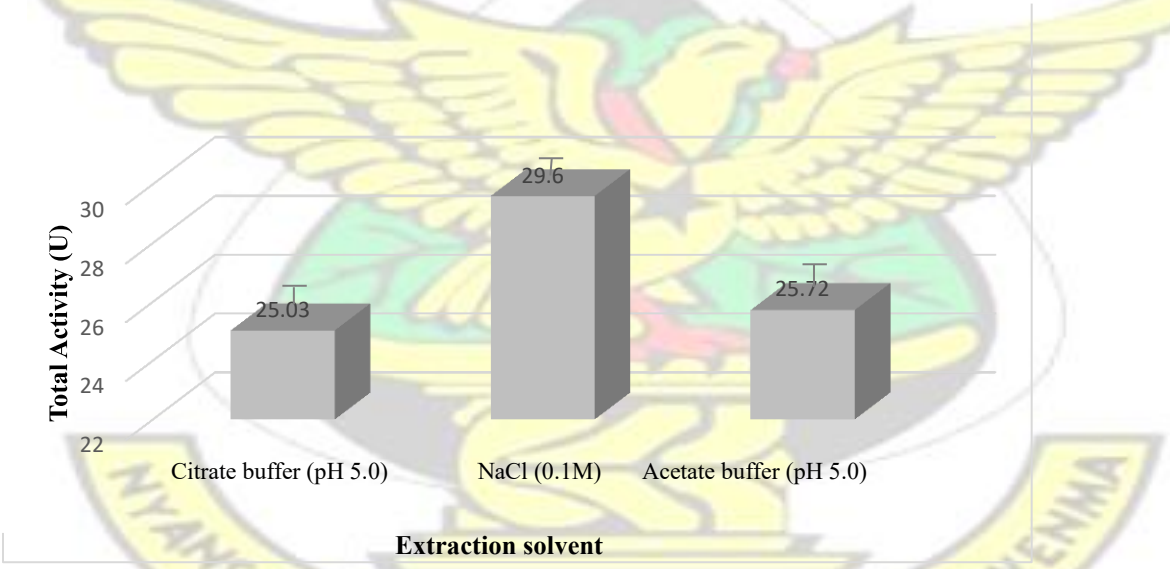

Figure 4.13. Effect of extraction solvent on total pectinase activity recovery from the fermented solid medium.

***Fermentation conditions -6 days of fermentation, 80 % corn cob: 20 % orange peel, pH 4.0 at 30 ^{o}C, 30 minutes agitation 10.46 x 10^{6} cells/ml.**

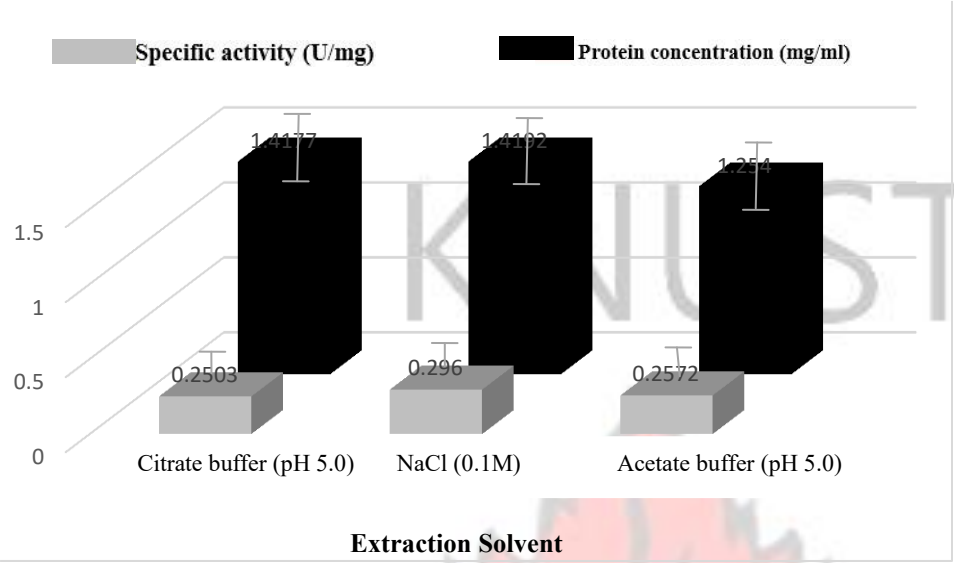

Figure 4.14. Effect of extraction solvent on protein concentration and specific activity of pectinase from the solid medium.

***Fermentation conditions -6 days of fermentation, 80 % corn cob: 20 % orange peel, pH 4.0 at 30 ^{o}C, 30 minutes agitation, 10.46 x10^{6} cells/ml.**

Comparing distilled water, acetate buffer (0.05 M), Tween 80 (0.05 %), Tween 40 (0.05 %) and glycerol (0.05 %) as solvents for pectinase extraction, acetate buffer was seen to be the best for pectinase extraction (Ahmed and Mostafa, 2013). Some enzymes produced under SSF have also been recovered from the solid phase by treatment with distilled water (Silva *et al*., 2005; Patil and Chaudhari, 2010), de-ionized water (Giese *et al*., 2008), surfactant Tween 80 (Rezende *et al*., 2002) and buffers (Rezende *et al*., 2002; Phutela *et al*., 2005; Linde *et al*., 2007). Castilho *et al*. (2000) used different extraction solvents to assay for polygalacturonase activity from which acetate buffer resulted in the best activity. Highest activity was also obtained with Na_2SO_4 (0.1 M) (Singh *et al*., 1999). These authors attributed the action of these solvents to breakage of the bond between carbohydrates and proteins (Solarito *et al.*, 2010).

In the present case, NaCl (0.1 M) as solvent for extraction was better than acetate and citrate buffers, and can be said to be the best extraction solvent in breaking the bonds thereby releasing

protein from the solid surface. The use of NaCl (0.1 M) for extraction with maximum activity (among the solvents studied) could be due to differences in the microorganism used for fermentation or the nature/type of substrates used for fermentation (Sing *et al*., 1999; Madhav and Pushpaltha, 2002; Magdy, 2011; Khan *et al*., 2012). NaCl is a much cheaper salt compared to others and thus can be considered an advantage in this work.

4.1.8. Pectinase production under the established optimized conditions

When all the optimal parameters were combined to produce the pectinase enzyme, a total activity of 29.57 U and specific activity of 0.296 U/mg were obtained (Table 4.1) **Table 4.1.** Activity of pectinase produced under optimal conditions

Pectinase activity (U/ml)	**Protein concentration (mg/ml)**	**Total activity (U)**	**Specific activity of pectinase (U/mg)**
0.3548	1.2000	29.57	0.2957

Therefore, pectinase production under the established optimal conditions showed 35.89 % increase in activity over corn cobs supplemented with orange peels alone (Figure 4.3). This therefore justifies the need for optimization of fermentation parameters during fermentation for increase in enzyme activity.

4.2. APPLICATION OF PECTINASE PRODUCED IN ORANGE JUICE EXTRACTION

The enzyme produced under the established optimal conditions was further concentrated by ammonium sulphate precipitation prior to its use in juice extraction.

4.2.1 Effect of $(NH_4)_2SO_4$ precipitation on pectinase activity

Ammonium sulphate precipitation of the crude protein extract was undertaken to increase pectinase activity. Increase in salt concentration had been proven to reduce solubility of proteins leading to precipitation of the proteins due to the insufficiency of water molecules interacting with protein molecules in the presence of ammonium sulphate (Jakoby, 1971).

Increase in ammonium sulphate saturation of the crude extract further enhanced pectinase activity with the best activity obtained at 60 % saturation (Figures 4.15 and 4.16) followed by a decline. From the results obtained, there was increase in pectinase activity from 29.57 U (for 0% $(NH_4)_2SO_4$ saturation) to 41.77 U (for 60 % $(NH_4)_2SO_4$ saturation) (Figure 4.15) and protein concentration also increased from 1.20 mg (for 0 % $(NH_4)_2SO_4$ saturation) to approximately 10 mg for 60 % $(NH_4)_2SO_4$ saturation .The decline in the activity of pectinase (Figures 4.15 and 4.16) beyond 60 % $(NH_4)_2SO_4$ saturation could be due to masking of the charged groups on pectin enzyme substrate recognition site by the salt (Bruna *et al*., 2008). Significant increase ($p<0.05$) in pectinase activity from 20 to 80 % $(NH_4)_2SO_4$ saturation was obtained upon salt precipitation up to 60 % saturation. There was also significant difference ($p<0.05$) in both pectinase activity and protein concentration between 50 % and 60 % $(NH_4)_2SO_4$ saturation of the crude extract, beyond which no significant difference was obtained. The increase in protein concentration beyond 60% $(NH_4)_2SO_4$ saturation was due to the effect of other proteins in addition to pectinase in the filtrate since enzymes are unique with regards to conditions at which they function best (Herron *et al.,* 2000).

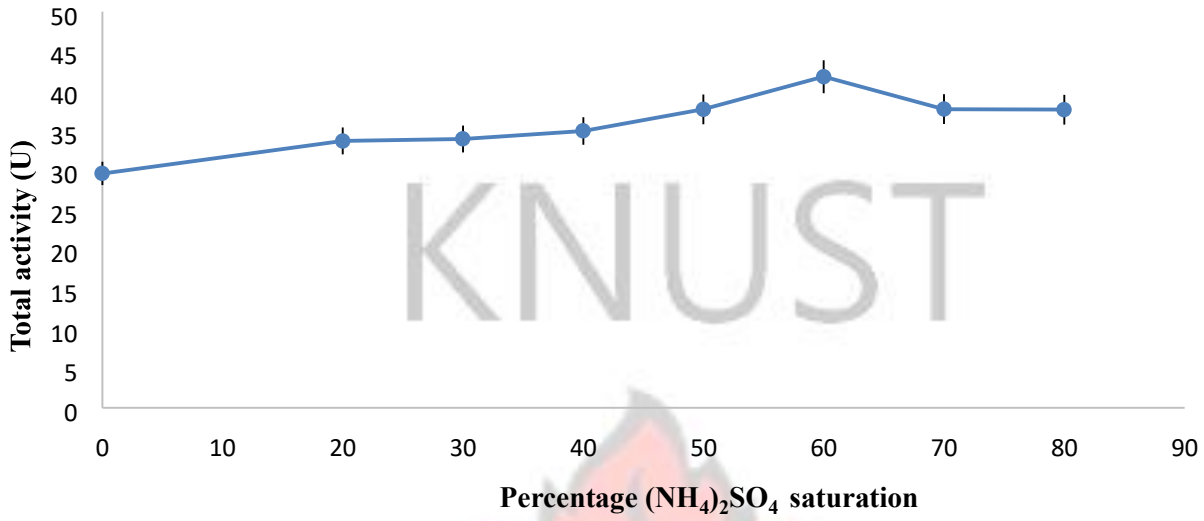

Figure 4.15. Effect of $(NH_4)_2SO_4$ saturation of the crude filtrate on total pectinase activity.

***Fermentation conditions -6 days of fermentation, 80 % corn cobs: 20 % orange peels, pH 4.0 at 30 ºC, 30 minutes agitation, 10.46 x10^6 cells/ml and 0.1 M NaCl as solvent for extraction.**

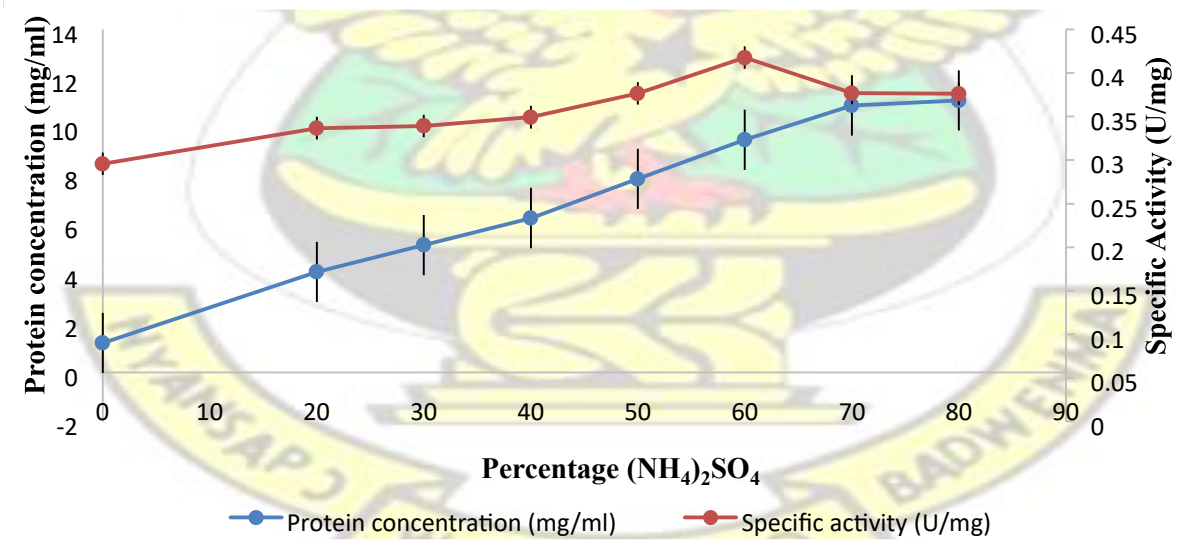

Figure 4.16. Effect of $(NH_4)_2SO_4$ saturation of the crude filtrate on protein concentration and specific activity of pectinase.

***Fermentation conditions -6 days of fermentation, 80 % corn cob: 20 % orange peel, pH 4.0 at 30 ^{o}C, 30 minutes agitation, 10.46 x10^{6} cells/ml and 0.1 M NaCl as solvent for extraction.**

4.2.2. Effect of enzyme reaction/holding time on volume of juice extracted

Holding or reaction time was undertaken to determine the optimum time for enzyme interaction with the orange mash substrate for effective extraction of juice. From Figure 4.17, 45minutes gave the optimum reaction time at which the highest volume of free-run juice was obtained beyond which no significant increase in juice volume extracted was obtained (Figure 4.17). Freerun juice as used here is simply the juice produced at the end of reaction and filtered (with Whatmann No. 1 filter paper -for six minutes) with no external applied pressure (Djokoto *et al*., 2006). Thus, 45 minutes reaction time was adequate for the enzyme to fully interact with the orange mash substrate.

In a similar work, however, 30 minutes was found to be the best reaction time for free-run juice extraction from pineapple using the same enzyme (Dzogbefia *et al*., 2001). The difference in optimum reaction time in the present work could be due to differences in the conditions used for pectinase production or could also be due to differences in the fruits from which the juices were extracted (Dzogbefia *et al*., 2001).

Figure 4.17. Effect of reaction/holding time on volume of orange juice extracted with pectinase.

***Fermentation conditions- 6 days fermentation, 80 % corn cob : 20 % orange peel, pH 4.0 at 30 ^{o}C, 30 minutes agitation, 10.46 x10^{6} cells/ml , 0.1 M NaCl as solvent for extraction, 60 % $(NH_4)_2SO_4$ protein saturation and 10 mg of the enzyme produced per 200 g orange mash.**

4.2.3 Effect of enzyme dosage on the volume of free-run juice collected

Figure 4.18 illustrates the effect of enzyme dosage on volume of juice produced. The optimum enzyme dosage for best juice extraction was four mls of 10 mg/ml (40 mg) total protein per 200 g of orange mash (0.02 % enzyme dosage). Forty mg total protein /200 g of orange mash resulted in 123.4 % increase in orange juice extracted while 10 mg (1 ml of 10 mg/ml protein) total protein per 200 g of orange mash led to 15 % increase in free-run juice over control. Therefore, the application of this enzyme enhanced hydrolysis of pectic substances in the mash thereby resulting in the release of more juice.

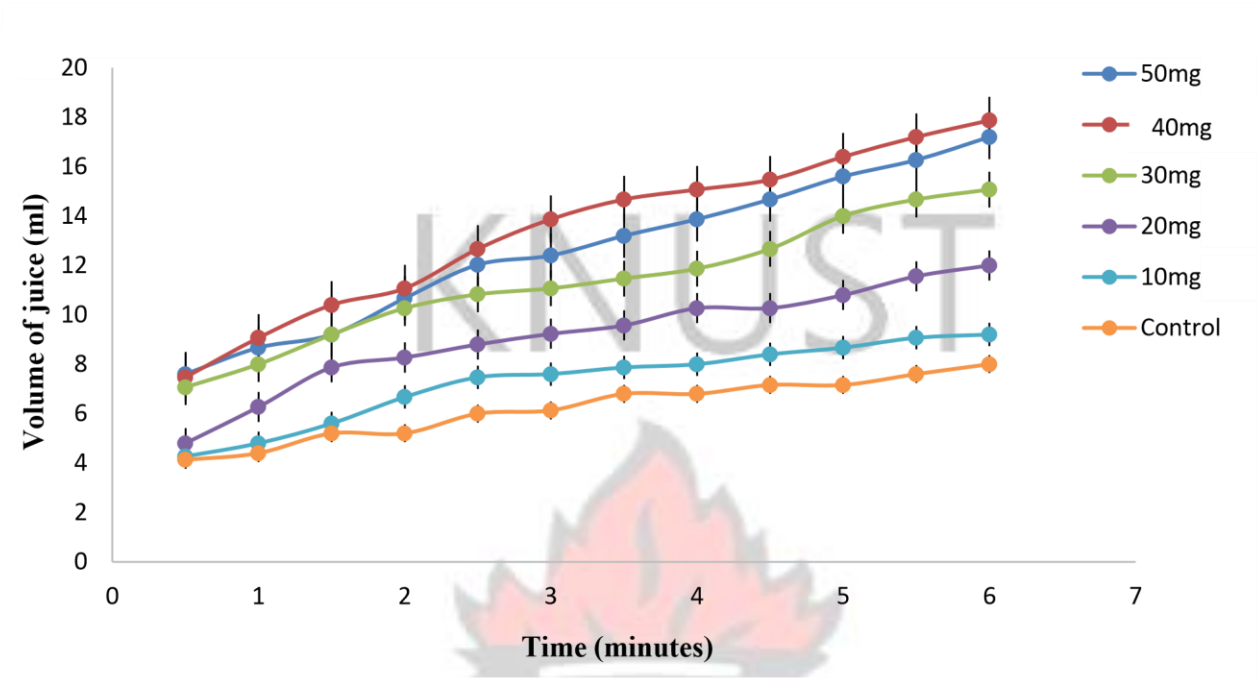

Figure 4.18. Effect of enzyme dosage on the volume of juice produced. ***Fermentation conditions- 6 days of fermentation, 80 % corn cobs: 20 % orange peels, pH 4.0 at 30 ºC, 30minutes agitation, 10.46 x10^6 cells/ml, 0.1 M NaCl as solvent for extraction, 60 % $(NH_4)_2SO_4$ protein precipitation and 45 minutes reaction time.**

At higher enzyme dosage (50 mg total protein/200 g of orange mash), however, a decline was obtained. The medium could have been saturated with the enzyme and the increasing amount of products formed could have reached inhibitory concentrations resulting in decrease in enzyme activity. Buamah *et al.* (1997) reported that *S. cerevisiae* produces the enzyme polymethylgalacturonase (PMG) which is able to hydrolyse pectin without the aid of pectin esterase. Higher levels of oligomers due to enzyme action in the medium may have reached inhibitory concentrations thereby inhibiting polygalacturonase activity (Lanzarini *et al*., 1989). This probably accounted for the decrease in orange juice extracted with increase in enzyme dosage above 40 mg total protein/200 g of orange mash.

The success of this study therefore shows the potential of using corn cobs supplemented with orange peels for pectinase production by *S. cerevisiae,* a microbe which is easier to handle as

compared to *Aspergillus niger* or *Bacillus* species. It also indicates the need for optimising parameters for effective pectinase action.

CHAPTER FIVE

5.0 CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSIONS

The established optimal conditions obtained during pectinase production for use in juice extraction (with enzyme dosage of 40 mg total protein per 200 g of orange mash), showed that, pectinase produced using *Saccharomyces cerevisiae* in solid state fermentation can be efficiently used to extract orange juice from orange mash. The use of this enzyme for juice extraction is encouraging due to the 123.4 % increase in juice yield obtained in comparison to orange mash with no enzyme (control).

The optimum enzyme dosage of 40 mg total protein per 200 g of orange mash is cost effective in that, higher concentration is not needed. The successful application of this enzyme in juice extraction also showed that in the traditional method of orange juice extraction, not all the juice is extracted. Therefore to minimise loss, local enzyme production is encouraged for small-scale industries that engage in juice extraction from fruits. In doing so, the rate of pollution from agro wastes such as orange peels and corn cobs could be minimised because the orange peels and corn cobs generated would be used for pectinase production with this less sophisticated technology thereby having the potential of reducing importation of pectinase leading to enhancement of the economy through job creation.

5.2 RECOMMENDATIONS

The following recommendations may be worth considering with regards to further understanding and enhancing the activity of pectinase:

- ✓ Other buffers and solvents are investigated for extraction of this enzyme to select the best for routine extraction.
- ✓ Future work should consider using Response Surface Methodology to fine-tune the optimisation process.
- ✓ A well designed fruit extraction instrument with portions for mashing of oranges and an area to hold the mash and enzyme for reaction time of 45 minutes before filtering is produced (in collaboration with the engineering department) to further ease the overall extraction process.

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APPENDIX A

TABULAR REPRESENTATION OF RESULTS

In the various tables below,

- **Pectinase (slope) at 235nm** represents the gradients of the mean values of the triplicates

(spectrophotometer) readings for each sub parameter.

Pectinase activity (u/ml) = Change in absorbance at 235nm per change/change in time

$$\frac{}{0.01 \times V}$$

Where;

V= final reaction volume, ml (0.1ml of enzyme solution plus 3.0ml of the pectin solution).

- **Specific activity of pectinases (U/mg)**

=Pectinase activity (U/ml)/ Protein concentration (mg/ml)

(I.e. total activity/100 DF)

- **DF** represents dilution factor.

Table A.1. Effect of Fermentation time (days) on pectinase activity and protein concentration.

Days	**Filtrate**	**Pectinase (slope) @ 235nm**	**Pectinase activity (u/ml) x10DF**	**Protein (Mean Absorbance) @ 540nm**	**Protein concentration (mg/ml)**	**Total activity x100DF**
3	1st	0.0002	0.065	0.048	0.429	15.20

	2nd	0.0001	0.032	0.009	0.233	13.70
6	1st	0.0004	0.129	0.075	0.565	22.83
	2nd	0.0002	0.065	0.031	0.344	18.90
9	1st	0.0004	0.129	0.076	0.570	22.60
	2nd	0.0002	0.065	0.049	0.434	15.00
12	1st	0.0003	0.097	0.075	0.565	17.20
	2nd	0.0001	0.032	0.060	0.489	6.50
15	1st	0.0001	0.032	0.086	0.620	5.20
	2nd	0.0001	0.032	0.062	0.499	6.41

Conditions= pH 4.5 and temperature of 30ºC.

Table A.2. Effect of percentage combination of corn cobs to orange peels on pectinase activity

Percentage Composition of corn cob to orange peel	**Filtrate**	**Pectinase (slope) @ 235nm**	**Pectinase activity (u/ml) x10DF**	**Protein (Mean Absorbance) @ 540nm**	**Protein concentration (mg/ml)**	**Total activity x100DF**

90:10	1st	0.0003	0.0968	0.0663	0.5214	18.57
	2nd	0.0001	0.0323	0.0080	0.2280	14.17
80:20	1st	0.0005	0.1613	0.1100	0.7413	21.76
	2nd	0.0002	0.0645	0.0290	0.3337	19.33
70:30	1st	0.0003	0.0968	0.1160	0.7715	12.55
	2nd	0.0002	0.0645	0.0800	0.5903	10.93
60:40	1st	0.0003	0.0968	0.1423	0.9039	10.71
	2nd	0.0002	0.0645	0.1010	0.6960	9.27
50:50	1st	0.0004	0.1290	0.1990	1.1892	10.85
	2nd	0.0003	0.0968	0.1240	0.8118	11.92

Conditions= pH4.5, temperature at 30ºC for 6 days.

Table A.3. Effect of pH on pectinase activity and protein concentration

pH	**Pectinase @ (slope) 235nm**	**Pectinase activity (u/ml) x10DF**	**Protein (Mean Absorbance) @ 540nm**	**Protein concentration (mg/ml)**	**Total activity x100DF**
3.0	0.0003	0.0970	0.1190	0.7867	12.33
3.5	0.0004	0.1290	0.1150	0.7665	16.83
4.0	0.0006	0.1940	0.1230	0.8070	24.04

4.5	0.0004	0.129	0.1080	0.7313	17.64
5.0	0.0003	0.0970	0.1020	0.7011	13.84
5.5	0.0002	0.0545	0.1060	0.7212	9.01
6.0	0.0001	0.0645	0.071	0.5450	5.93

Conditions=80% corn cobs: 20% orange peels at 30°C for 6 days.

Table A.4 Effect of fermentation temperature on pectinase activity and protein concentration

Temperature	**Filtrate**	**Pectinase (slope) @ 235nm**	**Pectinase activity (u/ml) x10DF**	**Protein (Mean Absorbance) @ 540nm**	**Protein concentration (mg/ml)**	**Total activity x100DF**
20 °C	1st	0.0003	0.0968	0.1090	0.7363	13.15
	2nd	0.0002	0.0645	0.0880	0.6310	10.22
30 °C	1st	0.0006	0.1940	0.1140	0.7614	25.48
	2nd	0.0003	0.0968	0.0910	0.6460	14.99
35 °C	1st	0.0003	0.0968	0.082	0.6004	16.12
	2nd	0.0002	0.0645	0.0750	0.5652	11.41
40 °C	1st	0.0002	0.0645	0.0710	0.5450	11.84
	2nd	0.0001	0.0323	0.0600	0.4890	6.50
50 °C	1st	0.0001	0.0323	0.0520	0.4490	7.19
	2nd	0.0000	0.0000	0.0001	0.1882	0.00

Conditions= 80% corn cobs: 20% orange peels; pH4.0 for 6 days.

Table A.5. Effect of inoculum size on pectinase activity and protein concentration

Cells/ml of *Saccharomyces cerevisiae* 52712	**Pectinase @ (slope) 235nm**	**Pectinase activity (u/ml) x10DF**	**Protein (Mean Absorbance) @ 540nm**	**Protein concentration (mg/ml)**	**Total activity x100DF**
0.128×10^7	0.0001	0.0323	0.1700	1.0432	3.10
0.255×10^7	0.0001	0.0323	0.1660	1.0232	3.16
0.385×10^7	0.0002	0.0645	0.1710	1.0483	6.15
0.530×10^7	0.0003	0.0968	0.1710	1.0584	9.15
0.700×10^7	0.0003	0.0968	0.179	1.0886	8.89
0.866×10^7	0.0005	0.1613	0.1820	1.1037	14.61
1.046×10^7	0.0009	0.2903	0.2050	1.2194	23.78
1.260×10^7	0.0002	0.0645	0.2110	1.2496	5.16
1.520×10^7	0.0003	0.0968	0.212	1.2576	7.70
1.850×10^7	0.0001	0.0323	0.2160	1.2748	2.53

Conditions= 80% corn cobs: 20% orange peels; pH4.0 at 30°C for 6 days.

Table A.6. Effect of agitation time on pectinase activity and protein concentration

Agitation (minutes)	Pectinase @ (slope) 235nm	Pectinase activity (u/ml) x10DF	Protein (Mean Absorbance) @ 540nm	Protein concentration (mg/ml)	Total activity x100DF
15	0.0008	0.2581	0.1698	1.0444	24.71
30	0.0011	0.3548	0.2442	1.4202	24.98
60	0.0010	0.3226	0.2396	1.3970	23.10

Conditions= 80% corn cobs: 20% orange peels; pH4.0 at 30ºC and 1.046 x 10^7cells/ml for 6 days.

Table A.7. Effect of extraction solvent on pectinase activity and protein concentration

Extraction solvent	Pectinase @ (slope) 235nm	Pectinase activity (u/ml) x10DF	Protein (Mean Absorbance) @ 540nm	Protein concentration (mg/ml)	Total activity x100DF
Citrate buffer (pH 5.0)	0.0011	0.3548	0.2437	1.4177	25.03
NaCl (0.1M)	0.0013	0.4194	0.2440	1.4192	29.60

Acetate buffer (pH 5.0)	0.0010	0.3226	0.2114	1.2540	25.72

Conditions= 80% corn cob: 20% orange peel; pH4.0; 30^{o}C; agitated for 30minutes and inoculum size of 1.046 x $10^{7}$for 6 days.

Table A.8. Effect of Percentage $(NH_4)_2SO_4$saturationof crude enzyme on pectinase activity

Percentage $(NH_4)_2SO_4$	**Pectinase @ (slope) 235nm**	**Pectinase activity (u/ml) x10DF**	**Protein (Mean Absorbance) @ 540nm**	**Protein concentration (mg/ml)**	**Total activity x100DF**
0	0.0011	0.3548	0.2000	1.20	29.57
20	0.0043	1.3871	0.7800	4.10	33.67
30	0.0055	1.7742	0.9900	5.20	33.92
40	0.0068	1.2060	1.2060	6.30	34.93
50	0.0092	2.9677	1.5230	7.90	37.66

60	0.0123	3.9677	1.8400	9.50	41.77
70	0.0127	4.0968	2.1150	10.90	37.69
80	0.0129	4.1613	2.1530	11.10	37.62

Conditions= 80% corn cobs: 20% orange peels; pH4.0; 30ºC; agitated for 30minutes, inoculum size of 1.046 x $10^7$for 6 days and NaCl (0.1M) as extraction solvent.

APPENDIX B.

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Worksheet size: 10000 cells.

Welcome to Minitab, press F1 for help.

B1. Effect of 6th day fermentation of both filtrates on:

i. Pectinase activity — Two-Sample T-Test and CI.

Sample N Mean StDev SE Mean

1 3 0.1287 0.0259 0.015

2 3 0.0650 0.0262 0.015

Difference = mu (1) - mu (2)

Estimate for difference: 0.063667

95% CI for difference: (-0.004051, 0.131385)

T-Test of difference = 0 (vs not =): T-Value = 2.99 P-Value = 0.058 DF = 3

ii. Protein concentration — Two-Sample T-Test and CI

Sample N Mean StDev SE Mean

1 3 0.565 0.103 0.059

2 3 0.344 0.113 0.065

Difference = mu (1) - mu (2)

-

T-Test of difference = 0 (vs not =): T- -

Estimate for difference: 0.221000

95% CI for difference: (0.059667, 0.501667)

Value = 2.51 P Value = 0.087 DF = 3

B2. Effect of (80:20) % corn cob to orange peel of both filtrates on:

I. **Pectinase activity Two-**

Sample T-Test and CI

Sample N Mean StDev SE Mean

1 3 0.1613 0.0248 0.014

2 3 0.0647 0.0321 0.019

Difference = mu (1) - mu (2)

Estimate for difference: 0.096667

95% CI for difference: (0.022178, 0.171156)

T-Test of difference = 0 (vs not =): T-Value = 4.13 P-Value = 0.026 DF = 3

II. **Protein concentration**

Two-Sample T-Test and CI

Sample N Mean StDev SE Mean

1 3 0.7413 0.0228 0.013

2 3 0.33370 0.00154 0.00089

Difference = mu (1) - mu (2)

Estimate for difference: 0.407600

95% CI for difference: (0.350907, 0.464293)

T-Test of difference = 0 (vs not =): T-Value = 30.93 P-Value = 0.001 DF = 2

B3. Effect of temperature (i.e. 30^{o}C) on both filtrates

Protein concentration

Two-Sample T-Test and CI

Sample N Mean StDev SE Mean

1 3 0.7614 0.0295 0.017

2 3 0.6460 0.0221 0.013

Difference = mu (1) - mu (2)

Two-

Estimate for difference: 0.115433

95% CI for difference: (0.047763, 0.183104)

T-Test of difference = 0 (vs not =): T-Value = 5.43 P-Value = 0.012 DF = 3

B4. Effect of Citrate buffer (pH 5.0) and NaCl (0.1M) on:

➢ Pectinase activity

Sample T-Test and CI

Sample N Mean StDev SE Mean

1 3 0.3548 0.0179 0.010

2 3 0.4194 0.0990 0.057

Difference = mu (1) - mu (2)

Estimate for difference: -0.064567

95% CI for difference: (-0.314488, 0.185355)

T-Test of difference = 0 (vs not =): T-Value = -1.11 P-Value = 0.382 DF = 2

➢ Protein concentration

Two-Sample T-Test and CI

Sample N Mean StDev SE Mean

1 3 1.4177 0.0863 0.050

2 3 1.4192 0.0365 0.021

Difference = mu (1) - mu (2)

Estimate for difference: -0.001500

95% CI for difference: (-0.234334, 0.231334)

T-Test of difference = 0 (vs not =): T-Value = -0.03 P-Value = 0.980 DF = 2

B5. Effect of NaCl (0.1M) and Acetate buffer (pH 5.0) on

➢ Pectinase activity

Two-Sample T-Test and CI

Sample N Mean StDev SE Mean

1 3 0.4194 0.0990 0.057

-

T-Test of difference = 0 (vs not =): T- -

2 3 0.3226 0.0141 0.0081

Difference = mu (1) - mu (2)

Estimate for difference: 0.096800

95% CI for difference: (0.151605, 0.345205)

Value = 1.68 P Value = 0.236 DF = 2

- **Protein concentration Two-Sample**

T-Test and CI

Sample N Mean StDev SE Mean

1 3 1.4192 0.0365 0.021

2 3 1.2537 0.0343 0.020

Difference = mu (1) - mu (2)

Estimate for difference: 0.165500

95% CI for difference: (0.073547, 0.257453)

T-Test of difference = 0 (vs not =): T-Value = 5.73 P-Value = 0.011 DF = 3

B6. Effect of 50% and 60% $(NH_4)_2SO_4$ saturation of crude extraction on:

- **Pectinase activity**

Two-Sample T-Test and CI

Sample N Mean StDev SE Mean

1 3 2.9677 0.0646 0.037

2 3 3.9677 0.0546 0.032

Difference = mu (1) - mu (2)

Estimate for difference: -0.999967

95% CI for difference: (-1.155447, -0.844486)

T-Test of difference = 0 (vs not =): T-Value = -20.47 P-Value = 0.000 DF = 3

- **Protein concentration Two-Sample**

T-Test and CI

Sample N Mean StDev SE Mean

1 3 7.900 0.166 0.096

Two-

2 3 9.497 0.323 0.19

Difference = mu (1) - mu (2)

Estimate for difference: -1.59633

95% CI for difference: (-2.49901, -0.69365)

T-Test of difference = 0 (vs not =): T-Value = -7.61 P-Value = 0.017 DF = 2

B7. Effect of 60% and 70% $(NH_4)_2SO_4$ saturation of crude extraction on:

- **Pectinase activity**

Sample T-Test and CI

Sample N Mean StDev SE Mean

1 3 3.9677 0.0546 0.032

2 3 4.097 0.116 0.067

Difference = mu (1) - mu (2)

Estimate for difference: -0.129133

95% CI for difference: (-0.446741, 0.188474)

T-Test of difference = 0 (vs not =): T-Value = -1.75 P-Value = 0.222 DF = 2

- **Protein concentration Two-Sample**

T-Test and CI

Sample N Mean StDev SE Mean

1 3 9.497 0.323 0.19

2 3 10.90 1.82 1.1

Difference = mu (1) - mu (2)

Estimate for difference: -1.40367

95% CI for difference: (-6.00426, 3.19693)

T-Test of difference = 0 (vs not =): T-Value = -1.31 P-Value = 0.320 DF = 2

B8. Effect of 15mins and 30mins agitations on:

-

T-Test of difference = 0 (vs not =): T- -

- **Pectinase activity**

Two-Sample T-Test and CI

Sample N Mean StDev SE Mean

1 3 0.2581 0.0246 0.014

2 3 0.3548 0.0127 0.0073

Difference = mu (1) - mu (2)

Estimate for difference: -0.096733

95% CI for difference: (0.165606, -0.027860)

Value = -6.04 P Value = 0.026 DF = 2

- **Protein concentration Two-Sample**

T-Test and CI

Sample N Mean StDev SE Mean

1 3 1.044 0.146 0.084

2 3 1.420 0.116 0.067

Difference = mu (1) - mu (2)

Estimate for difference: -0.375767

95% CI for difference: (-0.718077, -0.033456)

T-Test of difference = 0 (vs not =): T-Value = -3.49 P-Value = 0.040 DF = 3

B9. Effect of 30minutes and 60minutes agitation on:

- **Pectinase activity**

Two-Sample T-Test and CI

Sample N Mean StDev SE Mean

1 3 0.3548 0.0127 0.0073

2 3 0.3226 0.0262 0.015

Difference = mu (1) - mu (2)

Estimate for difference: 0.032200

95% CI for difference: (-0.040203, 0.104603)

T-Test of difference = 0 (vs not =): T-Value = 1.91 P-Value = 0.196 DF = 2

Two-

Protein concentration

Two-Sample T-Test and CI

Sample N Mean StDev SE Mean

1 3 1.420 0.116 0.067

2 3 1.3970 0.0207 0.012

Difference = mu (1) - mu (2)

Estimate for difference: 0.023167

95% CI for difference: (-0.270059, 0.316392)

T-Test of difference = 0 (vs not =): T-Value = 0.34 P-Value = 0.766 DF = 2

-

T-Test of difference = 0 (vs not =): T- -

Two-

B10. Effect of 60% $(NH_4)_2SO_4$ saturation of the crude extract in comparison to 30minutes agitations on Pectinase activity

Sample T-Test and CI

Sample N Mean StDev SE Mean

1 3 0.3548 0.0127 0.0073

2 3 3.9677 0.0546 0.032

Difference = mu (1) - mu (2)

Estimate for difference: -3.61287

95% CI for difference: (-3.75224, -3.47349)

T-Test of difference = 0 (vs not =): T-Value = -111.53 P-Value = 0.000 DF = 2

B11. Effect of 60% $(NH_4)_2SO_4$ saturation of the crude extract in comparison to NaCl (0.1M) as extraction solvent on:

- **Pectinase activity**

Two-Sample T-Test and CI

Sample N Mean StDev SE Mean

1 3 0.4194 0.0990 0.057

2 3 3.9677 0.0546 0.032

Difference = mu (1) - mu (2)

Estimate for difference: -3.54830

95% CI for difference: (-3.75608, -3.34052)

T-Test of difference = 0 (vs not =): T-Value = -54.35 P-Value = 0.000 DF = 3 ○

Protein concentration

Two-Sample T-Test and CI

Sample N Mean StDev SE Mean

1 3 1.4192 0.0365 0.021

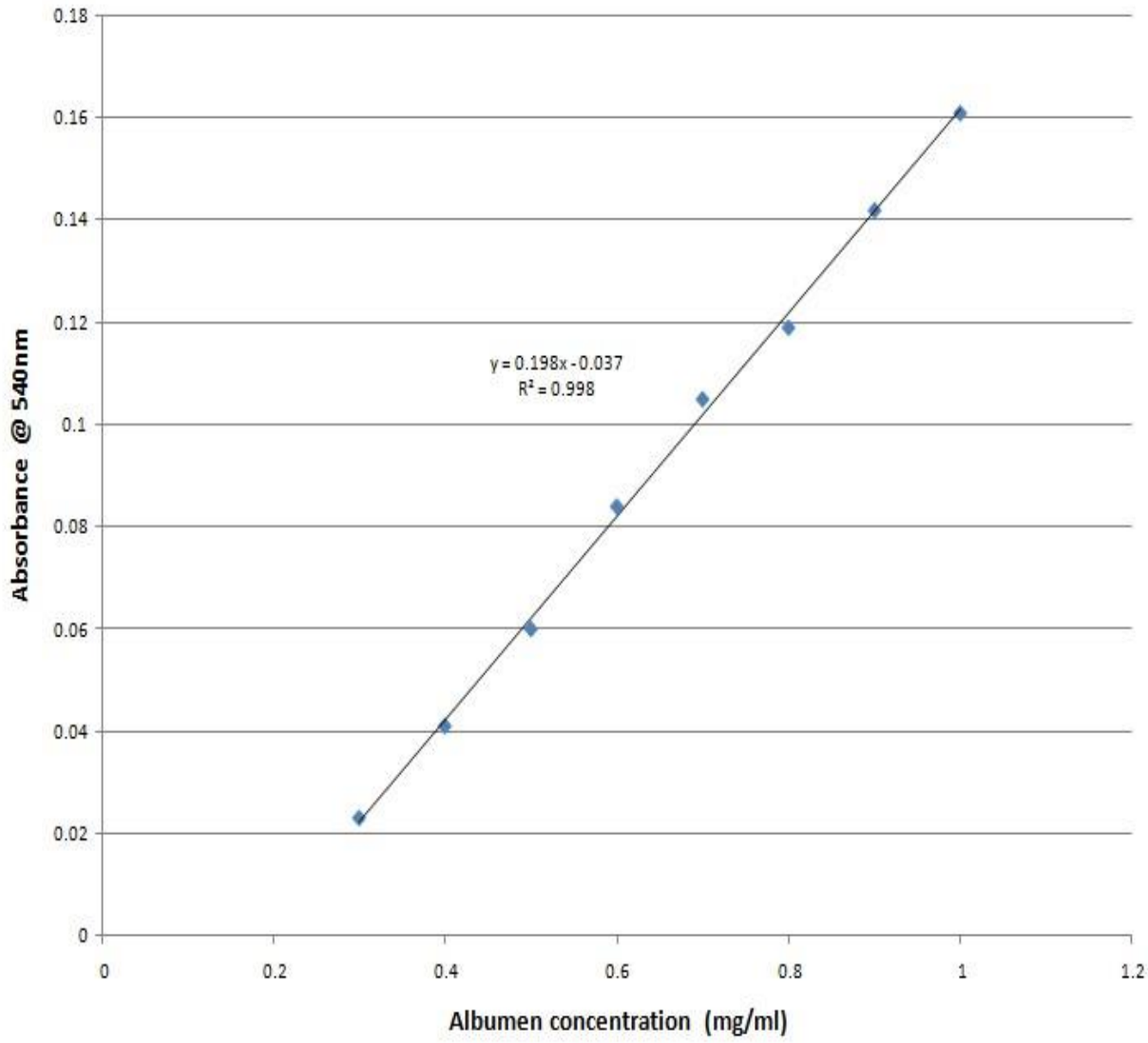
2 3 9.497 0.323 0.19

Difference = mu (1) - mu (2)

Estimate for difference: -8.07750

95% CI for difference: (-8.88614, -7.26886); T-Test of difference = 0 (vs not =): T-Value = -42.98 P-Value = 0.001 DF = 2

APPENDIX C



Protein Standard Curve.

APPENDIX D.

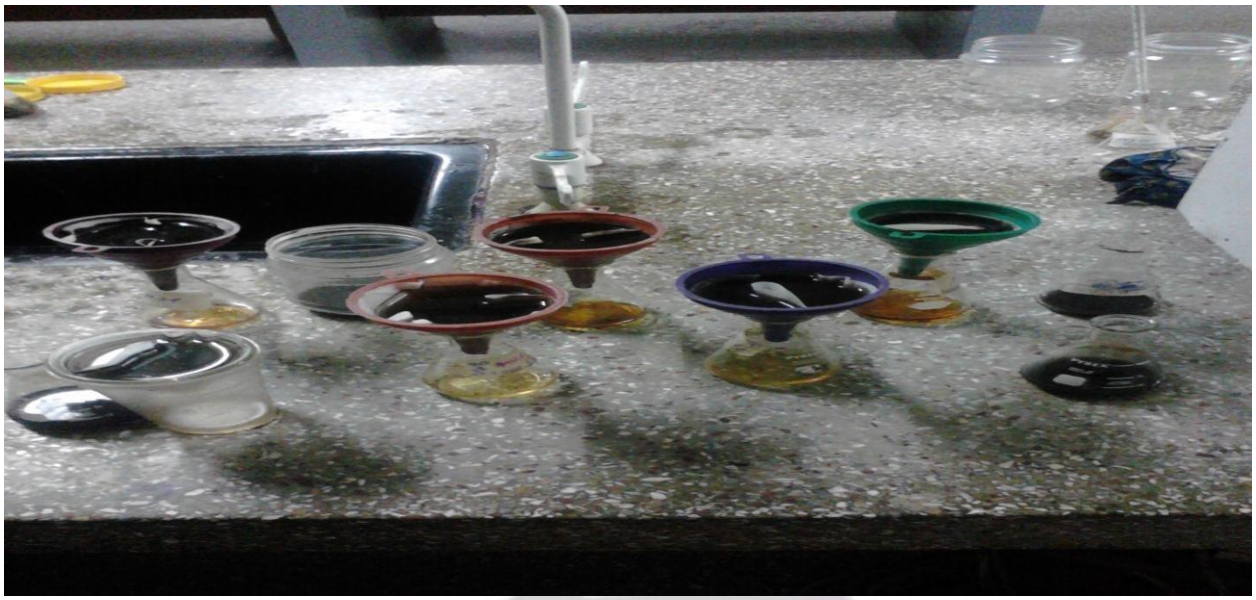
SOME IMAGES TAKEN IN THE COURSE OF THE WORK

D.1 Percentage ratio combination

D.2. Buffer at pH 5.0

D3. Culture of *Saccharomyces cerevisiae* ATCC 52712.

D.4. Filtration of the fermented substrate



D. 5.Filtrate extracts.

D.6. Spectrophotometer.

D.7. Inoculation hood and incubators.