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COMPARATIVE EFFECT OF CRUDE PECTINASE AND COMMERCIAL ENZYMES IN SHEA FAT EXTRACTION

BY

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CERTIFICATION PAGE

I hereby declare that this submission is my own work toward the MSc. and that, to the best of my knowledge, it contains no material previously published by another person or material which has been accepted for the award of any other degree of the University, except where due acknowledgement has been made in the text.

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ABSTRACT

Pectinases are one of the most used enzymes in food industry. Enzymes are widely used to improve upon process parameters such as product yield and rate of product formation. Such biotechnological applications are not currently exploited by most industries in Ghana. The objective of the study was to determine the efficacy of locally produced pectinases from *S. cerevisiae* and other commercial enzymes in the extraction processes of shea butter. The crude pectinase was produced using corn cobs as a substrate and the microorganism *Saccharomyces cerevisiae* ATCC 52712 in a solid state fermentation process for four days. The crude pectinase had optimal protein concentration of 7 mg/ml with enzyme specific activity of 0.130 U/mg. The commercial enzymes were Viscozyme L (beta endo- 1, 3 (4)-glucanase activity with declared activity of 100 FBG/g as well as side activities of xylanase, cellulase and hemicellulases) and Pectinex 5XL (polygalacturonase activity with declared activity of 4500 PECTU/ml as well as arabinase side activity). The following operational parameters: water to seed (w/s) ratio, pH, hydrolysis time, enzyme dosage and temperature were optimized for better oil recover. The optimum w/s ratio in the present study was 4:1 for the enzymes and 2:1 for the control. The study revealed that the crude pectinase works under a wide range of pH. Crude Pectinase exhibited maximum activity at pH range of 3 to 6. Commercial Pectinex and Viscozyme showed maximum activity at pH range of 4 to 6. The maximum oil yield for crude pectinases and commercial Pectinex was obtained at 90 minutes and that of Viscozyme treated seed was observed at 60 minutes incubation time. When all parameters were optimized, the control gave an optimum oil recovery of 40 % while the application of the crude pectinase gave an optimum oil recovery of 44 % with an enzyme dosage of 1.2 %. The commercial Pectinex and commercial Viscozyme gave optimum oil recovery of 58.6 % and 72.0 % respectively at enzyme dosage of 0.8 %. Analyses of the proximate composition of the residues revealed that with the exception of protein, no significant variations were observed for the fiber and ash content. The physicochemical characteristics of the extracted oils such as Density, Moisture, Sample melting point (SMP), Unsaponifiable matter (UM) with the exception of the fatty acids (FFA) and peroxide value (PV), were not significantly affected ($P > 0.05$) by the type of enzyme used. These results indicate the possible use of crude pectinase to improve shea fat extraction processes in Ghana if the enzymes are made available.

Keywords: Aqueous extraction, crude pectinase, corn cobs, Saccharomyces cerevisiae, solid state fermentation

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DEDICATION

I dedicate this thesis to my mum, I lost during my studies. Auntie Cecilia Abena Ampomaa Boateng you were a gift to us.

CHAPTER ONE

1.0 INTRODUCTION

Shea butter is a fatty extract from the seed of the shea nut (Salunkhe *et al.*, 1986), has approximately 50 % (w/w) of poly unsaturated fatty acids (PUFA) consisting of oleic (40.8 %), linoleic acid (6.9 %) and linolenic acid (1.6 %), and a degree of unsaturation of 0.59 % (Adomako, 1977, 1985). The shea tree, formerly *Butryospermum paradoxum*, now called *Vitellaria paradoxa*, is a wild plant and populates the dry Savannah belt of Senegal in the Western part of Africa through Sudan to the foothills of the Ethiopian highlands (Boffa, 1999). In Ghana, shea trees are commonly found in the Upper East, Upper West and the Northern Regions. Shea butter is obtained from the dried kernel of the shea nut and is usually seen as the major occupation of majority of the women in these regions. It is estimated that more than 600,000 women depend on the income from the sales of shea butter and its related products (*www.undp-gha.org/project*).

The vegetable fat of shea nut is second in importance only to palm oil in Africa (Hall *et al*., 1996). The fat is the source of edible oil for majority of the people in Northern Ghana and therefore the most important source of fats and glycerol in their diet (Agyente-Badu, 2010). A report from the American Shea Butter Institute (ASBI, 2004) shows that 100 % pure natural shea butter is an all natural vitamin A cream and is a superb moisturizer with exceptional healing properties. In the same development ASBI (2004) reported that 100 % shea butter has proved to be effective on a number of skin conditions such as dry skin, skin rash, skin peeling after tanning, blemishes and wrinkles, itching skin, sunburn, small skin wounds, skin cracks and tough or rough skin (on feet). Others are cold weather, frost

bites, stretch mark prevention during pregnancy, insect bites, muscle fatigue, aches and tension, skin allergies such as those from poison ivy or poison oaks, eczema, dermatitis and skin damage from heat. According to Russo and Etherington (2001), shea products are ideal for use as raw materials for cooking oil, margarine, cosmetics, production of soap, detergents and candles. Currently shea butter is being used as a substitute for cocoa butter in the chocolate and the confectionery industry (Kapseu *et al.*, 1999).

The extraction of shea fat is done by one of two extraction methods broadly described as traditional or modern. The traditional method of extraction is the preferred choice of most shea butter industries in Ghana. This method of shea butter production encompasses many manual unit operations. Each of these units apparently has some problems which renders the whole process tedious and laborious. To address this situation, researchers have identified the application of enzymes in the production of oil from oilseeds as an alternative and environmentally friendly technique for overcoming these challenges (Lusas *et al.*, 1982; Barrios *et al.*, 1990). Enzymes are the largest class of proteins and are catalysts that accelerate the rates of biological reactions (Garrett and Grisham, 1995).

Enzyme reactions may often be carried out under mild conditions; they are highly specific and normally have no side effect. The use of enzymes has emerged as a novel and an effective means to improve the oil yield in cold pressing and aqueous extraction techniques (Tano-Debrah and Ohta 1994) and it has been reported by many researchers; for instance Fullbrook (1983) in his earlier work reported on soybean and rapeseed; Buenrostro and Lopez-Munguia (1986) on avocado; McGleone *et al*., (1986) on coconut and Cheah *et al*., (1990) on palm oil. These studies revealed that treatment of the oil

bearing seeds with some enzymes after mechanical milling degrades the cellular structure of the material. Commercial enzymes which have been used for edible oil extraction are cellulase, protease, glucanase, amylase, hemicellulases and pectinases (Kamar *et al.*, 2000; Tano-Debrah and Ohta 1994; Rosenthal *et al.*, 1996).

The use of such commercial enzymes in the shea oil industry in Ghana will be difficult due to the cost involved in acquiring the enzyme, and also maintaining the activity of the enzyme as a result of frequent power fluctuations in the country. The alternative approach therefore will be to produce the enzyme locally and make it available to the shea industry on demand. Dzogbefia *et al.*, (1999) reported pectinase enzyme activity from *S. cerevisiae* (ATCC52712) and have utilized this enzyme effectively in fruit juice extractions (Dzogbefia *et al.*, 2001) and in cassava starch extraction (Dzogbefia *et al*., 2008). It is therefore attractive to investigate into the use of locally produced pectin enzymes in the shea butter industry to improve the technology of shea butter production in Ghana.

1.1 Problem Statement

The shea butter extraction industry has many challenges ranging from mesocarp removal, drying, shelling, winnowing, temperature control, crushing, and kneading. The traditional approach to shea butter production encompasses many manual unit operations such as roasting, milling, kneading, cooking and clarification prior to fat extraction. Each of these units apparently has some problems which render the whole process tedious, laborious, time consuming, energy sapping, environmentally unfriendly and grossly inefficient (Olaniyan *et al*., 2007). The use of chemicals in the extraction of the butter also ends up

destroying the beneficial ingredients. All these factors contribute to the low levels of yields of about 25 % and the product often being low in quality (Niess, 1983).

1.2 Goal

To determine the efficacy of locally produced pectinases from *S. cerevisiae* relative to two commercial enzymes in the extraction processes of shea butter.

1.2.1 Specific objectives

- To determine the level of protein and enzyme activity when corn cobs are used as substrate in the production of pectinases
- To establish optimum conditions of pH, temperature and water to seed ratio on oil extraction
- To determine effect of enzyme dosage on yield of butter
- To determine effect of reaction time on oil yield
- To compare quality of oil extracted with and without enzymes

1.3 Justification of Project

The commercialization of shea products represents an important source of income at different parts of the community chain and this is evident by the fact that the shea industry in Ghana has offered employment to about 600,000 women in Northern Ghana (*www.undp-gha.org/project*). Despite its great contribution to the local economy, shea butter remains underutilized due to lack of innovative ways of extracting the butter. Though the annual post harvest loss of shea nut in Ghana has not been documented, it is

generally believed that a larger portion of the kernels are left to decay. This is because its product diversity is limited.

It has been reported that the lower yield of oil obtained using aqueous extraction can be increased by using enzymes (Rosenthal *et al.*, 1996). Enzymes are normally used to enhance vegetable oil extraction. The use of enzymes to facilitate the extraction of oil from shea nut can increase the value and product utilization of the crop and reduce the post harvest loss. Secondly, the inefficiencies recorded in the traditional extraction methods (manual) as far as yield (25 %) and quality are concerned, can be improved with the application of enzymes which are cost intensive. The cost can further be reduced if enzymes are produced locally. Additionally, oil from shea nut contains poly unsaturated fatty acids (PUFA) and essential fatty acids which will contribute to the optimum nutrition of consumers. Finally, the dearth of knowledge on the local production of enzymes and their utilization in the extraction of oil from shea nut will be addressed.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Ecology of the Shea Tree

The shea tree also known as *Vitellaria paradoxa* (Maranz *et al.*, 2003) or *Butryospermum parkii* belongs to the *Sapotaceae* family. The plant mainly grows in the Savannah belt of West Africa from Senegal in the west to Sudan in the east and covers a distance of nearly 5,000 Km across the African continent. It occurs on an estimated 1million km^2, where annual rainfall ranges from 500 to 1200 mm (Salle' *et al.*, 1991; Boffa, 1995). The tree can be found growing naturally in the southern region of the Sahel and the northern regions of the Guinea Zone. It thrives very well in savannah areas where oil palm cannot grow due to low rainfall and today the shea tree produces the second most important oil in Africa after palm (Poulsen, 1981).

The major producing countries are West African countries (Mali, Burkina Faso, Benin, Senegal, Ivory Coast, Ghana and Nigeria). Using Land satellite remote sensing image in Mali, 18 million shea butter trees were counted over a distance of 20 km (Fleury, 1981). The tree usually grows to an average height of about 15 m. In Ghana, it virtually covers about two-thirds of the country, mostly in the wild state (Abbiw, 1990). The tree grows gradually from seeds, taking 12 to 15 years to bear fruit and about 30 years to mature (Adomako, 1985), but limiting or conditions such as bush-fire and harsh weather can reduce this. The tree starts flowering in November and fruits from April to August every year. When the fruits ripen, they fall to the ground and are gathered by hand, mostly by women and children. The crop is ellipsoidal in shape and has a white scar at one side.

The main size of the nut is about 35 mm long x 25 mm wide x 23 mm thick. There is a kernel inside the nut which fits properly into the shell and is about 32 mm long, 23 mm wide x 21 mm thick in size. It has a uniform shell of about 1 mm thick, the fresh fruit is green in colour, has a fleshy edible pulp which contains protein and carbohydrates, and is very sweet (Maranz *et al.*, 2003).

According to an FAO report, the fruit pulp is very rich in ascorbic acid, iron and calcium (FAO, 1988). Oil obtained from shea nut contains some fatty acids such as palmitic acid, stearic acid, oleic acid and linoleic acid. Shea butter is soft and solid at tropical temperature. It is yellowish white in colour and has a strong smell (Tessy, 1992).The fat content of the kernel and fatty acid profile are, however, extremely variable across the various areas in West Africa (Maranz *et al.,* 2004). Shea butter is the fat obtained from the shea kernel and is the most valued product from the shea tree (Hall *et al.,* 1996). The butter is also used to make soap and in the construction industry, it is used on the walls of houses to prevent them from being washed away during the rainy season. Shea butter can also be used as a cocoa butter substitute in the confectionery industry (Fleury, 1981).

2.2 Processes for Oil Extraction from Seeds

Edible fats and oils have been separated from animal tissues, oilseeds, and oil-bearing fruits for thousands of years. The purpose of all fats and oils recovery processes is to obtain triglycerides in high yield and purity and to produce co-products of maximum value. Several oil extraction methods have been developed but wet rendering, mechanical pressing and solvent extraction are the basic methods for oil extraction. Mechanical (hydraulic and screw) pressing has been practiced for thousands of years (Bargale, 1997).

Oilseed material is boiled in the wet rendering method for partial separation then the oil is skimmed off (Bredeson, 1983). The third and the most conventionally used method is the solvent extraction (Bargale, 1997).

2.2.1 Traditional pre-treatment and extraction of shea butter

Shea butter is a slightly yellowish or ivory coloured natural fat extracted from the fruit of the shea tree. Prior to the processing of the kernels into butter, the nuts undergo pre-treatment to render them suitable for the oil extraction process; this is important because the process of gathering fruits from the wild brings with it unwanted foreign materials.

The first stage of pre-treatment involves the removal of such unwanted materials. Shea fruits are then left in the open to ferment for between 3 – 5 days after which they are de-pulped to separate the fruit pulp (mesocarp and epicarp) from the nuts. The fermentation facilitates the removal of the fleshy pulp. The nuts are then sun dried to reduce the moisture content to about 15-30 %. The nut, which is made up of a hard outer shell with the kernel inside, is pounded in a mortar with a pestle, cracked between two stones or trampled upon with the feet to liberate the kernel (Salunkhe *et al.,* 1992). The kernels are thoroughly dried between 10-20 days depending on the weather conditions. They are then bagged and sold or processed into shea butter.

2.2.2 Traditional method of extraction of shea butter

Traditionally, the kernels are dried and then roasted in a pan. The kernels are stirred from time to time to ensure even roasting. After roasting they are allowed to cool and then ground into powder using a mortar and a pestle or an ordinary corn mill. The powder is

then mixed with cold water to form a paste which is then kneaded continuously with the fingers into a semi-solid mixture. Kneading continues until the original dirty brown colour of the paste turns grey. The fat suspends on top of the water and it is scooped and washed several times to ensure that the fat is free from impurities.

The grey fluffy matter is put into a cooking pot and heated. The grey matter, which is dirty or impure fat, melts and floats to the surface, leaving a brown solid residue to settle at the bottom of the pot. The oil is skimmed off into open calabashes and allowed to solidify. It is stirred continuously to facilitate solidification; it is then divided and wrapped in leaves for selling or for storage. The butter will last for many years if kept away from light and heat as it is resistant to oxidative rancidity (Fleury, 1981). The manual extraction method has been used for centuries; although this process does not produce high yields of shea butter, it is still the most used method of extracting shea butter in northern Ghana. Chemicals or synthetic agents are not added and therefore the butter extracted is pure or unrefined. According to Gray Ann (1997), the traditional method of extracting shea butter is a time consuming, arduous process using large quantities of water and firewood and averagely it takes 8.5- 9.5 hours for a woman to process 20 kg of shea paste.

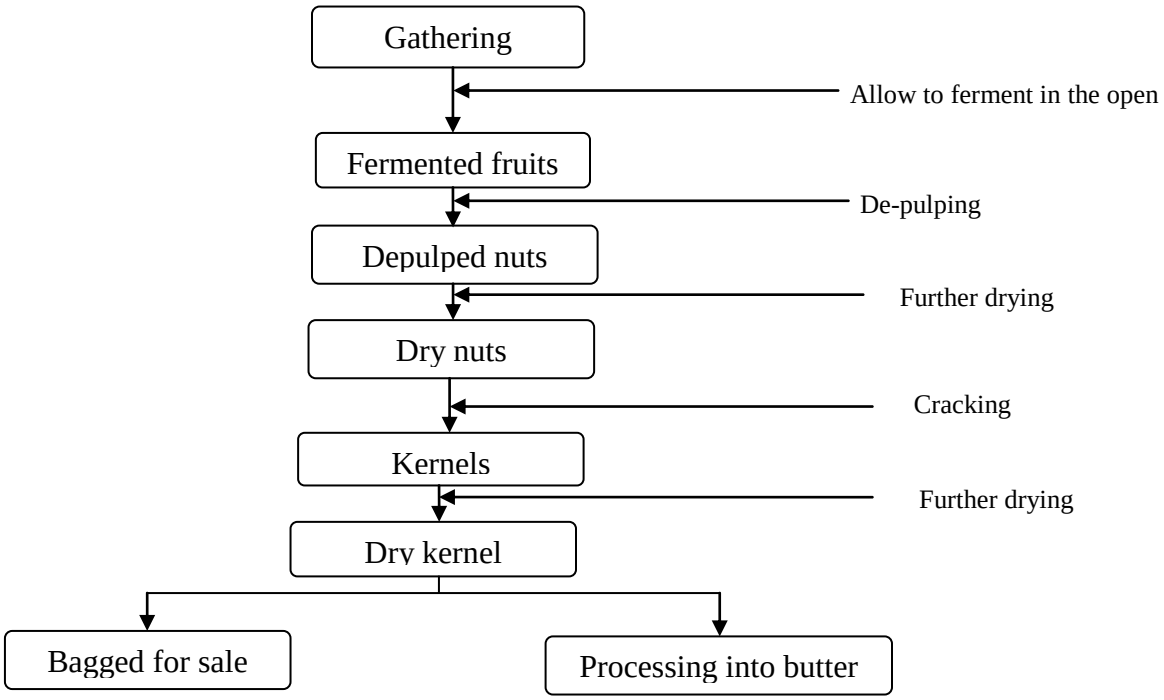

Figure 2.1: Flow chart for local collection and pre-treatment of shea nuts

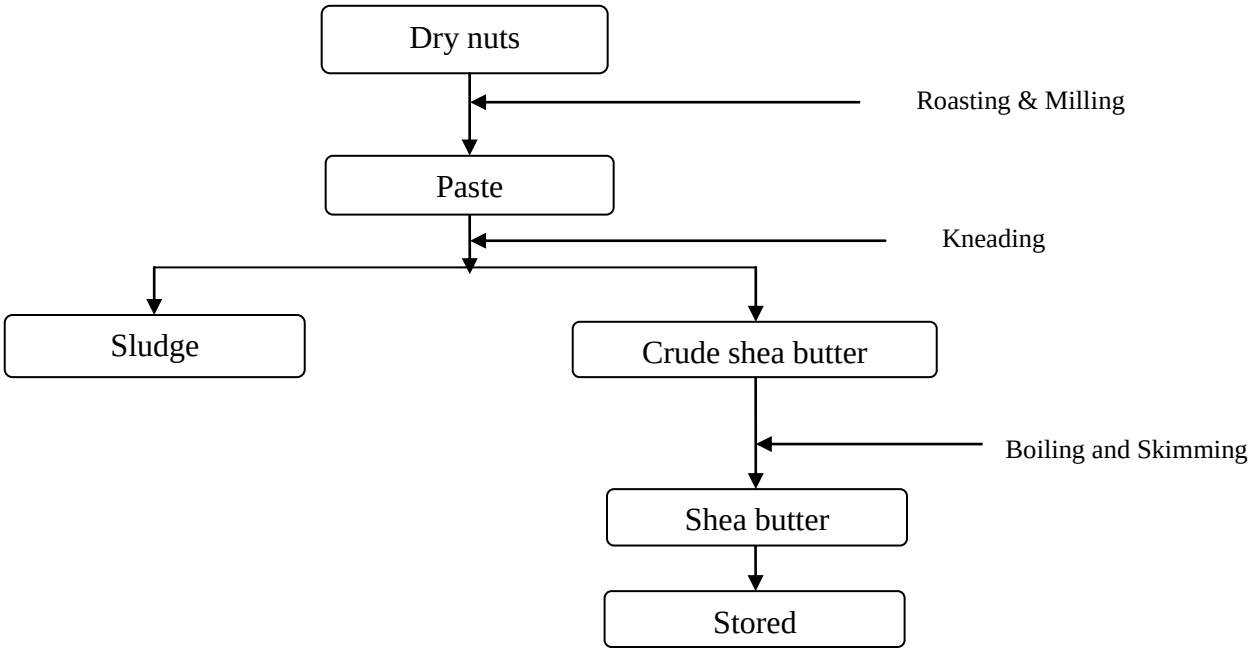

Figure 2.2: Flow chart for local processing of shea butter

2.2.3 Solvent extraction

Solvent extraction of oil from oilseeds is the most efficient and attractive method for oilseeds having low oil content (Anjou, 1972; Caviedes, 1996). This is the most economical, efficient and widely used process for high oil content seeds (e.g. sunflower, peanut, canola) and also for medium oil content seeds (cottonseed and corn germ) (Norris, 1964; Ward, 1982). Therefore, vegetable oils are mostly extracted by using solvent extraction (Kemper, 2005). Solvent extraction originated as a batch process in Europe in 1870 and developed to the continuous solvent extraction systems by technological developments (Mustakas, 1980). Extraction is normally carried out in a counter-current fashion to maximize oil recovery while minimizing solvent use (Balke, 2006).

The most common type of extractor used is the percolation type in which the solvent, hexane flows by gravity through a bed of meal (Anjou, 1972; Beach, 1983). When used hexane leaves the extractor with an oil concentration of 25-30 % and the defatted meal with a solvent content of 30-35 % (Anjou, 1972). The hexane in the meal is removed in a desolventizer-toaster which consists of steam heated trays. The meal is first heated by indirect heat in the upper trays to remove most of the solvent, followed by a direct steam injection in the middle trays, and finally by indirect heat in the lower trays to dry the meal (Anjou, 1972; Beach, 1983). The meal has dark colour with 6-11 % moisture content and 300 to 1800 ppm hexane (Beach, 1983; Dahlen and Lindh 1983).

This desolventization of meal is the main source of solvent losses (Dahlen and Lindh 1983). The solvent from the oil is removed in rising film evaporators and vacuum

distillation (Serrato, 1981). The hexane containing oil (miscella) is sent to a double effect shell-and-tube rising film evaporator, where 90 % of the hexane is removed (Anjou, 1972). The rest of the hexane and moisture in the oil is removed in stripping tower under vacuum (100 torr) at 110 ^{o}C (Anjou, 1972; Beach, 1983). The extracted oil is sent for degumming which removes the phosphatides by treating the oil with a small amount of water (2%) or a dilute acid solution (Caviedes, 1996). Mostly, the oil is super degummed (acid degummed) by using 2500 ppm H_3PO_4, or 1500 ppm citric acid or maleic acid and the oil is separated in a continuous centrifuge which is dried in vacuum at 80 ^{o}C (Caviedes, 1996).

Bargale (1997) summarized the limitations of the solvent extraction method; the process is not chemically safe for human health, although desolventization removes most of the solvent, some traces are always present in the meal. The solvents used for the oil extraction are highly flammable. Solvent extraction tends to be expensive since high initial capital and operating costs are required for handling even smaller quantities of oilseeds. The energy requirements are high and a lower quality of oil (with high content of phosphatides) is recovered. As a consequence of these drawbacks of solvent extraction method, several new approaches have been tried in order to develop a better technology (Bargale, 1997).

2.2.4 Pressing

Mechanical pressing (hydraulic and screw) is the oldest and simplest method for oil extraction from oleaginous seeds. No chemical is used for oil extraction and therefore the residue is free of chemical residue. The first records of oilseed presses were from

Sanskrit near 500 BC (Achaya, 1994) whereas in 1795, hydraulic pressing was invented in Europe (Mustakas 1980). It is a labour intensive technique and its use declined over the years.

Continuous screw-presses (expellers) have replaced the hydraulic equipment (Bargale, 1997). It consists of an extruder with a perforated body (slots or holes), a helical screw is used to convey and press out the oil (Balke, 2006). A major drawback of this process is the lower oil extraction. Heat pre-treatment improves the malleability of the seed, lower the shattering and denature some proteins to improve oil extractability (Balke, 2006). Before pressing, the seeds are flaked to crush the seed shell for better oil diffusion (Ward, 1982). The extracted oil is of superior quality but intense pressure and heat damages the seed protein (Balke, 2006). This method is more popular in developing countries for low operating costs than the solvent extraction (Bargale, 1997). The meal obtained is very compact and unsuitable for solvent extraction therefore, this method has been eliminated by modern expressors (Achaya, 1994; Head and Swetman, 1999).

On using the shea nut press, Neiss (1983), commented that no further processing of the oil is necessary. It should be noted that using a shea nut press, not only alleviates the time consuming process but also improves the fat output. For example, using a shea press, fat output will be between 40 to 45%, whereas fat output using the traditional method will be about 25 % (Neiss, 1983).

2.2.5.1 Aqueous extraction

The aqueous extraction process was developed as an alternative to the solvent oil extraction process in the 1950s (Rosenthal *et al*., 1996). It was thought to be safe and cheap with simultaneous recovery of oil and protein from oil-bearing materials (Cater *et al*., 1974). The hot water flotation method for oil extraction from oilseeds is a traditional process used in the rural areas of many developing countries (Southwell and Harris 1992), and has five basic steps: heating of the seeds, grinding, and extraction by boiling, oil recovery, and drying (Rosenthal *et al*., 1996). Aqueous extraction of peanuts was first described by Sugarman (1956). Peanuts were flaked and wet ground with an addition of water and alkali to maintain a pH of 7.5. The slurry was diluted with water at constant pH, heated to 80 °C and passed through a centrifuge to separate solids, emulsion phase and the aqueous protein solution. The solids were dried and the protein from the aqueous solution was precipitated by lowering the pH and separating the protein precipitate by centrifugation or filtration. The emulsion was broken by heating; controlling its pH and moisture content. High shear and pressure stress can be used to break emulsion. The stream was then sent to another centrifuge where oil, solids and aqueous phase were obtained.

Subrahmanyan *et al*. (1959) and Bhatia *et al*. (1966) extended Sugarman extraction system with slight modification in two points: milling was used to extract most of the oil, and the conditions under which oil was recovered. In this process 86 % of oil and 71 % of protein was recovered. The low oil recovery was attributed to a low efficiency in the three phase separator where 5 % of the oil was not recovered from the aqueous solution.

The oil had good quality with low colour and free fatty acid (FFA) contents. The protein product had a composition of 85.0 % protein, 9.0 % fat, 6.0 % moisture and 0.4 % ash and the carbohydrate meal had 16.2 % protein, 6.1 % fat, 12.0 % moisture, 41.6 % starch, 10.3 % crude fiber and 6.0 % ash contents.

Bhatia *et al*. (1966) improved this process and reported 91-94 % oil recovery and protein products with 4 % fat. Aqueous extraction of oil and protein from oilseeds has been studied for peanuts (Bhatia *et al*., 1966; Rhee *et al*., 1972; Lawhon *et al*., 1981), coconuts (Hagenmaier *et al*., 1972, 1973; Kumar and Bhowrnick 1995), soybeans (Lawhon *et al*., 1981), cottonseed (Sugarman 1956), sunflower (Hagenmaier 1974) and rapeseed (Embong and Jelen 1977; Staron and Guillaumin 1979).

2.2.5.2 Enzyme-assisted aqueous extraction (EAAE)

Aqueous enzymatic oil extraction is an emerging technology in the fats and oil industry, and it may offer many advantages as compared to conventional extraction (Rosenthal *et al*., 1996). It eliminates solvent consumption, which may lower investment costs (Barrios *et al*., 1990; Lusas *et al*., 1982) and energy requirements (Barrios *et al*., 1990). Simultaneous oil and protein recovery from most oilseeds is possible. Degumming operation can be eliminated and it may allow the removal of some toxins or antinutritional compounds from oilseeds (Caragay 1983). Several studies have been carried out on aqueous processing of oilseeds (Eapen *et al*., 1966; Hagenmaier *et al*., 1973; Kim 1989 and Rhee *et al*., 1972).

The oil globules are associated with proteins and a wide range of carbohydrates inside plant cells surrounded by a thick cell wall which has to be ruptured to release the protein and oil. Enzymatic hydrolysis of cell wall is an option for pre-treatment of oilseeds as it hydrolyses the complex lipoprotein and lipopolysaccharide molecules into simple molecules, thus releasing extra oil for extraction. Fullbrook (1983) first isolated the crude protein from melon seeds by enzymatic hydrolysis and released extra oil. Then this process was utilized for the processing of ground soybean and rapeseed. Bhatnagar and Johari (1987) reported an enhanced release of oil in crushed soybean, cottonseed and castor bean hydrolyzed in the presence of hexane.

Hydrolytic enzyme was initially investigated by Sherba *et al*. (1972) for partially releasing oil from full fat, heat-treated soya meal. Fullbrook (1983) hydrolyzed oilseeds in aqueous medium followed by solvent extraction. He also tried hydrolysis in the presence of a solvent to simultaneously extract the released oil. He observed that yields could be considerably improved if hydrolysis of the finely ground flour of soybean and rapeseed was carried out in the presence of solvent. By using 3 % enzyme mixture obtained from *Aspergillus niger*, 50 % more oil was obtained for rapeseed and 90 % of the original extractable oil for soybeans (Fullbrook, 1983). Olsen (1988) also performed aqueous hydrolysis of dehulled rapeseed with enzyme preparation including: pectinase, cellulase, and hemicellulase followed by extraction of the residual oil with petroleum ether. In these studies the enzymatic hydrolysis increased the permeability of the seed cell wall for more efficient extraction of the oil.

Aqueous enzymatic process appears to be an attractive approach compared to the conventional hexane-based process. Several reports suggest that oils obtained from aqueous or enzymatic extraction show superior quality (Bocevska *et al*., 1993; Dominguez *et al*., 1994; Che Man *et al*., 1996). However, Dominguez *et al*. (1994) reported that the composition and characteristics of oils obtained from enzyme assisted process was similar to the oil obtained by solvent extraction, but the color of the oil obtained by aqueous enzymatic process was lighter than solvent-extracted oil. EAAE has several advantages over a solvent extraction; no need for solvents, lower capital investment, higher safety standards, simple process, simultaneous recovery and purification of protein and oil, and less pollution (Cater *et al*., 1974; Kumar and Bhowmick 1995; Rosenthal *et al*., 1995). However, lower efficiency in oil extraction, higher risk of microbial contamination, need for a de-emulsification step and treatment of the resulting aqueous effluent have discouraged its commercial application.

As discussed earlier in comparison with *n*-hexane, the aqueous-based process is safer and more environmentally friendly. On the other hand, lower oil yields, the need for more difficult oil recovery steps and production of significant volumes of aqueous effluent are some of the disadvantages. In order to reduce aqueous effluent and save energy consumption, water used for extraction can be recovered and reused (Southwell and Harris 1992). Furthermore, recycling enzymes with water circulation can help to cut down the cost of enzyme (Barrios *et al*., 1990). Dominguez *et al*. (1995) reported that 30 % of active enzyme was eliminated in the effluent. It is only possible to recycle and reuse enzyme if its activity had not dropped significantly in the process. Furthermore, it

can be seen that enzymatic process might give a lower oil yield than solvent extraction. Protein obtained as a by-product, can be a valuable food ingredient. To improve the extraction yields more parameters have to be investigated.

2.3 Factors Affecting Oil Yield on Enzyme-assisted Aqueous Extraction (EAAE)

The unit operations in the aqueous process reported by Cater *et al*. (1974) and Lawhon *et al*. (1981) include grinding, extraction parameters, solid-liquid separation and product recovery.

2.3.1 Grinding of oilseeds

The whole oilseeds or fruits must be ground to fine particles for more efficient extraction of water-soluble components (Cater *et al*., 1974; Lawhon *et al*., 1981). Wet or dry grindings are normally used depending on the type of oleaginous materials (Cater *et al*., 1974). For high moisture content oleaginous materials (coconuts and palm kernel) wet grinding is used (Cater *et al*., 1974; Kim, 1989) and dry grinding is suitable for low moisture oilseeds such as sunflower seeds, peanut and soybean (Hagenmaier, 1974; Rhee *et al*., 1972; Rustom *et al*., 1991; Rosenthal *et al*., 1998). It has been reported that significantly higher protein and oil extraction yields were obtained from smaller particles of non-heat treated soybean flour than the larger sizes (Rosenthal *et al*., 1998). However, for peanuts, maximum recovery was observed with fine particle size, but a very stable emulsion was also observed in aqueous media (Rhee *et al*., 1972).

2.3.2 Extraction parameters

In enzymatic extraction, the ground seeds are mixed with water, and then agitated to increase extraction. The main parameters that influence the enzymatic extraction yields include enzyme mixture, enzyme concentration, and particle size of oilseed, pH, solid-liquid ratio, temperature and time (Cater *et al*., 1974; Lawhon *et al*., 1981). Buenrostro and Lopez-Munguia (1986) reported better oil recovery for avocado with 1 % (w/w) α-amylase, paste-to-water ratio of 1:5 at 65 ^{0}C for 1.5 h. The pH during the process depends on the composition of oilseed. In aqueous process, the aim is to obtain high oil and protein yields but it is not possible at a pH value near the isoelectric point of protein, because at this pH, protein can bind the oil in a very stable emulsion (Rosenthal *et al*., 1996). For peanut, a pH of 4-5 was found to be suitable for oil extraction with no emulsion phase and protein can be extracted easily at a pH of 8.0 (Rhee, 1972).

Different studies on enzymatic processes were conducted at different temperatures and the optimum temperature range (40-60 ^{0}C) was reported for several oilseeds (Rhee *et al*., 1972; Lawhon *et al*., 1981; Kim 1989; Rustom *et al*., 1991). Rosenthal *et al*. (1998) reported that the protein yield was decreased slightly when temperature was higher than 50 ^{0}C. No significant increase in the palm oil yield was reported when extraction temperature was raised above 45 °C (Kim, 1989). For peanut oil extraction, a temperature range of 60-64 ^{0}C, and a lower temperature of 40-44 ^{0}C was found to be suitable for protein extraction (Rhee *et al*., 1972). An unfavourable effect of temperatures greater than 60 ^{0}C was observed on protein extraction yield from peanuts (Rustom *et al*., 1991). This increased temperature, may cause denaturation of protein.

The extraction time especially, depends not only on the oilseeds type and temperature, but also on pH during the extraction process. According to Rhee *et al*. (1972) maximum oil and protein yields from peanut were obtained within 30 min, while Rustom *et al*. (1991) reported that increasing extraction times from 15-40 min had no significant effect on the yields. In another study, it was mentioned that an extraction time of 15 min was sufficient for protein and oil extraction from soybean flour (Rosenthal *et al*., 1998). Solid-to-liquid ratio is another important parameter that allows the products to be dispersed in the aqueous media. A high amount of water will produce a less stable emulsion and will ultimately simplify the separation process (Rosenthal *et al*., 1996).

2.4 Uses of Shea Tree and Butter

The shea tree is sacred to many ethnic groups and plays an important role in religious ceremonies (Vuillet, 1911; Millee, 1984).The fruit pulp, being a valuable food source, is also taken for its slightly laxative properties (Soladoye *et al.*, 1989). The leaves are used as medicine to treat stomach ache in children (Millee, 1984). A leaf decoction is also used as an eye bath (Abbiw, 1990; Louppe, 1994). The leaves are a source of saponnin, which lathers in water and can be used for washing (Abbiw, 1990). When a woman goes into labor, branches may be hung in the doorway of her hut to protect the newborn baby. Branches may also be used for covering the dead prior to their burial (Agbahungba and Depommier, 1989).

In most savannah areas the roots are used as chewing sticks (Isawumi, 1978). In the treatment of jaundice, the roots and root bark are ground into paste and taken orally (Ampofo, 1983). These are also used for the treatment of diarrhoea and stomach ache

(Millee, 1984). Mixed with tobacco, the roots are used as a poison by the Jukun of northern Nigeria. Chronic sores in horses are treated with boiled and pounded root bark (Dalziel, 1937).

Macerated with the bark of *Ceiba pentandra*, and salt, bark infusions have been used to treat cattle with worms in the Tenda region of Senegal and Guinea (Ferry *et al.*, 1974). The infusions have been used to treat leprosy in Guinea Bissau (Dalziel, 1937) and for gastric problems as well as for diarrhea or dysentery (Soladoye *et al.*, 1989). Bark infusion is used as an eyewash to neutralize the venom of the spitting cobra (Soladoye *et al.*, 1989) and also, in Ghana, as a footbath to help extract jiggers. Greenwood (1929) noted that the stripping of bark for medicinal purposes may have a severe impact on the health of shea trees and may even be fatal. The wood is only used when individual trees are not valued for butter production. The latex is heated and mixed with palm oil to make glue (Hall *et al.*, 1996). It is chewed as a gum and made into balls for children to play with (Louppe, 1994). In Burkina Faso, Bobo musicians use it to repair cracked drums and punctured drumheads (Millee, 1984). It contains only 15-25 % of carotene and, therefore, is not suitable for the manufacture of rubber (André, 1947).

Traditionally, shea butter is probably the primary cooking fat for a large proportion of the rural populations of Northern Ghana. The butter is used in pastry for its high dough pliability and in confectionery as a cocoa butter substitute (Hall *et al.*, 1996). The shea butter has been exploited as food, for skin pomade which protects the skin against sun burns, it can also be used as a hair dressing cream to moisturize a dry scalp and stimulate hair growth, for the production of soap, detergents and candles, for the prevention of

stretch marks especially during pregnancy and help scars to fade, as a shaving cream to reduce irritation, as a moisturizer for hair treatment, for blemishes and wrinkles to promote even skin tone, for skin cracks, for dermatitis; it is also used as eczema cream. Currently it is being used as a substitute for cocoa butter in the chocolate and the confectionary industry (Kapseu *et al.,* 1999).

Because of its characteristics, shea butter is used as a base for cosmetic and pharmaceutical preparations for the treatment of dry hair, skin, burn and multiple skin ailments (Ezema and Ogujiofor, 1992) and also to massage pregnant women and little children (Marchand, 1988). Shea butter contains tocopherols, collectively known as vitamin E and represents an important class of antioxidants (Maranz and Wiesman, 2004).

2.5 Physicochemical Properties of Shea Butter

Proximate composition of the kernel on dry matter basis has been documented by Tano-Debra and Ohta (1994) as shown in the table below;

Table 2.1: Proximate composition of shea Kernel

Parameter	**(%)**
Total lipids	59.04
Crude fats	54.85
Proteins	7.81
Total carbohydrates	34.77
Ash	2.57
Starch	7.59
Hemicellulose	10.84
Cellulose	5.95
Pectin substances	2.93
Total fiber content	20.35

The physicochemical characteristics of shea butter have also been documented by Tano-Debra and Ohta (1994) as shown in Table 2.2.

Table 2.2: Physicochemical characteristics of shea butter

Parameter	**(%)**
Melting range	34 – 36
Iodine value	58.53
Saponification value	180.37
Unsaponifiable matter content	7.48
The predominant fatty acids	
Palmitic	3.55
Stearic	44.44
Oleic	42.41
Linoleic	5.88
Linolenic	1.66

2.6 Pectic Substances

Pectic substances are complex high molecular mass glycosidic macromolecules found in higher plants. They are present in the primary cell wall and are the major components of the middle lamellae, a thin extracellular adhesive layer formed between the walls of adjacent young cells. In short, they are largely responsible for the structural integrity and cohesion of plant tissues (Whitaker, 1990).The fact that the middle lamella is largely composed of pectin substances has been proven by the comparable uptake of ruthenium red by the use of alkaline hydroxylamine (Gee *et al.*, 1959; McCready *et al.*, 1955). They make up between 20 % and 35 % of plant cell wall, (Abbott and Boraston, 2008) and account for 0.5-4.0 % of the fresh weight of plant material (Dayanand and Patil, 2003). The biological functions of pectin is to cross-link cellulose and hemicellulose fibers and thus providing rigidity to the cell wall, regulation of ion and water exchange and also for development and defense (Abbott *et. al.,* 2008).

Contrary to proteins, lipids and nucleic acids, being polysaccharides, pectin substances do not have a defined molecular weight. The relative molecular masses of pectic substances range from 25 to 360 kDa (Jayani *et al.,* 2005)

Table 2.3: Molecular weights of some pectin substances

Source	Molecular Weight (kDa)
Apple and lemon	200-360
Pear and prune	25-35
Orange	40 50
Sugar beet and pulp	40-50

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

2.6.1 Classification of pectic substances

The American Chemical Society classified pectic substances into four main groups as follows (Alkorta *et al.,* 1998):

(i) *Protopectin*: is the water insoluble pectic substance present in intact tissue. It is highly esterified with methoxyl groups and produces the hard texture of fruits and vegetables. Protopectin on restricted hydrolysis yields pectin or pectic acids.

(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 acids*: is the polygalacturonan chain that contains >0 and <75% methylated galacturonate units. Normal or acid salts of pectinic acid are referred to as pectinates.

(iv) *Pectin (Polymethyl galacturonate)*: is the polymeric material in which, at least, 75 % of the carboxyl groups of the galacturonate units are esterified with methanol. It confers rigidity on cell wall when it is bound to cellulose in the cell wall (Holloway *et al.,* 1983).

2.6.3 Industrial uses and applications of pectin

Because of its colloidal properties, pectin has been applied as jelling agent in the production of jams and thickening of foods. Examples of such foods include yoghurt, cake, ketchups and fruit jelly (Holloway *et al.,* 1983). Madhav and Pushpalatha (2002) have also reported that pectin is also used in pharmaceutical preparations as filler, as an agglutinator in blood therapy and also to glaze candied fruits. In addition, it can be used to increase the foaming power of gases in water. There are also reports that pectin has been used to increase insulin resistance, relieve diarrhoea, used as a regulator and protector of the gastrointestinal tract, as an immune system stimulant and as anticancer and antinephrotic agent (Holloway *et al.,* 1983).

2.7 Industrial Uses of Enzymes

A large number of industries, including environmental and food biotechnology industries utilize enzymes at some stage or the other. Enzymes are biological catalysts, mainly proteins that catalyse chemical reactions in the cell of living organisms. They are among the most important products obtained for human needs through microbial sources (Pandey *et al.,* 1999). They are widely used in industries to improve upon process parameters such as product yield and rate of product formation. Many chemicals used in various industries have inherent drawbacks from a commercial and environmental point of view (Bargale, 1997).

Some of the drawbacks of chemicals are that they are nonspecific and such reactions may result in poor product yields and therefore high temperatures and pressure may be needed to drive these reactions which may lead to high cost of energy and in some cases

unwanted by-products may even be difficult or costly to dispose of (Aehle, 2007). Some of these drawbacks can be eliminated by the use of enzymes.

Enzyme reactions may often be carried out under mild conditions, they are highly specific and involve high reaction rates. They work under mild conditions of temperature and pH and are readily destroyed (denatured) under extremes of temperature and pH. Additionally, small amounts of energy are needed in order to carry out enzymatic reactions even on industrial scale (Aehle, 2007). Both solid and liquid enzymes take small storage space. Mild operation conditions enable uncomplicated equipment to be used, and enzyme reactions are generally easily controlled. An enzyme may also reduce the impact of manufacturing on the environment by reducing the consumption of chemicals, water and energy and the generation of other wastes (Aehle, 2007).

2.7.1 Pectinolytic enzymes

Pectin enzyme is an important hydrolytic enzyme that plays a vital role in the hydrolysis of pectin molecules and finds variety of applications in food processing industries (Bargale, 1997). Pectin enzymes are also known as pectinases and are heterogeneous mixture of related enzymes which hydrolyze pectin substances in most plants (Lonsane, *et al.,* 1985). The enzymes that hydrolyze pectic substances are known as pectic enzymes, pectinases or pectinolytic enzymes. The three major types of pectinases are Pectinesterases (PE), Depolymerizing enzymes (Polymethylgalacturonases, Polygalacturonases and Polygalacturonate lyases) and Protopectinase (Saroglu *et al.,* 2001). Pectinases are produced during the natural ripening process of some fruits and they help to soften the cell walls in combination with cellulases. Their assumed natural

role in plants includes fruit maturation, growth, abscission and pollen development (Capounová and Drdak, 2002). Depolymerases act on pectic substances by two different mechanisms, hydrolysis, in which they catalyze the hydrolytic cleavage with the introduction of water across the oxygen bridge and trans-elimination lyses, in which they break the glycosidic bond by a trans-elimination reaction without any participation of water molecule (Codner, 2001). Depolymerases can be subdivided into four different categories, depending on the preference of enzyme for the substrate, the mechanism of cleavage and the splitting of the glycosidic bonds (Rexova-Benkova *et al*., 1976). Polygalacturonase and polymethylgalacturonase breakdown pectate and pectin, respectively by the mechanism of hydrolysis. However, polygalacturonate lyase and polymethylgalacturonate lyase breakdown pectate and pectin by elimination, respectively (Whitaker, 1984).

Protopectinases (PPase) are enzymes that catalyze the solubilization of protopectin (Briton *et al.*, 1927). Pectinesterase (PE) often referred to as pectinmethylesterase, pectase, pectin methoxylase, pectin demethoxylase and pectolipase, is a carboxylic acid esterase and belongs to the hydrolase group of enzymes (Whitaker, 1984). It catalyzes the deesterification of methyl ester linkages of galacturonan backbone of pectic substances to release acidic pectins and methanol (Cosgrove, 1997). The resulting pectin is then acted upon by polygalacturonases and lyases (Prade *et al.*, 1999). The mode of action of PE varies according to its origin (Micheli *et al.*, 2001). Fungal PEs act by a multi-chain mechanism, removing the methyl groups at random. In contrast, plants PEs tend to act either at the non-reducing end or next to a free carboxyl group, and proceed along the

molecule by a single chain mechanism. Polygalacturonases (PGases) are the pectinolytic enzymes that catalyze the hydrolytic cleavage of the polygalacturonic acid chain with the introduction of water across the oxygen bridge. They are the most extensively studied among the family of pectinolytic enzymes (Prade *et al.,* 1999). PGases isolated from different microbial sources differ markedly from each other with respect to their physicochemical and biological properties and their mode of action (Whitaker, 1984). Among the PGases obtained from different microbial sources, most have the optimal pH range of 3.5–5.5 and optimal temperature range of 30–50 ^{o}C. Two endo-PGases (PG I and PG II), isolated from *Aspergillus niger* have optimal pH range of 3.8–4.3 and 3.0–4.6, respectively (Singh and Rao, 2002). Most of the PGase reported, work efficiently in acidic pH range but a few alkaline PGases have been also reported from *Bacillus licheniformis* (Singh and Roa, 1999) and *Fusarium oxysporum f. sp. Lycopersci* (Pietro *et al.,* 1996) with optimum pH of 11.

Lyases (or transeliminases) perform non-hydrolytic breakdown of pectates or pectinates, characterized by a trans-eliminative split of the pectic polymer (Sakai *et al.,* 1993). The lyases break the glycosidic linkages at C-4 and simultaneously eliminate H from C-5, producing a D 4:5 unsaturated products (Albersheim *et al.,* 1960). Lyases can be classified into the following types on the basis of the pattern of action and the substrate acted upon by them; Polygalacturonate lyases (Pectate lyases or PGLs) are produced by many bacteria and some pathogenic fungi with endo-PGLs being more abundant than exo-PGLs. PGLs have been isolated from bacteria and fungi associated with food spoilage and soft rot (Micheli, 2001). Very few reports on the production of

polymethylgalacturonate lyases (pectin lyases or PMGLs) have been reported in literature. They have been reported to be produced by *Aspergillus japonicus* (Ishii *et al.,* 1975), *Penicillium paxilli* (Szajer *et al.,* 1982), *Penicillium sp*. (Alana *et al.,*1990), *Pythium splendens* (Chen *et al,*. 1998) *Pichia pinus* (Moharib *et al.,* 2000), *Aspergillus sp*. (Sunnotel *et al.,* 1982) and *Thermoascus auratniacus* (Martins *et al.,* 2002).

Many purification studies have also been carried out on lyases, which led to increase in specific activities of the enzymes with significant recoveries (Miyazaki, 1991). Pectinesterase, often referred to as pectinmethylesterase, pectase, pectin methoxylase, pectin demethoxylase and pectolipase, is a carboxylic acid esterase and belongs to the hydrolase group of enzymes (Whitaker 1984). It catalyzes the deesterification of methyl ester linkages of galacturonan backbone of pectic substances to release acidic pectins and methanol (Cosgrove, 1997). The resulting pectin is then acted upon by polygalacturonases and lyases (Prade *et al.,* 1999). The mode of action of PE varies according to its origin (Micheli, 2001). Fungal PEs act by a multi-chain mechanism, removing the methyl groups at random.

In contrast, plants PEs tend to act either at the non reducing end or next to a free carboxyl group, and proceed along the molecule by a single chain mechanism (Prade *et al.,* 1999). PE is found in plants, plant pathogenic bacteria and fungi (Hasunuma *et al.,* 2003). It has been reported in *Rhodotorula sp*. (Vanghan 1969), *Erwinia chrysanthemi* B341 (Pitka¨nen *et al.,* 1992), *Saccharomyces cerevisiae* (Gainvors and Berlarbi, 1996), *Lachnospira pectinoschiza* (Cornick *et al.,* 1994), *Pseudomonas solanacearum* (Schell, 1994), *Aspergillus niger* (Madonaldo *et al.,* 1998), *Lactobacillus lactis subsp*. *Cremoris*

(Karam and Berlarbi, 1995), *Penicillium frequentans* (Kawano *et al.,* 1999), *E. chrysanthemi* 3604 (Laurent *et al.,* 2000) and others. PE activity is highest on 65–75% methylated pectin, since the enzyme is thought to act on methoxyl group adjacent to free carboxyl groups (Whitaker, 1984). PE action has a very little effect on viscosity of pectin containing solutions unless divalent ions are present, which increase viscosity due to cross-linking. PEs are highly specific enzymes.

Some PEs attack only at the reducing chain, while others attack the non-reducing end (Sakai *et al*., 1993). The molecular weights of most PEs are in the range of 35–50 kDa. pH values at which PEs are active range from 4.0 to 8.0. Fungal PEs have a lower pH optimum than that of bacterial origin (Assis *et al.,* 2004). Optimum temperature range for maximal activity for majority of PEs is 40–50 ^{0}C (Silva *et al.,* 2005). Immobilization studies have also been carried out on PEs (Assis *et al.,* 2004).

A large number of microbial strains have been studied for the production of pectinase (Silva *et al.,* 2005).The main sources of the microorganisms that produce pectinolytic enzymes are yeasts, bacteria and large varieties of fungi and particularly *Aspergillus ssp.* Few yeasts have the ability for pectinases production. Endo Polygalacturonase production by yeasts was first reported in 1951 using *S.fragilis* (Luh *et al.,* 1951). Other genera of yeasts like *Candida, Pichia, Saccharomyes* and *Zygosaccharomyces* also have the ability to produce pectinolytic enzymes (Luh *et al.,* 1951).

2.7.2 Industrial applications of microbial pectinases

Over the years, pectinases have played a vital role in several conventional industrial processes, such as textile, plant fiber processing, tea, coffee, oil extraction, treatment of industrial waste water, especially those containing pectinaceous materials (Jayani *et al.,* 2005). It is also known that of all the enzymes employed worldwide for industrial purposes, the pectinases account for about 10 % (Stutzenberger, 1992). Among the many industrial uses are, fruit juice extraction and wine making, textile processing and bioscouring of cotton fibres, degumming of plant bast fibers, retting of plant fibers, purification of plant viruses and oil extraction.

2.7.2.1 Fruit juice extraction and wine making

The fruit juice and wine making industries are by far the ones that enjoy the most industrial application of the pectinases. The industry is estimated to employ approximately 25% of global sale of all food enzymes as pectinases **(**Jayani *et. al.,* 2005). It is reported that the use of pectinases in combination with xylanases and cellulases have been found to reduce filtration time by approximately up to 50% and in such fruits as banana, grape and apple, it has been found to increase the fruit juice volume when the fruits' pulps were treated with the pectinases **(**Kaur *et. al.,* 2004**).** Their use in combination with other enzymes such as the cellulases, arabinases and xylanases has also helped increase pressing efficiency of the fruits for juice extraction. Citrus fruits have also been softened and easily peeled when their peels were (vacuum) infused with pectinases (Baker and Wicker, 1996).

2.7.2.2 Textile processing and bioscouring of cotton fibers

Pectinases have been used in conjunction with amylases, lipases, cellulases and hemicellulases to remove sizing agents from cotton in a safe and eco-friendly manner, replacing toxic caustic soda used for this purpose earlier (Hoondal *et al.,* 2000). Bioscouring is a novel process for removal of non-cellulosic impurities from the fiber with specific enzymes. Pectinases have been used for this purpose without any negative side effect on cellulose degradation (Hoondal *et al.,* 2000).

2.7.2.3 Degumming of plant bast fibers

Bast fibers are the soft fibers formed in groups outside the xylem, phloem or pericycle. These fibers contain gum, which must be removed before it can be used for textile making (Hoondal *et al.,* 2000). The chemical degumming treatment is polluting, toxic and non-biodegradable. Biotechnological degumming using pectinases in combination with xylanases presents an eco-friendly and economic alternative to the above problem (Kapoor *et. al.,* 2001).

2.7.2.4 Retting of plant fibers

Pectinases have been used in retting of flax to separate the fibers and eliminate pectins (Hoondal *et al.,* 2000).

2.7.2.5 Waste water treatment

Vegetable food processing industries release pectin-containing waste waters as by-product. Pretreatment of these waste waters with pectinolytic enzymes facilitates removal

of pectinaceous material and renders it suitable for decomposition by activated sludge treatment (Hoondal *et al.*, 2000).

2.7.2.6 Coffee and tea fermentation

Pectinases treatment accelerates tea fermentation and also destroys the foam forming property of instant tea powders by destroying pectins (Carr, 1985). They are also used in coffee fermentation to remove mucilaginous coat from coffee beans (Hoondal *et al.*, 2000).

2.7.2.7 Paper and pulp industry

During papermaking, pectinase can depolymerise pectins and subsequently lower the cationic demand of pectin solutions and the filtrate from peroxide bleaching (Reid and Richard, 2000).

2.7.2.8 Animal feed

Pectinases are used in the enzyme cocktail used for the production of animal feeds. This reduces the feed viscosity, which increases absorption of nutrients, liberates nutrients, either by hydrolysis of non-biodegradable fibers or by liberating nutrients blocked by these fibers, and reduces the amount of faeces (Hoondal *et. al.*, 2000).

2.7.2.9 Purification of plant viruses

In cases where the virus particle is restricted to phloem, alkaline pectinases and cellulases can be used to liberate the virus from the tissues to give very pure preparations of the virus (Salazar and Jayasinghe, 1999).

2.7.2.10 Oil extraction

Citrus oils such as lemon oil can be extracted with pectinases. They destroy the emulsifying properties of pectin, which interferes with the collection of oils from citrus peel extracts (Scott, 1978). Enzymatic processes had been tried on the seed cell walls to facilitate oil extraction (Cintra *et al*., 1986; Frevert *et al*., 1990; Ho *et al*., 1992; Ohlson, 1992; Sosulski and Sosulski 1993; Rosenthal *et al*., 1995). The use of hydrolytic enzymes such as cellulases, hemicellulases, and pectinases in aqueous enzyme-assisted extraction process break the structure of cotyledons of cell walls which makes the structure more permeable. Protease and carbohydrase enzymes are mainly used for this process. The proteins in the cell membranes and inside the cytoplasm are hydrolyzed by the proteolytic enzymes (Rosenthal *et al*., 1996). Proteolytic enzymes can potentially hydrolyze the lipid body membranes and can also affect the cytoplasmic network thus making the inner structure less tightly bound/compact which facilitates the protein and lipid removal from the cell (Rosenthal *et al*., 1996).

The released oil can be easily separated from the cotyledon cells by an aqueous medium. An enzyme-aided process has also been used for soybeans (Rosenthal *et al*., 1995) and coconuts (Cintra *et al*., 1986). The enzyme efficiency based on oil yield enhancement was: mixed activity enzyme > β-glucanase > pectinase > hemicellulase > cellulase (Rosenthal *et al*., 1996). For palm oil recovery from the centrifuge sludge of a palm oil mill, cellulase, α-amylase, pectinase and protease enzymes were utilized (Ho *et al*., 1992). A surfactant Sodium dodecyl sulfate (SDS) was tried to release the oil into the aqueous phase because the oil remained attached to the plant material even with high speed centrifugation (Ho *et al*., 1992). Although they optimized conditions for cellulase;

pH 4.8 and 30 °C for 5 hr with an enzyme concentration of 0.5 % (w/v), followed by a washing with 0.03 M SDS solution, still it was not economically viable.

2.7.2.11 Improvement of chromaticity and stability of red wines

Pectinolytic enzymes added to macerated fruits before the addition of wine yeast in the process of producing red wine resulted in improved visual characteristics (colour and turbidity) as compared to the untreated wines. Enzyme treated red wines presented chromatic characteristics, which are considered better than the control wines. These wines also showed greater stability as compared to the control (Revilla and Guanzalez-san, 2003).

2.7.3 Microorganisms as sources of pectolytic enzymes

Yeast polygalacturonases and bacterial pectate lyases are involved in the softening of cucumber and olives in brine (Bell *et al.*, 1968). Yeast polygalacturonases have also been found to be responsible for the breakdown of pectin in fermenting apple pomace. A highly resistant polygalacturonase from *Rhizopus spp* is responsible for the texture breakdown in canned apricots (Luh *et al.*, 1978). Some best known bacteria such as *Bacillus*, *Clostridium* and *Erwinia spp* have also been found to produce pectolytic enzymes (Meyrath and Volavsek, 1975). Commercial enzyme preparations have been made and used as processing agent, using moulds (Rombouts and Pilnik 1978). The most potent strains from the large genus of *Aspergillus* were selected for this purpose. Enzymes from some highly pathogenic microorganisms have been found to have higher activity (Tomizawa and Takashashi, 1970).

Pathogenic strains from *Xanthomonas* have been shown to produce pectolytic enzymes of considerably higher activity than weak pathogenic or invasive ones. Using carrot extract preparation, *Erwinia aroidea* was found to produce pectolytic enzymes with high activity (Tomizawa and Takashashi, 1970). The activity of enzyme from this microorganism was increased using repeated enrichment procedure (Meyrath and Volavsek, 1975). Despite all this progress, the use of the pathogenic microorganisms, which gave the higher activity, suffers a serious setback. The fear of having some of the viable cells left in the enzyme preparation led to serious reservations as to the use of the pathogenic microorganisms. This need not necessarily be the case in the future if adequate measures are taken to remove all viable cells (Meyrath and Volavsek, 1975).

Yeasts offer several advantages as compared to other microorganisms when it comes to its industrial applications; they do not produce toxins and they secret protein extracellularly. They are glycosylate proteins, a function bacterium are unable to perform. They have been known to be used from a technical and industrial standpoint to produce wine and beer and also to leaven bread (Stanier *et al* 1995). Though yeasts have generally been associated with alcohol production, certain strains have been found to possess pectolytic activity. The action of yeasts in degrading cocoa pulp to sweating during cocoa bean fermentation is one evidence of the presence of pectolytic activity (Ansah and Dzogbefia 1990; Buamah 1995).

2.7.4 Production of enzymes

The use of enzymes in food related issues is an ancient technology that has seen much advancement over the years. In some cases the precise role or even the identity of the

enzyme was not known over most of the period of usage. They were often used as component of intact cells or as extracts containing a mixture of many enzymes (Whitaker, 1990).

2.7.5 Selection of source of enzyme

The first step in the production of any microbial enzyme is the selection (usually by screening hundreds of species and strains) of an organism that produces the desired enzyme in good yield. The selected strain may be improved by mutation. It may also be possible to use genetic manipulation to make the organism increase the production of its own enzyme or transform another organism (usually microorganisms) to produce the required enzymes (Whitaker, 1990). The organism is maintained as a pure strain under rigidly controlled conditions, including cultivation on agar slopes and stored at 4^0C. In order to maintain uniformity in enzyme production, it is important to ensure that it is free from contaminants, and variants. In order to optimize production, parameters affecting enzyme synthesis must be investigated and optimized (Whitaker, 1990).

To consider an enzyme like pectolytic enzymes for use in extraction, certain properties of the enzymes must be taken into consideration. Some of these properties are presence of pectolytic activity, optimum pH and pH range of activity, optimum temperature and temperature range of activity, pH and temperature range at which the extraction process would take place. The enzyme must be stable in the environment in which it is supposed to act. It must be safe to use, pose no danger to human health, and it must also pose no danger to the environment. It must not have unnecessary or undesired enzyme activities and it must be economical to use (Aunstrup, 1977). It must also be characterized and

classified. The yeast used for this project, *S. cerevisiae* is classified under the American Type Culture Collection as ATCC 52712. It has been shown to have pectolytic activity (Sanchez *et al.,* 1984; Buamah *et al.,* 1997) and its pH range of activity is 3.5-5.0. Sanchez *et al.,* (1984) reported an optimum temperature of 25 ^{0}C but Buamah *et al.,* (1997) reported a value of 40 ^{0}C for pectolytic enzymes of *S. cerevisiae* (ATCC52712).

2.8 Solid State Fermentation

Solid State Fermentation (SSF) is the cultivation of microorganisms under controlled conditions in the absence of free water (Hesseltine, 1977). Examples of products of Solid State Fermentation include industrial enzymes, fuels and nutrient enriched animal feeds, pharmaceutical and agricultural products, (Ward, 1992). The application of modern biotechnical knowledge and process control technologies can lead to significant productivity increases from this ancient process.

Even though, submerged fermentation has been extensively employed for the production of enzymes, solid state fermentation is mainly advocated to improve the production level of enzymes, however, it has its own problems, (Perez-Guerra, *et al.,* 2006). Below are some of the advantages and disadvantages of solid state fermentation in comparison to submerged fermentation.

2.8.1 Advantages of solid state fermentation over submerged fermentation

Mienda *et al.* (2011) in their report on 'Microbiological Features of Solid State Fermentation and its Application' outlined the following advantages.

- Culture media are often quite simple. The substrate usually provides all the nutrients necessary for growth.
- Solid state fermentation produces higher yields of enzymes than those obtained in the corresponding submerged cultures.
- The low availability of water reduces the possibilities of contamination by bacteria and yeasts. This allows working in aseptic conditions in some cases.
- Provide similar environmental conditions to those of the natural habitats for fungi, which constitute the main group of microorganisms used in solid state fermentation (SSF).
- The low moisture availability may favour the production of specific compounds that may not be produced or may be poorly produced in liquid state fermentation in comparison to liquid state fermentation.
- Small volumes of polluting effluents are produced.
- The enzymes are less sensitive to catabolic repression or induction.

Aside all these advantages solid state fermentation has some few disadvantages when compared with the submerged fermentation (Mienda *et al.*, 2011).

- Microorganisms that can grow at low moisture levels are preferred, when it comes to selection of microorganisms
- Usually the substrate requires pre-treatment before they are used. Some of the pretreatment parameters are particle size, rasping or chopping, homogenization, grinding, physical, chemical or enzymatic hydrolysis and cooking or vapour treatment.

- Little studies have been done on scale up of solid state fermentation and this present a lot of challenges in its engineering.

Substrates normally used for SSF are by-products of agro-industry or agro-wastes. The selection may include screening a large number of agricultural wastes for microbial growth and product formation and find ones that are suitable. They should include starch, cellulose, pectin, lignin, lignocelluloses that give the substrate its solid properties. These macromolecular components provide an inert matrix or support within which the carbon and energy sources are adsorbed (Ward, 1992).

The substrates (agro wastes) are pre-treated and prepared into suitable forms for use;

- The substrate size is reduced to increase availability by chopping, grinding, chemical and enzymatic hydrolysis and many more.
- It is supplemented with nutrients such as phosphorus, nitrogen, salt and setting the pH and moisture content through addition of mineral solution.
- Vapour treatment of the macromolecules degrade and eliminate some of the major contaminants (Ward, 1992).

Several agro-wastes including rice and wheat bran, cassava peels, sugarcane and cassava bagasse, corncobs, sawdust, fruit pulps (e.g. apple pomace), fruit peels, spent brewing grains, coffee pulp etc. are used commonly as substrates for SSF processes (www.scientificpsychi .com).

2.8.2 Corn cobs as substrate for pectin enzymes production

The corn cob is the central core of a maize *(Zea mays)* ear. It is the hard cylindrical core that bears the kernel of an ear of corn. They are of little nutritional value and are used as fibre in ruminant feed. They are an important source of a furfural which is used in industrial processes (www.renewables.morris.umn.edu). Corn cobs, because of their limited uses, are considered as cheap substrates and they contain pectin and other important components and hence can provide nutrients as well as support for the growth of microorganisms (www.bioapplication.blogspot.com). Corncobs are made up of starch and fibre and the fibre contains major components as shown in Table 2.4.

Table 2.4: Major component of corncobs fibre

Corn cob fibre	**%**
Cellulose	32.3- 45.6
Hemicelluloses	39.8
Lignin	13.9
Pectin	3.5
Starch	3.5

Source: (www.renewables.morris.umn.edu)

2.9 Fermentation Media

Ingold (1981) reported the following on fermentation media. A medium for growth of a selected microorganism must contain in aqueous solution or suspension, certain constituents, namely:

- A suitable organic substance as a source of carbon
- A nitrogen source
- Certain inorganic ions in appreciable amounts
- Other inorganic ions as mere traces

- Certain organic growth factors in very low concentrations

Apart from these basic nutritional needs, growth may be strongly influenced by environmental factors particularly, temperature, pH and by the development of staling substances as by-products of fungal metabolism (Ingold, 1981).

2.9.1 Fermentation techniques

Some enzymes, e.g. *Aspergillus* proteases may be produced on semi-solid media in trays or similar equipment, where the special conditions of low water content and high degree of aeration (at the surface), apparently favor the formation of the enzymes (Palmer, 1995). Most microbial enzymes are produced by submerged cultivation in conventional equipment under aseptic fermentation where the degree of aeration, pH and temperature can be controlled (Aunstrup, 1997; Palmer, 1995). The cultivation medium must contain the essential nutrients needed by the microorganism, together with an inducer necessary for the synthesis of the required enzyme (Palmer, 1995). In liquid culture, with restricted volume, the medium changes in composition as substances are used and by- products of metabolism are liberated into it (Palmer, 1995).

Batch processes give the highest enzyme concentration and the utilization of medium components. Incremental amounts of carbohydrates or proteins are fed during fermentation. This allows the utilization of higher concentrations of substrate without catabolite repression or repression by high osmotic pressure, which may be caused by rapid and readily utilized carbon source (carbohydrates, protein) or metabolic end-products (Owen, 1989; Palmer, 1995).

2.9.2 Enzyme purification

The precise treatments employed in the purification of an enzyme depend on whether the enzyme is extracellular or intracellular enzyme and the purpose for which it is prepared.

2.9.3 Extracellular enzymes

A liquid enzyme preparation may be made by removing the cell mass (cells and cellular debris), concentrating the enzyme by evaporating the enzyme or ultra filtration, and adding a preservative. To achieve a higher activity for the enzyme, the enzyme can be precipitated with salt or solvents, separated by centrifugation or filtration and re-dissolved. The resulting product is attractive, of light colour and often has fewer odours than the original product. The disadvantages are higher costs and occasionally lower stability of the partially purified enzyme (Palmer, 1995).

The enzyme could also be purified by selection of suitable combination of absorbent gels, electrophoresis, ion exchange chromatograph, gel filtration, affinity chromatography, and high performance liquid chromatography. Sometimes it is unnecessary and uneconomical to involve combinations of such detailed methods, because much purified enzyme is not required and substances that affect the enzymes usefulness are absent. The stability of liquid products is often a problem. The acceptable loss of activity at 25 ^{0}C is less than 10% per year. Stabilizers, biocides and or pH regulation may be used to achieve stability (Aunstrup, 1997; Palmer, 1995).

2.9.4 Immobilization of enzymes

An immobilized enzyme is one which has been attached to or enclosed by an insoluble support medium (termed a carrier) or one where the enzyme molecules have been cross-linked to each other, without loss of catalytic activity. Binding forces like physical adsorption, ionic binding, or covalent binding may be present (Palmer, 1995).

Immobilization of industrial enzymes is an attractive solution to the problems of conversion costs, purity of reaction products, and the introduction of continuous processes. Enzymes are immobilized on carriers such as glutaraldehyde polymers, glass, ceramics, DEAE cellulose, cellulose acetate fibres, or acrylamide (Auntrap, 1977). Practical examples on the study of immobilized pectic enzymes are the immobilization of endo-polygalacturonase and pectin esterases. Endo-polygalacturonases were immobilized on inorganic supports like glass, silica, talc, bentonite, graphic, and on organic supports like cellulose, silk and chitin (Lanzarini *et al*., 1989).

2.9.6 Enzyme activity and action

Factors governing enzyme activity include enzyme concentration (in respect of substrate concentration), application temperature, and reaction (Baumann, 1981). Skillful manipulation of these factors enables the adaptation of enzyme technology to many food industry processes. Doubling the enzyme concentration will produce approximately the same result in half the time, and vice versa. For most pectinases, 10 ^{0}C increases in temperature approximately doubles the enzyme activity within the range of 10-50 ^{0}C. Under certain conditions some pectinases can withstand temperatures up to 60 ^{0}C and others below 10 ^{0}C (Baumann, 1981).

There is close interaction between reaction time and temperature. The higher the temperature, the sooner the enzyme becomes exhausted. Adverse conditions such as unfavorable pH, high polyphenol, alcohol and sulphur dioxide content, or other enzyme inhibitors in the substrate will accelerate inactivity (Baumann, 1981). Another essential mechanism underlying pectolytic enzyme activity is the mechanism of pectic substance breakdown, which largely depends upon the type of enzyme and the composition of the substrate (Baumann, 1981).

2.9.7 Safety measures and control methods

Any enzyme that is produced commercially and marketed must be approved by a regulatory body. The producer will have to submit data on handling hazards and methods of production in order to ensure that good manufacturing practices are adhered to. The United States of America Food and Drugs Administration, considers pectinases prepared in accordance with "good manufacturing practices'' as safe. Pectinase derived from *Aspergillus niger*, are generally recognized as safe (GRAS) in the United States (Neubeck, 1975). The process of fermentation is the most important stage in enzyme production since it is at this stage that the enzymes are produced. The purity of the enzyme is regulated by controlling the purity and stability of the enzyme producing microorganism strain. The purification step, which is carried out aseptically is also controlled to limit unavailable microbial contamination. Since the purification step is not aseptic, frequent control of contaminated levels is necessary to keep the microbial level low (Aunstrup, 1997).

A new enzyme product has to pass a number of biological tests in order to be declared as safe. These tests take 3- 4 years to complete. It is very expensive and begins when the production method of the enzyme is well developed (Aunstrup, 1997). Biological tests such as oral toxicity, cutaneous activity, pathogenicity of the microbial strain used, and antigenicity of the enzyme is carried out. Each batch of enzyme produced has to pass through these tests in order to verify its safety properties and activity (Aunstrup, 1997).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Source of material

The pure yeast strain, *S. cerevisiae* (ATCC 52712), yeast extract, peptone, dextrose agar, NaCl, NH_4SO_4, urea, Na_3PO_4, acetate buffer, n-hexane used were obtained from the Biochemistry and Biotechnology Department, KNUST-Kumasi. The corn cobs used in the solid state fermentation were obtained from a farm in Anwomaso, a suburb of Kumasi. Dried shea kernels were obtained from New Vision Shea Village in Nakolo, a suburb of Kasena-Nankana in the Upper East Region of Ghana. Crude Pectinase and two commercial enzymes were used in this research. The commercial enzyme preparations used for the enzyme treatment studies were Viscozyme L (beta endo-1,3(4)-glucanase activity with declared activity of 100 Fungal β-glucanase (FBG/g) as well as side activities of xylanase, cellulase and hemicellulases) and Pectinex 5XL (polygalacturonase activity with declared activity of 4500 Pectinase Unit (PECTU/ml) as well as arabinase side activity). These commercial enzyme preparations were obtained from Novozymes, North America Inc., USA.

3.1.2 Preparation of corn cobs

Corn cobs were washed, dried, milled using a hammer mill into an average particle size of 0.3 mm and then autoclaved at 121°C for 15 min.

3.2. Methods

3.2.1 Microorganism

The pure yeast strain, *S. cerevisiae* (ATCC 52712) was cultured on a prepared slant of yeast extract/ peptone /dextrose agar. The tubes were incubated at 30°C for 3-4 days to allow *S. cerevisiae* to grow.

3.2.2 Preparation of media

Nutrient agar and nutrient broth were prepared as follows: The nutrient agar was made of malt extract agar, (48 g/l) containing 5 % yeast extract and was dissolved in distilled water by heating. The nutrient broth was made of malt extract broth, (17 g/l) containing 5% yeast extract and was dissolved in distilled water. The nutrient broth was dispensed in 100 ml aliquots into 250 ml conical flasks. Both media were sterilized by autoclaving at 121 ^{o}C for 10 min. Agar slants and plates were obtained by transferring 20 ml and 10 ml of liquid nutrient agar into sterile test tubes and petri dishes respectively. Petri dishes and tubes containing the media were left for three days in a laminar hood. Petri dishes and tubes that had no contamination after the three days were used since they passed the sterility check.

3.2.3 Yeast maintenance and Propagation

3.2.3.1 Microorganism

The strain of yeast used for this study was *Saccharomyces cerevisiae* (ATCC 52712) and was maintained at 4 ^{o}C on slopes of malt extract agar supplemented with 5 % yeast extract. Subculturing was regularly done during the study.

3.2.3.2 Inoculum preparation

Nutrient broth and nutrient media that passed the sterility test were inoculated aseptically with yeast cells from thawed slants using sterile inoculating pin. Three days following inoculation, slopes which showed marked growth were kept at 4 ^{o}C for later use.

3.2.3.3 Estimation of yeast cell number

The number of yeast cells in a culture was determined from a standard graph of absorbance at 540 nm against cell number (Sanchez *et al.,* 1984). Colonies of yeast cells were picked from the slants and used to inoculate 100 ml of sterile malt extract broth containing 5 % yeast extract in 250 ml conical flask. Four milliliter of the freshly inoculated broth was taken and its absorbance read at 540 nm against an uninoculated blank. Subsequent absorbance readings were taken at 12, 24, 36, 48 and 60 h after inoculation. The turbidity of the yeast culture measured as the absorbance were correlated with the total number of yeast cells as determined by quantitative pour plate count at the respective time intervals above. A standard graph of absorbance against cell number was then plotted (Sanchez *et al.,* 1984).

3.3.1 Solid state fermentation for pectin enzyme production

The yeast was pre-cultured in a malt extract broth for three days at room temperature to ($5.25x10^{6}$) cells per milliliter prior to inoculation. A 250 ml Erlenmeyer flask served as the containment for the solid state fermentation. Ten ml solution of NaCl (0.3 g), NH_4SO_4 (1.4 g), Na_3PO_4 (2.0 g) and urea (0.3 g) were added to 20 g of milled corn cob in the Erlenmeyer flask and inoculated with 10 ml of *S. cerevisiae* cell suspension

($5.25x10^7$) and the moisture content adjusted from 40 % to 80 %. The resulting media were incubated at 30 °C for 9 days and cell growth was monitored.

3.3.2 Extraction of pectin enzyme from the fermented medium

The enzyme was extracted from the fermented medium by adding 100 ml of sterilized acetate buffer (pH 5.0), filtered and then centrifuged at 4000 rpm for 15 min. The supernatant obtained was then used as the crude enzyme solution. The total protein and the pectinase activity were then determined (Albersheim P., 1996).

3.3.3 Determination of total protein content in extract

The total protein content of the crude enzyme solution was determined using the Biuret protein assay method (Gornall *et al.,* 1949).

3.3.3.1 Preparation of standard curve using egg albumin

Ten grams of egg albumin were dissolved in 10 ml of distilled water, to form a stock solution. From the stock protein solution (10 mg/mL), ten protein standard solutions were prepared by adding 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.8 and 0.9 ml and making up volume to 1.0 ml with distilled water to give concentrations of 1mg, 2 mg, 3 mg, 4 mg, 5 mg, 6 mg, 7 mg, 8 mg, and 9 mg/ml.

3.3.3.2 Preparation of Biuret Reagent

One litre of Biuret reagent according to Gornall *et al.*, (1949) was prepared. A 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) were weighed. The mixture was dissolved in 500 ml of

H_2O and 300 ml of 10 % NaOH added. The volume was brought up to 1 liter. The reagent was then transferred to an amber bottle and stored at 2-8 °C. A volume of 4 ml Biuret reagent was added to each test tube and thoroughly mixed (Gornall *et al.,* 1949).

3.3.4 Measuring the protein concentration

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. For the analysis of total protein in the crude enzyme solution, 4 ml of Biuret reagent from a stock prepared in the laboratory was added to 1 ml of test solution in a sterile test tube and the mixture properly mixed. The tubes were then incubated at 37 °C for 10 minutes and finally cooled and the absorbance measured at 540 nm. The concentrations were then extrapolated from the calibration curve constructed using egg albumin (Gornall *et al.,* 1949).

3.3.5 Determination of pectinase activity

The pectinase activity was determined using the method described in an assay prepared at the 55^{th} meeting of Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2000). This assay is based on the enzymatic 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 conditions of the assay (pectin 0.5%, pH, 5.8 and 30 ^{o}C) JECFA (2000).

Procedure

In a properly washed and cleaned test tube, 0.1 ml of the enzyme solution was added to 3.0 ml of 0.5 % pectin solution, pre-warmed to 30±0.1°C. A volume of acetate buffer (pH 5.0) was added and the resulting mixture was mixed thoroughly by shaking the test tube gently. After short mixing, the absorbance at 235 nm was registered each minute over 8 minutes using distilled water as blank. Determination was carried out in triplicates. The absorbance (sample-water blank) data was plotted as the abscissa and the time of the reaction as the ordinate. The slope of the reaction, ($\Delta A/\Delta t$), was determined in the linear section of the function.

Pectinase activity units were calculated as:

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

Where:

V = final reaction volume, ml (0.1 ml of sample or standard solution plus 3.0 ml of substrate solution)

3.3.5 Calculation of specific activity of pectinases

The specific activities of the pectinases produced on both substrates on the optimal day of fermentation were calculated using the formula below;

$$\text{Specific activity of pectinases (U/mg)} = \frac{\text{Pectinases activity (U/ml)}}{\text{Protein concentration (mg/ml)}}$$

3.4.1 Laboratory Pre-treatment of the shea kernel

The methods described by Agyente-Badu (2010) were used with some few modifications to extract the shea butter in the laboratory. The kernels were first roasted / baked at an oven temperature of 120^0C for 1h and were cracked using a cracking machine. The cracked kernels were further milled into a fine paste /mass using the ordinary corn mill. Samples of this were used in the oil extraction studies (Agyente-Badu, 2010).

3.4.2 Solvent fat extraction

Part of the raw finely ground kernels was used for fat extraction in a soxhlet extractor. Two grams of the finely ground kernels was placed in a cellulose paper cone and extracted using light petroleum ether (40–60 ^{o}C) in a 5-L Soxhlet extractor for 8 h (AOAC 1994). The oil extracted was stored under refrigerator at 4 ^{0}C, until used for further analyses.

3.4.3 Enzyme-assisted aqueous extraction (EAAE)

A set of accurately weighed 50 g meal samples in conical flasks was mixed with distilled water in a ratio of 1:4 (wt/vol). The mixture was boiled for 5 min and allowed to cool to room temperature (Abdulkarim *et al*., 2005). Different enzyme dosages (0, 20 mg, 40 mg, 60 mg and 80 mg) total proteins were added. The mixture was incubated at different periods of time (1.0, 2.0, 3.0, and 4 h) and temperature was maintained at 45^0C. To the treated and untreated meal obtained after the enzymatic pretreatment, 100 ml of boiling distilled water was added and the suspension stirred vigorously with a glass rod to inactivate the enzymes and help separate the oil from the residue. The mixture was allowed to cool down to room temperature and the oil in the form of emulsion was

recovered by centrifugation of the aqueous mixture at 4000 rpm for 20 minutes to separate the cream (or emulsion), supernatant and solid phase (or meal). The creamy phase floating atop was transferred into a separating funnel and allowed to separate into the oil and water layers. The water layer was then drained off and the oil recovered decanted into a beaker. The beaker was then placed in an air oven at a temperature of 100 ^{0}C for an hour to dry and clarify. The residual was dried overnight in a vacuum oven (90-95 ^{0}C). The dried meal was analyzed for residual oil and protein content.

3.5.1 Optimization of Parameters for EAAE

Crude pectinase and two commercial enzyme preparations were used for the enzyme treatment studies. The commercial enzymes were Viscozyme L (beta endo-1,3 (4)-glucanase activity with declared activity of 100 FBG/g as well as side activities of xylanase, cellulase and hemicellulases) and Pectinex 5XL (polygalacturonase activity with declared activity of 4500 PECTU/ml as well as arabinase side activity). These commercial enzyme preparations were obtained from Novozymes North America Inc., USA. Commercial enzyme-mixtures were utilized to evaluate the effect of enzyme on oil recovery from finely ground kernels during EAAE. An amount of the ground kernel was treated with the enzymes at optimum experimental parameters to find the best enzyme-mixture. The enzyme activity during EAAE is strongly affected by the pH, enzyme dosage, temperature, hydrolysis time, and water to seed w/s ratio (Abdulkarim *et al*., 2005). All these operational parameters were optimized for better oil recovery.

3.5.1.1 Effect of Temperature

Ground seed was mixed with distilled water. The effect of temperature was studied with an optimum pH for each enzyme mixture at optimum level of seed to water ratio and enzyme concentration. The temperature was controlled at 20, 30, 40, 50, 60 and 70 ^{0}C with a magnetic stirring hot plate and the extraction was carried out different intervals of time.

3.5.1.2 Effect of water to seed ratio

For each run, 50 g of the ground kernel were mixed to get water to seed ratios of 2:1, 4:1, 6:1 and 8:1. Shea fats were extracted using different intervals of time to evaluate the effect of water ratio in EAAE.

3.5.1.3 Effect of hydrolysis time

Experiments were carried out for four different extraction times: 1, 2.0, 3.0, and 4.0 h. The ground seed was mixed with distilled water at water to seed ratio of 4:1. The temperature was maintained at 50 ^{o}C for each period.

3.5.1.4 Effect of pH

The effect of pH was studied at five different pH levels: 3, 4, 5, 6, and 7. For each trial, ground seed sample was mixed with distilled water to make desired s/w ratio with an optimum concentration of the enzyme mixture. The temperature was maintained at 50 ^{o}C on a magnetic stirring hot plate for different intervals of time.

3.5.1.5 Effect of enzyme dosage

For each run, 50 g of the ground kernel were mixed with 200 ml of distilled water to obtain seed/ water ratio of 1:4 in a 250 ml beaker and then stirred on a magnetic hot plate at 50 ^{o}C for different periods of time. Five different enzyme dosages 0, 20, 40, 60 and 80 mg (total protein) were used to evaluate the effect of enzyme concentration on the oil yield during EAAE.

3.6 Quality Evaluation of Oils and Oilseed Residues

The oils and the residues, after oil extraction were characterized by using various analytical methods (AOAC, 1994).

3.6.1 Proximate Composition of Oilseed Residues

After oil extraction, the residue was analyzed for protein, fiber, and ash contents.

3.6.1.1 Protein

Protein content (N x 6.25) was determined according to the AOAC (1994) method. Total protein was determined by the Kjeldahl method as modified by Pousga *et al*, (2007). The analysis of protein content in a compound by Kjeldahl method is based upon the determination of the amount of reduced nitrogen present. Thirty grams (30 g) of each sample was weighed into a filter paper and put into a Kjedahl flask, 10 tablets of Na_2SO_4 were added with 1 g of $CuSO_4$ respectively. Twenty millilitres (20 ml) of concentrated H_2SO_4 were added and then digested in a fume cupboard until the solution became colourless. It was cooled overnight and transferred into a 500 ml flat bottom flask with 200 ml of distilled water. This was then cooled with the aid of packs of ice block. About

60 to 70 ml of 40 % of NaOH were poured into the conical flask which was used as the receiver with 50 ml of 4 % boric acid using methyl red as indicator. The ammonia gas was then distilled into the receiver until all the gas evaporated. Titration was done in the receiver with 0.1 N H_2SO_4 until the solution became colourless. The formula used is:

$$\% \text{ Crude protein} = \frac{\text{Vs- Vb X 0.01401 X N acid (6.25)}}{\text{Original weight of sample used}} \text{ X } 100$$

Where Vs = Volume (ml) of acid required to titrate sample;

Vb = Volume (ml) of acid required to titrate blank; N acid = normality of acid. 6.25 = Factor to convert nitrogen content into protein content.

3.6.1.2 Crude fiber

Crude fibre content was determined according to methods adopted by Pousga *et al.*, (2007). Twenty grams (20 g) of each shea residual samples were defatted separately with Diethyl ether for 8 hours and boiled under reflux for exactly 30 min with 200 ml of 1.25 % H_2SO_4. It was then filtered through cheese cloth on a fluted funnel. This was later washed with boiling water to completely remove the acid. The residue was then boiled in a round bottomed flask with 200 ml of 1.25 % Sodium hydroxide (NaOH) for another 30 min and filtered through previously weighed couch crucible. The crucible was then dried with samples in an oven at 100 °C, left to cool in a desiccator and later weighed. This was later incinerated in a muffle furnace at 600 °C for 2 to 3 h and left to cool in a desiccator and weighed. The formula used is presented as:

$$\% \text{ Crude fibre} = \frac{\text{weight of fibre (g) X 100}}{\text{Weight of original sample (g)}}$$

3.6.1.3 Ash content

The ash represents the inorganic component (minerals) of the sample after all moisture has been removed as well as the organic material. The ash content was determined according to Pousga *et al.,* (2007). The method was based on the decomposition of all organic matter such that the mineral elements would not be lost in the process. Approximately 1g of finely ground sample was weighed into porcelain crucible which had been ignited. The crucible was placed in a muffled furnace and heated at 500ºC for four hours, removed and cooled. The ignited residue was moistened with 2 ml distilled water and slowly and carefully 5 ml of 8 N HCl (2 parts of conc. HCl was mixed with one part of water). It was transferred again into the cool muffle furnace and the temperature was increased step wise to 550 ± 5 ^{o}C. The temperature was maintained for 8 hours until white ash was obtained.

It was then brought out and allowed to cool in a desiccator and weighed again. Percentage weight was calculated as weight of ash multiplied by 100 over original weight of the samples used. The formula used is presented as:

$$\% \text{ Ash Content} = \frac{\text{Weight of ash (g)}}{\text{Weight of original sample}} \times 100$$

3.6.2 Characterization of extracted oils

3.6.2.1 Physico-chemical parameters of oils

Iodine value, free fatty acid (FAA), density, unsaponifiable matter, peroxides and saponifiable values of the control, solvent and enzyme-extracted oils were determined by various standard methods (AOAC,1994).

3.6.2.2 Determination of free fatty acid (FFA)

The free fatty acid formed was determined as oleic acid (BP, 1993) by titrating with standard 0.1M potassium hydroxide solution using phenolphthalein to a neutral point. The FFA was expressed as milligram of potassium hydroxide required to completely neutralize one gram shea butter, using the formula below:

$$\text{Free Fatty Acid Content} = \frac{5.610V}{W}$$

Where V is the volume of 0.1M KOH used and W the weight of fat used for the assay (BP, 1993).

3.6.2.3 Determination of peroxide value (PV)

The hydroperoxide or peroxide formed after the extraction of the oil was estimated iodometrically. The sample was reacted with a saturated aqueous solution of potassium iodide, and the iodine liberated by the peroxides was titrated with a standard solution of 0.1M sodium thiosulphate. The peroxide Value (PV) was expressed in units of milliequivalent of oxygen per kilogram of fat (BP, 1993).

$$\text{Peroxide Value (PV)} = \frac{10\ V}{W}$$

Where V is the titre and W, weight of sample used.

3.6.2.4 Determination of Iodine Value (IV)

Treated samples of shea butter were reacted with iodine bromide and the excess iodine liberated, titrated with standard 0.1M sodium thiosulphate. A blank titration was also

performed. The iodine value was expressed as milligrams of iodine to completely saturate all the double bonds in the shea butter using the formula:

$$\text{Iodine Value (IV)} = \frac{1.69V}{W}$$

Where V was the difference in volume between the blank and the sample titre, while W was the weight of the sample used (BP, 1993).

3.7 Statistical Analysis

Values are reported as mean ± SD of each sample for each enzyme extraction, analyzed individually in triplicates. Mean values in the same row followed by the same superscript letters are not significantly different ($P > 0.05$). One way ANOVA was used to determine significant differences between groups, considering a level of significance of less than 5% ($P < 0.05$) by using Statgraphics centurion XV. Graphs were drawn using the Microsoft Office Excel (2007) version.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSIONS

In a preliminary study the moisture content of the corn cob prior to the solid state fermentation was adjusted to different levels using distilled water. It was adjusted from 40 % to 80 % moisture content. Moisture content at 60 % showed enough growth coverage of *Saccharomyces cerevisiae* in the bottle. At moisture content of 60 % the *Saccharomyces cerevisiae* growth covered almost all the surface area of the 250 ml Erlenmeyer. The rest of the experienment was therefore performed at the moisture content of 60 %. The results representing the variation in the moisture content is found in appendix 3.

4.1 Changes in total Protein, Pectinase activity and Specific Activity during Fermentation

Protein concentration and pectinase activity profile during fermentation are shown in Fig 4.1. An increase in both protein and enzyme activity was observed from the second day of fermentation, reaching a peak by the 4^{th} day and thereafter began to decline. Enzyme activity was totally lost by the 8^{th} day of fermentation while the protein concentration reached the minimal level by 12 days of fermentation (Fig. 4.1). The trend observed in both protein concentration and enzyme activity can be explained in terms of a microbial growth in a culture medium.

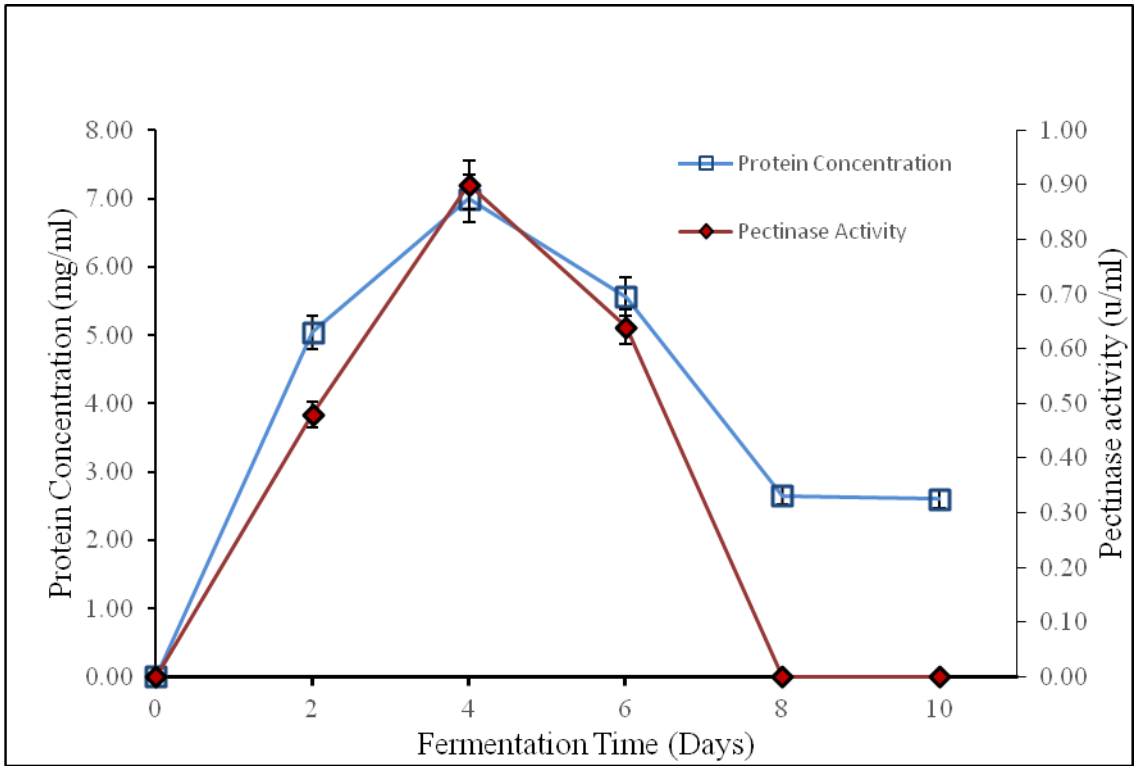

Figure 4.1: Changes in concentration of protein and pectinase activity produced by *Saccharomyces cerevisiae* with fermentation time

From the first to the second day, the *Saccharomyces cerevisiae* cells were adjusting to their new environment by producing the necessary enzymes such as pectinases, lipases and cellulases to be able to utilize the nutrients available, as a result of this, little protein concentration was observed. From the 2nd to the 4th day, there was an increase in protein concentration. These exponential increases in protein concentrations represented the log phase of the growth curve of *Saccharomyces cerevisiae* where the yeast cells have sufficiently adapted to the new fermentation medium producing more enzymes, utilizing the nutrients available and multiplying rapidly. The decrease after the 4th day indicated that the microorganisms have used all the nutrients available. After the 10th day, the *Saccharomyces cerevisiae* cells entered into their stationary phase, and subsequently the decline or death phase due to depletion of nutrients in the medium leading to gradual

death of the microorganisms (Prescott *et al*, 1999). Pectinase activity monitored during the fermentation followed a similar pattern to total protein production attaining a peak activity on the 4th day after which it declined rapidly to zero by the eighth day (Fig. 4.1). This result indicates that most of the protein produced by the yeast in the medium is pectinase in nature.

In a growth medium, microorganisms require carbon, nitrogen, minerals, sometimes growth factors and oxygen (if aerobic) as elements for cell biomass and as energy for biosynthesis and cell maintenance. Depletion of these nutrients tends to affect cell biomass and energy required for biosynthesis, cell maintenance and product formation (Owen, 1989). The decline in protein production and pectolytic enzyme activity after the 4^{th} day could be accounted for on the following basis: batch culture may be considered as a closed system (except for aeration) containing limited amount of medium constitiuents in which the inoculated culture passes through a number of phases. There is a repressive effect of rapidly utilized carbon source (substrate depletion), decrease in essential mineral elements and growth factors, decrease in viscosity of medium (in this case due to breakdown of pectin) and effect of toxic metabolic waste or catabolite repression (Owen, 1989). Substrate depletion occurs when all the substrates have been used up and therefore the organisms do not have sufficient growth nutrients to multiply, resulting in declined growth (Owen, 1989). The observation that enzyme activity followed the same trend as protein concentration is an indication that most of the protein in the culture medium is pectinase (Fig 4.1).

Specific activity is the number of enzyme units per ml divided by the concentration of protein in mg/ml. Specific activity values are therefore quoted as units/mg. Specific activity gives a measurement of enzyme purity in the mixture. It is the amount of product formed by an enzyme in a given amount of time under given conditions per milligram of total proteins. The value becomes larger as an enzyme preparation becomes purer, since the amount of protein (mg) is typically less, but the rate of reaction stands the same or may increase due to reduced interference or removal of inhibitors (Nelson *et al.*, 2000). The specific activity for the various pectinase activities was calculated. The specific activity curve as shown in Fig 4.2 followed the shape of the pectinase activity curve. It was also at its peak on the fourth day.

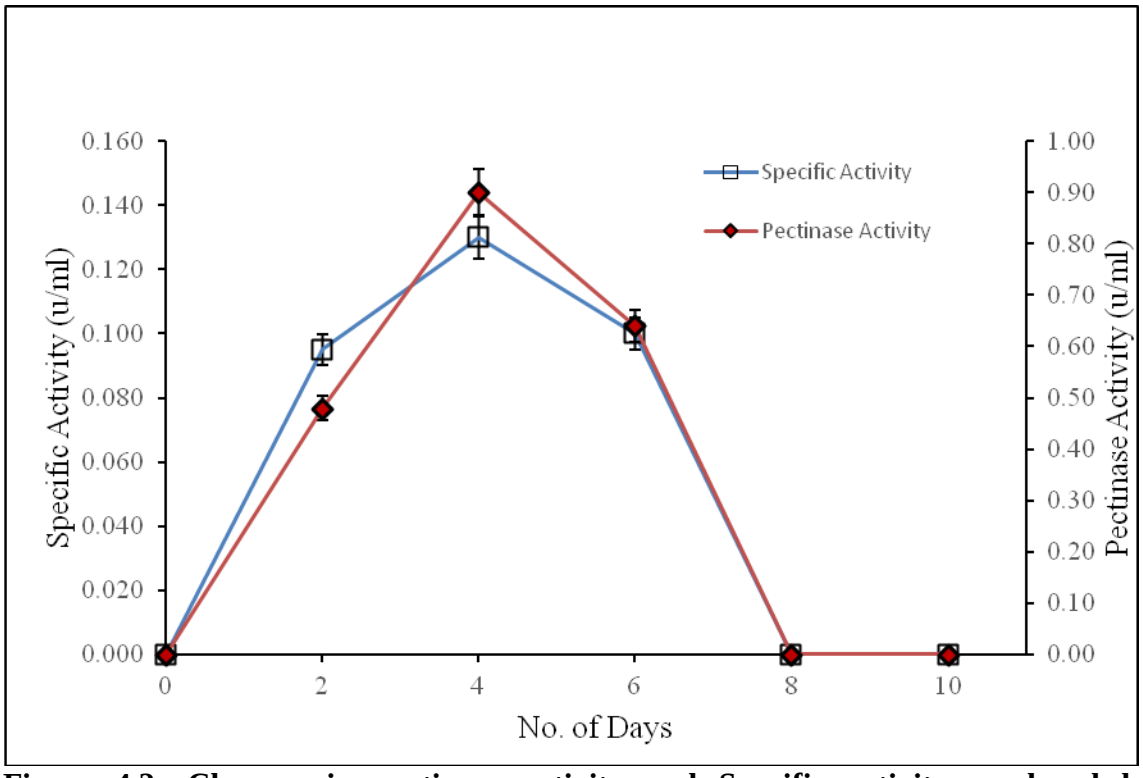

Figure 4.2: Changes in pectinase activity and Specific activity produced by *Saccharomyces cerevisiae* with fermentation time

Most crude enzymes usually may have low specific activities due to the fact that they have not been purified. In our work, the peak specific activity (S.A) was low at 0.130 U/mg. The fourth day gave the highest pectinase activity and therefore was obvious that the same day may give the highest specific activity. In similar work reported by Dzogbefia *et al.* (2008), a low specific activity of 0.62 U/ mg extract was also obtained.

4.2 Stability of Protein and Enzyme Activity on Storage

Based on the results shown in Figure 4.1, subsequent enzyme productions were carried out at 4 days of fermentation. The stability of the protein and enzyme was monitored by storing the crude preparation in refrigerator at 8 ^{o}C. The protein concentration and enzyme activity of the crude pectinase was measured on weekly bases for four weeks. The protein concentration remained unchanged (7.0 mg/ml) for the first two weeks, after which it declined to 5 mg/ml on the twenty first day, and further reduced to 3 mg/ml by the twenty eighth day (Fig. 4.3). Similarly the activity of the crude pectinase was the same from the first day of production to the second week, the enzyme acticity began to decline after the second week. No activity was observed after the fourth week of storage (Fig. 4.4).

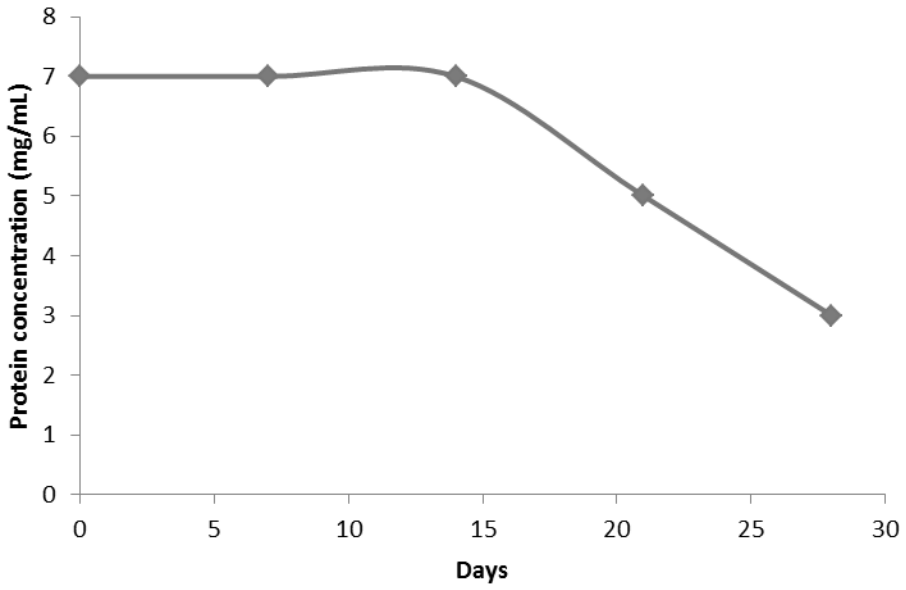

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Figure 4.3: Stability of protein concentration of enzyme extract on storage

The ability to maintain the protein concentration is very important; since enzymes are proteins and a decline in the amount of protein may affect the activity of the enzyme. From Figures 4.3 and 4.4, both protein and enzyme activity were maintained for the first two weeks and afterwards declined. It will therefore be important that the pectinase enzyme is produced upon request by a local industry and the users must use it within two weeks to get the efficacy of the enzyme or the strength of its activity will reduce. Proteins stored in solution at 8 °C can be dispensed conveniently as needed but require more diligence to prevent microbial or proteolytic degradation; such proteins may not be stable for more than a few days or weeks.

By contrast, lyophilization allows for long-term storage of protein with very little threat of degradation, but the protein must be reconstituted before use and may be damaged by the lyophilization process (www.piercenet.com).

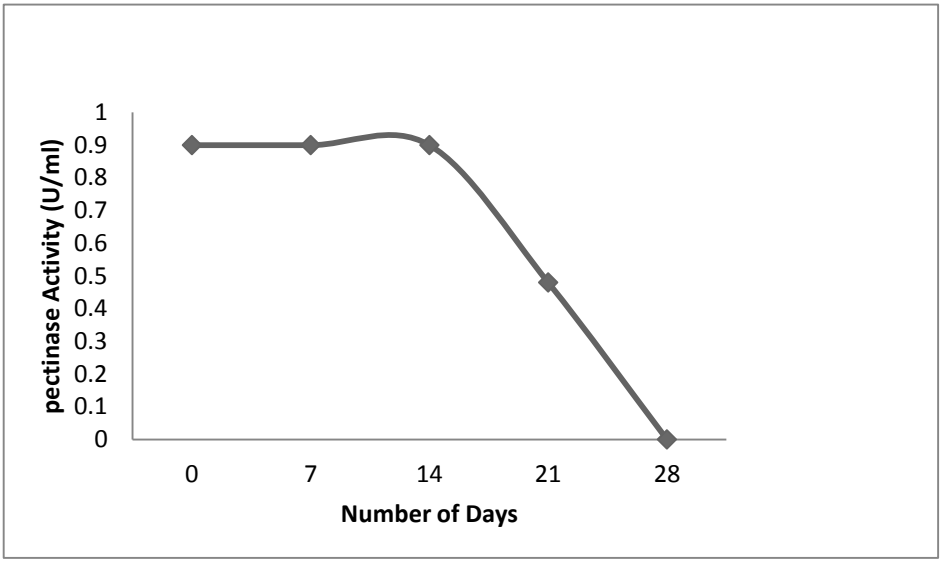

Figure 4.4: Stability of enzyme extract during storage

From the above results the crude pectinase activity is not effective after two weeks of production, thus the enzyme should not be stored beyond 14 days. One of the common practical problems facing an emzymologist is the loss of enzymatic activity in a sample due to enzyme instability (Gomori, 1992). Enzymes, like most proteins are prone to denaturation under many laboratory conditions and specific steps must be taken to stabilize these macromolecules as much as possible (Copeland, 1994). An enzyme can be maintained in stable conditions for several days at 4°C, however, it should be used in smaller quantities to prevent denaturing (Gomori, 1992). If a particular enzyme is not stable under these conditions, any sample remaining at the end of an experiment must be

discarded. To avoid wasting enzyme sample material, samples should be stored in small volumes, of high concentration aliquots (Gomori, 1992). This way the volume of sample that is needed for each day's experiments can be thawed, while the bulk of the sample aliquots remain frozen. Once thawed, the enzyme should be kept at ice temperature (4 °C) for as long as possible before equilibration to the assay temperature (Copeland, 1994). For long term storage for 1 month to 1 year, some researchers choose to bead single-use aliquots of the protein in liquid nitrogen for storage in clean plastic containers under liquid nitrogen. This method involves adding the protein solution dropwise (about 100 µl each) into a pool of liquid nitrogen, then collecting the drop-sized frozen beads and storing them in cryovials under liquid nitrogen (www.piercenet.com). Many compounds such as Protein Stabilizing Cocktails, Cryoprotectants such as glycerol or ethylene glycol, protease inhibitors, anti-microbial agents such as sodium azide (NaN3),metal chelators such as EDTA, reducing agents such a dithiothreitol (DTT) and 2-mercaptoethanol (2-ME) may be added to protein solutions to lengthen shelf life (www.piercenet.com).

In agreement to our findings, sigma-Aldrich and Worthington-Biochem all advised that pectinases should be stored at a range of 2 °C to 8 °C (www.sigmaaldrich.com: www.worthington-biochem.com)

4.2 Oil Extraction of shea seeds during Enzyme-Assisted Aqueous Extraction (EAAE)

4.2.1 Effect of pH on oil recovery

Enzyme activities are strongly dependent upon the pH: thus the effect of pH on the enzyme activity during oil extraction was determined. Figure 4.5 shows the effect of pH of the extraction medium on oil yield during EAAE. The pH range of 4-6 was found to be generally suitable in the present study. NaOH/HCl was occasionally added to the dispersion to maintain the pH. Crude Pectinase and commercial Pectinex exhibited maximum activity at pH range of 4 to 6 whereas Viscozyme and the control showed maximum activity at pH of 6.0.

In earlier extraction trials at unmodified pH 5.4 of the dissolved meal, yield was persistently high and closer to that at pH 6.0 which is the optimum pH of the control. In subsequent experiments, therefore, pH adjustments were not made. This also suggests that in such aqueous extraction processes without the use of commercial enzymes, pH adjustments may not be necessary. Very high and low pH's can result in higher viscosity due to cell degradation (Abdulkarim *et al.,* 2006). This makes it more difficult to separate solid-liquid phase by centrifugation which results in decrease in the oil yield. It may also result in deterioration of oil quality through saponification (Rhee *et al*., 1972) and cause several changes to the amino acids, such as the formation of lysinoalanine and lanthionine (De Groot and Slump 1969).

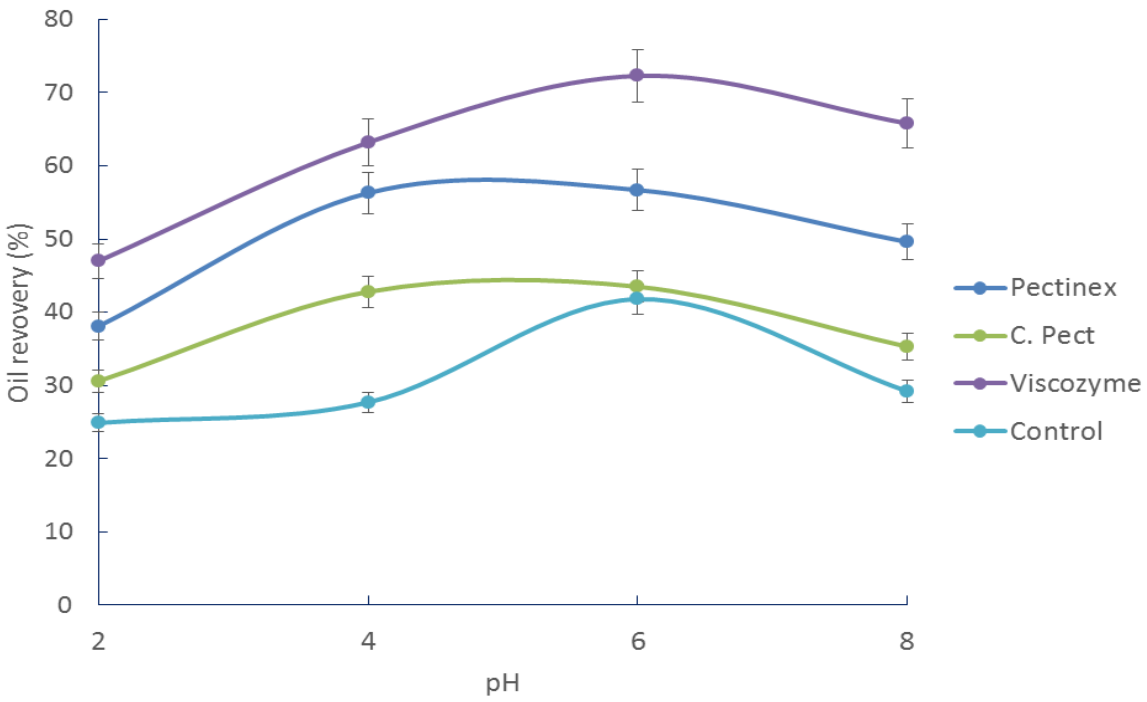

Figure 4.5: Effects of pH on oil recovery from shea seeds during EAAE

A close relationship exists between the oil and protein extraction in some oilseeds because the conditions for the maximum oil extraction generally coincide with the highest protein yields (Rosenthal *et al*., 1996). Highest oil yield occurs at pH values corresponding to maximum protein extractability in the aqueous system and the lowest yield is obtained when the protein solubility is at its lowest, i.e., around the isoelectric point (Rosenthal *et al*., 1996). In general, for most oilseeds, maximum oil and protein yields are obtained at a pH away from the isoelectric point. Optimum pH values for oil extraction were 4.0, 7.0, or 10.0 for peanut (Subrahmanyan *et al*., 1959; Rhee *et al*., 1972; Hagenmaier *et al*., 1973), 10.0 for sunflower (Rhee *et al*., 1972) and 6.6 for rapeseed (Embong and Jelen 1977).

From the above results it can be concluded that modification of pH in extraction of shea fat using the aqueous method is not necessary; similarly, in the application of crude pectinase for shea fat extraction, pH modification may not be necessary since the pH of the unmodified dissolved meal which was 5.4 fell within the observed optimum range of 4.0 – 6.0

4.2.2 Effect of enzyme dosage on oil recovery

Figure 4.6 illustrates the effect of enzyme dosage on oil yield from shea seeds. The optimum enzyme dosage for crude Pectinase was 0.12% while commercial Pectinex and commercial Viscozyme were found to be 0.08 %. It has been observed elsewhere that the oil yield increases with little increment in enzyme dosage (Abdulkarim *et al.*, 2005). The high enzyme cost associated with high enzyme dosage is a major obstacle for the economy of the process. Depending on the enzyme and the experimental conditions, different oil yields were obtained.

Previous studies showed that only 0.02 % of the local pectinase was the dosage required for optimal pineapple juice, pawpaw juice and cassava starch extractions (Dzogbefia *et al.*, 2001: Djokoto *et al.*, 2006 and Dzogbefia *et al.*, 2008).

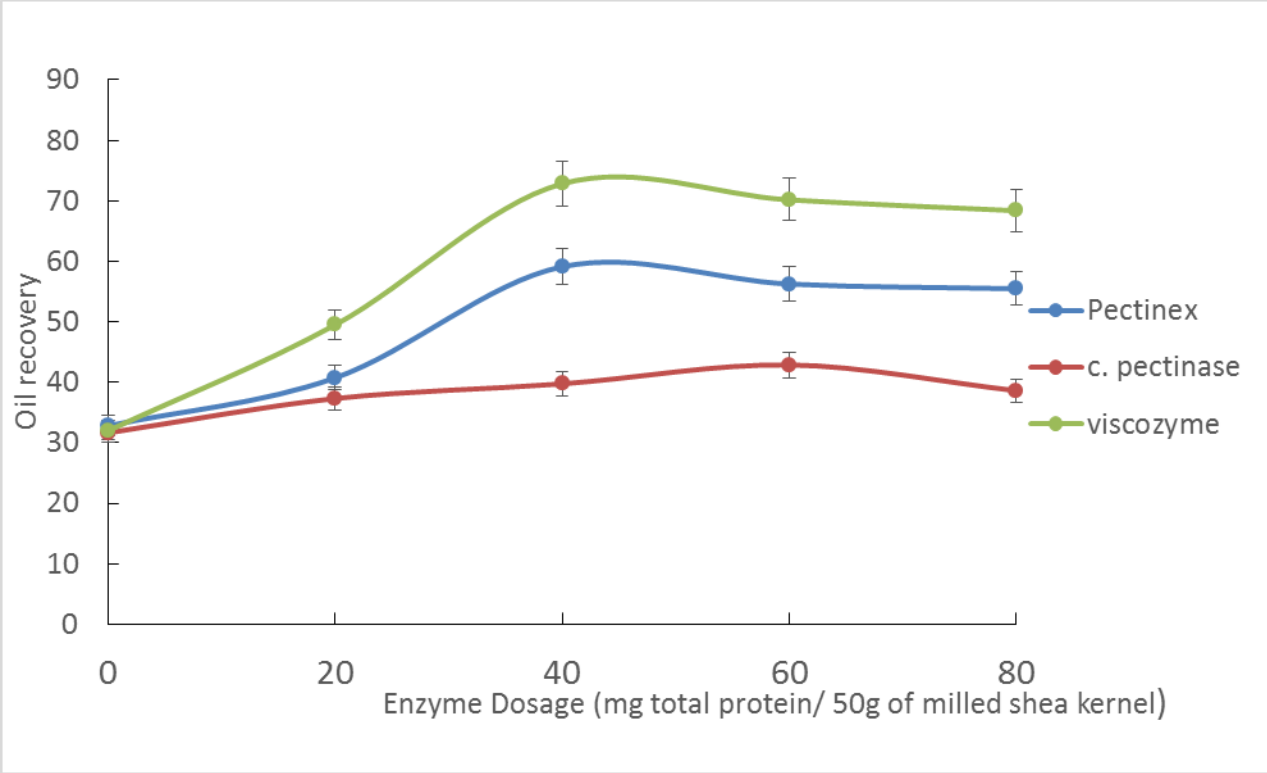

Figure 4.6: Effect of Enzyme Dosage on Oil Recovery from shea seeds during EAAE

Enzymes are substrate-specific, therefore their activity varies according to the nature of the oilseeds. The effect of enzymatic pre-treatment depends on the oilseed structure and the cell wall composition; it therefore varies according to the kind of oilseed and type of enzyme used (Dominguez *et al*., 1994). Enzyme mixtures offered higher oil recovery due to their combined effect on colloidal and lipoprotein seed structures (Dominguez *et al*., 1994). This can be attributed to the presence of several potent components in these enzyme mixtures, e.g. arabanase, cellulase, β-glucanase, hemicellulase, xylanase and pectinase (in Viscozyme). Similar results as ours have also been reported by Hernandez *et al*. (2000) for rice bran.

Enzyme mixtures were found to be more effective than the single enzyme crude pectinase used as shown in Figure 4.6. Tano-Debrah and Ohta (1995) also examined combined enzymatic effects of acid protease, cellulase, hemicellulase and glucanase, and Sumizymes LP (protease), cellulase and hemicellulase and obtained recoveries of 74.1 and 72.7 % for the extraction of shea and cocoa fat, respectively. A 70 % recovery during aqueous-enzymatic extraction of sunflower kernel oil was obtained by using a combination of cellulase and pectinase (Novozymes) (Dominguez *et al*., 1995). The palm mesocarp was treated with cellulase preparation and 57 % palm oil was recovered during the aqueous process (Cheah *et al*., 1990). Lanzani (1975) reported peanut oil yields of 74-78 % by aqueous extraction using protease, cellulase, and α-1,4 galacturonide glycano hydrolase as compared to control (72 %). Olsen (1988) utilized pectinase, cellulase, and hemicellulase combinations to degrade rapeseed during aqueous extraction process. By using cellulolytic enzymes on steamed rapeseed flour, 35 % of the oil was extracted (Marek *et al*., 1989). In this work, the crude pectinase used is a single enzyme and was not purified; therefore it gave a lower yield as compared to the combined and purified enzymes (Fig 4.6).

Enzyme dosage, even in the range of 1-3 g/100 g seed, has considerable effects on oil yields depending on the seed nature. For rapeseed 2 g/100 g appears to be the optimum (Dominguez *et al*., 1994). Fullbrook (1983) reported increment in melon seed oil recovery over 100 % with respect to control samples during aqueous extraction. Bhatnagar and Johari (1987) made an improvement (more than 20 % of the total oil) in cottonseed oil extraction yield, while for castor and sunflower the increment was less

than 10 %. It is known that an increase in the enzyme concentration increases the rate at which the oil is separated, but the optimum level must be established. Cellulase (at 25–30 g/100 kg) enhanced the oil extraction of olive, but its efficiency was considerably reduced at higher or lower concentrations. Dominguez *et al*. (1994) reported 50 g/100 kg as an optimum concentration for acid protease. Tano-Debrah and Ohta (1995) observed a rapid increase in extracting shea fat at enzyme concentration (0.0 to 1.0 %).

Similarly, Dominguez *et al*. (1995) used 0.5–4 % (w/w) enzyme (Celluclast + Pectinex) concentration to extract sunflower oil and found that a 2 % (w/w) enzyme concentration was most favorable. No increment in the oil recovery was observed beyond this dosage. Abdulkarim *et al*. (2006) reported a gradual increase in oil recovery with the increase in the enzyme dosage from 0.5 to 2.0 % and obtained highest *M. oleifera* seeds oil recovery with 2.0 % enzyme. No increase in the oil yields were observed on further increase in enzyme dosage to 2.5 %. In agreement with our findings, there were no significant increments after enzyme dosage of 2.0 %.

4.2.3 Effect of shea meal hydrolysis time on oil recovery

The effect of hydrolysis time on EAAE is shown in Figure 4.7. It can be seen that hydrolysis time beyond 30 minutes had appreciable effect on oil recovery, but the oil yield decreased after 2.5 h. As evident in Figure 4.7, an increase in the incubation time from 1.5 h to 2.5 h did not improve oil yield. It can be seen that oil yields increased slightly when extraction time increased. The maximum oil yield for crude Pectinase and commercial Pectinex were observed at hydrolysis time of 90 min and that of Viscozyme and the control gave their maximum yield at 60 min of hydrolysis. Kim (1989) reported

30 min period as an optimum time for palm oil extraction, and 40 min period for extracting both soybean oil and protein (Lusas *et al*., 1982) whereas Embong and Jelen (1977) found 15 min blending followed by a prolonged period of l-4 h of stirring more appropriate for rapeseed oil extraction.

In agreement with our observations (Figure 4.7), Abdulkarim *et al*. (2006) reported increase in the oil recovery with an increase in the incubation time after an hour. A very sharp increase in oil recovery was observed from 0 to 2 h, after which the rate slowed down. Similarly, Dominguez *et al*. (1994) also reported that 0.33–2.00 h was sufficient to exhibit a significant increase in oil recovery. A gradual increase in the reaction time from 60 min to 90 min increased the oil recovery to an optimum level, (Fig 4.7) However, the optimal recovery time selected by most local shea butter producers was 2 h since most of the rural industries stir the shea meal for that long, and this time is considered too long if this technology is to be commercially exploited as yields decreased by this time period (Fig 4.7). A shorter incubation time will be more advantageous in industrial extraction processes (Abdulkarim *et al*., 2006).

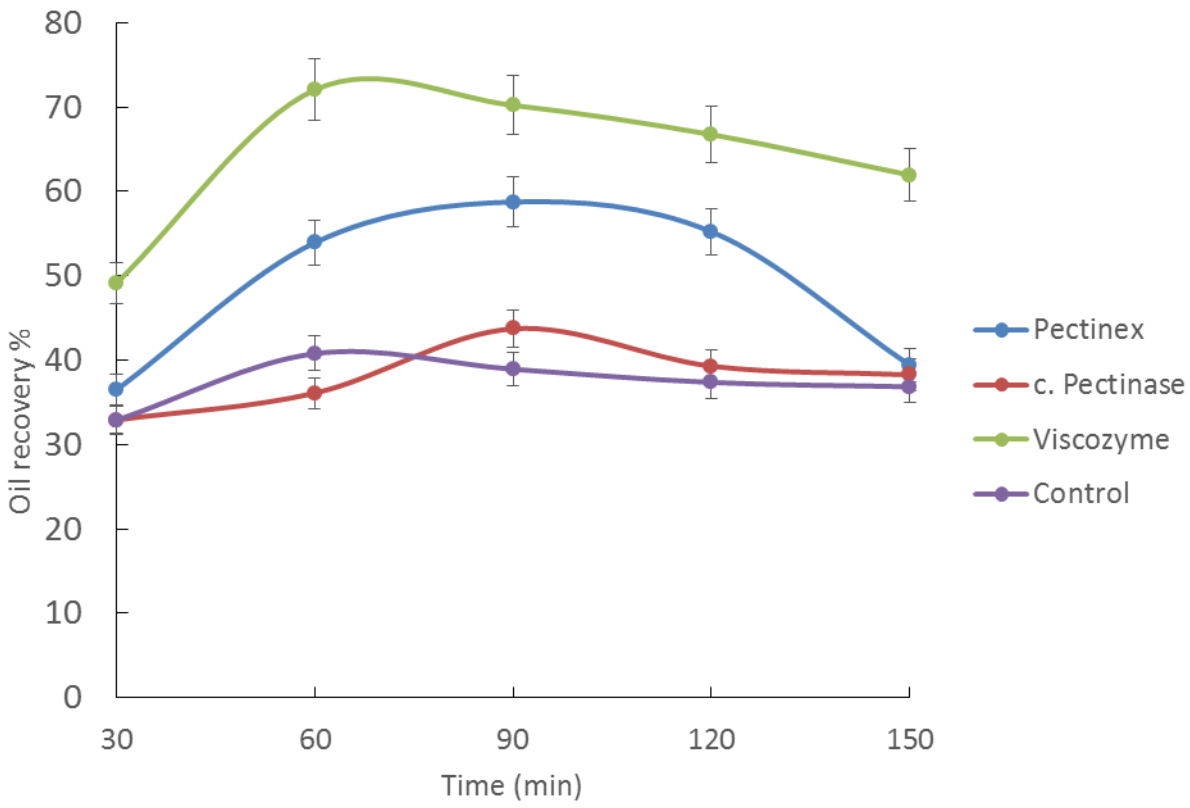

Figure 4.7: Effect of shea meal hydrolysis time on oil recovery from shea seeds.

4.2.4 Effect of temperature on oil recovery from shea seeds

The effect of temperature on oil yields during EAAE is shown in Figure 4.8. When the extraction temperature was raised from 30 to 40^{o} C, the oil yield increased significantly forall the enzymes. All the enzymes gave maximum oil recovery at 40^{o} C. No enhancement in the oil yield was observed above 40 ^{0}C. At high temperatures it was difficult to maintain homogeneity during extraction and it also resulted in protein denaturation. Therefore, a temperature range of 40-50 ^{0}C was deemed to be satisfactory for the extraction. Temperature exhibits considerable effect on oil yields from different oilseeds during hot water floatation method (Southwell and Harris 1992). Lusas *et al*. (1982) reported that the temperature is critical for oil extraction from soybeans during

aqueous extraction process. They observed maximum oil recovery between 40-60 °C. In some other studies, different temperatures have been employed in aqueous extraction process with other oilseeds. Hagenmaier (1974) extracted sunflower oil at room temperature.

A temperature range (60-65 °C) was selected for the extraction of peanut oil (Subrahmanyan *et al*., 1959; Rhee 1972) and 70 °C for the extraction of rapeseed oil (Embong and Jelen 1977) whereas 80 °C was maintained during coconut oil extraction (Hagenmaier *et al*., 1972). Lanzani *et al*., (1975) treated sunflower, rapeseed and peanut using increasing temperatures (40, 50 and 65 °C) for a period of 3 h during enzyme-assisted aqueous extraction whereas, Fullbrook (1983) utilized a temperature of 50 °C for 60 min, 63 °C for 120 min and a short period of 13 min at 80 °C to inactivate enzymes for rapeseed and soybean during aqueous extraction. Aparna *et al*. (2002) used temperatures of 37, 40, 50 and 60 ^{0}C during the enzyme-assisted extraction of peanut oil and observed a temperature of 40 ^{0}C as the optimum for best oil recovery. Barrios *et al*. (1990) used temperatures of 40, 50, 55 and 60 ^{0}C for coconut oil extraction and reported the highest oil recovery at 50 ^{0}C. Thus the results obtained in this study are consistent with other findings.

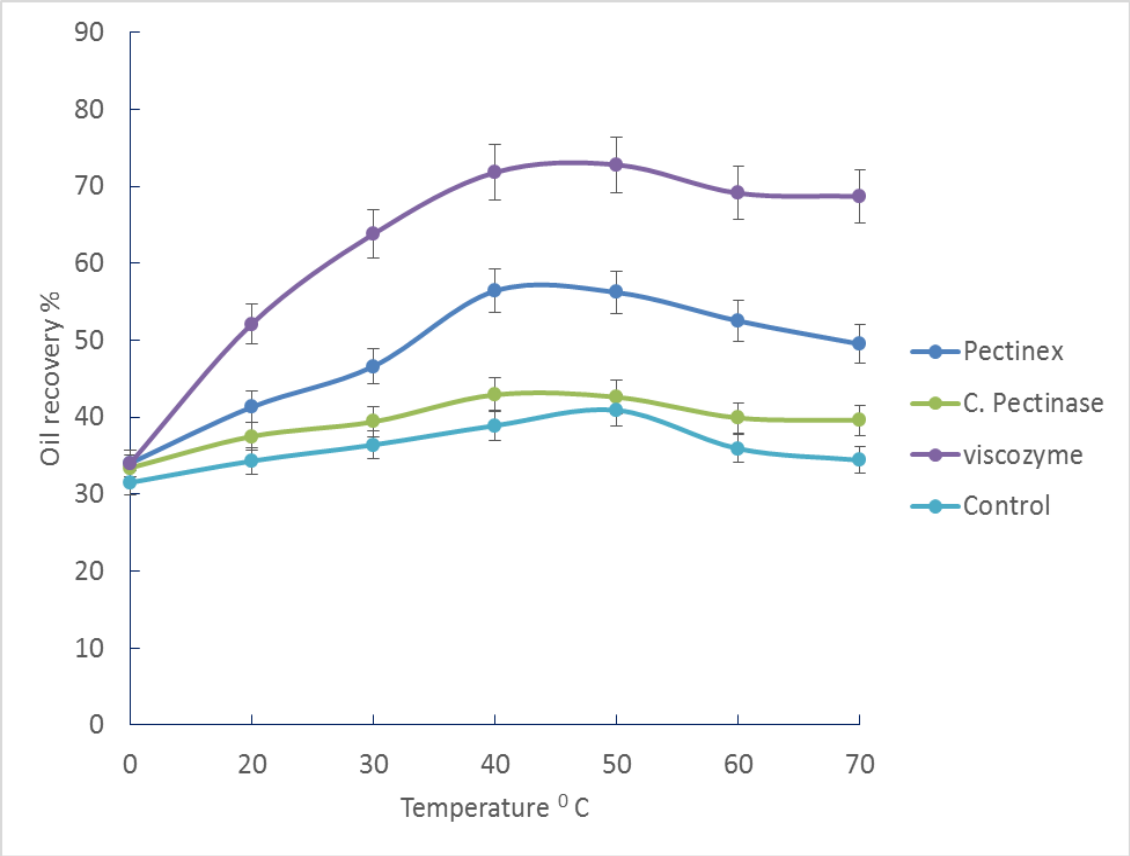

Figure 4.8: Effect of temperature on oil recovery from shea seeds.

4.2.5 Effect of water/seed (w/s) ratio on oil recovery

The effect of w/s ratio (w/w) on oil recovery was also investigated. The optimum water to seed ratio was found to be 4:1 for crude pectinase, Pectinex and Viscozyme while that of the control was found to be 2:1 (Figure 4.9). At a higher w/s ratio (8:1), the viscosity of the mixture decreased. This made it difficult to maintain mixture homogeneity and it decreased the oil recovery. In the present study, a w/s ratio of 4:1 was found to be adequate for this process when enzymes were applied. The amount of water added during the treatment affects the oil recovery. The low water/seed ratio of 2:1 of the control experiment may be due to the fact that, kneading is best done with little water and more

water would not give enough fat suspension. The application of enzymes may need enough water for the enzymes to work effectively; this may explain why more water was needed in the case of the enzyme extraction processes. Buenrostro and Lopez-Munguia (1986) reported that the maximum yield of avocado oil was obtained with a 5:1 w/s ratio, while for coconut the best yields were obtained at a 4:1 w/s ratio (Cintra *et al*., 1986). Tano-Debrah (1997) also reported that the maximum yield of coconut oil and shea kernel was 4:1 which is consistent with our results.

Oil yield was reported to be increased slightly with dilution, but decreased again at higher dilutions McGleone *et al.* (1986). This may be attributed to both emulsion formation and emulsion stability. When the w/s ratio was decreased to 2:1, in the case where enzymes were applied, the oil yield decreased drastically which may be due in part to the difficulty of keeping the mixture in suspension during treatment. In agreement with this study, McGleone *et al.* (1986) also reported similar trends. However, Picuric-Jovanovic *et al*. (1997) reported that the extraction yields depend on the ratios, because the enzyme accessibility to the cell walls should be more effective at lower ratios. The w/s ratio is normally selected so as to obtain less stable emulsions and generate less effluent; however, to obtain very clear yields devoid of impurities, it is usually necessary to use large quantities of water (Cintra *et al*., 1986).

Literature reports revealed that w/s ratios from 5:1 to 12:1 for peanut oil extraction (Rhee *et al*., 1972; Bhatia *et al*., 1966), 10:1 for sunflower (Hagenmaier 1974) and 12:1 for soybeans (Lusas *et al*., 1982) were found as the optimum water seed ratios. Embong and

Jelen (1977) reported an optimum ratio varying between 2.5:1 and 3.5:1 for rapeseed oil extraction.

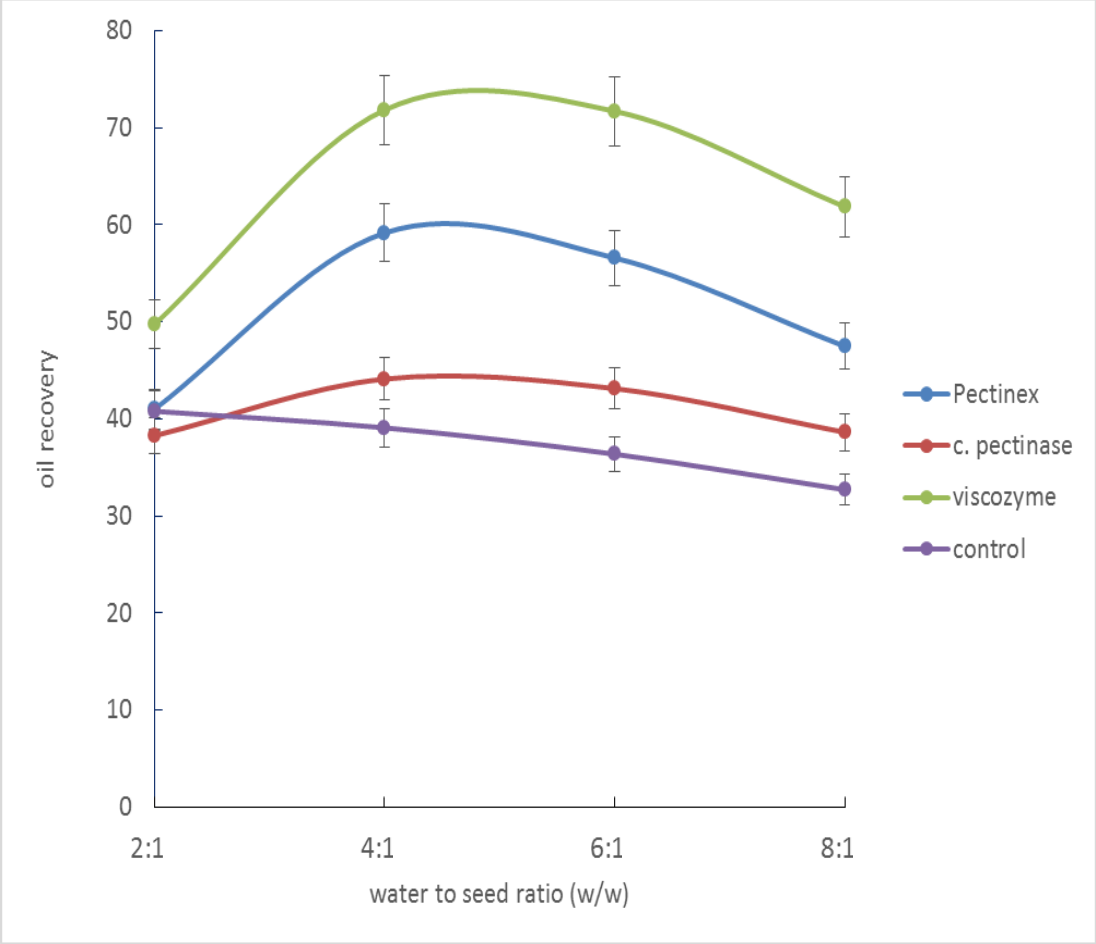

Figure 4.9: Effect of water seed ratio on oil recovery from shea seeds

4.2.6 Oil recovered after optimization of all parameters

All the parameters that were investigated were optimized and used to extract the oil; the table below summarizes the optimum conditions for all the enzymes and control.

Table 4.1: Summary of all optimum conditions for all enzymes and control

Parameter	Control	Crude Pectinase	Pectinex	Viscozyme
Enzyme dosage	0.0	0.12%	0.08%	0.08%
Water: seed	2:1	4:1	4:1	4:1
pH	5.4	4-6	4-6	6
Optimum Temp	50 ^{0}C	40-50 ^{0}C	40-50	40-50
Hydrolysis time	60 min	90 min	90 min	60 min
Recovery %	40	44	58.60	72.00
Oil content	25.33	26.43	36.35	44.52

The enzyme pre-treatment appreciably increased the oil yields at optimized set of experimental conditions as shown in Figure 4.10. Viscozyme gave the highest amount of oil recovery of 72.0 % followed by Pectinex which gave 58.6 %. The oil recovered by the application of the crude pectinase was significantly ($P \leq 0.05$) different from that of the control. The crude pectinase gave 44.0% oil recovery while that of the control was 40.0 %.

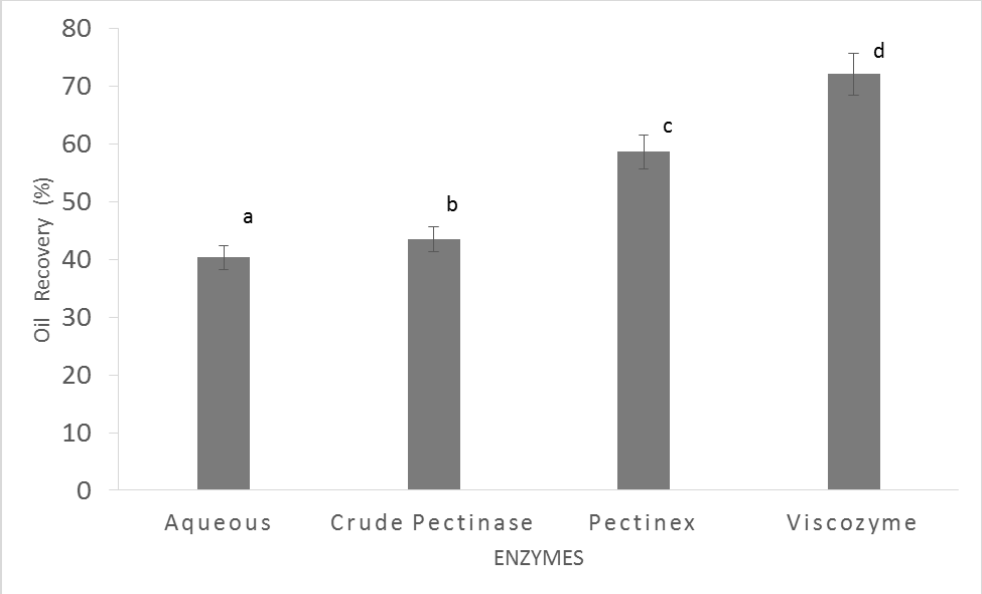

Figure 4.10: Performance of crude pectinase verses commercial enzymes in shea fat extraction under optimum conditions

Enzymes are substrate specific; therefore their activity varies according to the nature of the oilseeds. The effect of enzymatic pre-treatment depends on the oilseed structure and the cell wall composition and therefore varies according to the kind of oilseed and type of enzyme used (Dominguez *et al*., 1994). Enzyme mixtures offered higher oil recovery due to their combined effect on colloidal and lipoprotein seed structures (Dominguez *et al*., 1994). This can be attributed to the presence of several potent components in these enzyme mixtures, e.g. arabanase, cellulase, β-glucanase, hemicellulase, xylanase and pectinase (in Viscozyme), and polygalacturonase activity as well as arabinase side activity in Pectinex. Similar results as ours have also been reported by Hernandez *et al*. (2000) for rice bran.

In agreement with our results that enzyme mixtures were found to be more effective than the single enzyme, Tano-Debrah and Ohta (1995) also examined combined enzymatic effects of acid protease, cellulase, hemicellulase and glucanase, and Sumizymes LP (protease), cellulase and hemicellulase and obtained recoveries of 74.1 and 72.7 % for the extraction of shea and cocoa fat, respectively. A 70 % recovery during aqueous-enzymatic extraction of sunflower-kernel oil was obtained by using a combination of cellulase and pectinase (Novozymes) (Dominguez *et al*., 1995). The palm mesocarp was treated with cellulase preparation and 57 % palm oil was recovered during the aqueous process (Cheah *et al*., 1990). Lanzani (1975) reported peanut oil yields of 74-78 % by aqueous extraction using protease, cellulase, and α-1,4-galacturonide glycano hydrolase as compared to the control (72 %). Olsen (1988) utilized pectinase, cellulase, and hemicellulase combinations to degrade rapeseed during aqueous extraction process. By

using cellulolytic enzymes on steamed rapeseed flour, 35 % of the oil was extracted (Marek *et al*., 1989). Dominguez *et al*. (1995) observed an enhancement up to 30 % in the sunflower oil recovery during aqueous extraction by using mixtures of cellulase and pectinase. A carbohydrase complex was found to be the most effective enzyme when a group of commercial enzymes were utilized for aqueous extraction of corn germ oil (Bocevska *et al*., 1993). Similarly, 80 % oil recovery has been reported by using a commercial carbohydrase complex in the aqueous process of rapeseed (Deng *et al*. 1992). The low effect of the crude pectinase may also be due to the fact that the preparation was not further purified.

4.2.7 Simple benefit-cost analysis

Few processes in the production of enzymes make it very expensive. The cost of particular enzyme is directly related to the steps involved (www.researchgate.com). The following are the steps involved: production, extraction, purification, concentration, stabilization/ immobilization. Each of these steps comes at a cost. 50 ml bottle of Viscozyme costs Euro 103.00, while a 250 ml of Pectinex costs $ 74.99 (www.sigmaaldrich.com). It will cost twice the same amount of money for the enzymes to get to the buyer due to high cost of importation and taxes. Using reagents and instruments from a well equipped laboratory, it will cost a researcher few Ghana Cedi to produce crude pectinase since the main substrate used is very common and cheap in Ghana.

In situations where one wants to apply commercial pectinase, it would be good if a purified crude pectinase is used since the yield obtained by the crude pectinase is closer

to that of the application of the crude pectinase than the Viscozyme. The yield of the application of Viscozyme far outweighs that of the application of the crude pectinase and it would be good if a researcher can look at the production of crude Viscozyme so that we can have an effective analysis of the cost benefit other than that, buying a commercial Viscozyme would be recommended.

4.3 Comparison of the Quality of Extracted Shea Butter and Residues

4.3.1 Proximate composition of shea residues

Table 4.2 shows comparison of Proximate Composition of Enzyme-assisted Aqueous Extracted Shea butter.

Crude protein content from the residue of the application of the Pectinex and Viscozyme are very close (12.98 % and 12.06 %), but higher than that of the control and the crude enzyme (Table 4. protein was significantly ($P \leq 0.05$) different from one residue to the other as shown in Table 4.1. The highest protein in the aqueous phases of shea seeds was extracted with Pectinex and Viscozyme and this could be due to the fact that they are commercial enzymes with high enzyme activities. After the enzymes have been able to work on the cell wall, they are able to release proteins into the aqueous medium. The protein content in the shea kernel was found to be low in the crude Pectinase. Viscozyme was found to be the best enzyme mixture for protein extraction in the aqueous phase. The extracted proteins can potentially be used as food ingredients.

No significant ($P > 0.05$) variations were observed for the fiber and ash contents among enzyme-treated and control oilseed residues. The statistical analysis of the crude fibre

content indicated that it was not different ($P \geq 0.05$) across the various residues. The crude fibre content ranged from 19.82 from residues extracted using Pectinex to 22.13 from the residue of the control.

The total ash content presented in Table 4.1 did not vary significantly among samples across the residues. The total ash content ranged from a minimum of 1.610 % with the control and a maximum value of 2.20 % with residue from Pectinex. The total ash content was statistically ($p \geq 0.05$) not different between the residues from the application of different enzymes although it was higher in the residue of the Pectinex.

Table 4.2: Comparison of proximate composition of shea residues

Parameter	Control (%)	Crude Pectinase (%)	Commercial Pectinex (%)	Commercial Viscozyme (%)
Protein content	10.54 ± 0.49^{b}	10.46 ± 0.49^{b}	12.98 ± 0.47^{a}	12.06 ± 0.31^{a}
Fiber content	22.13 ± 0.32^{a}	20.78 ± 0.19^{bc}	19.817 ± 0.64^{c}	21.38 ± 0.77^{ab}
Ash content	1.61 ± 0.13^{b}	1.88 ± 0.13^{b}	2.20 ± 0.26^{a}	1.75 ± 0.05^{b}

*Values are mean ± SD, calculated as percentage on dry seed weight basis for three shea kernel seed samples for each enzyme, analyzed individually in triplicate. Mean values in the same row followed by the same superscript letters are not significantly different ($P > 0.05$).

4.3.2 Physicochemical characteristics of oils

Various physical and chemical parameters were investigated on the oil obtained from the extraction using the various enzymes and the control as shown in Table 4.3. There were no significant ($P > 0.05$) differences for the moisture, density, and sample temperature values between the crude enzyme-assisted-extracted oils and the commercial enzyme assisted extracted oils. The level of free fatty acids was found to be significantly ($P < 0.05$) higher in all the enzyme-extracted seed oils as compared to aqueous extracted oil. This increase in the free fatty acids may be attributed to the longer hydrolysis time and enzymatic effect during the Enzyme-Assisted Extraction.

Table 4.3: Comparison of physico-chemical properties of enzyme-assisted aqueous extracted shea butter

Enzymes	Moisture	FFA	PV	Density/g	SMP/c	UM
Control	0.18±0.01[a]	6.50±0.15[a]	4.90±0.14[a]	993.51±4.79[a]	23.50±0.71[a]	4.91±0.43[a]
Crude Pectinase	0.17±0.01[a]	7.72±0.17[b]	5.33±0.18[b]	1016.89±2.71[b]	22.50±0.71[a]	5.35±0.30[a]
Pectinex	0.17±0.02[a]	7.77±0.09[b]	5.63±0.18[bc]	1018.30±3.59[b]	23.50±0.71[a]	5.70±0.28[a]
Viscozyme	0.17±0.02[a]	8.69±0.34[c]	5.85±0.07[c]	1000.07±11.16[a]	23.50±0.71[a]	6.67±0.30[b]

Values are means of duplicate ± standard deviation. The superscript showed that at $P < 0.05$, a significant difference exists. Means within each column that do not share a letter are significantly different. FFA, free fatty acid, PV, peroxide value, SMP, sample melting point, UM, unsaponifiable matter content

CHAPTER FIVE

5.0 CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The study showed that crude pectinase, produced from the fermentation of corn cob using *Saccharomyces cerevisiae* can be used to extract shea fat, but not as efficiently as combination of enzyme mixtures. Whereas commercial enzyme mixtures required 0.08 % dosage for optimal yield, the use of crude pectinase alone was at a dosage of 0.12 % for optimal results. With the exception of free fatty acids and peroxide value, enzyme treatment has no significant effect on the physico-chemical properties of the butter.

5.2 Recommendations

- Future work should consider the purification of the crude Pectinase using ammonium sulphate to increase its Specific Activity making it a technical enzyme.
- Further research should be conducted on producing other crude enzymes and their combinations for the extraction of shea kernel using fungal strain as *Aspergillus niger.*

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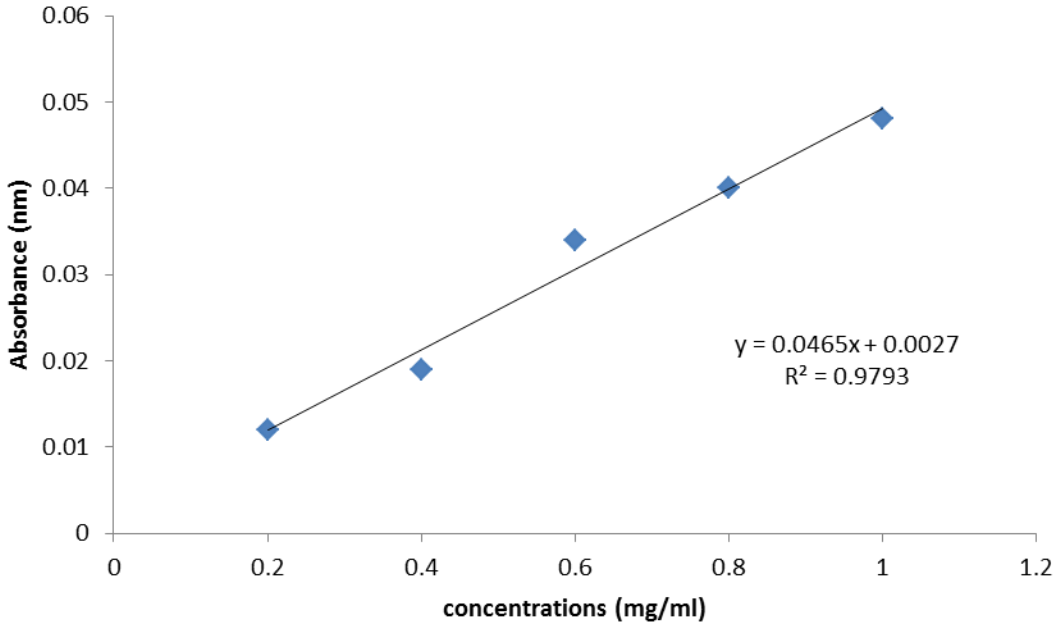
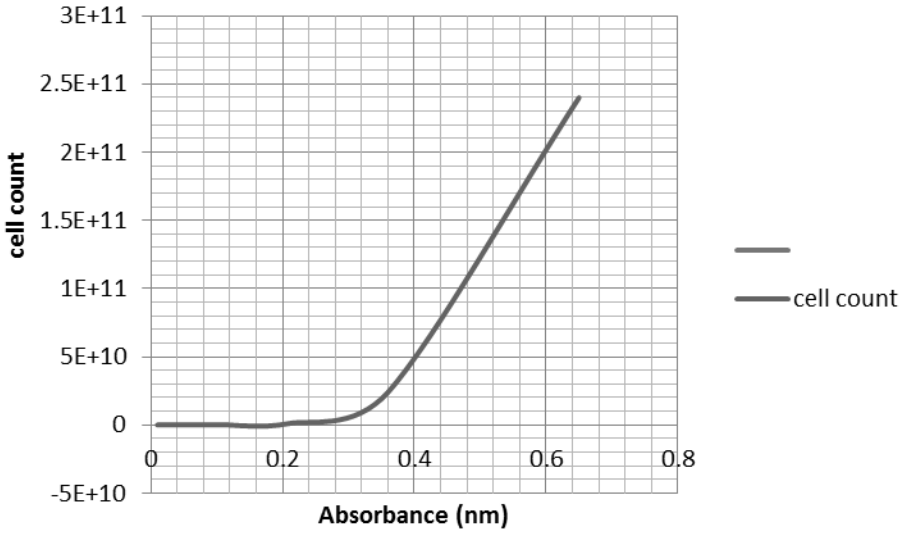
APPENDICE

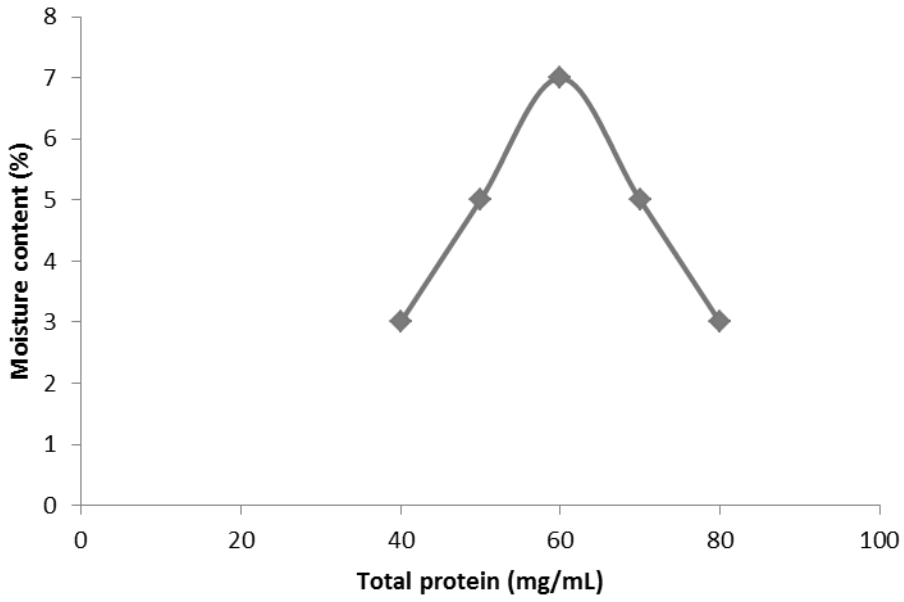
APPENDIX 1: Standard Curve



APPENDICE 2: Growth curve of *Saccharomyces cerevisiae*



APPENDICE 3: Moisture Content of Corn Cob



APPENDICES 4: A Table Representing the Protein Concentrations, Pectinase Activities and Specific Activities

Days	Protein concentration (mg/ml)	Pectinase activity (U/ml)	S.A (U/mg)
0	0	0	0
2	5.0	1.95	0.40
4	7.0	6.40	0.90
6	5.60	4.20	0.75
8	2.70	0	0
10	2.60	0	0

Report: **1-Way ANOVA Report**
Created by Daniel's XL Toolbox version 6.52 (http://xltoolbox.sourceforge.net)
Created on: 2014-05-05 23:21:11 -0000

Data source
Worksheet: Sheet1
Workbook: raw data.xlsx

Groups

Name	Count	Avg.	SD
TIME	5	90	47.43416
Pectinex	5	48.742	10.09077
c. Pectinase	5	38.052	3.996587
Viscozyme	5	64.003	9.168268
Control	5	37.354	2.938612

Test for equal variance

The XL Toolbox performs a modified version of Levene's test,
as described by Glantz et al. in "Primer of applied regression & analysis of variance", ch. 7

F	DFn	DFd	P
6.752052464	4	20	0.001311

FAIL - equal variance CANNOT be assumed (p <= 0.05).
The ANOVA results should be interpreted with extreme caution,
as it is very likely that a basic precondition of this analysis is not met.

Analysis of variance

	SS	DF
Between	9709.234	4
Within	9841.956	20
F	4.932573	
P	**0.006234**	****

Posthoc test: Bonferroni-Holm

Group 1	Group 2	Critical	P	Significant?
Viscozyme	Control	0.005	0.000262	**Yes**
C. Pectinase	Viscozyme	0.005556	0.000404	**Yes**
Pectinex	Viscozyme	0.00625	0.036773	No
TIME	Control	0.007143	0.03829	No
TIME	C. Pectinase	0.008333	0.040551	No
Pectinex	Control	0.01	0.041663	No
Pectinex	C. Pectinase	0.0125	0.058773	No
TIME	Pectinex	0.016667	0.093628	No
TIME	Viscozyme	0.025	0.263279	No
C. Pectinase	Control	0.05	0.761091	No

Groups

Name	Count	Avg.	SD
dosage	5	40	31.62278
Pectinex	5	48.872	11.48405
c. pectinase	5	38.042	4.122626
viscozyme	5	58.572	17.4852

Test for equal variance

The XL Toolbox performs a modified version of Levene's test,
as described by Glantz et al. in "Primer of applied regression & analysis of variance", ch. 7

F	DFn	DFd	P
2.599227232	3	16	0.088139

PASS - equal variance may be assumed ($p > 0.05$).

Analysis of variance

	SS	DF
Between	1325.406	3
Within	5818.448	16
F	1.2149	
P	**0.336423**	

Posthoc test: Bonferroni-Holm

Group 1	Group 2	Critical	P	Significant?
c. pectinase	viscozyme	0.008333	0.03389	No
Pectinex	c. pectinase	0.01	0.082446	No
dosage	viscozyme	0.0125	0.283648	No
Pectinex	viscozyme	0.016667	0.330133	No
dosage	Pectinex	0.025	0.571688	No
dosage	c. pectinase	0.05	0.894194	No

Report: **1-Way ANOVA Report**

Created by Daniel's XL Toolbox version 6.52 (http://xltoolbox.sourceforge.net)

Created on: 2014-05-05 23:20:17 -0000

Data source

Worksheet:	Sheet1
Workbook:	raw data.xlsx

Groups

Name	Count	Avg.	SD
ratio	4	0.045139	0.001793
Pectinex	4	51.0525	8.371313
c. pectinase	4	41.0275	3.018036
viscozyme	4	63.7775	10.46478
control	4	37.2325	3.527493

Test for equal variance

The XL Toolbox performs a modified version of Levene's test,
as described by Glantz et al. in "Primer of applied regression & analysis of variance", ch. 7

F	DFn	DFd	P
4.436503163	4	15	0.014505

FAIL - equal variance CANNOT be assumed (p <= 0.05).
The ANOVA results should be interpreted with extreme caution,
as it is very likely that a basic precondition of this analysis is not met.

Analysis of variance

	SS	DF	
Between	9132.831	4	
Within	603.427	15	
F	56.75602		
P	**7.03E-09**	****	

Posthoc test: Bonferroni-Holm

Group 1	Group 2	Critical	P	Significant?
ratio	c. pectinase	0.005	1.65E-07	**Yes**
ratio	control	0.005556	7.42E-07	**Yes**
ratio	Pectinex	0.00625	1.86E-05	**Yes**
ratio	viscozyme	0.007143	1.86E-05	**Yes**
viscozyme	control	0.008333	0.002978	**Yes**
c. pectinase	viscozyme	0.01	0.005829	**Yes**
Pectinex	control	0.0125	0.022725	No

Pectinex	c. pectinase	0.016667	0.06516	No
Pectinex	viscozyme	0.025	0.106304	No
c. pectinase	control	0.05	0.15318	No

Report: **1-Way ANOVA Report**
Created by Daniel's XL Toolbox version 6.52 (http://xltoolbox.sourceforge.net)
Created on: 2014-05-05 23:19:41 -0000

Data source
Worksheet: Sheet1
Workbook: raw data.xlsx

Groups

Name	**Count**	**Avg.**	**SD**
TEMP	7	38.57143	24.10295
Pectinex	7	48.07786	8.191137
C. Pectinase	7	39.30857	3.220377
viscozyme	7	61.75357	14.09363
Control	7	36.03857	3.105805

Test for equal variance
The XL Toolbox performs a modified version of Levene's test,
as described by Glantz et al. in "Primer of applied regression & analysis of variance", ch. 7

F	**DFn**	**DFd**	**P**
4.28455103	4	30	0.007346

FAIL - equal variance CANNOT be assumed (p <= 0.05).
The ANOVA results should be interpreted with extreme caution,
as it is very likely that a basic precondition of this analysis is not met.

Analysis of variance

	SS	**DF**
Between	3107.083	4
Within	5200.166	30
F	4.481226	
P	**0.00587**	****

Posthoc test: Bonferroni-Holm

Group 1	Group 2	Critical	P	Significant?
viscozyme	Control	0.005	0.000502	**Yes**
C. Pectinase	viscozyme	0.005556	0.001452	**Yes**
Pectinex	Control	0.00625	0.003412	**Yes**
Pectinex	C. Pectinase	0.007143	0.02173	No
Pectinex	viscozyme	0.008333	0.046468	No
TEMP	viscozyme	0.01	0.048422	No
C. Pectinase	Control	0.0125	0.077078	No
TEMP	Pectinex	0.016667	0.342649	No
TEMP	Control	0.025	0.787432	No
TEMP	C. Pectinase	0.05	0.937398	No

Report: **1-Way ANOVA Report**
Created by Daniel's XL Toolbox version 6.52 (http://xltoolbox.sourceforge.net)
Created on: 2014-05-05 23:18:55 -0000

Data source
Worksheet: Sheet1
Workbook: raw data.xlsx

Groups

Name	Count	Avg.	SD
PH	5	6	3.162278
Pectinex	5	47.3	9.877517
C. Pect	5	36.309	6.644049
Viscozyme	5	61.474	9.418045
Control	5	29.788	6.907487

Test for equal variance
The XL Toolbox performs a modified version of Levene's test,
as described by Glantz et al. in "Primer of applied regression & analysis of variance", ch. 7

F	DFn	DFd	P
0.939878233	4	20	0.461248

PASS - equal variance may be assumed ($p > 0.05$).

Analysis of variance

	SS	**DF**
Between	8575.737	4
Within	1152.487	20
F	37.20536	
P	**5.34E-09**	********

Posthoc test: Bonferroni-Holm

Group 1	**Group 2**	**Critical**	**P**	**Significant?**
PH	Viscozyme	0.005	1.58E-06	**Yes**
PH	C. Pect	0.005556	1.56E-05	**Yes**
PH	Pectinex	0.00625	2E-05	**Yes**
PH	Control	0.007143	0.000112	**Yes**
Viscozyme	Control	0.008333	0.0003	**Yes**
C. Pect	Viscozyme	0.01	0.001221	**Yes**
Pectinex	Control	0.0125	0.011723	**Yes**
Pectinex	Viscozyme	0.016667	0.048747	No
Pectinex	C. Pect	0.025	0.072844	No
C. Pect	Control	0.05	0.166653	No

Summary Statistics for Yield

Parameters	*Count*	*Average*	*Standard deviation*	*Coeff. of variation*	*Standard error*	*Minimum*	*Maximum*	*Range*
Aqueous	3	40.2333	0.802081	1.99357%	0.463081	39.4	41.0	1.6
Crude Pectinase	3	44.0667	1.24733	2.83055%	0.720147	42.65	45.0	2.35
Pectinex	3	58.6	1.1	1.87713%	0.635085	57.5	59.7	2.2
Viscozyme	3	72.1867	0.685954	0.950251%	0.396036	71.4	72.66	1.26
Total	12	53.7717	13.2372	24.6174%	3.82125	39.4	72.66	33.26

Parameters	*Stnd. skewness*	*Stnd. kurtosis*
Aqueous	-0.26265	
Crude Pectinase	-1.04786	
Pectinex	0.0	
Viscozyme	-1.15021	
Total	0.587143	-1.11184

The StatAdvisor

This table shows various statistics for Yield for each of the 4 levels of Parameters. The one-way analysis of variance is primarily intended to compare the means of the different levels, listed here under the Average column. Select Means Plot from the list of Graphical Options to display the means graphically.

ANOVA Table for Yield by Parameters

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
Between groups	1919.7	3	639.899	659.74	0.0000
Within groups	7.7594	8	0.969925		
Total (Corr.)	1927.45	11			

The StatAdvisor

The ANOVA table decomposes the variance of Yield into two components: a between-group component and a within-group component. The F-ratio, which in this case equals 659.74, is a ratio of the between-group estimate to the within-group estimate. Since the P-value of the F-test is less than 0.05, there is a statistically significant difference between the mean Yield from one level of Parameters to another at the 95.0% confidence level. To determine which means are significantly different from which others, select Multiple Range Tests from the list of Tabular Options.

Multiple Range Tests for Yield by Parameters

Method: 95.0 percent LSD

Parameters	*Count*	*Mean*	*Homogeneous Groups*
Aqueous	3	40.2333	X
Crude Pectinase	3	44.0667	X
Pectinex	3	58.6	X
Viscozyme	3	72.1867	X

Contrast	*Sig.*	*Difference*	*+/- Limits*
Aqueous - Crude Pectinase	*	-3.83333	1.85432
Aqueous - Pectinex	*	-18.3667	1.85432
Aqueous - Viscozyme	*	-31.9533	1.85432
Crude Pectinase - Pectinex	*	-14.5333	1.85432
Crude Pectinase - Viscozyme	*	-28.12	1.85432
Pectinex - Viscozyme	*	-13.5867	1.85432

* denotes a statistically significant difference.

The Stat Advisor

This table applies a multiple comparison procedure to determine which means are significantly different from which others. The bottom half of the output shows the estimated difference between each pair of means. An asterisk has been placed next to 6 pairs, indicating that these pairs show statistically significant differences at the 95.0% confidence level. At the top of the page, 4 homogenous groups are identified using columns of X's. Within each column, the levels containing X's form a group of means within which there are no statistically significant differences. The method currently being used to discriminate among the means is Fisher's least significant difference (LSD) procedure. With this method, there is a 5.0 % risk of calling each pair of means significantly different when the actual difference equals 0.