STUDIES ON THE MALTING PROPERTIES AND PROTEOLYTIC ENZYME ACTIVITIES OF A NIGERIAN MAIZE VARIETY

BY

AGBO, ANTHONY OGBONNIA

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A DISSERTATION SUBMITTED TO THE DEPARTMENT OF APPLIED MICROBIOLOGY AND BREWING, FACULTY OF BIOSCENCES, NNAMDI AZIKIWE UNIVERSITY, AWKA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF DEGREE OF DOCTOR OF PHILOSOPHY (Ph. D) IN INDUSTRIAL MICROBIOLOGY.

SUPERVISOR: PROF. F. J. C. ODIBO

MAY, 2021

CERTIFICATION

This is to certify that this research work titled "**Studies on the Malting Properties and Proteolytic Enzyme Activities of Nigerian Maize Variety**" was carried out by Agbo, Anthony Ogbonnia, with Registration number NAU/PG/Ph. D/2012487001P under the supervision of Prof. F. J. C. Odibo in the Department of Applied Microbiology and Brewing, Faculty of Biosciences, Nnamdi Azikiwe University, Awka Anambra State, Nigeria and that this work is original and has not been submitted in part or full to this University or any other Institution for the award of a degree or a Diploma.

…… ……....

Agbo, Anthony Ogbonnia Date

APPROVAL

This Dissertation titled "**Studies on the Malting Properties and Proteolytic Enzyme Activities of a Nigerian Maize Variety**" by Agbo, Anthony Ogbonnia with Registration Number NAU/PG/Ph. D/2012487001P has been examined and approved for the award of Doctor of Philosophy (Ph.D) in Industrial Microbiology in the Department of Applied Microbiology and Brewing, Faculty of Biosciences, Nnamdi Azikiwe University, Awka.

…… ……

Prof. F. J. C. Odibo Date

(Supervisor)

…… ……

Prof. E. I. Chukwura Date

(HOD, Applied Microbiology and Brewing)

…… ……

Dr. S. C. Onuorah Date

(Postgraduate Sub Dean)

……. …….....

Prof. S. C. Udedi Date

(Dean, Faculty of Biosciences)

…… ……

Prof. A. N. Moneke Date

(External Examiner)

…….. ……...

Prof. P. K. Igbokwe Date

(Dean, School of Postgraduate Studies)

DEDICATION

This research work is dedicated to the Holy Trinity - God the Father, the Son and the Holy spirit for the immeasurable blessings upon us.

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Abstract

Maize (*Zea mays* L.) plays a significant role in food security and economy of many countries. The Nigerian maize cultivar, Oba Super 2 (OS2) was assessed for its potential to replace barley in beer brewing in Nigeria since it is inexpensive. The aim of this work was to study the malting properties and proteolytic enzyme activities of this Nigerian maize variety. The objectives were to: determine the malting properties of the unmalted maize, optimize the malting conditions for the production of well modified maize variety for brewing, study the time course for protease modulation, purify and characterize the proteases in relation to pH, temperature, effectors and inhibitors as well as determine the cleansing properties of the proteases incorporated into detergent. Certified Oba Super 2 maize grains were obtained from Premier Seed Limited, Zaria, Nigeria. The grains were malted at varying steeping (S) periods (30, 36 and 42 hours), different germination (G) periods (0, 1, 2, 3, 4 and 5 days) and varying kilning (K) temperatures (45, 50 and 55^{o}C). The properties of the unmalted and malted maize grains were determined using standard methods. The crude enzyme from the malt was purified and characterized using standard procedures. Data were analysed using analysis of variance (ANOVA) at $p < 0.05$ and the means were compared using least significant difference (LSD) and Duncan's multiple range test (DMRT). The 1000-corn weight, moisture content, germination energy, germinative capacity, water sensitivity, broken kernel, protein (N × 6.25) and fat (ether extract) of the unmalted grain were; 280g, 11.5%, 94%, 98%, 87%, 0.82%, 9.06%, and 4.20%, respectively. The malting loss (ML) values of 20.12, 21.10 and 23.0% were significantly higher ($p < 0.05$) at different steeping periods (hour) S_{30}, S_{36} and S_{42}, on the fifth day of germination (G_5). The cold water extract (CWE) values of 43.25, 44.56 and 51.87% were significantly higher ($p < 0.05$) at S_{30}, S_{36} and S_{42} on the fourth (peak) day of germination (G_4) kilned at K_{50}. The peak values for hot water extract (HWE) at 243.25, 238.60 and 250.05 L^{o}/kg were significantly higher ($p < 0.05$) on the G_4 at K_{45}, K_{55} and K_{50}, respectively, while free alpha amino nitrogen (FAN) values of 49.50, 49.75 and 53.55 mg/l were significantly higher ($p < 0.05$) on the G_4, all kilned at $K_{50.}$ The peak values for diastatic power (DP) at 33.40, 36.55 and 38.08 ^{o}L were significantly higher ($p < 0.05$) on the $G_{5,}$ kilned at K_{50}, while the total soluble nitrogen (TSN) values were not significantly higher ($p < 0.05$) at varying steeping hour 30, 36 and 42 on the G_5, at $K_{55, 50}$ and $_{55}$.The cold water soluble protein (CWS-P) values of 48.83, 47.65 and 49.55% were significantly higher ($p < 0.05$) on the G_3 kilned at K_{50}, while total non-protein nitrogen (TNPN) were significantly higher ($p < 0.05$) on the G_4 kilned at $K_{50.}$ The time course of protease activity

(peak) at 0.834 U/ml was significantly higher ($p < 0.05$) on the G_3 at K_{50}. The yields of the purified proteases, Oba Super 2A (OS2A) and Oba Super 2B (OS2B) were 3.26% and 4.24%, respectively. Their specific activities were 16.21 and 16.75 U/mg proteins, at purification folds of 3.805 and 3.932, respectively. The native polyacrylamide gel electrophoresis (PAGE) revealed single migrating protein bands corresponding to relative molecular masses of 62.81 KDa and 63.44 KDa for OS2A and OS2B, respectively. Using casein as substrate, the purified OS2A protease was optimally active at 50^{o}C and pH 9 whereas the maximum stability was achieved at 60^{o}C and pH 9 but retained about 50% of its original activity after 30 min at 90^{o}C. The optimum temperature and pH of OS2B were 50^{o}C and pH 9, respectively, with maximum stability at 50^{o}C and pH 8, retaining 68% of its activity at 90^{o}C. Appreciable stimulation of OS2A protease was achieved by Cu^{2+}, Ca^{2+}, Mn^{2+}, Ba^{2+}. Mercury (Hg^{2+}) was slightly stimulatory, while other metal ions studied (Ag^{+}, Mg^{2+}, Zn^{2+}, Fe^{2+} and Co^{2+}) were inhibitory. The OS2B activity was appreciably activated by Cu^{2+}, Ca^{2+}, and Mn^{2+}, but fairly by Hg^{2+} and Ba^{2+}, while Ag^{+}, Mg^{2+}, Zn^{2+} and Fe^{2+}, were highly inhibitory. Phenylmethylsulphonyl fluoride (PMSF), ethylenediaminetetra acetic acid (EDTA), iodoacetic acid (IAA), ethylene bis (oxyethylene nitrilo) tetra acetic acid (EGTA) and para-chloromercuribenzoate (p-CMB) were inhibitory to the two proteases. Significant inhibitory effects were recorded by PMSF at 60 and 66% for OS2A and OS2B, respectively. Both proteases showed an enhanced activity on treatment with 1.0% of hydrogen peroxide (H_2O_2) and dimethyl sulfoxide (DMSO) after incubation for 30 min, but decreased activities as the concentration of these agents increased from 1.0 - 5.0%. The proteases were inhibited in the presence of dithiothreitol (DTT) and 2-mercaptoethanol (2-ME) as concentration increased from 1.0 - 5.0%. However, OS2A activity was stimulated in the presence of 1.0% DTT at 117% residual activity. Both OS2A and OS2B proteases were stable at different levels in the presence of surfactants and (Triton X- 100, SDS) and detergent (Ariel). The detergents (Klin and Omo) and surfactants (Tween- 80 and 20), however, inhibited the enzymes. Excellent de-staining properties were displayed by both proteases at 1.0% detergent (Ariel), as shown by the white clothes stained with blood. The proteases exhibited broad specificities (39.5-100%) in the hydrolysis of various proteins; casein, bovine serum albumin (BSA), gelatin and egg albumin (EA). The proteases obeyed the Michaeli's-Menten's equation. Protease OS2A had highest affinity for gelatin (K_M = 0.022 mg/ml, *Vmax* 0.213 mg/ml/min) while protease OS2B demonstrated highest affinity for casein (K_M = 0.071 mg/ml, *Vmax* 0.234 mg/ml/min). Protease OS2A had a catalytic efficiency value of 0.977 U/ml/min for gelatin, followed by BSA (0.458 U/ml/min), casein (0.317 U/ml/min) and egg albumin (0.114 U/ml/min) whereas OS2B recorded 0.256 U/ml/min for casein, BSA (0.156

U/ml/min), egg albumin (0.079 U/ml/min) and gelatin (0.034 U/ml/min). This study showed that the Nigerian maize variety, Oba Super 2 exhibited excellent malting properties for brewing. The maize malt can serve as a source of protease which could be further assessed for use in relevant industries, especially in brewing, pharmaceutical and detergent. On overall assessment, the partially purified proteases presented a promising application for industrial utilization.

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CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

The term cereal is derived from Latin word ‘cerealis’ meaning ‘grain’ which is botanically a type of fruit called caryopsis, composed of the endosperm, germ and bran (Sarwar *et al.*, 2013). Cereals have been of great significance in human history usually being the first crop to be grown and being the staple foods both directly by humans or indirectly via livestock feed since the beginning of civilization (Nuss and Tanumihardjo, 2010; FAO, 2012). They are grains of the grass family (a monocot family *Poaceae*, also known as *Gramineae*), which usually have long thin stalks, such as maize, rice, wheat, triticale, millets, barley, oats, rye and sorghum (Sarwar *et al.*, 2013; Head, 2016).

Cereals are important sources of energy, protein, fiber, a range of micronutrients such as vitamin E and some B vitamins, minerals such as magnesium (Mg), zinc (Zn), calcium (Ca), iron (Fe), and a range of bioactive substances (Topping, 2007; Sarwar, 2009; Awika, 2011; Saleh *et al*., 2018).

Research has shown that consumption of cereals, specifically whole grains may have a role in the prevention of chronic diseases (Gani *et al*., 2012) such as cardiovascular or coronary heart disease (Truswell, 2002; Kelly *et al*., 2017), diabetes (Liu, 2003) and colorectal cancer (Peter *et al.*, 2003; Topping, 2007).

Maize (*Zea mays* L.) is an American- Indian word for corn literally means “that which sustains life”. It originated from the Western Hemisphere possibly the Americans (Southern Mexico) but is now cultivated in parts of Africa, Europe and Asia (Gibson and Benson, 2002; University of Utah Health Science, 2016).

Maize plays a significant role in food security and economy of many countries of the world, including Nigeria (Wu and Guclu, 2013; Olaniyan, 2015). It is a major staple food crop grown in many parts of the world especially in Nigeria (Nuss and Tanumihardjo, 2010; Shiferaw *et al*., 2011; Franklyn, 2013). In Nigeria and sub-Sahara Africa (SSA), it ranks first next to millets and sorghum and third most important grain standing next to wheat and rice in global production (Smale *et al*., 2011; Iken and Amusa, 2014; Ranum *et al*., 2014; Olaniyan, 2015).

Maize is used in developing countries for human consumption in form of processed products e.g cooked whole grains, fried and weaning foods (Abdulrahaman and Kolawole, 2006; Osuntogun and Aboaba, 2007; Amankwah *et al.,* 2009; Belitz *et al*., 2009; Maji *et al*., 2011). In developed countries, it is used for animal feed and industrial purposes such as brewing, production of bioethanol, biofuels and enzymes (Oyewole and Agboola, 2011; Wallington *et al*., 2012).

Nutritionally, maize is rich in fibers, β-glucan, phenolics, antioxidants, enzymes, carbohydrate, minerals, protein, and vitamins (Belitz *et al*., 2009; Delcour and Hoseney, 2010).

Barley remains the main raw material for brewing due to hydrolytic enzymes (amylolytic, cytolytic and proteolytic) development during malting, high diastatic power (DP), low starch gelatinization temperature, low free alpha amino nitrogen (FAN) etc. (Goode *et al*., 2005; Schmitt *et al*., 2013). Since barley is a temperate grain crop, its cultivation in the tropics and sub-tropical regions is far less viable compared to the tropical cereals (Taylor *et al*., 2013). Indeed, tropical grains such as sorghum (Malomo and Alamu, 2013; Sani and Fatoki, 2017), millet (Eneje *et al*., 2012), wheat (Sarwar *et al*., 2013), and maize (Adedeji *et al*., 2011) etc., have been used as a replacement for barley due to economic considerations, government policy on high import duty and local availabilities etc. (Taylor *et al*., 2013).

Malting is the germination of cereals under controlled conditions of moisture, aeration and temperature and then kilned thereby, promoting the development of hydrolytic enzymes

(Awoyinka and Adebawo, 2008; Mohammed and Addy, 2014). It is a complex process that promotes changes in the biochemical, sensorial and nutritional characteristics of grains. It is the most effective way to improve the nutritional value of cereals by transforming the low content of hydrolytic enzymes, hard texture, and undesirable taste into soft texture with desirable flavour being rich in enzymes (Gernah *et al*., 2011). It equally contributes to the reduction of phytic acid (Ghavidel and Davoodi, 2011; Gupta *et al*., 2015), some flavonoids like proanthocyannin and proanthocyanidin (Santos-Buelga and Scalbert, 2000; Cortes *et al*., 2006; Awoyinka and Adebawo, 2008; Ghavidel and Davoodi, 2011). The mineral contents (Mg, Zn, Fe, and Ca) as well as lipid and free alpha amino nitrogen (FAN) contents of the wort during mashing are enhanced which are also important for stabilization of yeasts during fermentation (Tizazu *et al*., 2011; Kruger *et al*., 2012).

Among the hydrolytic enzymes that are developed during malting are the proteases. Protease development depends on numerous factors such as, the variety of grains, steeping, couching, germination and kilning processes (Ghavidel and Davoodi, 2011). These processes influence the malt quality parameters including: wort viscosity, free alpha amino nitrogen (FAN), total soluble nitrogen and fine-coarse extract difference which increases with increased germination time due to more extensive protein hydrolysis (enzymatic and physical solublisation) (Faltermaier *et al.*, 2013).

Proteolytic enzymes (EC 3.4, 21-24 and 99) also known as proteases or peptidases are extracellular enzymes capable of hydrolysing the grain endosperm storage proteins to peptide bonds and amino acids in favour of assimilable nitrogenous materials essential for adequate yeast metabolism during fermentation and liberation of starch granules embedded in the protein matrix, thus promoting extract yield and wort fermentability (Schmitt *et al*., 2013).

Proteases are classified according to their origin (animal, plant, microbes), preferential site of cleavage (exo- and endopeptidases, amino- or carboxylpeptidases), pH optimum (acid,

neutral, alkaline), mechanism of catalysis (alkaline (serine), aspartic, sulfhydryl or metallo-protease, glutamic, threonine, cysteine, and asparagine peptide lyases) and protease with unknown catalytic mechanism (Rawlings *et al.,* 2012; Oda, 2012).

Proteases are ubiquitous in occurrence, being found naturally in all living organisms, from microbes to higher organisms (Prathamesh, 2011; Pant *et al*., 2015). They are of immense attraction to researchers due to their pivotal position with respect to their physiological and commercial applications (Li *et al.,* 2013; Saranraji *et al*., 2017). They catalyze a number of functions, from mobilization of storage proteins during seed germination to the initiation of aging programmes and cell death (Coffeen and Wolpert, 2004; Schmitt *et al*., 2013). In biological systems, they participate in viral maturation, protein degradation (fungal peptidase), blood pressure regulation (rennin) etc. (Antonelli and Tunizani, 2012). They have multiple applications in various sectors such as pharmaceuticals, medicine, leather, fruit juice, biological research etc. (Negi and Banerjee, 2006; El-Shafei *et al.,* 2010; Gaur *et al.,* 2014; Kusuma *et al.,* 2016). They are the most important enzymes and account for nearly 60-65% of total world-wide enzyme production and sales (Sarrouh *et al*., 2012; Protease market by source, 2016).

In the past decades, proteases from plant source such as fruit peels (Ademola and Malomo, 2017), agro waste (Ekpa *et al*., 2010), deteriorated fruit (Ajayi *et al*., 2014); bulb (Ndidi and Nzelibe, 2012), some improved tropical grains (Arasaratnam and Kalpana, 2010; Smale *et al*., 2011), had been an integral part of food and non- food industries.

Maize grains could be potential source of proteases. Therefore, utilization of a Nigerian maize variety (Oba Super 2) might contribute at decreasing the cost of enzyme production and sales.

Since extensive works had been documented on the proteases purified from some improved tropical grains, there is paucity of information especially for this local maize variety and so the need for elaborate research in this area.

1.2 Statement of the Problem

In the last four (4) decades, there has been much interest in the production and use of enzymes from various sources (Oyeleke *et al*., 2011). However, microbial proteases had been the most exploited but they pose a lot of health challenges due to their tendency to harbour residual endotoxins (Pant *et al*., 2015), but local maize varieties have not been fully explored.

Presently, Nigeria with her teeming population of over 200 million, enzyme producing industry is still a mirage. She therefore, depends on leading scientific nations for her daily needs with attendant import duty challenges.

Maize grains are widely available and inexpensive but under- utilized owing to poor development of malting technology for brewing and enzyme production. Although, some improved grains such as sorghum (Sani and Fatoki, 2017), maize (Awoyinka and Adebawo, 2008), millet (Eneje *et al*., 2012) had been conditioned through malting to bring up their properties to that of barley malt (Ghasemi *et al*., 2012), very little success had been achieved in terms of malting yield for brewing purposes and protease production.

1.3 Justification for the Study

Enzyme purification has emerged as an area of major importance and interest within food and non-food industries, due to our increased demand (Protease Market by Source, 2017). Proteases are of immense attraction to researchers due to their commercial value and multiple applications in various sectors including; pharmaceuticals, leather, detergent industries, medicine and biological research (Sarrouh *et al*., 2012). Therefore, the search for alternative and reliable sources of proteases with high activity and stability, more efficient and eco-friendly such as maize grain, is important. Embarking on such lofty venture would contribute meaningfully to economic development.

Our country is overwhelmed with population of unemployed youths and so, manpower needs of enzyme industries would be guaranteed. Tied to this, there would be decrease in import duty, thereby boasting our foreign exchange earning.

Extensive work had been documented on malting performances and purification of some improved cereals, including wheat (Faltermaier *et al*., 2013), sorghum (Sani and Fatoki, 2017) and maize (Iwouno and Odibo, 2015). Despite these efforts, biochemical processes of grains and proteolytic potentials, especially maize malt, have not been exhausted. Besides, little has been reported on the purification and characterization of the proteases from local variety of maize. It is against this background that local variety of maize, Oba Super 2 capable of high malting yield and enhanced protease production was used in this study.

1.4 Aim of the Study

The aim of this work was to study the malting properties and proteolytic enzyme activities of a Nigeria maize variety, Oba Super 2.

1.5 Research Objectives

The specific objectives of the present study were to:

1. determine the properties of unmalted Oba Super 2 maize variety,

2. optimize the malting conditions for the production of well modified maize variety for brewing,

3. study the time course of protease modulation/production in this variety,

4. purify the maize protease(s),

5. study the kinetics of the protease(s) in relation to pH, temperature, enzyme effectors and inhibitors, and

6. determine the cleansing properties of the partially purified protease(s) incorporated into detergents.

CHAPTER TWO

LITERATURE REVIEW

2.1 Cereal Crops

The term cereal is derived from Latin word ‘cerealis’ meaning ‘grain’ which is botanically a type of fruit called caryopsis composed of the endosperm, germ and bran (Sarwar *et al.,* 2013). Cereals have been of great significance in human history, being the first crop to be grown (rather than gathered from the wild) and being the staple foods both directly by humans or indirectly via livestock feed since the beginning of civilization (FAO,2012). Cereals are collections of edible seeds of the grass family (a monocot family *Poaceae,* also known as *Gramineae*) which usually have long and thin stalks. A number of cereals such; barley, oats, rye, maize, millets, wheat and sorghum are grown in different countries of the world.

Among the cereals, wheat is the most important grain in the world export market. Available records showed that 21% of the total production is exported compared to 14% for maize (corn) and only 3% for rice. Cereals are grown on nearly 60% of the cultivated land in the world. Maize, wheat, rice and millet take up the greatest part of the land cultivated and produce the largest quantities of cereal grains (FAOSTAT, 2015).

The cultivation of all cereals is, in principle similar. They are annual plants and consequently one planting yields one harvest. The demand on climate is however, different. ‘Warm season’ cereals (maize, rice, millets, sorghum) are grown on tropical low lands throughout the year and in temperate climates during the frost-free season. ‘Cool-season’ cereals (wheat, rye, barley and oats) grow best in moderate climate and can be differentiated into winter or spring varieties (Koehler and Wieser, 2013).

The term cereal is not limited to these grains, but also to food stuff prepared from the starchy grains of cereal- like flours, breads, noodles and pasta (Sarwar *et al.*, 2013). Cereals and their products are important sources of energy, carbohydrates, proteins, fiber, a range of micronutrients such as vitamins E, some of the B vitamins, minerals such as Mg, Zn, Ca, Fe, etc., and also contain a range of bioactive substance utilized both by developed and developing countries (Topping, 2007; Sarwar, 2009; Awika, 2011; Gupta *et al*., 2015; Saleh *et al*., 2018).

There are growing interests in the potentials health benefits these cereals may provide. Research has shown that consumption of cereals, specifically whole grains may have a role in prevention of chronic diseases such as coronary heart diseases (Truswell, 2002; WHO/FAO, 2003; Kelly *et al*., 2017), diabetes (Liu, 2003), and colorectal cancer (Peter *et al.*, 2003; Bingham *et al.*, 2003).

2.1.1 Chemical Composition of Cereals

Cereals are composed of 11-14% moisture, 56-73% carbohydrate, 2-7% lipid, 1-3% minerals, 2-13% fiber and 7-11% protein. In general, cereals are quite similar in gross composition; being low in protein and high in carbohydrate (Belitz *et al.*, 2009). Okonkwo and Agharandu (2017) reported that carbohydrate is clearly predominant in all the cereals tested, followed closely by protein, fat, ash and crude fiber. Maize, however, possessed the highest concentration of most nutrients tested (Table 2.1).

Table 2.1: Proximate Composition (%) of Cereals

Cereal	Crude Protein	Crude Fats	Ash	Crude Fiber	Moisture	Available Carbohydrate	Dried Matter
Rice	8.76	0.14	0.78	0.84	9.24	80.22	90.76
Sorghum	9.85	1.94	1.83	1.63	10.17	74.57	89.87
Maize	10.79	4.07	3.17	1.85	10.37	69.74	89.63
Wheat	12.39	3.34	2.38	1.73	9.79	70.33	90.21

Source: Okonkwo and Agharandu (2017)

2.2 Maize (*Zea mays L)*

Maize (*Zea mays* L.) is the American- Indian word for corn literally means "that which sustains life". It originated from the Western Hemisphere, possibly the Americans (Southern Mexico), but is now cultivated in many parts of Africa, Europe and Asia (Doebley, 2004; University of Utah Health Science, 2016). It is introduced into Africa by the Portuguese in the 16th century and had become African's most important staple food crop grown in diverse agro-ecological zones and farming system (Oluwalana, 2014). It is consumed by people with varying preferences and socio-economic background in sub-Sahara Africa (SSA) (Nuss and Tanumihardjo, 2010; Smale *et al.*, 2011; Onyibe *et al*., 2014). Maize is predominantly the cereal crop of the southern Nigeria, just as sorghum and millets are those of the north. It is a sun - loving crop admirably suited to tropical conditions. Of the cereal crops, maize ranks third in the world tonnage after wheat and rice (Table 2.2). These three most important cereals have been reported to comprise at least 75% of the world's grain production and therefore humanity has become dependent upon cereal grains for the majority of their food supply (Imtiaz *et al*., 2011).

Table 2.2: Cereal Production Worldwide

Cereal	Area Cultivated (Hectares)	10^3 Ton Production
Wheat	236, 586	497, 475
Rice	143, 602	428, 738
Maize	126, 670	415, 738
Barley	79, 410	161, 194
Sorghum	45, 565	68, 214
Oats	26, 360	43, 776
Millets	40, 008	29, 134
Rye	16, 955	28, 702
Others	8, 477	11, 967

Source: FAO (2011)

In Nigeria and sub-Sahara Africa (SSA), maize ranks first next to millets and sorghum. It provides nutrients for humans and animals and serves as basic raw material for the production of starch, oil and protein, alcoholic beverages, food sweeteners, corn flakes, corn meal, bread etc. (Smale *et al*., 2011) (Table 2.3). Starch from maize can also be made into plastic, fabrics, adhesives and many other chemical products (Oyewole and Agboola, 2011; FAO, 2011; Imtiaz *et al.,* 2011).

Table 2.3: Production of Cereals (Thousand Metric Tons) in Nigeria and Sub-Sahara Africa

Cereals	Thousand Metric Tons	% World Production
Maize	24,798	43.0
Millets	22,967	38.9
Sorghum	17,400	28.2
Rice	11,321	2.0
Wheat	3,140	0.5

Source: Smale *et al*. (2011)

Africa with its vast land covering 3 billion hectares (ha), has 1.3 billion ha of agricultural land, out of which only 252 million ha (19.36%) is arable (FAO, 2011). Agriculture is the 'engine' for growth in Africa. With subsistence agriculture practiced by majority small holder farmers, yield gaps are high and poor soils, among other constraints added to the difficulties for production of maize and other tropical cereals for the teeming population. These cereals are grown over an area of 98.23 million ha producing 162.4 metric tons (FAOSTAT/FAO, 2015) (Table 2.4).

The central role of maize as a staple food in SSA is comparable to that of rice or wheat in Asia with consumption rate being highest in Eastern and Southern Africa (ESA). An estimated 208 million people in SSA depends on maize as a source of food security and economic well-being. Maize occupies more than 33 million ha of SSA's estimated over 200 million ha of cultivated land. The average maize grain yields are still low and meeting the projected increased demand for maize grain in Africa present serious challenges (FAOSTAT/FAO, 2015).

Table 2.4: Area and Production of Selected Cereal Crops

Cereals	Area (Ha.)	Production (Tons)
Maize	34,075,972	70,076,591
Millets	19, 998, 008	16, 008, 838
Paddy rice	11, 206, 813	28,798,202
Sorghum	23,142,595	23,350,064
Wheat	10,224,952	24,706,201
Total	98,226,080	162,422,507

Source: FAOSTAT/FAO Statistics Division (2015)

2..2.1 Properties of Maize Grain

2.2.2 Anatomy of Maize Grain

All grains develop from flowers or florets, although the structure of the various cereal grains are different, the anatomical structure of all cereals is basically similar. Maize grain (naked caryopsis) consists of pericarps (fruit coat) and seed. The seed is comprised of seed coat, germ (embryo) and endosperm. The basal part attached by a short stalk to the rachis is narrow and the apex broad. The large scutellum (10-13% of the grain) and the endosperm are with the pericarp and testa which are fused to form a 'hull' (Encylopedia Britannica, 2013) (Plate 2.1). This part of the maize floret is lost in threshing. The part of the hull overlying the germ is called the tip cap. Different parts of the maize kernel contain different nutrients in various quantities (Table 2.6). The endosperm constitutes about 80% of the grain. It has 75% of the maize protein. The aleurone portion is the richest fraction of the grain. The aleurone layer is the horny endosperm, which is translucent, hard and flinty. The interior is filled with the floury endosperm which is starchy. It contains about 20% protein which surpasses that of the germ (about 10%) and fat underneath. The germ also contains nearly all the oil and most of the vitamins and

mineral. The germ or the embryo is located at the lower end of the grain where it is attached to the cob. It is normally large about 9.5-12% of the whole grain (Delcour and Hoseney, 2010).

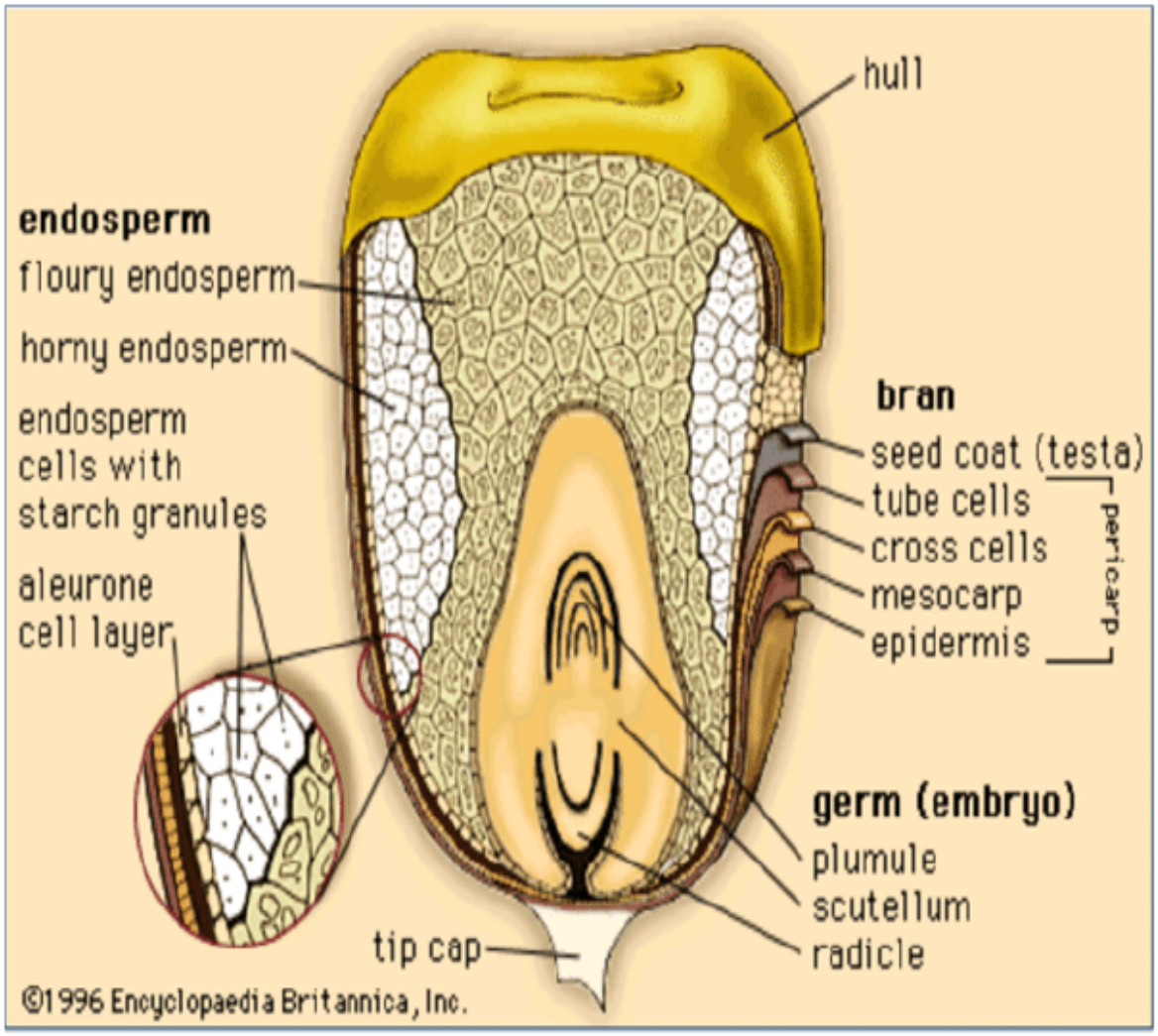

Plate 2.1: Generalized structure of maize kennel.

Source: Encyclopedia Britannica (2013).

2.2.3 Chemical Composition

Tropical cereal grains are similar in general proximate chemical composition to barley with only a few clear differences. These chemical compositions of cereal grain are characterized by the high content of carbohydrates and low protein. Available carbohydrate, mainly starch

deposited in the endosperm amount to 56-74%, protein 7-12%, lipid 2-7%, moisture 11-14% and fiber, mainly located in the bran, 2-13% (Belitz *et al.*, 2009).

A mature maize grain consists of carbohydrate (mainly starch) and do contain significant quantities of other compositions including; nitrogenous compound (mainly proteins), lipids (fats), mineral salts and water together with small quantities of vitamins, enzymes and other substances (Belitz *et al*., 2009; Ingbian and Oduyela, 2010). The major constituents of maize are carbohydrates (mainly starch) 70-80%, protein 8-11%, and fats 5%. The remainder of the kernel, 5-10% consists of fibrous components, free sugars, free amino acids and polypeptides (Belitz *et al*., 2009; Ikram *et al*., 2010) (Table 2.5).

2.2.4 Carbohydrate

Carbohydrate is one of the major constituents of maize (mainly starch) containing 70-80%. The high starch content which consists of concentrated energy food gives highest conversion of dry substance to meat, milk and eggs. Apart from high starch content, a mature grain of maize consists of nitrogenous compound (mainly proteins), lipids (fats), mineral salts and water together with small quantities of vitamins, enzymes and other substances (Belitz *et al*., 2009) (Table 2.5).

Table 2.5: Chemical Composition of Maize Grain

Parameters	Average Values
Moisture•	11.3
Protein (N×6.25)	8.8
Lipid•	3.8
Available Carbohydrate•	65.0
Fiber•	9.8
Minerals•	1.3
Vitamin B1 (thiamine)••	3.6
Vitamin B2 (riboflavin)••	2.0
Vitamin B6••	4.0
Nicotinamide••	15.0
Panthothenic acid••	6.5
Folic acid••	0.3
Total Tocopherols••	66.0

Source: Belitz *et al*. (2009)

• = g/100 g •• = mg/kg

Many workers had documented reports on the important difference in the chemical composition of the main parts of the maize kernel (Table 2.6). The seed coat or pericarp is characterized by a high crude fiber content of about 87% which constituted mainly of hemicelluloses 67%, cellulose 23% and lignin 0.1%. Crude fiber in the kernel comes mainly from the seed coat. The weight distribution in maize kernel and their particular chemical composition and nutritive value are of great importance when maize is processed for consumption (Delcour and Hoseney, 2010; Koehler and Wieser, 2013). The average chemical composition of major components of typical dent maize according to Delcour and Hoseney, (2010) is as shown in Table 2.6.

Table 2.6: Average Chemical Composition of Major Components of Typical Dent Maize

Components	Weight (%)	Oil ether extract (%)	Protein (N×5.7) (%)	Ash (%)
Whole maize	100.0	4.6	9.5	1.2
Endosperm	81.1	0.7	7.7	0.2
Bran	5.0	2.1	5.1	1.1
Germ	12.7	33.0	19.8	9.1
Tip cap	1.2	4.0	10.2	1.2

Source: Delcour and Hoseney (2010).

2.2.5 Protein Content

Protein is found in all tissues of a normal corn with higher concentrations occurring in the embryo, scutellum and aleurone layer than in the starchy endosperm (as a whole), pericarp and testa (Table 2.7). Within the endosperm, the concentration of protein increases from the centre of the periphery. The major types of proteins found in the germ are albumins and globulins (50%), glutelins (30-40%) and prolamin or zein 5% (Watson, 2003). About 45-50% of the maize endosperm proteins is prolamin or zein and contains neither lysine nor tryptophan, two of the essential amino acids. Glutelins contain up to 3.3- 3.7 g per 100 g protein of lysine and tryptophan, respectively. In a nutshell, when considered as a whole, the protein of maize (like that of sorghum) is low in lysine, very low in tryptophan but reasonsably fair in sulphur-containing amino acids, methionine and cysteine. It is excessively high in leucine and high in the aromatic amino acids, phenylalanine. Tryptophan, however, limits its biological value to the extent of some 60% of the ideal (Vasal, 2000).

Table 2.7: Distribution of Protein in Maize

Part of Grain	Proportions of Kernel (%)	Protein (N×6.25) (%)	Total Nutrient in Kernel (%)
Pericap	6.5	3.0	2.2
Aleurone	2.2	19.2	4.7
Endosperm	79.6	-	71.0
Outer	3.9	27.7	11.9
Middle	58.1	7.5	48.2
Inner	17.6	5.6	10.9
Embryo	1.1	26.5	3.2
Scutellum	10.6	16.0	18.9
Whole grain	100	9.0	100

Source: Watson (2003)

2.2.6 Lipid Content

Lipids are mainly stored in the germ, to a smaller extent, in the aleurone layer and to a lesser extent, in the endosperm. The average lipid content of the maize kernel falls between 4 and 5%. Almost 35% of the maize lipids are found in the germ. Maize fat is a semi-drying oil, highly unsaturated since linoleic and oleic acids predominate with smaller amounts of saturated fatty acids principally palmitic and stearic acids (Table 2.8).

Table 2.8: Components of Fatty Acids of Maize Fats

Fatty acid	%Total	Weight of Fats
Linoleic	50.1	60.8
Oleic	39.1	36.3
Palmitic	7.3	7.8
Stearic	3.0	3.5
Arachidic	0.4	0.5 - 2.0
Hynoceric	-	1.0 - 3.0
Iodine value	111 - 130	100 - 140

Source: Delcour and Hoseney (2010)

2.2.7 Vitamin Content

Like minerals, vitamins are concentrated in the outer layers of the grains, in particular in the aleurone layer as well as in the germ. Therefore, milling of cereals into white flour will remove most of the vitamins. The use of whole grain or products enriched in vitamin- containing tissues is of nutritional benefit. Whole maize kernel contains(mg/kg); choline 537, folic acid 0.30, niacin 23.0, panthothetic acids 5.0, riboflavin 1.0, thiamine 4.0, pyridoxine 7.0 and vitamin B_{12} 8.0. Water soluble and fats soluble vitamins appear to be present in maize at concentrations below those recommended for growth, with the exception of vitamins A, niacin, thiamine and pyridoxine (Belitz *et al*., 2009; Delcour and Hoseney, 2010). Okonkwo and Agharandu (2017), reported that maize grain contains the highest concentration of vitamin C (7.85 mg/100g),

followed by vitamin A (4.29 IU/ g), vitamin B3 (2.67 mg/100 g), vitamin E (1.6 IU/ g), vitamin B2 (0.53 mg/ 100 g) and vitamin B1(0.13 mg/100 g).

2.2.8 Mineral Content

The major portion of the minerals (90%) is located in the outer layer of the grains namely in the bran, the aleurone layer and the germ. Consequently, products made from whole grains should increasingly be introduced into human nutrition to benefit from the mineral content of cereals. The mineral content of maize has been extensively reported and documented by various workers. The average mineral compositions of maize are; (%) calcium 0.03, phosphorus 0.32, potassium 0.35 magnesuim 0.17, iron 0.003, soduim 0.01 and sulphur 0.12 of the whole kernel (Delcour and Hoseney, 2010).

2.3 Maize Varieties

There are many varieties of maize with widely differing characteristics. A selection pressure by both human and nature has resulted in various maize varieties, generally classified on the basis of size and by properties of their germ endosperm. Genetic improvement has played a key role in the development of genotypes with high technological and nutritional values. Maize hybrids which include; high lysine, high oil, waxy, white and sugary among others are a result of selection for improved chemical composition of grain (Doebley, 2004; Zilic *et al*., 2011). Another classification criterion is the sweetness or amount of sugar. The amount of residual sugar depends on the variety of the maize and when it is harvested from the field. For instance, sweet maize stores poorly and must be eaten fresh, canned, or frozen before the kernel ages, becoming small, tough and starchy (Gibson and Benson, 2002). The most common types of maize include; flint (*Zea indurata*), pop (*Zea mays indurata Strut*), dent (*Zea mays*), sweet (*Zea mays Saccharat*), flour or soft (*Zea amylcea Strut*), waxy (*Zea cereitina Kalesh*) (Zilic *et al.*, 2011). Some varieties mature in 60 to 330 days from planting, produce one to four ears per plant, 10 to 1,800 kernels per ear and yield from 0.5 to 23.5 tons of grain per hectare. Kernel may be

coloureless or white, yellow, red, purple, dark brown, blue or variegated with these colours in mottled or striated patterns. The physical appearance of each grain type is determined by its pattern of endosperm composition as well as quantity and quality of the endosperm. A classification of maize based on endosperm characteristics distinguishes six varieties (Delcour and Horeney, 2010; Zilic *et al*., 2011).

2.3.1 Flint (*Zea indurata*)

Grains of flint maize have mostly hard, glassy endosperm with smoother hard seed coat (pericarps). Usually, yellow flint maize has high contents of proteins and β-carotene. In other maize types, β-carotene with the highest pro-vitamin A activity is present in relatively low concentration. Flint maize is similar to pop but with larger grain. It probably developed from pop types by selection for grain size and greater yield. This type is produced in areas where cold tolerance is required or where storage and germination condition are poor. It currently accounts for 14% of commercial production (Cortes *et al.*, 2006).

2.3.2 Flour or Soft (*Zea amylcea Sturt*)

Flour maize endosperm is made of soft starch with thin pericarps. It remains the preferred form of direct human consumption as it consists of soft starch that is easily ground to produce meal that can be consumed directly. It currently accounts for 12% of commercial production.

2.3.3 Pop (*Zea mays indurata Sturt*)

The original domesticated type consists of a small spherical grain with floury (soft) starch corn and flinty (hard) endosperm shell. Moisture entrapped in the floury starch expands upon heating and burst through the hard shell creating the popular confection. Heating the grain turns this moisture into steam which expands, splits the pericarps and causes the endosperm to

explode turning the grain inside out. Most commercial varieties expand 30-40 times their volume. Pop accounts for less than 1% of commercial production.

2.3.4 Dent (*Zea mays*)

Dent maize with flinty sides and soft cores of starch that cause the end of the grains to collapse or dent during drying falls between the flinty and flour types. Although the majority of the dent maize types have yellow endosperm; white, red, and blue dents are very popular in human food products.

These colorations (red and blue) come from phenolic compounds which have antioxidant properties (Cortes *et al.,* 2006). Red and blue maize have a coarser, sweeter, nuttier taste than other maize grown for flour or meal. Although most of the products (cooking oil, various maize grits, meal, flour starches, sweeteners alcohol, paper adhesives, cosmetics, citric acid, glutamic acid, etc.,) are made from dent maize, other types of maize are becoming more and more important. This is the most produced types of maize on global basis, accounting for 73% commercial production.

2.3.5 Sweet (*Zea mays Saccharat*)

Unlike dent maize, sweet maize is grown primarily for fresh consumption not feed or flour, although United States Department of Agriculture (USDA) researchers have developed a technique to produce a high fibre, no calorie flour from sweet maize (USDA, 2014). The endosperm consists of soluble sugars with little starch, and an intermediate form of sugar polymer called phytoglycogen. Commercial production is negligible (1%) though the crop has value as a processed vegetable in industrial economies.

2.3.6 Waxy (*Zea cereitina Kalesh*)

Waxy maize is a starch variant of normal maize which contain 100% amylopectin whereas normal maize contains 75% amylopectin and 25% of amylose. The high proportion of amylopectin exerts considerable effect on starch physical properties (Fergason, 2001). Waxy barley and maize starch have been found to exhibit much greater swelling than their normal counterparts which had 27.5% and 29.4% amylose respectively, despite the waxy starch having 1-2 degree higher gelatinization temperature. Waxy maize is used by wet-maize millers to produce waxy starch, utilized by the food industry as a stabilizer (Ortega and Serna-Saldivar, 2004; Taylor *et al.*, 2006) and in paper industry as an adhesive (Watson 2003; Ptazek *et al.*, 2009).

Among the most important types of maize are high lysine maize, namely opaque-2 (Hasjim *et al.*, 2009), quality protein maize (QPM) and high oil content genotypes with more than 6% of oil high polyunsaturated essential fatty acids (Prasama *et al.*, 2001). Genetically modified (GM) herbicide-resistant maize (eg Bt corn), a variant maize that has been genetically altered to express one or more proteins from *Bacillus thuringeiensis* has become the major types of maize grown in many countries, including U.S. where 85% of the crop is GM. European and African countries originally banned GM maize, but while still very controversial, this position is changing as benefits of Bt corn become accepted. In fact, as of 2011, herbicide-resistant GM maize was grown in 14 countries (James, 2011). By 2012, the European Union (EU) authorized importation of 26 varieties of herbicide-resistant GM maize and about 30 million tons of GM crops was reported to have been imported. The GM maize M0N810 was cultivated in almost 89,000 ha in five European countries, particularly in regions with high infestation levels of maize borer (a pest affecting both the quality and quantity of the harvest) (GMO, 2014). Bouis and Welch (2010) reported that there appear to be no nutritional difference between normal variety and Bt maize; therefore, its presence or absence should have no effect on fortification technology or policy.

Nigeria has a wealth of improved maize varieties and management technologies that can double or triple traditional yields. Earlier maturing, drought and disease resistant high yielding varieties of maize offer exciting new possibilities for multiple cropping. In the future, conservation tillage offers great hope for checking soil erosion, conserving moisture, building up organic matter and reducing the bulk breaking work and drudgery of hard weeding and land preparation (Iken and Amusa, 2014).

2.3.7 Oba Super 2

Oba Super 2 maize is an annual herbaceous hybrid plant developed by crossing or breeding two different inbred parent lines with desired characteristics. Hybrids are wonderful plants but the seeds are often sterile or they do not have seeds that are genetically true to the parent plant. Not only will the plants not be true-to-type, but they will be considerably less vigorous. Hybrid seeds can be stabilized, becoming open pollinated varieties (OPV), by growing, selecting and saving the seeds over many years. Oba Super 2 (hybrid) is an earlier maturing, a drought tolerant variety, resistance/tolerance to *Striga hermonthica* and other foliar disease and insects (Drought Tolerant Maize for Africa (DTMA, 2014). It is a high quality protein maize (QPM), having about 70% higher in essential amino acids- lysine and tryptophan (Hasjim *et al*.,2009; Giwa and Ikujeniola, 2009), and higher polyunsaturated essential fatty acids, reported to have been in sub-optimal amount in normal maize varieties. Thus, the nutritional quality of its protein is superior to that of normal maize varieties (Prasama *et al*., 2001).

Oba Super 2 is tolerant to low nitrogen as well as excellent stay-green leaves at maturity, desired by livestock farmers (Nuss and Tanumiharjo, 2011; DTMA, 2014). Hybrids have been characterized to have significant ($p \leq 0.05$) assimilatory surface, with more than 100 grains (7.13 g) and ear weight (0.08 kg), harvest index (37.60%) than OPV maize cultivar (Sawan 1) (Tollenaar and Lee, 2006). Open pollinated varieties (OPV), on the other hand, are those which

if properly isolated from other varieties in the same plant species will produce seeds that are genetically 'true to type'. However, the yield potential of hybrid maize is higher compared with OPV due mainly to higher assimilatory surfaces and high leaf angle that could facilitate diffusion of light into the lower portion of the canopy. Genetic improvement in conjunction with good management practices has played a fundamental role on this increase (Tollenaar and Lee, 2006). Abayomi *et al*. (2006) reported that the yield advantage observed in Oba Super 2 maize could be linked to their higher leaf growth, leaf area duration and effective leaf area.

2.4 Importance/ Utilization of Maize

In developing countries including Nigeria, the importance of maize as harvested food crop with potential to mitigate food insecurity and alleviate poverty cannot be over emphasized (Olaniyan, 2015). Maize is a preferred staple food for over 900 million poor consumers, 120-140 million poor farm families and about one third (1/3) of malnourished children (Centro Internacional de mejora miento de maiz trigo (CIMMYT) and International Institute of Tropical Agriculture (IITA), CIMMYT/IITA, 2010). In SSA, shortage of maize invariably leads to famine and starvation. It is estimated that by 2025, maize would have become the crop with the greatest production in developing countries of the world, and by 2050, the demand for maize will double (CIMMYT and 11TA, 2010).

Maize is highly responsive to production inputs, and as such, its food and industrial uses are many, and its production potential can hardly be matched by any other cereals. It is therefore, a solution to hunger, which can salvage the famine population (Olaniyan, 2015). Maize is commonly used by man as food crop, as feed stuff for biofuels, as roughage feed for livestock and also included in poultry feeds (Iken and Amusa, 2014).

2.4.1 Maize as Food Crop

It is the most important cereal crop in the economy of African countries and is one of the most important commodities used as food aid. It is cheaper than other cereals such as rice, sorghum and wheat, more affordable to the vast majority of the population and therefore occupies a prominent position in the agricultural development agencies of several countries in Africa especially Nigeria. All parts of the crop can be used as food and non-food products (11TA, 2009). It is also a basic ingredient for some indigenous drinks and food products. In developed world, maize is largely used as livestock feed (poultry, pigs and ruminant animals) and raw material for industrial products, while in developing country, it is mainly used as food (Yan *et al.,* 2011; Yangcheng *et al.,* 2013; Koehler and Wieser, 2013).

It is an important source of carbohydrate, protein, iron, vitamin B, and minerals. As food, the whole grain, fresh or dried, may be processed traditionally by wet and dry milling methods to give a variety of food products. Wet milling yields starch and by products such as maize gluten (feed ingredient). Preparation of maize alone or in combination with other food materials serve as staple food or snacks in Nigeria, such as; ogi (in hot or cold forms), tuwo, maasa, akple, gwate, nakia, egbo, abari, donkwa, ajepasi, and kokoro (Abdulrahaman and Kolawole, 2006).

2.4.2 Maize as Livestock Feed

The bulk of the concentrated feed to farm animals consist of grains, and maize is the most important and preferred one due to its low cost in comparison with other cereals, low fiber content and high starch content which consist of concentrated energy food that gives highest conversion of dry substance to meat, milk and eggs. Maize stover which is the plant residue after the ears had been removed contains 30 to 40% of the plants' total nitrogen, about 75% of the potassium, sulphur, and magnesium and almost 100% of calcium. It is used by many farmers in developing countries as roughage feed for livestock (Iken and Amusa, 2014).

Silage maize is important feed in temperate areas (U.S, Canada, and Europe), and consists of entire plant, which is cut, chopped and placed in a structure for anaerobic fermentation to occur. The fermented product is stored and used as livestock feed (FAO, 2006).

2.4.3 Maize in Industry

Maize is used as primary feed stuff to generate bioethanol and biogas (biofuels) additive for gasoline (Yan *et al.,* 2011; Wallington *et al.,* 2012; Koehler and Wieser, 2013; Yangcheng *et al.,* 2013). It is a basic raw material for production of starch. Starch from maize can be made into plastics, fabrics and adhesives (Oyewole and Agboola, 2011).

2.4.4 Maize in Brewing Industry

Maize has been employed in the brewing industry exclusively to complement and supplement (as adjunct or unmalted) the major brewing ingredient which is barley malt. The grain can be used both as a cooker mash or mash tun adjuncts, and kettle or copper adjuncts (Sadosky *et al.,* 2002). Adjuncts can increase brew house capacity by allowing for shorter brewing cycles as well as providing for consistent wort quality. Other attributes are; easy to handle and use, produce cleaner fermentation with beer yeast heads, better hot and cold breaks and reduced maturation time. Research has shown that two classes of adjuncts are derived mainly from maize and rice but, owning to the periodic scarcity of rice, its unstable and exorbitant price, maize adjuncts are more common. These adjuncts include; corn meal and grists, corn flakes, refined corn grits etc. (Poreda *et al*., 2014).

Many researchers have reported on the use of some Nigerian dry milled maize products for beer brewing and they concluded that one can produce good quality corn grits for beer brewing particularly if yellow maize is used (Esslinger, 2009; Poreda *et al.,* 2014; Iwouno and

Odibo, 2015;). The reasons for incorporating unmalted grains in beer are straight forward. According to Sadosky *et al.* (2002), the resulting changes to the wort, mash, and final beer add complexity to the brew. The changes when un-malted grains are incorporated into the brewing process includes; change in the wort composition such as viscosity and β-glucan content, which affect the filtration rate and foam stability, altered mash pH affect the rate of enzymatic processes as well as the flavor, foam stability, colour, rate of fermentation, final beer stability and increased haze, reduced nitrogen content and modified nitrogen composition slowing the rate of fermentation.

Reports on the malting of barley (Agu *et al.*, 2006; Kumar and Malta, 2011), and wheat (Sarwar *et al.*, 2013) have advanced and are well documented, but that of maize is yet to receive similar attention so far.

2.5 Malting Processes

The essence of malting is to obtain a modified grain which will yield high extract, produce complement of enzymes to convert endogenous and exogenous protein to an appropriate mixture of polypeptides and amino acid. Malting process involves; choice of suitable grain, storage until it is required for use, steeping, germinations and kilning (Aehle, 2004).

2.5.1 Choice of Grain

There are arrays of maize cultivars, thus suggesting their genetic potential in utilizing them for food and industrial uses. Quality of the grain is very important for meaningful selection since many characteristics are cultivars dependent. The locality of its production determines the quality of grain (Olaniyan and Lucas, 2004). Also soil composition may affect grain varieties because cultivars grown at different locations showed a mixed difference in amylolytic potentials. Some maize cultivars obtained from different localities showed marked differences in

tannin content, phytic acid content and high sugar content, steep out moisture, malting loss, diastatic power and other malting properties (Verheye, 2010).

Grains colour had been reported to be a striking feature in the choice of grains. Iwouno and Odibo (2015), in their study on two maize varieties (yellow and white), reported that yellow variety gave a very high extract than the white cultivar. Grain size to a large extent determines the extract yield. Large grains are known to produce high extract than the smaller ones. Grains with mealy endosperm are more suitable than ones with steely endosperm. Unhealthy grain will definitely present low yields as a result of poor complement of enzymes even after kilning, low level of total nitrogen and uneven degradation of the endosperm. Grains with good yield potentials, disease resistance, pest free should be used while dormant and pre-germinated grains should be avoided (Abayomi *et al*., 2006; DTMA, 2014).

2.5.2 Steeping

This involves immersing the grains in water to imbibe a suitable amount of water at a temperature of about 30-40^{o}C, until they absorb sufficient moisture to support growth and biochemical changes during germination (Mohammed and Addy, 2014). Additives such as formaldehyde and lime water could be added to improve germination. Steeping lasts 1-3 days depending on grain's condition. During steeping, the moisture of the steeped grain increases rapidly at first but progressively slows down and in the absence of germination it effectively ceases. Water uptake by un-germinated grain is a physical process which is independent of the grains viability but is accelerated if the grains are so badly damaged that their surface layer and testa are broken (Goldhammer, 2008).

Steeping also serves two other functions; dirt, chaff and broken kernels are removed from the grain by washing and floatation. This process is often also used to inactivate the tannins

which bind to the malt's enzymes- amylases and protease, thus resulting in reduced sugar production and poor modification of protein. A process of inactivating the tannins by soaking cereals grains for 4-6 hours in a very dilute solution of formaldehyde is very important. However, the use of formaldehyde has not been viewed favorably in recent time because of its potential health risk. Alternative method of inactivating tannins by soaking in dilute alkaline (sodium hydroxide, sodium hydrogen carbonate etc.) seems to be a safer and almost equally effective method and is now used commercially (Okolo *et al.*, 2010).

The rate of water uptake, temperature of the steep liquor, variety and types of the grain's endosperm greatly affect steeping. Most of the physiological difficulties which reduce malting performances are overcomed through proper manipulation of steeping with regard to improved aeration, optimal submersion, required out of steep moisture and appropriate hydration of the grains (Okolo and Ezeogu, 1996). However, under-steeping reduces nitrogen solubilization and also retard cell wall breakdown in the endosperm. Also, high out-of-steep moisture (46-47%) should be avoided for it leads to high protein solubilization in the endosperm. Low germination temperature (12-14^{o}C) should be used since it limits protein solubilization and makes it good for alcoholic production. Steeping is affected by a lot of factors including; steep liquor, additives in steep liquor, ions in steep and brewing liquor.

2.5.2.1 Steep Liquor

This is basically the water use for steeping and it must meet the basic requirement of good brewing water. According to Ogu *et al*. (2004), a good brewing water must meet the standards for potable water, should be clear, colourless and free from objectionable taste. If it is surface water, it must be treated to reduce organic matter, the alkalinity in the supply should be reduced to 50 parts per million (ppm) or less, and if the alkalinity is 50 ppm or less, pH is not important and values ranging from 4-9 are acceptable.

2.5.2.2 Additives in Steep Liquor

Additives can be added to influence malt quality. The nature of the steep liquor in terms of its ionic content greatly affects malt quality. Certain salts when dissolved in steep liquor stimulate the germination of seeds. According to Okolo and Ezeogu (1996), alkaline steeping dissolves resin material and tannin more readily than acid or neutral salts; thus causing an increase in the water uptake. The use of lime reduces phenol and promotes germination of dormant grain or grain harvested under adverse weather condition and has suffered microbiological contaminations. Hypochlorite imparts a taint flavor to the resulting malt and beer and their use is thus unsuitable on the commercial scale (Okolo and Ezeogu, 1996).

The use of formalin increases germination while that of formaldehyde reduces the anthocyanogen content of wort and so increases the non-biological stability of the resulting beer. The use of sulphuric acid with gibberellic acid (GA) have been reported to reduce malting loss and increase extract but with poor yeast attenuation (Okolo and Ezeogu, 1996). Oxidizing agent e.g potassium bromate stimulate both dormant and non-dormant grains. Many phenolic compounds and growth regulators e.g kinetin and GA, stimulate germination energy. The use of solutions containing ferrous sulphate, uranyl, acetate and other metal ions particularly those which co-ordinate sulphur, sulphydryl-binding reagents such as N-ethylmaleimide and oxidizing agents discourage water sensitivity.

2.5.2.3 Ions in Steep and Brewing Liquor

The main ions present in most malting and brewing waters are; Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, SO_4^{2-}, Cl^{-}, CO_3^{2-}, HCO_3^{-} and NO_3^{2-}. There may be little amounts of Fe^{2+}, Cu^{2+} and other heavy metals plus silica. No toxic ions such as Pb^{2+} should be present. Of the principal ions, Ca^{2+}, HCO_3^{-} and CO_3^{2-} have the most profound effect upon the malting and brewing process. Ions and their individual concentrations have great influence in the steeping processes (Bamforth, 2005; Ayenor and Ocloo, 2007).

The activity and stability of enzyme during mashing are influenced by the ion and therefore the extract yield. It has been reported that sorghum treated with Ca^{2+}promotes extract yield (Ogbonna *et al*., 2003). Also, pH and phosphate concentration in the mash and derived wort are strongly affected by the ions present. Generally, ions influence the pH of the mash, act as buffering agents, influence the extraction of proteins, vitamins and other minerals, affect the activity of enzymes with resultant changes in the extract recovered, the fermentability of the wort, the efficiency of hop extraction and nature of the finished beer (Ogbonna *et al.*, 2003).

2.5.3 Germination

Here, most of the dramatic advances in malting technology take place during this phase. The grains after steeping are germinated under controlled conditions. Briggs *et al.* (2004) stated that during germination, the moist grains are allowed to grow under controlled conditions in the dark with no further addition of water. Germination is controlled by regulating the moisture level and temperature of the germinating grains. The objectives of the germination process are thus; to bring the reserve food material into suitable state that can be hydrolyzed at mashing by the enzymes produced at this stage, vitamin C is elaborated, phosphorus availability is increased, lysine and tryptophan are synthesized, elaboration of amylases, thus resulting in lower viscosity and thinning down of the starch slurry, elaboration of proteases that degrade storage protein and overall improvement in flavor profile (Goldhammer, 2008; Ghavidel and Davoodi, 2011).

During germination, the enzymes that will modify the endosperm reserves and cell wall materials to useful extracts are thus developed. Germination rate and modification intensity are controlled by regulating the moisture content and the temperature of the grain (Pelembe *et al.*, 2004). While barley undergoes complete modification, other grains such as maize, sorghum, millet, etc. are poorly modified because of the limited attack of the enzyme on the endosperm. Studies on the progressive partial degradation of these grains have revealed that like in the barley, the extent of such modification is under the effects of steeping condition, environmental

conditions and variety of the seed (Pelembe *et al.,* 2004). Germination is influenced by a number of factors including; temperature, duration, aeration, dormancy, plant hormones and to a lesser extent malting apparatus (Raimi *et al*., 2012).

2.5.3.1 Temperature

In maize grains, germination carried out at room temperature (25-30^{o}C) for about 5 days have been reported (Palmer *et al.,* 2003; Iwouno and Odibo, 2015). Germinating at a lower temperature (below 25^{o}C) produces a very unsatisfactory result in maize, but in barley, germination temperature of 15-18^{o}C yields wort containing higher proportion of nitrogen than applying a higher temperature. The use of higher temperature, more sophisticated steeping schedules as well as additives which can offset some of the undesirable effects of elevated temperature, have however, resulted in reduced germination period (3 - 5 days). Malt composition at elevated temperature differs from malts produced from a long cool germination (Palmer *et al.,* 2003; Badau *et al.,* 2005a)

2.5.3.2 Duration

This depends on the degree of modification required and the various treatments accorded to the germinating grain. Un-germinated grains do not have any diastatic power (DP), but the DP increases with a peak after some days of germination. For instance, DP of pearl millet increased with a peak value obtained after 5 days of germination (Pelembe *et al.,* 2004). Malts made under this condition would have been well modified to a powdery and chalky state. It should be noted that low germination temperature leads to malt production with lower DP, but malting at high temperature only for a short period e.g 30^{o}C for 72 h does not give maximum development of amylases and other hydrolytic enzymes (Briggs *et al.,* 2004; Ayernor and Ocloo, 2007).

2.5.3.3 Aeration

The composition of air is one of the determining factors of germination. Exposing the germinating grain to oxygen (0_2) in the steep liquor or sometimes from 'air- rest' or aeration leads to increase in germination. Poor aeration slows respiration and prevents the germinative power of the embryo (Ezeogu and Okolo, 1995). A resting grain has a slow level of respiration, but suddenly rises as the grain is wetted. Oxygen absorption is exponential and in 1 hour, most of the 0_2 has been removed from the steep liquor. The embryo and aleurone layer contribute to the utilization of 0_2, resulting in the influx of sugars from the endosperm and release of absorbed air or carbon (iv) Oxide, (CO_2) by the seed tissues.

2.5.3.4 Dormancy

This factor is of great interest to the maltsters because the grains fail to germinate even under favourable condition of germination. This delay in germination hinders the activation, synthesis and secretion of the various hydrolytic enzymes thus, causing impaired extract yield. These types of grains are called idlers' and they are unsuitable for malting and brewing (Ayernor and Ocloo, 2007). Grains dormancy may be due to water sensitivity, restriction of access to 0_2 or $C0_2$ removal from the embryo by the testa, presence of microflora or it may be hormonal in nature. Dormancy is eliminated during malting by using high drying and storage temperature, while water sensitivity is eliminated by the use of 'air-rest' steeps (Okolo and Ezeogu, 1995).

2.5.3.5 Plant Hormones

The metabolism of germinating grain is affected by a number of plant hormones. The gibberellin plays a vital role in enzyme synthesis regulation. These hormones are formed in the embryo of cereal seeds and act as phytohormones regulating enzyme synthesis (Marx *et al.*, 2003). Hydrolytic enzymes such as α–amylase, endo-β-glucanases, pentosanses and proteases synthesis are increased in the presence of gibberellin. The use of gibberellin in malting is not regarded as an additive since they are synthesized in the embryo of barley grain. However, the

effectiveness of this hormone in regulating the hydrolytic enzymes synthesis has not been proved.

2.5.3.6 The Malting Apparatus

Malting apparatus is considered to a lesser extent as a factor that affects germination. The use of wooden trays has been reported to reduce germination rate due to heat generated. Temperature control in the traditional out-door operation is difficult. Pilot malting enables programmed temperature control as well as humidity of the air. (Irakoze *et al.*, 2010).

2.5.4 Kilning

Kilning is the final stage of the malting process which involves the drying of the green malt at high temperature ranging from 45^{o}C-60^{o}C for 8-30 h depending on brewer's aim. This process preserves the malt, add colour and flavor to the finished malt (Bamforth, 2005).

The main purposes of kilning are; to prevent further growth and stop action of the hydrolytic enzymes, to reduce the moisture content to a level where the grains will not be susceptible to microbial attack, and to introduced such characteristics properties as flavour, colour, aroma and malt friability. The finished malt contains enzymes like α–amylase, β-amylase and maltase which can break down starch to maltose, glucose and other simple fermentable sugars as well as protease for degradation of storage protein. The kilning times and temperatures are dependent on the type of grains and to a great extent, on the malt desired (Bamforth, 2005).

2.6 Malt Production

Malt is defined as a modified cereal grains (barley, maize, sorghum, millet etc.), resulting from induced germination under controlled conditions of moisture, pH and temperature for a limited period of time. It represents a rich source of carbohydrates, degraded protein, various B-vitamins, minerals, inorganic materials, starch splitting enzymes. During malt extraction process, suitable wort is produced for beer production (Mahboobeh *et al.,* 2014). Malting process

transforms the low content of enzymes, hard texture and undesirable taste of cereal grains into soft grains with desirable flavor being rich in enzymes. It is influenced by various physicochemical factors including grain variety, sulphur and nitrogen content, oxygen and carbon(iv)oxide content, carbohydrates, enzymes, antioxidants, protein and lipids of grains as well as steeping, germination and kilning regime. Malting is a complex process requiring many enzymes with α- amylase, β amylase, α-glycosidease, limit dextrinase as well as proteinase, being the most important among others (Ghavidel and Davoodi, 2011; Faltermaier *et al*., 2013).

Malt has found a special application in food and enzyme industries worldwide. Malt and its extract are used as sweeteners, flavorings, colorings, fermenting agent in malt vinegar and beer brewing, malt concentrate, maltose syrup, infant formula. Malt also shows medicinal properties including lowering blood sugar level, functioning against intestinal diseases, stimulation of lactating glands, strengthening hairs and prevent them to become grey (Topping, 2007; Kumar *et al.*, 2011). Malt and its products from grains is considered a good food index and thus, the greatest added value (Feyzipour and Hoseini, 2010).

Production of high quality and reduction of wastes resulting in increased malting yield are feasible through optimizing the physicochemical conditions under which malt is produced. The ungerminated grains are the dormant seeds of grass plants; barley *(Hordeum spp.*), wheat *(Triticum spp*.), maize *(Zea mays)*, sorghum (*sorghum bicolor*) etc. Through the malting process, the grains are germinated under controlled conditions and kilned to produce the desired malt. However, the correct extent of germination is the key for producing good malt (Elkhalifa and Bernhardt, 2010).

2.7 Modification of Cereal Grains

Malting degrades the grain storage protein reserves in favour of assimilable nitrogenous materials essential for adequate yeast growth during fermentation (Ogbonna *et al.*, 2003). During germination, the embryo grows at the expense of reserve materials stored in the kernel. As soon

as the grain makes contact with suitable condition during steeping (moisture, oxygen and adequate temperature), all enzymatic apparatuses are gradually activated to break the reserves of starch and proteins to form a new plant. Here lie the crucial roles of malting, which are enriching the malt with enzymes (cytolytic, amylolytic, proteolytic etc.), modification of kernel endosperm, formation of flavor and aroma compounds. Starch degrading enzymes (such as α-amylase, β-amylase, α-glucosidase and limit dextrinase) produced during germination are better characterized than the proteolytic counterparts (Schmitt *et al.,* 2013). However, with proper manipulations of the experimental variables, protease can be enhanced. A well modified malt enhances the brewing potential of such cereal grains.

Germination is halted when the malt is rich in enzymes, has achieved sufficient endosperm modification and had consumed as little reserve materials (starch, proteins) as possible during embryo development. At this point, germination is arrested by kilning (drying) at temperatures ranging from 45^{o}C-60^{o}C for 8-36 hours depending on brewer's aim. This process preserves the malt, adds colour and flavor to the finished malt. Kilning is controlled to prevent inactivation of the enzymes developed during germination (Bamforth, 2005).

A lot of methods are used to enhance the brewing potential of cereals malt including; manipulation of steeping sequence (alkaline steep treatment, air-rest cycle, cold and hot water extract and warm water final steep), appropriate cultivar selection, manipulation of germination time, temperature, kilning and mashing temperature as well as addition of exogenous enzymes (Ogbonna *et al.*, 2003; Owuama and Adeyemo, 2009; Ukwuru, 2010).

Manipulation of steeping sequences were targeted primarily to increase grain germinability, increase protein and enzyme synthesis, and reduce polyphenol influence on protein content of malts (Ogbonna *et al.,* 2003). When malts are manipulated the protein quality characteristics such as percentage protein, the nitrogen solubility index and the content of the first limiting amino acid, lysine etc., are improved (Ogbonna *et al.,* 2003). More so, it reduces

the polyphenol content of the malt which is known to inhibit the development of enzymes and protein reserves (George *et al.,* 2005).

Germination produces malt for brewing although germinated or malted grains can also be used in bakery products, non- alcoholic drinks and weaning food formulations (Amankwah *et al.,* 2009). Flour from germinated seeds had been reported to have better nutritional properties than flours from non-germinated cereals (Amro *et al.,* 2006). Irakoze *et al.* (2010) highlighted the different biochemical modification that occurred in grains during steeping and germination which gave improved nutritional properties. The viability of germination can be realized if high soluble solid yield and low malting loss can be maintained.

Malting loss includes losses due to leaching of solids during steeping and losses due to increased metabolic growth during germination. Increase in malting loss may subsequently decrease the level of water soluble nutrients in the malt. To forestall such occurrence, adequate moisture must be attained to hasten metabolic development of the roots and shoots. Attainment of suitable moisture content of the seed is achieved if the optimum steeping time and temperature, as well as germination time and temperature are better manipulated to influence the soluble solid yield (Irakoze *et al.*, 2010).

During germination, the correlation between viscosity or falling number and amylase activities had been investigated. Amylase breaks down the amylose and amylopectin components of the starch, producing smaller dextrins, maltose and glucose thus reducing the viscosity. Thus, viscosity decreased significantly as time and temperature of germination increased. Similar observations were noted in germinated millets (Jingjiun *et al.,* 2010). Also, the tannins which are located in the seed coat are reported to form complexes with hydrolytic enzymes and inactivate them. The changes in the seed coat permeability may be much greater and rapid, thus allowing higher solid losses. Tannin lost as a result of its leaching into the endosperm or growth medium along with the imbibed water form complexes with reserve seed

protein and enzymes (Amro *et al.,* 2006). Jingjiun *et al. (*2010) reported that treatment processes and fermentation on protein content as well as digestibility fluctuated and varied between cultivars. Germination of the grains increased the protein content and digestibility (except of course ground grains) among the cultivars. Protein content digestibility increased as a result of fermentation of the germinated and coarse ground grains and compared favorably higher than cultivars without such treatments (Amro *et al.*, 2006).

2.8 Enzyme Activation and Formation in Grains

Activation of enzymes and formation of new enzymes are processes that are vital during malting of all cereals. In barley for instance, some enzymes are already present in abundance in bound state (Trevor, 2004). These enzymes are released or formed in increasing amount as malting progressed. Enzymes and enzyme complexes contained in cereals and their malt include; starch degrading enzymes (especially α- and β-amylases), cytolytic enzymes (β- glucanases and cytases), protein degrading enzyme (proteolytic enzyme or proteases) and phosphoric acid splitting enzymes (phosphatases). These enzymes are present with the exception of α-amylase. The development of these enzymes are as a result of activation of gibberellins (hormones) which are distributed with the punctuation of water from the scutellum along the aleurone layer which caused the release and formation of enzymes. These hormones caused the release of amino acids and production of hydrolytic enzymes (amylase, β-glucanase and proteases) (Gupta *et al*., 2010; Faltermaier *et al*., 2013).

2.9 Cereal Enzymes

Cereals have an array of enzymes that hydrolyses the high molecular weight compounds of the grains during germination and subsequent processes. These enzymes are classified into two, depending on their point of action viz: exo- and endo-acting enzymes. The endo- enzymes attack at the large entities and solublize the marco-molecules, thus reducing their size such that they are not prone to cause problems in wort processing. The exo-enzymes on the other hand,

serve the purpose of producing low molecular weight profile from the products of endo-enzymes action, thus making them readily assimilable by yeast for its metabolism and improvement of beer quality (Trevor, 2004). There are two important enzymes in cereals- the proteases and beta (β)-glucanase. Proteases break down proteins to give amino acid, essential for yeast growth. β-Glucanase breaks down β- glucan which cause filtration difficulties and low extract if poorly degraded. Other enzymes in cereals that are relevant to the brewer are; α- amylase, β-amylase, amyloglucosidase, α-glucosidase, dextrinases, pullulanases, endo-β-glucanase, pectolytic and lipolytic enzymes (Trevor, 2004; Oyewole and Agboola, 2011).

2.10 Enzymes in the Brewing and other Processes

Enzymes promote the hydrolysis of the endosperm cell wall, starch, proteins and lipids during malting and mashing to give wort which is then fermented by yeast into beer (Ogbonna *et al.*, 2003; Trevor, 2004). Yeast itself can be considered as a package of complex enzymes which catalyze the reactions necessary for converting fermentable sugars into alcohol (Iran, 2006). Enzymes are usually highly specific, that is, each enzyme will act on only a limited range of substrate frequently. The main factors affecting enzyme activity are temperature and pH. Other factors that are less important are ions (Ca^{2+}, Na^{+}, Mn^{2+}, HCO_3^{2-}, SO_4^{2-} etc.), moisture content and oxygen. Thus, an increase in temperature increases the rate of reaction up to an optimum. During malting, the formation of many enzymes is promoted and is dependent upon the moisture and oxygen content of the cereals (Bamforth, 2005; Ayenor and Ocloo, 2007).

During mashing, one of the most important enzymes, α-amylase, is stabilized by Ca^{2+}. In other words, calcium is essential such that without calcium ions, Ca^{2+}, α–amylase is rapidly destroyed at normal mashing temperatures and pH. The presence of Ca^{2+} in sufficient amount stabilizes the enzyme but only destroyed in the presence of copper, Cu^{2+} (Bamforth, 2005).

Much of the enzyme activity is activated during malting. Maltsters are concerned with the breakdown of the endosperm and the mobilization of the enzymes of the grain during

germination. Enzymes such as α -amylase, β-glucanase, endo-protease, exo-peptidase and carboxypeptidase are present in the starch endosperm of cereals and are activated during malting. Other enzymes such as exo-protease, β-amylase and pentosanases are formed in the aleurone layer of the cereal during malting. The formation and activation of these enzymes is promoted by increasing moisture and oxygen during the steeping, and is then arrested by kilning (Yamasaki, 2003; Bamforth, 2005).

2.11 Proteolytic Enzymes

In the course of malting, proteolytic enzyme is among the hydrolytic enzymes that developed. Proteolytic enzymes (EC 3.4.1., 21-24 and 99) also termed proteases or peptidases, or peptide hydrolases, or proteinases are capable of hydrolyzing peptide bonds in proteins thereby increasing their solubility. In practical terms, peptidases and proteases are essentially equivalent to each other in terms of chemical features, with difference lying only on the size of the peptide chain used preferentially as substrates (Antao and Malcata, 2005). There are two major protease groups; the exoproteases and endoproteases or endopeptideases. Exoproteases catalyze protein hydrolysis from the terminal amino acids of their target protein molecules (aminopeptideases and carboxypeptidase) or act only near the ends of polypeptide chains. In contrast, the endoproteases act at specific amino acid residue within their substrate molecules, thus breaking proteins up from the inside. Endoproteases include trypsin, chymotrypsin, pepsin, papain and elastase. These enzymes can be found in all living organisms, from microorganisms to animal, humans and plants.

Since virtually all biological processes involve proteins, all proteins undergo proteolysis (Jisha *et al*., 2013). Proteases can be seen as one of the major biological regulators (Schilling and Overall, 2007). Proteases are physiologically necessary for living organisms. In plants, the presence of proteolytic activity has been reported in several cell compartments, such as vacuoles, mitochondria, cell wall, chloroplasts cytosol etc. (Hamilton *et al*., 2003). This explains why they

are ubiquitous and found in wide diversity of sources such as plants (Gaur and Wadhwa, 2008), animals and microorganisms (Huang *et al*., 2013; Gaur *et al*., 2014). Besides being necessary from the physiological point of view, proteases are potentially hazardous to their proteinaceous environment and the respective cell or organism must precisely control their activity. When uncontrolled, proteases can be responsible for serious diseases. While these proteases are highly beneficial, they are also very dangerous if left unchecked. To limit these dangers, they must be strictly controlled both in time and place. Nature therefore, evolved controls for proteases. The control of proteases is generally achieved by regulated expression, secretion, activation and degradation of mature enzymes, and by the inhibition of their proteolytic activity (Hamilton *et al*., 2003).

Plants are important sources of easily available proteases. In tuberous plants, they are present in the leaves and tubers. Among the grains such as maize, wheat etc. they are primarily present in the endosperm (Chen *et al*., 2004; Mohamed, 2005; Ekpa *et al*., 2010).

2.11.1 Uses of Proteases

Proteases are important economic enzymes. They have a first place in the world market of enzymes, with estimated sales of approximately US$3 billion which accounts for about 60% of the total enzymes market and among the most valuable commercial enzymes (Leary *et al*., 2009; Mohanasrinivasan *et al.,* 2012; Sarrouh *et al.,* 2012; Li *et al.,* 2013). This is because they play an important role in biotechnology, considering the fact that proteolytic reaction changes the chemical, physical, biological or immunological properties of proteins (Ademola and Malomo, 2017). They are employed in industry, medicine, pharmaceutical, and as a basic

biological research tool. Digestive proteases are part of many laundry detergents and are also used extensively in the bakery industry as bread improver (Walsh, 2002).

Proteases are equally used as coagulating agent in cheese, dairy products, in the tendering of meat, production of sweeteners, chocolate syrups, bakery products, alcoholic beverages, infant food, precooked cereals, soft drink, chill proofing of beer and modification of gluten flavor development. Proteases are used in the processing of marine foods and treatment of marine wastes, for example; the production of protein hydrolysates, silages, peeled and squid softening, water recovery, and food stuff processing (Kristinsson and Rasco, 2000; Vazquez and Murado, 2008).

They also play very significant roles in non-food industries. They are used as laundry and dish washing detergents, stone washing jeans, pulp and paper manufacture, leather dehairing and tanning, de-sizing of textile, de-inking of paper and decreasing of hides (Enzymes Technical Association, 2001). Proteases are equally used as therapeutics (Crack *et al.,* 2011)

2.11.2 Classes of Proteases and their Physiological Relevance

Proteases are classified according their origin (animal, plant, microbial), preferential site of cleavage (exo-and endo-peptidases, amino-or carboxyl- peptidases), pH optimum (acid, neutral, alkaline) or mechanism of catalysis (serine, aspartic, sulfhydry or metallo-proteases, glutamic, cysteine and asparagines peptide lyases). Majority of these enzymes are synthesized in the organisms in non-active form (zymogen or proenzymes) and they are activated at the site of their action by proteolytic cleavage of their molecules (Jones 2005; Dubey *et al.,* 2007).

Jones (2005) reported four distinct mechanistic classes of proteases. Here, each protease class is differentiated by its mode of catalysis and its inhibition by one or more characteristic class specific inhibitors. Rawlings *et al.* (2012) classified proteases based on the catalytic mechanism and presence of amino acid residues at the active site as; aspartic, cysteine, glutamic,

metallo, asparagines, serine, threonine and proteases with mixed or unknown catalytic mechanism.

The enzyme subclass of proteases (EC 3.4) is in turn divided into sub subclasses. Enzymes belonging to sub-subclass EC 3.4.21 (serine) possess a Ser residue in the active site; EC 3.4.22 (cysteine) have a Cys residue instead; EC 3.4.23 (aspartic) depends on an Asp residue for their catalytic activity; and those belonging to EC 3.4.24 (metalloproteases) use a metal ion (normally Zn^{2+}) in their catalytic mechanism (IUBMB, 1992; Jisha *et al*., 2013). At present, researchers have identified seven (7) broad groups according to the character and catalytic active site and condition of action. These groups include; serine, threonine, cysteine, aspartate, glutamic acid, asparagine peptide lyases and metalloprotease (Dubey *et al*., 2007; Oda, 2012).

2.11.2.1 Cysteine Proteases

They are found in higher plants where they are involved in protein breakdown. These groups exert their catalytic activity via a cysteine residue present in their active sites. They use cysteine thiol. Catalysis occurs by the formation of a covalent intermediate between the sulphur atom of the protease cysteine residue (that is, the nucleophile or electron donor species) and the substrate molecules carbonyl group (that is the target electron pair residue of the substrate protein molecule). This class of protease is specifically inhibited by trans-expoxsuccinyl- l - leudcylamido-(4-guanidino) butane (E-64) and stimulated by reducing agents such as dithiothreitol (DTT) (Cleland's reagent) and beta (β)- mercaptoethanol (Oda, 2012).

2.11.2.2 Metallo Proteases

Metalloproteases require the presence of metal ion (usually zinc) at their active sites and specifically inhibited by the metal ion chelator 1, 10 phenanthroline which has a high affinity for zinc. These enzymes do not induce hydrolysis via the formation of covalent intermediates. Instead they use a mechanism whereby their metal ion is electrophilically bound to a molecule of

H_20, thus polarizing it and creating a strong nucleophilic draw to attract and hydrolyze the target protein through its carboxyl group (Beynon and Bond, 2001; Dubey *et al*., 2007).

2.11.2.3 Aspartic Proteases

These proteases, as with the metallproteases, catalyze substrate hydrolysis without the formation of covalent intermediates. Instead, hydrolysis is brought about by the action of two active sites- the aspartic side chains and an active site bound water molecule (again, the H_20 molecule acts as the nucleophile). These groups of proteases are specifically inhibited by pepstatin A. They use an aspartate carboxylic acid (Oda, 2012).

2.11.2.4 Serine Proteases

This mechanistic class is probably the most widely studied protease class in the field of enzymology (Beynon and Bond, 2001). They use a serine alcohol. These enzymes catalyze substrate hydrolysis via the hydroxyl group of the serine residue at their active sites in a very similar manner to that of the cysteine proteases; that is through the formation of covalent intermediates. The characteristics class specific inhibitors of serine proteases (and to a lesser extent, the cysteine proteases) are phenylmethylsulphonylfluoride (PMSF) and diisopropylfluorophosphate (DFP). However, p-amidinophenylmethanesulfonylfluoride (APMSF), 4-(2-aminoethyl) benzenesulphonylfluoride (AESBSF), antipain, aprotinin, chymostatin and soybean trypsin inhibitor (STI) are also frequently employed (Oda, 2012).

2.11.2.5 Threonine Proteases

This is a family of proteolytic enzyme harbouring a threonine (Thr) residue within the active site. The prototype member of this class of enzymes are the catalytic subunits of the proteasome, however the acyltransferase convergently evolved the same active site geometry and mechanism. It uses the secondary alcohol of their N-terminal threonine as a nucleophile to

perform catalysis (Cheng and Grishin, 2005). Threonine must be N-terminal since the terminal amine of some residues act as a general base by polarizing an order of H_2O which deprotonates the alcohol to increase its reactivity as a nucleophile. Catalysis takes place in two steps: First, the nucleophile attacks the substrate to form a covalent acyl-enzyme intermediate releasing the first product. Secondly, the intermediate is hydrolyzed by water to regenerate the free enzyme and release the second product (Ekici *et al.,* 2008).

2.11.2.6 Glutamic Proteases

These are a group of proteolytic enzymes containing a glutamic acid residue within the active site. This type was first described in 2004 and became the 6^{th} catalytic type of protease. This mechanistic class uses a glutamate carboxylic acid (Fujinaga *et al.,* 2004). Members of these groups had been previously assumed to be an aspartate protease, but structural determination showed they belong to a novel protease family. These groups are found primarily in pathogenic fungi affecting plant and humans (Oda, 2012).

2.11.2.7 Asparagine Peptide Lyases

A seventh catalytic type of proteolytic enzyme, asparagine peptide lyase, was described in 2011. These enzymes use asparagines to perform an elimination reaction (not requiring water). Its proteolytic mechanism is unusual since, rather than hydrolysis, it performs an elimination reaction (Rawlings *et al.,* 2011). During this reaction, the catalyzed asparagine forms a cyclic chemical structure that cleaves itself at asparagines residues in proteins under the right conditions. Given its fundamentally different mechanism, its inclusion as a peptidase may be debatable (Rawlings *et al*., 2011).

2.11.3 The Role of Proteases in Malting

During seed germination, proteases provide precursors for the synthesis of new proteins (the soluble nitrogen) required not only for the growing embryo that triggers off the activation of

latent enzymes and synthesis of many hydrolytic enzymes which require amino acid, by hydrolyzing endosperm storage proteins. In brewing, it catalyses the degradation of large, insoluble storage proteins into soluble proteins, peptides and amino acids, which affect haze formation, head formation and retention and yeast nutrition (Ogbonna *et al.*, 2003).

Proteases have a myriad of biological responsibilities and have major roles in grains germination (Jones, 2005). However, despite the huge body of research dedicated to the protease enzymes as a whole, comparatively little research has been carried out into their functions in the germinating grain. Jones *et al.* (2000) reported that the levels of protease activity in mature resting grains is low, a situation which changes significantly at the onset of germination with protease activity beginning to appear at day one and reaching its maximum by approximately day three (3) to four (4) of the malting process (depending upon the malting parameters). Jones (2005) investigated the effects of pH and class-specific protease inhibitors on the levels of storage protein hydrolysis during grain germination and revealed strong evidence for the cysteine class proteases playing a key role in the degradation of proteins, thus making them very important to the production of high quality malt.

Proteases are vital components in the malting process as not only do they provide yeast with a source of free nitrogen, sulphur and amino acids, but are also important contributors to the clarity, flavors and filterability of beer and are also involved in beer foam stability and haze formation (Bamforth, 2009). Their involvement in these processes is a direct result of their roles in storage protein solubilisation, a process which releases the proteins and amino acids that affect these aspects of beer and whisky quality. Furthermore, protease inhibitors such as the serine protease inhibitor protein z, have been implicated as important components of beer foam stability. Thus, a full knowledge of the protease spectrum of the germinating grain is an important and potentially valuable tool for the brewing and distilling industries. This will not only result in the production of higher quality products, but also possible changes to the malting

process which could ultimately result in more cost effective malting practice (Robinson *et al.,* 2007)

Jones *et al.* (2000) reported that high temperature involved in the process of kilning resulted in the inactivation of proteolytic enzymes. In contrast, the same authors in their comparative studies of the proteolytic activities of green malt and malt kilned up to 85^{o}C, claimed that is not true, because there is no measurable effect on the levels of proteolysis between the two malts (Jones *et al.*, 2000). Thus, it indicates that kilning does not lead to protease inactivation within the grain, rather at 85^{o}C there was increase in the level of protease activity within the grains.

Proteases are found in all tissues of higher plants where they are involved in not only storage protein degradation and formation (through hydrolysis and post-transcriptional modifications) but also in apoptosis and organ senescence (aging), response to oxidative stress cell cycle control, flower development, morphogenesis and embryogenesis (Salas *et al.*, 2008).

It was reported that not all cysteine proteases are synthesized *de novo* during germination. Some cysteine and aspartic proteases are already present in the mature grain and are stored in specially designed organelles within the aleurone layer (protein storage vacuoles) and are released into the endosperm during germination in response to GA signaling. Enzymes from these protein storage vacuoles have been implicated in the mobilization of the grain's protein stores during germination (Mukhtar and Haq, 2012).

Among the proteases, alkaline (serine) proteases account for a major share of the enzyme market all over the world (Amoozegar *et al*., 2007). Alkaline proteases are those proteases that are active in the neutral to alkaline pH range. They have a serine centre (serine protease) or are of metallo type (metallo protease) (Gupta and Lorenz, 2002). Serine proteases are of considerable interest in view of their activity and stability at alkaline pH and thus they have

applications in a number of industries including detergents, pharmaceuticals, food etc. (Ire *et al*., 2011; Raimi *et al*., 2011).

Serine protease from the latex of *Euphorbia supina* exhibited a strong caseinolytic activity, unlike its stems and leaves, inspite of its broad specificity, cleaved preferentially the carboxylic amino acids residues (Arima *et al*., 2000a). Two serine proteases from barley (*H. vulgare* L.): hordolisin and subtilisin-like serine protease (SEP-1), have not proven important in degradation of storage proteins- hordein during grain germination (Fontanini and Jones, 2002). A serine protease that initiates the proteolysis of β-conglycinin storage proteins in soybean (*G.max*) was isolated from seedling cotyledons (Liu *et al*., 2001). Similarly, a serine protease designated as KLSP, was purified from mature leaves of common beans (*P. vulgaris* L.) which requires Arg in the P1 position for cleavage of the peptide bond (Popovic *et al*., 2002). The involvement of plant serine proteases, namely subtilases in many physiological processes has been well documented (Palmar *et al*., 2002). These processes include; microsporogenesis, symbiosis, hypersensitive response, signal transduction and differentiation, senescence and protein degradation/processing (Beer *et al*., 2000; Coffeen and Wolpert, 2004; Barnaby *et al*., 2004). However, several authors (Beilinson *et al*., 2002; Fontanini and Jones, 2002) have claimed that the roles of the vast majority of subtilisin-like proteases remained unknown.

The activities of proteases in malts have been measured with protein substrates such as hemoglobin, casein, gliadin or gelatin or with synthetic substrates including N-substituted peptides and related peptide derivatives (Agu and Palmer, 2000). Protease activity is influenced by the amino acid sequence of these artificial substrates as well as their tertiary structure. However, it remains uncertain whether assaying for activity with these parameters could reflect proteolysis occurring during malting and mashing (Palmer, 2000). It is therefore more advantageous to have technique whereby cereals and malt enzymes could be investigated using hordein, their main natural substrate. Hordein is an important reserve protein in starchy

endosperm, which is broken down by proteases during germination to provide the nitrogen pool required for embryo growth as well as for the synthesis of many hydrolytic enzymes essential for the mobilization of carbohydrate reserve. The importance of grain protein component as an essential determinant of grain quality for malting, brewing, enzyme and feed industries etc. is widely noted (Mohammed and Addy, 2014).

2.12 Protein Extraction

Proteins in all cereals are grouped into four (4) differentiated by their varying solubility in different solvents. They are albumin (soluble in water and dilute saline solution), globulin (soluble in dilute saline but insoluble in pure water), prolamin (hordein) (soluble in 60-75% ethanol) and glutelin (soluble in weak alkali) (Aehle, 2007). Proteins have been extracted from several sources, including plant, animal and microorganisms, with a lot of challenges. Extraction from plant materials usually presents some difficulties due to non-protein constituents such as phenolic compounds and their tannin derivatives. These compounds interfere with and even prevent the extraction of the proteins (Ogbonna *et al*., 2003). Some antioxidants or extractants such as cysteine hydrochloride (cyst HCl), 2-mercaptoethanol (2-ME), dithiothreitol (DTT), diothioerythreitol (DTE), glutathione, thioglycollate and a host of others are used as phenol-complexing agents or oxidase inhibitors. Several reports favour the use of varying concentrations of cysteine hydrochloride and 2-ME (Ogbonna *et al*., 2003) for the extraction of proteolytic enzymes from malt. The effects of these extractants to a large extent depend on the period of germination. Ogbonna *et al*. (2003) for instance noted that the highest carboxypeptidase activity was recorded from sorghum samples germinated for 5 days extracted with buffer containing cysteine HCl as the extractant. Similarly, the authors observed that the proteinase activity was lowest with a 3-day sample when 2-ME was used as extractant.

2.13 Purification and Characterization of Malt Proteases

Our knowledge of chemical reactions and intermediates in metabolic pathways as well as the regulatory mechanism that operate at level of catalysis emanate, to a great extent from the studies of purified enzymes. Downstream processing for the production of pure enzymes can generally constitute a major percentage of overall production cost, especially if the end purity requirements are stringent (Jaouadi *et al*., 2008). Information concerning their kinetics, co-factors, active sites, structures and mechanisms of action, also requires highly purified enzymes. Purification processes strongly depend on the market, processing cost, final quality and available technology (Proteases Market by Source, 2016). Enzyme extracts must be purified in order to determine their structure and biochemical properties. Proteases constitute one of the most important groups of industrial enzymes to have established roles in detergent, food, leather, waste processing, biological research tool (Gupta *et al*.,2002; Ire *et al*., 2011).

In industrial setting, proteases are purified and sold as products such as drugs, vaccines, diagnostic tools or food additives. Enzyme purification is carried out to isolate a specific enzyme from crude cell extract containing many other macromolecules. This is achieved by precipitation or salting out, dialysis, ion exchange chromatography or chromatography on size exclusion supports, hydrophobic interaction chromatography, gel filtration chromatography, polyacrylamide gel electrophoresis etc. It separates the desired enzyme from hundreds of chemically and physically similar proteins. The need for large scale cost effective purification of protein has resulted in evolution of techniques that provide fast, efficient and economical protocols with fewer processing steps (Amritkar *et al*., 2004).

The physicochemical characteristics of proteases such as thermostability and pH profile should match its application. Hence, the diversity of the application creates the need to search for novel enzymes with novel and improved properties. In view of this, the rate of hydrolysis of proteases depend to large extent on many process conditions; including temperature, pH, nature

of substrate, substrate concentration, enzyme concentration, presence of specific metal ion and other stabilizing agents (Amritkar *et al*., 2004).

Protease production by several members of the gramineae (Ogbonna and Okolo, 2005; Jones and Lookhart, 2005; Arasaratnam and Kalpana, 2010; Kumar and Matta, 2011), several bacteria (Pant *et al.*, 2015), viruses (Allaire *et al.*, 1994; Huang *et al*., 2013), and fungi species (Jani *et al.*, 2012; Mohanasrinivasan *et al.*, 2012), have been reported. Proteases have characteristics of biotechnological interest and have thus become important industrial enzymes (Pant *et al.*, 2015). Microorganisms are known to play a vital role in technology of production of intracellular and extracellular enzymes at industrial scale (Gupta *et al.*, 2002).

Microbial sources are the most exploited owing to the ease of downstream processing to produce microbe-free enzymes, their broad biochemical diversity and their susceptibility to genetic manipulation, among others (Jani *et al*., 2012; Gaur *et al*., 2014; Pant *et al*., 2015). The production of proteases from animal source depends on the availability of livestock for slaughter, which in turn, is governed by political and agricultural policies (Rao *et al*., 1998). Plants source on the other hand, is time-consuming process since it requires much land for cultivation and suitable climatic conditions. However, it could be a potential source of protease due to ease of purification, low levels of interfering substances during purification, and good yield (Rao *et al*., 1998; Konno *et al*., 2004).

Alkaline proteases from microbial sources have been documented by a number of researchers. Jani *et al*. (2012) produced protease from actinomycete isolated from water sample. This alkaline thermophilic organism, *Saccharomonospora viridis* produced extracellular protease at high temperature above 55^{o}C. Maximum production of protease was observed after 96 hours at 55^{o}C and pH maximum of 9.5, indicating that the protease is alkaline as well as thermostable. Similarly, Oyeleke *et al*. (2010) reported maximum protease yield for *A. flavus and A. fumigatus at* 30^{o}C. Optimum pH for the activity of protease was recorded at pH 8 and 5,

respectively. Kusuma *et al*. (2016) had studied protease production by *Bacillus cereus* NC77, a soil isolate grown in casein agar and shaken flask. The enzyme was purified by $(NH_4)_2SO_4$ precipitation, followed by dialysis and further concentrated by ion- exchange chromatography. The SDS-PAGE revealed molecular weight of the protease to be 29 kDa. The enzyme was purified 6.13-fold to give a 75.42% yield relative to the total activity in crude extract and the specific activity of 2.64 U/mg protein. The kinetic property of the purified protease has shown that the optimum pH range was 7 and 8, while optimum temperature was 90^{o}C.

Despite the above findings, there is paucity of information on documented works on protease production by several members of the *gramineae* especially local maize variety. Although, few reports on protease production by some of these *gramineae* are available (Ogbonna *et al*., 2003; Kumar and Matta, 2011), but even when purified, they are quite often not readily available in sufficient quantities for food applications and industrial use.

Report on the kinetic studies of malt amylase and protease of a local rice variety had been recorded by Arasaratnam and Kalpana (2010). They stated that the optimum pH and temperature for the activities of these enzymes were 5 and 7 and 60^{o}C and 50^{o}C, respectively. DeBarros and Larkins (1990) reported the purification and characterization of zein-degrading protease from endosperm of germinating maize seeds. They concluded that the apparent molecular weight and net negative charge of each of these proteases are very similar. Ogbonna *et al*. (2003), reported that sorghum malt variety KSV8 contains two distinct proteases separated by gel filtration chromatography on sephadex G-100, purified and characterized as KSV8-1 (higher molecular weight) and KSV8-11(lower molecular weight). The enzyme was purified 5-fold to give a 14.1% yield relative to the total activity in the crude extract and a final specific activity of 1348.9 U/mg protein. SDS-PAGE showed single migration protein band of relative molecular weight of 16 kDa. Purified protease had optimum activity at 50^{o}C and maximum temperature stability between 30^{o}C and 40^{o}C. The pH optimum was 5 with maximum stability at pH 6. The

protease was inhibited by Ag^{+}, Ca^{2+}, Co^{2+}, Fe^{2+}, Mg^{2+}, iodoacetic acid (IAA) and p-chloromercuribenzoate (p-CMB) and stimulated by Cu^{2+}, Sr^{2+}, phenylmethylsulfonylfluoride (PMSF) and 2-mercaptoethanol (2-ME) while Mn^{2+} and ethylenediaminetetraacetic acid (EDTA) had no effect. Similarly, both the metallo-type protease (Ogbonna and Okolo, 2005) and cysteine-type protease (Ogbonna *et al*., 2004), have been purified from sorghum malt varieties. The sorghum malt, SK 5912 was purified 8.4- fold to give 13.4% yield relative to the crude activity. The optimum temperature activity was 50^{o}C and pH was 6 (Ogbonna *et al*., 2004). Kumar and Matta (2011), reported on the changing protein profile in developing and germinating barley seeds. Jones and Lookhart (2005) presented comparative reports of the purification of endoproteinases from various cereal grains.

In addition to protease purification from these grain malts, some plants have been reported as potential producers of these enzymes that occur in distinct parts, ranging from the seeds to the latex and the fruits (Antao and Malcata, 2005). They have indeed been extracted from Africa milkbush (*Synadenium grantii* Hook) (Menon *et al*., 2002), *Euphorbia supina* (Arima *et al*., 2000a); from flowers, stems, leaves and roots of *Arabidopsis thaliana* (Hamilton *et al*., 2003) and mushroom (*Hypsizigus marmoreus*); from the storage roots of sweet potato (*Ipomoea batatas* [L.] Lam) and maize (*Zea mays* L.) (Chen *et al*., 2004); from the sprouts of bamboo (*Pleioblastus hindsii* Nakai) (Arima *et al*., 2000); leaves of tobacco (*Nicotana tobacum* L.) (Mesdaghi and Dietz, 2000) and common bean (*Phaseolus vulgaris* L. cv Cesnjevec) (Popovic *et al*., 2002).

Serine protease has been isolated and characterized from *S. grantii* Hook by a combination of ammonium sulfate precipitation, gel filtration on Sephacryl S-200 anion exchange chromatography on resource Q-column. The enzyme had optimum activity and stability at pH 7 and temperature at 60^{o}C. It had molecular weight of 76 ± 2 KDa and was completely inhibited by PMSF and diethyl pyrocarbonate (Menon *et al*., 2002). The stability of

this enzyme at neutral pH is essential of their use in removal of protein haze in brewing industry and meat tenderization. Arima *et al*. (2000a) had purified serine protease B from *E. supina* using ion exchange on DEAE-Sepharose, gel filtration on Sephacryl S-300. The protease had molecular weight of 80 KDa and optimum pH at 8 with casein as substrate. The enzyme exhibited broad specificity at P1 position; preference for C-terminal sides of hydrophobic amino acids and was inhibited by diisopropylfluorophosphate (DFP). An alkaline serine protease of this nature has been found useful as key ingredient in detergent industry (Raimi *et al*., 2011).

Similarly, Ara 12 was purified from the tissue of *Arabidopsis thaliana*, which showed optimum pH at 5 and temperature at 80^{o}C. Its molecular weight (MW) was 76.1 KDa and was inhibited by DFP, PMSF and soybean trypsin inhibitor (STI), after purification by combination of anion-exchange chromatography, hydrophobic interaction chromatography (HIC) on phenyl-Sepharose (Hamilton *et al*., 2003). This protease is particularly useful in the cheese making industry as it contributes to the hydrolysis of casein during cheese ripening and cause the development of complex flavor and texture. Chen *et al*. (2004) purified a serine protease IBSP82 from storage roots of sweet potato, *Ipomoea batatas* [L.] Lam. This was achieved by combination of ammonium sulfate precipitation, gel filtration on Superdex 75/ Superdex 200 HR, and tricine- sodium SDS-PAGE. The purified protease showed 82 KDa MW and optimum pH activity was 7.9 and temperature optimum at 40^{o}C using bovine hemoglobin as substrate. The enzyme exhibited substrate specificity for hydrophobic and aromatic amino acid residues at p1 position, but was inhibited by PMSF.

Protease purification from maize root had been reported by some workers. James *et al*. (1996) purified serine protease, RSIP from *Zea mays* L., by a combination of ion exchange chromatography on Q-Sepharose, gel filtration on Sephacryl S-200, chromatography on Mono-p and HIC on phenyl Superose. The enzyme gave MW of 59 KDa on SDS-PAGE and 60 KDa on gel filtration. The optimum pH was 6.5 for casein, BSA, and ovalbumin and 6.1 for azocasein.

The enzyme was inhibited by DFP, PMSF, STI and 3,4- dichloroisocumarin. Similarly, MRP (protease 1) was purified also from maize root. The four steps purification which combined ammonium sulfate precipitation, ion exchange chromatography on carboxylmethyl cellulose, DEAE-Sephadex and gel filtration on Sephadex G-100, gave 54 KDa MW. After characterization, the optimum pH was 4 (hemoglobin) and 9-10 (azocasein). The enzyme showed specificity towards protein substrates preference for N-terminal sides with Ala residue and was completely inhibited by PMSF (Goodfellow *et al*., 1993). Ademola and Malomo (2016) have purified and partially characterized proteases from *Citrus sinensis* fruit peel by combination of 95% ammonium sulphate precipitation, dialysis and column chromatography. The enzymes were optimally active at 40 - 48^{o}C, but retained activity at 60 - 70^{o}C. The pH optimum for these proteases was between 7 and 8. The enzymes were stable at neutral to alkaline pH especially between 6 and 9, retaining more than 60% of its activity. The stability of these enzymes at neutral and alkaline pH is essential for their use in a range of commercial applications especially in removal of protein hazes in brewing industry, meat tenderization, fur and leather industries.

CHAPTER THREE

MATERIALS AND METHODS

3.1 MATERIALS

The reagents, substrates, salts, solvents, resins and other chemicals employed in this work were of analytical grades.

3.1.1 Sample Collection

Hybrid maize variety, Oba Super 2 represented as OS2, certified on Ist February, 2017 was obtained from Premier Seed Nigeria Limited, Zaria, Kaduna-State, Nigeria.

3.2 METHODS

3.2.1 Thousand Corn Weight

Thousand corn weight determination was done according to the recommended method of the Institute of Brewing and Distilling (2005). Fifty grammes of the grain sample was weighed into 500 ml beaker after removing foreign matter and half corns. The number of grains sample was counted and recorded. The weight of thousand grains (G) was determined by noting the total weight in one thousand grains and dividing by the sample counted;

$$G = \frac{Wx1000xDM}{Nx100}$$

Where; N- Total number of grains counted

DM- Dry matter percentages (%) of maize

W- Total weight of maize

3.2.2 Moisture Content

The recommended method of the Association of Official Analytical Chemists (AOAC, 2016) was used. Ten (10) grammes of the maize were ground finely. Five grammes of sample were weighed immediately into a moisture dish. The dish was closed and the weight of the dish taken immediately with the sample. The cover was removed and dish placed in a pre-heated Gallenkamp Hot Oven, model Ovb-300-010 for 3 h at 105^{o}C. The lid was replaced and dish allowed to cool in a desiccator for 20 minutes at room temperature. The dish was re-weighed to three decimal places. The moisture percentage (M) of the maize sample was determined by the expression;

$$M = \frac{W1 - W2}{W1}\ x\ 100$$

Where;$w_{1=}$ $Weight\ of\ sample\ before\ drying$

$$w_{2=} Weight\ of\ sample\ after\ drying.$$

The average value gave the percentage moisture content of sample.

3.2.3 Germinative Capacity

The recommended method of the American Association of Cereal Chemists (AACC, 2010) with modifications by Nnamchi *et al*. (2014) was used. Two hundred kernels of maize were steeped for 48 h in 200 ml of 0.75% (v/v) hydrogen peroxide, H_20_2 at 18-21^{o}C. The steep liquor was strained off and replaced with 200 ml of fresh H_20_2 (aq.) at the same temperature and left for additional 24 hours. The steep liquor was again strained off and germinated grains counted. The germination capacity (G.C) in percentage, H_20_2 (%) was determined using the formula;

$$G.C(\%) = \frac{200 - n}{2}$$

$$where; n - number\ of\ corns\ which\ did\ not\ germinate$$

3.2.4 Germination Energy

The recommended method of the Association of Official Analytical Chemists (AOAC, 2016) was applied. One hundred (100) kernels of maize were placed in 200 ml flask and filled with distilled water. Intermittently, floating kernels were forced below the surface of the water. The water was drained off after 24 hours and replaced with fresh water. After 48 hours of steeping, the water was drained off and the kernels rinsed with distilled water and drained again. A sheet of whatman No. 1 filter paper was moistened by dipping it in distilled water for few seconds and allowed to drain. The moistened filter paper was placed on a glass sheet and steeped grains distributed in compact single layers of kernels in the center of one half of the filter paper. The other half of the filter paper was pressed down finally on the layer of the maize kernels and this was placed in a desiccator containing water in the bottom to maintain humidity near saturation. The desiccator was kept on a bench at room temperature. After 24 hours the germinating maize grains were examined and those kernels with roots were removed and counted. The percentage of sprouted kernel at the end of 72 hours was reported as Germination Energy, G.E (%) = 100 – X, where X is the number of grains which did not sprout in 100 grains.

3.2.5 Water Sensitivity

Determination of water sensitivity was done according to the recommended method of the Institute of Brewing (IOB, 1989) as described by Nnamchi *et al*. (2014). Two filter papers were placed in the bottom of a petri-dish and 8 ml of deionized water was added. Four milliliters (4 ml) of deionized water was also added to another petri- dish containing also two filter papers. One hundred (100) kernels of grains were placed in each of the petri-dishes, so that each was in good contact with the wet filter paper. In the 8 ml test, only the vertical side touched the paper to

avoid drowning of the embryo. Each dish was with its lid. The dishes were incubated for 72 hours at room temperature. The difference between the percentage in 4 ml and 8 ml test after 72 hours was reported as water sensitivity percent.

3.2.6 Nitrogen Estimation for Protein Determination

The estimation of nitrogen was done by Kjeldahl method as described by the recommended method the AOAC (2016). Maize sample was ground finely using Buhler maig mill of setting 2. Then, 1.5 grammes of the sample was weighed using aluminum foil and quantitatively transferred into a dried Kjeldahl flask. Ten (10) grammes of mercury, Hg catalyst consisting of; potassium sulphate (K_2SO_4), copper (ii) sulphate pentahydrate ($CuSO_4.5H_2O$) and titanium (iv) oxide (TiO_2) were ground together in the ratio of 100:3:3, respectively and added together with 20 ml of concentrated tetra oxosulphate (vi) acid (H_2SO_4) and gently swirled to mix and wet the content of the flask thoroughly. The flask was placed in an inclined position on a digestion rack and heated for 1h 20 minutes to boil, until the content of the flask become clear. The flask and its content were cooled to room temperature and 200 ml of water were added and content mixed. The mixture was cooled to 24^oC and 25 ml of sodium thiosulphate solution added to precipitate the mercury (Hg). Then, 70 ml of 0.1N sodium hydroxide (NaOH) solution was slowly added so that two distinct layers were formed. The flask was connected to a condenser by the bulb trap and the content was swirled to ensure rapid mixing and heated until all of the ammonia, NH_3 (aq) had distilled into an excess 2% boric acid solution (about 25 ml) containing about 0.5 ml of screened indicator (methyl red/bromocresol green solution). The NH_3 (aq) was titrated with the standard sulphuric acid (Y) to a grey end-point. The moisture content had earlier been determined as described. The percentage nitrogen (N) in dry maize sample was calculated thus;

$$N(\%) = \frac{Y\ x\ 14}{W\ x\ DM}$$

Where; Y= ml of 0.1M acid required to neutralize the ammonia after subtracting reagent blank

W= weight of sample (in gram) taken.

DM= percentage of dry matter.

% Protein on dry weight basis = N x 25.

3.2.7 Fat Content

The fat content of the maize was done according to the method of the Institute of Brewing (IOB, 1989) described by Pearson (2003). Ten grammes of ground maize were quantitatively transferred to lower section of a Jacob's Singer separator flask. Ammonium hydroxide (1.25 ml) was added and the sample mixed thoroughly. Eleven (11) ml of 95% ethyl alcohol was added and the sample shaken vigorously for 30 seconds. Petroleum ether (25 ml) was added and the sample shaken again for another 30 seconds. The separatory flask was stoppered at the upper section. The sample was kept standing until the upper section became very clear. The mixed ether layer was drawn off into a weighed 'fat' flask.

The lipid remnant in the flask was extracted with 5 ml of 95% alcohol and 15 ml of ethyl ether, shaking vigorously after each addition and allowing the mixture to settle. The clear solution was drawn off into the same tarred flask. Water was added to the separator flask and the remaining ether solution was drawn into the same tarred flask as carefully as possible. The ether in the tarred flask was evaporated off after each extraction, slowly on a steam bath while the subsequent extraction was allowed to settle. The outside of the flask was wiped off and the flask placed in a thermostatically controlled oven (Gallenkamp model 046300) at 100-105^{o}C for 5minutes. The flask was weighed with a similar flask as a counter poise after cooling in a desiccator. The fat content of the maize grain was calculated thus;

$$Fat\ content\ (\%) = \frac{weight\ of\ fat\ in\ tarred\ flask}{weight\ of\ maize\ sample} x100$$

3.3 Grain Malting

Malting is a controlled process involving many steps such as, cleaning/sorting, steeping, couching, germination and kilning.

3.3.1 Grain Sorting and Cleaning

The grain sample was sorted and manually cleaned using tray; impurities, broken kernels and foreign matter were also screened out. One (1.0) kg of the sample was surface sterilized by immersion for 40 minutes in sodium hypochlorite (NaOCl) solution having 1% *(v/v*) available chlorine to check microbial contamination and washed several times in tap water (Ogbonna *et al*., 2003). The grains were subsequently drained and washed four (4) times in tap water.

3.3.2 Steeping

Steeping of the sample was done at a temperature of 30^{o}C for different periods; 30, 36, and 42 hours in a ratio of 1:2 (*w/v*) grains to water in a plastic container as described by Bryce *et al*. (2010). The steep cycle involved alternating 12 hours wet steep with 30 minutes air-rest period. The steep water was changed at every cycle to prevent fermentation and microbial growth after which the grains were drained and subjected to germination using the method of Ayernor and Ocloo (2007). The steep water temperature fluctuated between 25 to 28^{o}C.

3.3.3 Couching

After steeping, the grain sample was couched (heaped) on jute bag previously sterilized with dry heat to generate enough heat required for germination to commence. At the end of the steeping cycles, the samples were once again sterilized.

3.3.4 Germination

Germination was conducted in shallow wooden trays with fine mesh floor kept in germination boxes in the dark. Samples were germinated at fluctuating temperature of 25- 30^{o}C.

From the first day of germination to the fifth day, samples were removed and kilned at different temperatures of 45, 50, and 55^{o}C for 30 hours using hot air oven, model Ovb-300-010.

3.3.5 Kilning

Germination was arrested by kilning at different temperature regimes as earlier stated in an oven. Kilned samples were manually de-rooted by rubbing against a sieve or plastic screen and the malt stored for analyses.

3.4 Malt Analyses

The parameters analyzed on the malt include:

3.4.1 Moisture Content

This analysis was done as earlier described in subsection (3.2.2).

3.4.2 Malting Loss

The malting loss (M.L) was determined using the Institute of Brewing (IOB, 1989) method of analysis as described by Nnamchi *et al*. (2014). Twenty (20) grains (unmalted) were counted and weighed (W_1). Also, the same quantities of malted grains were removed from first to fifth day of germination and weighed (W_2). The loss in weight was the difference between the unmalted and malted one (after removal of rootlets and culms). The percentage loss was then calculated thus;

$$Malting\ loss\ (\%) = \frac{W1-W2}{W1}\ x\ 100$$

Where; $w_{1=}\ Weight\ of\ unmalted\ grains$

$w_{2=}Weight\ of\ malted\ grains.$

The experiments were carried out in duplicates and the results were presented as mean ± standard deviation (SD), n = 2, statistically significant at $p < 0.05$. Comparison of means along the column, values followed by the letter "a" indicated significant difference (SD) at $p < 0.05$ while values followed by the letter "b" indicated no significant difference at $p > 0.05$.

3.4.3 Cold Water Extract (CWE)

The CWE of the malt was done according to Institute of Brewing (IOB, 1989) method of analysis as described by Nnamchi *et al*. (2014). Ten grammes of ground malt were digested with 200 ml of distilled water containing 12 ml of 0.1N ammonia for 3 hours at 20^{o}C and stirring at 30 minutes intervals. The solution was filtered through a Whatman No. 1 filter paper and the relative density of the filtrate measured at 15.5^{o}C. The CWE (%) was determined as follows;

$$\text{CWE (\%)} = \frac{G}{3.86}\ x\ 20.$$

Where; G= Excess degree of gravity of the filtrate taking water at 15.5^{o}C as 1000,

i.e G=1000 (S.G - 1) or $\text{CWE (\%)} = \frac{S.G}{3.86}\ x1.000\ x\ 20.$

The experiments were carried out in duplicates and the results were presented as mean ± standard deviation (SD), n = 2, statistically significant at $p < 0.05$. Comparison of means along the column, values followed by letter "a" indicated significant difference at $p < 0.05$ while values followed by the letter "b" indicated no significant difference at $p > 0.05$.

3.4.4 Hot Water Extract (HWE)

The HWE was determined according the recommended methods of the IOB (1989) as described by Nnamchi*et al*. (2014). Fifty (50) grammes of ground malt was measured into 1000 ml beaker and placed in water bath at 65^{o}C preheated for 10- 15 minutes. Distilled water (360 ml) was added into the beaker at a temperature not exceeding 68^{o}C to ensure an initial mash mix

of 65^{o}C. All lumps in the mash were rapidly eliminated by stirring. The mash was kept at a temperature of 65^{o}C in GSL water bath for exactly 1 hour after the initial mix. The beaker was immersed at least to the level of the mash surface and stirred at 10 minutes' interval and was removed from the water bath after 1 hour and quickly cooled to 20^{o}C with ice block. The mash was then transferred to a 515 ml flask through a funnel exactly 25 minutes after removal from the bath, washing out the beaker with water and making up the contents of the flask with distilled water to 515 ml. The contents of the flask were mixed by shaking vigorously for five (5) minutes. Thereafter, the volume of the prepared mash was adjusted to 515 ml as described.

The entire mash was filtered with the first 50 ml of filtrate, returned to the filter and the specific gravity of the filtrate collected was determined at 15.5^{o}C within 1 hour of collection. The HWE expressed in Lintner degrees per kilogram (^{o}L/kg), calculated thus; Extract (as is) = G x 10.13 (Lintner degree/kg)

Where; G = Excess degree of gravity of the filtrate taking water at 15.5^{o}C as 1000

i.e G = 1000 (S.G - 1)

$$\text{Extract (dry)} = \frac{\text{Extract (as is)} \times 100}{100 - \mu}$$

Where; μ = Moisture (%) of sample.

The experiments were carried out in duplicates and the results were presented as mean ± standard deviation (SD), n = 2, statistically significant at $p < 0.05$. Comparison of means along the column, values followed by letter "a" indicated significant difference at $p < 0.05$ while values followed by the letter "b" indicated no significant difference at $p > 0.05$.

3.4.5 Free Alpha (α-) Amino Nitrogen (FAN) (Ninhydrin Method)

The FAN was determined using ninhydrin as described by AOAC (2016). One (1.0) gramme of the malt sample was extracted with 40 ml of 5% trichloroacetic acid (TCA) at 30^{o}C for 1 hour. This was centrifuged using Eppendorf centrifuge 5804 R for 25 minutes at 4000 revolutions per minute (rpm) and 1.0 ml of the clear supernatant was diluted to 25 ml in deionized water. Two (2.0) milliliters of the diluted sample was added to 1.0 ml of ninhydrin colour reagent in a test tube. This was stoppered and placed in boiling water bath for 10 minutes and cooled for 20 minutes at room temperature. Five (5) milliliters of diluting solution (potassium iodate solution) was added, thoroughly mixed and then the absorbance measured at 570 nm within 30 minutes of mixing using Jenway 6405 Uv/vis spectrophotometer against a reference glycine treated as exactly as test solution. The absorbance of diluted glycine standard solution was measured in duplicate samples. Then, FAN (mg/l) was measured as;

$$FAN\ (mg/l) = \frac{Absorbance\ of\ test\ solution\ x\ 2\ x\ dilution\ factor}{Mean\ \ Absorbance\ of\ standard\ solution}$$

The experiments were carried out in duplicates and the results were presented as mean ± standard deviation (SD), n = 2, statistically significant at $p < 0.05$. Comparison of means along the column, values followed by letter "a" indicated significant difference at $p < 0.05$ while values followed by the letter "b" indicated no significant difference at $p > 0.05$.

3.4.6 Diastatic Power (D.P) in Degree Lintner (^{o}L)

The D.P was determined using Fehling's solution according to the recommended method of the Institute of Brewing (IOB 1989) as described by Eneje *et al*. (2012) and diastatic power reported as degree Lintner (^{o}L). Fischer chemical starch was used to prepare 2% starch buffered (0.1 M acetate buffer, pH 5.0) solution for this determination. A malt infusion was prepared as done in CWE, but the extract was allowed to settle and not filtered. Three (3) milliliters aliquot of the supernatant was removed using pipette into 100 ml of 2% buffered starch solution at 25^{o}C and contained in a 200 ml flask. The flask was shaken and left at this temperature for 1 hour.

The reaction was terminated by adding 30 ml of 0.1N of NaOH and the volume made up to 200 ml at 20^{o}C with distilled water.

Five (5) milliliters of mixed Fehling's solutions A and B were pipetted into a 150 ml narrow- necked boiling flask. The digested solution was added to the cold Fehling's solution from a burette and the flask content was mixed and boiled with moderate heat for 2 minutes. The boiling and addition of starch solution was continued for another 2 minutes until the blue colour of Fehling solution was discharged. Three (3) drops of methylene blue indicator was added and the titration completed. The end-point was indicated by the decolorization of the methylene blue indicator and the reaction liquid, just became reddish or pink. The blank was earlier prepared by titrating the undiluted 2% starch solution against 1 ml of mixed Fehling's A and 2 ml Fehling's solution B using the technique described under the method above with methylene blue indicator. The D.P expressed in degree Lintner (^{o}L) was calculated as thus;

$$DP = \frac{2000 - 200}{XY - SX}$$

$$where; X = number\ of\ ml\ of\ malt\ extract\ used\ to\ digest\ starch,$$

$$Y = number\ of\ ml\ of\ converted\ starch\ to\ reduce\ 5\ ml\ of\ Fehling's\ solution,$$

$$S = titer\ of\ the\ starch\ blank.$$

The experiments were carried out in duplicates and the results were presented as mean ± standard deviation (SD), n = 2, statistically significant at $p < 0.05$. Comparison of means along the column, values followed by letter "a" indicated significant difference at $p < 0.05$ while values followed by the letter "b" indicated no significant difference at $p > 0.05$.

3.4.7 Total Soluble Nitrogen (TSN) (%)

The total soluble nitrogen (amount of the total nitrogen solublized during mashing) was determined by Kjeldahl method as described in total nitrogen estimation with slight modification

by Ijasan *et al*. (2011). The malt infusion was prepared by digesting 10 grammes of the malt with 50 ml of distilled water and 10 ml of the wort was used to determine the total soluble nitrogen of the sample as calculated thus:

$$TSN\ (\%) = \frac{Titer\ (ml)x\ 14.008}{DM}$$

$$where; TSN = total\ soluble\ nitrogen\ content\ of\ malt\ in\ \%\ dry\ matter$$

$$DM = percentage\ dry\ matter.$$

$$For\ nitrogen\ in\ any\ material, 1.0\ ml\ of\ titre = 14.008\ mg\ nitrogen$$

The experiments were carried out in duplicates and the results were presented as mean ± standard deviation (SD), n = 2, statistically significant at $p < 0.05$. Comparison of means along the column, values followed by letter "a" indicated significant difference at $p < 0.05$ while values followed by the letter "b" indicated no significant difference at $p > 0.05$.

3.4.8 Cold Water Soluble Protein (CWS-P)

The soluble protein in the cold water extract was measured using the Biuret reagent expressed as: mg CWS- Protein % dry matter (Ogbonna *et al*., 2001). The Biuret reagent was prepared by dissolving 0.3 g sodium potassium tartrate in 300 ml of 0.5 M potassium hydroxide and 200 ml of 0.024 M copper (ii) sulphate pentahydrate, $CuS0_4.5H_20$. The mixture was diluted with 500 ml of iso-propanol and kept in an amber bottle. The Biuret reagent (5 ml) was added to 2.5 ml of the sample, mixed and placed in water bath at 40^{o}C for 30minutes and cooled at room temperature for 5 minutes. The absorbance was read within 30 minutes at 550 nm using Jenway 6405 Uv/vis spectrophotometer against a blank without copper (ii) sulphate pentahydrate in the reagent solution. The CWS-P was calculated as expressed:

$$CWS - P\ (\%) = 0.855\ x\ Absorbance\ at\ 550nm$$

The experiments were carried out in duplicates and the results were presented as mean ± standard deviation (SD), n = 2, statistically significant at $p < 0.05$. Comparison of means along

the column, values followed by letter "a" indicated significant difference at $p < 0.05$ while values followed by the letter "b" indicated no significant difference at $p > 0.05$.

3.4.9. Total Non-Protein Nitrogen (TNPN)

The TNPN content of the malt was determined using supernatants from malt FAN analysis. The TNPN in the extract was measured using the semi-micro Kjeldahl distillation method as reported by Pearson (2003). One (1.0) milliliter of the extract sample was digested in small digestion flask using 0.8 g catalyst mixture made up of 3.5% (*w/v*) copper sulphate, 0.028 g; 0.5% (w/v) selenium dioxide, 0.004 g and 2 ml concentrated H_2SO_4 placed on a preheated hot plate. Sample and catalyst mixture in the acid were heated until fuming stopped and greenish clear solution appeared. The sample was removed, cooled, and the contents diluted with deionized water (25 ml). Diluted sample was transferred to a steam-out apparatus in a Marks Harmstill distillation flask and 15 ml of 40% (w/v) NaOH added. Ammonia steam was distilled into 2% (w/v) boric acid solution (10 ml) containing 0.2 ml screened methyl red indicator. The distillate was then titrated against 0.02 M hydrochloric acid, HCl. The percentage TNPN was calculated thus;

$$TNPN\ (\%) = \frac{Titre\ x\ 100\ x\ Normality\ of\ HCl\ x\ 0.014}{Weight\ of\ sample}$$

The experiments were carried out in duplicates and the results were presented as mean ± standard deviation (SD), n = 2, statistically significant at $p < 0.05$. Comparison of means along the column, values followed by letter "a" indicated significant difference at $p < 0.05$ while values followed by the letter "b" indicated no significant difference at $p > 0.05$.

3.5 Enzyme Extraction

Enzyme extraction was done as described by Ogbonna *et al*. (2003). Maize malt, 100 g, was extracted with 250 ml of 0.1 M citrate- phosphate buffer, pH 7.0 containing: 0.5% (*w/v*) cysteine hydrochloride (cyst. HCl) and 1.075 g sodium chloride (NaCl). The mixture was

agitated in a rotary shaker at room temperature at 120 rpm for 2 hours. The extract was centrifuged with Eppendorf centrifuge 5804 R at 5000 rpm for 30 minutes at 4^{o}C and the supernatant was recovered as crude protease.

3.6 Protease Activity Determination

Determination of the proteolytic activity was carried out using the modification of the methods of Lowry *et al*. (1951) as described by Upton and Fogarty (1977). The reaction mixture consisted of 0.1 ml of 1.0% (*w/v*) casein in 0.1 M citrate-phosphate buffer, pH 7.0 and 0.1 ml of the crude enzyme solution. Incubation of the enzyme/substrate mixture was at 50^{o}C for 30 minutes in a GSL water bath. The reaction was terminated by the addition of 2.0 ml of 5% (*w/v*) trichloroacetic acid (TCA). The reaction mixture was filtered with Whatman No.1 filter paper and 1.0 ml of the filtrate collected. Thereafter, 5 ml of 0.5 M sodium trioxocarbonate (iv), (Na_2CO_3) solution was added to 1 ml of the filtrate, followed by the addition of 0.5 ml of three (3)-fold diluted Folin-Ciocalteau reagent. The mixture was allowed to stand at room temperature for 30 minutes prior to the measurement of absorbance at 660 nm using a Jenway 6405 Uv/Vis-Spectrophotometer (EU). A blank tube was prepared following the above procedure but the TCA solution was added before the enzyme. One unit (U) of the protease activity is the amount of the enzyme required to liberate one milligramme (1 mg) of tyrosine from the substrate (casein) per minute at 50^{o}C under the condition of the assay.

3.7 Protein Measurement

Protein content was measured the dye-binding method of Bradford (1976) as described by Hammond and Kruger (1988), using bovine serum albumin (BSA) as the standard and measuring the the absorbance at 595 nm. The Bradford reagent was prepared by dissolving 50 mg of Commasie Blue 250G (Serva) in 47.5 ml of ethanol. Fifty (50) milliliters of 85% phosphoric acid were added and the volume made up to 500 ml with deionized water. The reagent was filtered through filter paper and stored in a refrigerator in a brown bottle. According

to Bradford (1976), one (1) volume of the Bradford reagent was diluted with four (4) volumes of deionized water (1 in 5 dilution) prior to use.

The protein content was carried out by preparing different concentrations (0.2- 1.0 mg) of the stock BSA (1mg/ml). Then, 2.5 ml of 5-fold diluted Bradford reagent (with water) was added respectively to 0.05 ml of each concentration and to the tube that had no BSA (control). At the same time, equal volume (0.05 ml) of the enzymes was added to the mixture. The protein contents of the reaction mixtures were measured at OD 595 nm after 5 minutes using a Jenway 6405 Uv/Vis Spectrophotometer against a blank (deionized water). The values obtained from BSA (0-1.0 mg/ml) were used to prepared a standard curve. The protein contents of the samples were then extrapolated from the standard curve with the values obtained from absorbance at 595 nm.

3.8 Enzyme Purification

Crude extract was partially purified by standard protein purification methods which include; ammonium sulphate, precipitation (salting out), followed by dialysis at 4^{o}C against 5 M sucrose solution, ion- exchange chromatography (IEC) on carboxymethyl (CM) Sepharose and hydrophobic interaction chromatography (HIC) on phenyl Sepharose CL-4B gel.

3.8.1 Ammonium Sulphate Precipitation Profile Studies

The ammonium sulphate precipitation profile studies were carried out by pipetting 10 ml of the crude enzyme into each of nine (9) different tubes. Then, 1.06, 1.64, 2.26, 2.91, 3.61, 4.36, 5.16, and 6.03 grammes of ammonium sulphate salt were measured out into each of the enzyme tubes and mixed gently to achieve homogeneity. Each mixture corresponds to 20, 30, 40, 50, 60, 70, 80, and 90% ammonium sulphate saturations, respectively. The enzyme mixture was concentrated overnight at 4^{o}C, and then centrifuged and the supernatants were decanted into 9 different tubes. The pellets were re-dissolved with 10 ml of 0.1 M citrate phosphate buffer, pH

7.0. The supernatants and precipitates were assayed for enzyme activities. A plot of enzyme activity against percentage saturation was carried out to obtain the ammonium sulphate saturation suitable to precipitate protein with highest activity. The saturation that precipitated protein with highest activity was used and the mass of the salt was scaled up to correspond with volume of the crude protein (Duongly and Gabelli, 2014).

3.8.2 Enzyme Concentration

The purification process was initiated by concentrating the enzyme overnight at $4^{o}C$ with 70% saturated solid $(NH_4)_2SO_4$ which precipitated the protease. This was carried out by dissolving gradually 89.38 g of the solid ammonium sulphate in 205 ml of crude extract in a bowl of ice- block. A magnetic stirrer was inserted to achieve homogeneity. The mixture was later centrifuged and the supernatant decanted to collect the precipitate. The pellet was re-dissolved with 10 ml of 0.1 M citrate-phosphate buffer, pH 7.0 and 20 ml of the enzyme was recovered. The supernatant and precipitate were assayed for enzyme activity and protein content measured by direct determination of the optical density (OD) at 280 nm using Jenway 6405 Uv/vis spectrophotometer against a water blank.

3.8.3 Ion-Exchange Chromatography on Carboxymethyl (CM) Sepharose

The ion- exchange chromatography on carboxymethyl (CM) Sepharose purification procedure was carried out according to the method described by Khan *et al*. (2008). The ion-exchanger (CM Sepharose) was mixed thoroughly before being packed into the column (Pharmacia: 1.8 × 26.0cm). Then, 100 ml of 0.5 M sodium hydroxide (NaOH) was used to sanitize the column, and was washed off with 100 ml of distilled water. The column was later equilibrated with 100 ml of 0.1 M citrate-phosphate buffer, pH 7. After a wash with 100 ml of this buffer, about 20 ml of the enzyme was applied on a CM Sepharose (fast flow) column and eluted with 250 ml of the same buffer and equal volume of a linear gradient of 0.5 M sodium chloride (NaCl) solution at a flow rate of 70 ml/hour. A total of fourteen (14) tubes were

collected before the ion-exchange gradient was connected. Subsequent samples were taken until 36 fractions (10 ml each) were collected and assayed for proteolytic activity. The protein content was determined as earlier stated. The active fractions (4-11), designated Oba super 2A (OS2A) and fractions (26-31), designated Oba super 2B (OS2B), which showed high enzyme activities were pooled separately and re-concentrated by overnight dialysis at 4^{o}C against 5 M sucrose solution. The protein content of each dialysate was determined and the enzyme activity assayed and subjected to further purification using the hydrophobic interaction resin.

3.8.4 Hydrophobic Interaction Chromatography (HIC) on Phenyl Sepharose CL-4B Gel

In hydrophobic interaction chromatography, the column (2.3 x 8.5cm) packed with phenyl Sepharose CL-4B gel (Pharmacia), was sanitized with 0.5 M NaOH (70 ml) and washed with 100 ml of deionized water. The column was made hydrophobic by the application of 3 M NaCl solution in 70 ml of 0.1 M citrate- phosphate buffer, pH 7. The enzymes were equally made hydrophobic by dissolving 0.81 g of 3 M NaCl in 4.6 ml enzyme (for OS2A) and 1.14 g of 3 M NaCl in 6.5 ml enzyme (for OS2B). Elution was done with 100 ml of varying concentrations of NaCl solution, but equal volumes of buffer as follows: 3 M NaCl for fractions 1-7, 2 M NaCl for fractions 8-14, 1 M NaCl for fractions 15-21, 0.5 M NaCl for fractions 22-27, and finally, using the elution buffer alone for fractions 28-36, all at a flow rate of 82 ml/h and 80 ml/h for OS2A and OS2B, respectively. The protein content of the 36 fractions were determined and their enzyme activity assayed. Fractions 26-32 (for OS2A) and fractions 27-32 (OS2B) which showed high enzyme activities were pooled and re-concentrated by overnight dialysis at 4^{o}C against 5 M sucrose solution. The recovered partially purified protease was stored at freezing temperature for kinetic studies (Gaur *et al*., 2014).

3.9 Native Polyacrylamide Gel Electrophoresis (Native PAGE)

The Native polyacrylamide gel electrophoresis (PAGE) of the enzyme was done according to the method of Laemmli (1970). The homogeneity of the partially purified protease was tested using 12% polyacrylamide slab gel in the absence of denaturing agent (sodium dodecyl sulphate, SDS) and reducing agent (dithiothrietol, DTT) (Native PAGE, Sigma Aldrich). Ten (10) microliter (µl) of the enzyme sample in 20 µl of sample buffer, consisting of 1 ml Tris-HCl buffer (0.5 M, pH 6.8), 0.8 ml glycerol, and 0.4 ml of 0.5% (*w/v*) bromophenol blue were mixed thoroughly. Native- PAGE was then performed with a stacking gel consisting of 3.4 ml deionized water, 0.63 ml Tris buffer (1.5 M, pH 6.8), 0.88 ml acrylamide mix (40% *w/v*), 50 µl ammonium per sulphate (APS) (10% *w/v*) and 5 µl tetramethylethylenediamine (TEMED), and a separating gel consisting of 3.3 ml deionized water, 2.5 ml Tris buffer (1.5 M, pH 8.8), 4 ml acrylamide mix (40%, *w/v*), 100 µl APS (10%, *w/v*) and 5 µl TEMED. A tracking dye of 0.5% bromophenol blue sodium salt (Sigma Aldrich) in sample buffer was used to monitor the progress of molecules moving through the gel. A current of 40 mA and 180 volts was used to run the electrophoresis at room temperature until the tracking dye emerged using a FB Fisher Scientific Electrophoresis system (Germany).

Protein in the gel was visualized by staining with Coomassie Brilliant blue stain consisting of 0.125 g Coomassie Brilliant blue R250, 62.5 ml methanol, 12.5 ml glacial acetic acid and 50 ml deionized water for 2-3 hours. Dye that is bound to protein is removed by destaining in solution consisting of 50 ml methanol, 50 ml glacial acetic acid and 400 ml deionized water.

3.10 Estimation of the Relative Molecular Weight

The relative molecular weight of the partially purified enzyme was determined by Native gel electrophoresis. The average molecular weight was estimated using UNSCAN-IT software version 6.1 (Silk Scientific). The molecular mass markers used were an assortment of proteins of

various molecular weights (Sigma Aldrich cloudbursts electrophoretic markers); lysozyme (14.2 KDa), soybean trypsin inhibitor (21 K Da), trypsinogen (bovine) (24 K Da), egg albumin (45 KDa), bovine serum albumin (monomer) (66 K Da) and bovine serum albumin (dimer) (132 KDa). Proteins were detected using a calibration curve drawn on the basis of the relative mobility values and molecular weights of these reference proteins.

3.11 Kinetic Studies

All the experiments in these kinetic studies were conducted in duplicates and the mean values presented.

3.11.1 pH Activity and Stability Determination

The effect of pH on the activity of the partially purified protease was studied over pH ranges of 3.0-11.0 using different buffers: 0.1 M citrate- phosphate buffer pH (3.0-7.0), and 0.1 M phosphate-NaOH buffer (pH 8 -11) as described by Sharma *et al*. (2014). The reaction mixture was comprised of 0.1 ml of the partially purified enzyme plus 0.1 ml of 1.0% (*w/v*) casein in buffers pH 3-11. The mixture was incubated for 30 minutes at 50^{o}C and enzyme activity was determined using the standard method as earlier described.

The pH stability profile of the protease was determined by pre-incubating equal volume of the enzyme (0.1 ml) with each of the prepared buffers (pH 3-11) at room temperature for 3 hours. Thereafter, equal volume of 1.0% *(w/v*) casein in appropriate buffers was added to the pre-incubated mixture and further incubated at 50^{o}C for 30 minutes. The residual enzyme activity was then assayed as stated

3.11.2 Temperature Activity and Stability Determination

The temperature activity and stability profiles of the partially proteases were determined over the range of 30-90^{o}C by adding 0.1 ml of the enzyme to 0.1 ml of 1.0% (*w/v*) casein in 0.1 M phosphate-NaOH buffer, pH 9.0 and incubated for 30 minutes at the test temperatures as described by Sharma *et al*. (2014). Thereafter, each reaction mixture was promptly chilled in ice-block and the residual activity determined as described.

The enzyme thermostability was determined by pre-incubating separately 0.1 ml of the enzyme at various temperatures between 30 and 90^{o}C for 30 minutes and then promptly chilled on ice or freezing temperature. Then equal volume of 1.0% (*w/v*) casein in 0.1 M phosphate-NaOH buffer, pH 9.0 was added and each reaction mixture was further incubated at 50^{o}C for 30 minutes after which the residual activity was assayed.

3.11.3 Effect of Metal Ions on Enzyme Activity

The effects of the cations: Fe^{2+}, Ca^{2+}, Co^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+}, Ba^{2+}, Ag^{+}, Zn^{2+}, and Cu^{2+}, on the activity of the partially purified proteases were evaluated as described by Harshada and Geeta, (2015). The enzyme and the individual metal chloride, sulphate or nitrate (5 mM) were pre-incubated in 0.1 M phosphate-NaOH buffer pH 9.0 at 30^{o}C for 3 hours. Then, another equal volume of 1.0% (*w/v*) casein in buffer was added to the pre-incubated mixture prior to re-incubation at 50^{o}C for 30 minutes and assayed for their residual activity. The control tube which had no metal ion was taken as 100%.

3.11.4 Effect of Some Inhibitors on Enzyme Activity

The effects of some inhibitors (1.0 mM): ethylenediamine tetraacetic acid (EDTA), phenylmethyl sulfonylfluoride (PMSF), iodoacetic acid (IAA), para-chloromercuribenzoate (p-CMB), ethylene bis (oxyethylene nitrilo) tetra- acetic acid (EGTA) on the activity of the purified enzyme were examined. The enzyme (0.1 ml) was pre- incubated with 0.1 ml of 1.0 mM of each

inhibitor at 30^{o}C for 10 minutes. Thereafter, equal volume of 1.0% (*w/v*) casein in 0.1 M phosphate- NaOH buffer, pH 9.0 was added to the reaction mixture and incubated for 30 minutes at 50^{o}C to determine the activity. The control was pre-incubated with inhibitor- free enzyme and the residual activity was calculated (Sharma and Kantishree, 2011).

3.11.5 Effect of Oxidizing Agents (O. As) on the Enzyme Activity

The effect of O. As on partially purified protease was done by incubating with different concentrations: 1, 2, 3, 4, and 5% (*v/v*) of hydrogen peroxide, H_2O_2 and dimethyl sulfoxide (DMSO), respectively. Equal volume of the enzyme solution (0.1 ml) and various concentrations of the O. As were incubated at room temperature for 30 minutes. Thereafter, equal volumes of 1.0% (*w/v*) casein in 0.1 M phosphate-NaOH were added and incubated at optimal conditions for enzyme activity and assayed for activity (Ananthan, 2014).

3.11.6 Effect of Reducing Agents (R. As) on the Enzyme Activity

The impact of R. As on the activity of enzyme was studied by incubating 1, 2, 3, 4, 5% (*v/v*) of dithiothreitol (DTT), and 2-mercaptoethanol (2-ME), respectively. The reaction mixture comprises: 0.1 ml of enzyme plus 0.1 ml of various percentages of R. As and incubated for 30 minutes at room temperature. Equal volume of 1.0% (*w/v*) casein in 0.1 M phosphate-NaOH was added and incubated at optimal conditions for enzyme activity and then assayed for activity (Kunamneni *et al*., 2003).

3.11.7 Effect of Detergents and Surfactants on Enzyme Activity

The compatibility and stability of the partially purified proteases with surfactants and detergents were studied. A modified method of Francois and More (2015) was used. The ionic and non-ionic detergents used were Klin, Ariel, Omo while the non-ionic surfactants were Triton X-100 (trioctyl phenoxy polyethoxy ethanol), Tween - 80 (polyethylene glycol sorbitan monooleate), and Tween -20 (polyoxyethylene sorbitan monooleate) and anionic surfactant used

is sodium dodecyl sulphate (SDS). These detergents 1.0% (*w/v*) and surfactants 1.0% (*v/v*) were prepared and equal volumes (0.1 ml) pre-incubated with the enzyme at room temperature for 30 minutes prior to the addition of equal volume of 1.0% casein in 0.1 M phosphate-NaOH buffer, pH 9. The reaction mixture was re-incubated for 30 minutes at 50^{o}C and the activity assayed. The control sample (tube without any detergent/surfactant) was taken as 100%.

3.11.8 De- Staining Property of Proteases

The de-staining property of the partially purified enzymes were studied by soaking six pieces of white cotton clothes (4cm × 4cm) in blood (0.1 ml) for 1 hour and then allowed to dry. The stained materials were inserted into three different flasks designated A, B and C for OS2A and D, E and F for OS2B separately as described by Kunamneni *et al*. (2003). Flask A contained 100 ml of distilled water plus stained material without detergent and enzyme. Flask B contained 100 ml of distilled water, stained material and 1 ml of detergent (7 mg/ml). Flask C contained 100 ml of distilled water, stained material, 1 ml of detergent (7 mg/ml) and 1 ml of OS2A partially purified protease. The same treatment procedures were applied for OS2B. The untreated cloth materials stained with blood were taken as control for proteases. The flasks were incubated at 40^{o}C for 15 minutes and the incubated clothes were rinsed with water and dried. At the end of drying, visual examination of various pieces showed the effect of the enzyme in the removal of stains.

3.11.9 Relative Rates of Substrates Hydrolysis

The substrates used were casein, egg albumin (EA), bovine serum albumin (BSA) and gelatin. Each substrate, 1.0 % (*w/v*) in 0.1 M phosphate - NaOH buffer pH 9 was separately incubated with equal volume (0.1 ml) of the partially purified enzyme at 50^{o}C for 30 minutes. Then, their relative rates of hydrolyses were determined by assaying for their activities. The substrate in which the enzyme exhibited the highest hydrolytic activity was taken as 100% (Srividya, 2012).

3.11.10 Effect of Substrates Concentrations on Protease Activity

Partially purified protease activities were assayed in reaction containing different concentrations (0-1.0 mg/ml, that is 0.2, 0.4 0.6, 0.8, and 1.0 mg/ml) of the test protein substrates; casein, egg albumin (EA), gelatin, and bovine serum albumin (BSA) in 0.1 M phosphate-NaOH buffer, pH 9 as described by Srividya, (2012). Then, equal volume of the enzyme (0.1 ml) was incubated with the various substrate concentrations for 30 minutes at 50^{o}C to assay their activities (Balakireva *et al*., 2018). The kinetic constants (maximum velocity, V_{max} and Michaelis-Menten constant, *Km*) of the enzyme for each substrate was estimated by double reciprocal plots of the data according to Lineweaver-Burk plot (1934) as described by Lehninger (1976).

3.12 Statistical Analysis

The data obtained in this study were subjected into statistical analyses using IBM statistical package for social science (SPSS) statistics 22 software. Results were presented as mean of duplicates ± standard deviation (± S.D), n = 2 and significant level was defined at $p < 0.05$. Analysis of variance was performed by one-way ANOVA procedures. Comparisons of the mean values were determined by the least significant difference (LSD) and Duncan's multiple range test (DMRT). The graphs were plotted using Microsoft Office Excel 2016.

CHAPTER FOUR

RESULTS

4.1 Determination of the Properties of the Unmalted Maize Grain

Table 4.1 showed the results of some chemical and physical properties of the unmalted maize variety. The results showed that Oba super 2 (OS2) variety had large 1000 corn weight of 280 grammes. The moisture content (%), germination energy (%), germinative capacity (%), water sensitivity (%), broken kernel (%), protein content (%) and fat (ether extract) (%) of the unmalted grains recorded were; 11.5, 94, 98, 87, 0.82, 9.06 and 4.20, respectively.

Table 4.1: Some Properties of Unmalted Maize, Oba Super 2 (OS2) Grain Studied

Properties	Oba Super 2 (OS2)

Thousand corn corn weight (g)	280
Moisture (%)	11.5
Germination energy (%)	94
Germinative capacity (%)	98
Water sensitivity (%)	87
Broken kernel (%)	0.82
Protein (N × 6.25) (%)	9.06
Fat (ether extract) (%)	4.20

Key: g = grammes % = percentage

4.2 The Effects of Different Steeping (S) period (hours) and Germination (G) period (days) on Malting Loss (%)

Table 4.2 showed the effects of different steeping period (hours) and germination period (days) on the malting loss (ML) (%) of the grain sample. It is interesting to observe from the results of the malting loss through the roots and shoots growth that these parameters increased

progressively with longer days of germination period at different steeping cycles. The malting losses were slightly higher for the 42 hours (h) steep cycle but the increase in all the cycles were consistent.

At 30 hours (h) steeping, the ML obtained at day 0,1,2,3,4 and 5th germination, were in increasing order (%); 7.10, 10.25, 14.22, 16.68, 19.58 and 20.12, respectively. Similar order of increase was recorded at 36 hours (h) steeping period with 8.10, 10.05, 14.80, 16.70, 20.12 and 21.10 %, at germination period (days) of G_0 to G_5. At 42 hours (h) steeping, the ML (%) of 8.90, 10.50, 14.40, 17.80, 21.90 and 23.0 were recorded between germination time, G_0 to G_5.

The result of analysis of variance (ANOVA) of the effects of steeping period (hours) and germination period (days) on the ML of the grain indicated significant difference ($p < 0.05$) between the parameters [steeping period (hours) and germination period (days)] on Oba super 2 (OS2) maize malt.

The least significant difference (LSD) and Duncan multiple range test (DMRT) revealed that, there was significant difference (increase) ($p < 0.05$) between the germination time at zero (G_0) to fifth day (G_5), steeped at 30 and 36 h, respectively. Also, sample steeped for 42 h showed significant difference ($p< 0.05$) between zero (G_0) to third day (G_3) of germination. However, there was no significant difference ($p> 0.05$) between the fourth (G_4) and fifth (G_5) day of germination.

Table 4.2: Effects of Different Steeping (S) period (hours) and Germination (G) period (days) on Malting Loss (%)

Germination period (days)	S_{30}	S_{36}	S_{40}

	Malting Loss (%)		
G_0	7.10±0.00[a]	8.10±0.00[a]	8.90±0.14[a]
G_1	10.25±0.12[a]	10.05±0.07[a]	10.50±0.14[a]
G_2	14.22±0.14[a]	14.80±0.28[a]	14.40±0.14[a]
G_3	16.68±0.04[a]	16.70±0.42[a]	17.80±0.28[a]
G_4	19.58±0.11[a]	20.12±0.17[a]	21.90±0.14[b]
G_5	20.12±1.14[a]	21.10±0.07[a]	23.00±0.14[b]

Key: S_{30}, S_{36}, S_{42} = Steeping period of 30, 36 and 42 hours; G_0, G_1, G_2, G_3, G_4 and G_5 = Germination period (days); of day zero (0), 1, 2, 3, 4 and 5, respectively.

4.3 Cold Water Extract (CWE) (%) of the Malt

It was observed that the cold water values increased with longer periods of steeping hours and germination days. This parameter reached it maximum value after 4^{th} day of germination, 42 hours of steeping and kilned at $50^{o}C$.

The result of analysis of variance (ANOVA) of the cold water extract (CWE) values indicated significant difference ($p < 0.05$) among the parameters [steeping period (hours), germination period (days) and kilning temperature (^{o}C)] on Oba Super 2 (OS2) maize malt.

However, the least significant difference (LSD) and Duncan multiple range test (DMRT) revealed that, there was no significant difference ($p > 0.05$) among germination period at zero day (G_0) to fifth day (G_5), kilning temperatures of 45 - $55^{o}C$ at steeping hour 30 (S_{30}).

At steeping period of 36 h, there was significant increase ($p < 0.05$) between the kilning temperatures 45 - $55^{o}C$ at germination day zero, while at first to fifth day of germination (G_1 - G_5), there was no significant difference ($p > 0.05$).

At steeping regime of 42 h, LSD and DMRT revealed that no significant difference ($p > 0.05$) exists among kilning temperatures of 50 and 55; 50; 45, 50 and 55 at germination time of G_1, G_2 and G_3, respectively. However, there was significant difference (increase) ($p < 0.05$) among the kilning temperature of 45 - 55; 45; 45 and 55; 45 - $55^{o}C$ at germination days of G_0, G_1, G_2, G_4 and G_5, respectively.

Table 4.3: Cold Water Extract (CWE) (%) of the Malt

Malting Conditions	S_{30}	S_{36}	S_{42}
G_0K_{45}	26.99±0.01[b]	26.08±0.03[a]	26.37±0.05[a]
K_{50}	28.58±0.11[b]	29 75±0.07[a]	28.18±0.07[a]
K_{55}	27.19±0.16[b]	28.84±0.21[a]	28.18±0.07[a]
G_1K_{45}	37.53±0.07[b]	36.86±0.08[b]	37.56±0.06[a]
K_{50}	36.69±0.02[b]	36.07±0.04[b]	38.63±0.04[b]
K_{55}	37.45±0.21[b]	36.88±0.49[b]	38.70±0.14[b]
G_2K_{45}	40.90±0.07[b]	36.50±0.14[b]	43.40±0.14[a]
K_{50}	41.85±0.07[b]	38.80±0.14[b]	46.61±0.01[b]
K_{55}	41.65±0.07[b]	38.98±0.02[b]	42.08±0.04[a]
G_3K_{45}	41.45±0.21[b]	40.52±0.12[b]	47.81±0.01[b]
K_{50}	42.50±0.28[b]	43.14±1.50[b]	47.80±0.14[b]
K_{55}	42.19±0.42[b]	43.35±0.21[b]	46.67±0.04[b]
G_4K_{45}	41.80±0.11[b]	43.51±0.16[b]	49.93±0.05[a]
K_{50}	43.25±1.60[b]	44.57±0.07[b]	51.87±0.05[a]
K_{55}	43.03±2.06[b]	44.42±0.04[b]	50.85±0.07[a]
G_5K_{45}	42.37±0.23[b]	41.32±0.04[b]	50.57±0.05[a]
K_{50}	41.95±0.71[b]	41.82±0.01[b]	49.61±0.16[a]
K_{55}	43.14±1.18[b]	41.17±0.04[b]	49.26±0.06[a]

Key: $S_{30,}$ S_{36}, S_{42} = Steeping period of 30, 36 and 42 hours; $G_{0,}$ G_1, G_2, G_3, G_4 and G_5 = Germination period (days); of day zero (0), 1, 2, 3, 4 and 5, respectively; K_{45}, K_{50} and K_{55} = Kilning temperature (^oC) of 45, 50 and 55, respectively.

4.4 Hot Water Extract (HWE) (%) of the Malt

Table 4.4 showed that the HWE values equally exhibited increasing trends with longer periods of steeping and germination, thus reaching maximum, also on the 4^{th} day of germination and decline. Also, the highest HWE development was noticed on sample steeped for 42 hours, 4th day of germination and kilned at $50^{o}C$.

The result of analysis of variance (ANOVA) of the hot water extract (HWE) values indicated significant difference ($p < 0.05$) among the parameters [steeping period (hours), germination period (days) and kilning temperature (^{o}C)] on Oba Super 2 (OS2) maize malt.

The LSD and DMRT at steeping hour 30 showed that, there was no significant difference ($p > 0.05$) among the kilning temperatures 55; 55; 45 and 55; 45 and 50 at germination period (days) of G_0, G_1, G_2, and G_3, respectively while significant difference ($p < 0.05$) exists among the kilning temperatures (^{o}C) of 45 - 50; 45 - 50; 50; 55; 45 - 55 at germination time of zero to fifth day, respectively.

At steeping period (hour) of 36, the LSD and DMRT revealed that no significant difference exists among the kilning temperatures 50; 50; 45 and 55 at germination days of G_1, G_2, G_4 and G_5, respectively. However, difference exists significantly ($p < 0.05$) between the kilning temperature of 45 - 55; 45 - 50; 45 and 55; 45 - 55; 50 - 55 and 45 - $50^{o}C$ at germination time (days) of G_0 - G_5, respectively.

At hour 42, the LSD and DMRT depicted no significant difference ($p > 0.05$) among kilning temperatures 50 - 55; 45 - 50; 45 and 55; and 45 at germination day of G_0, G_2, G_3 and G_5, respectively while significant difference ($p < 0.05$) existed among the kilning temperatures 45; 45 - 55; 55; 50; 45 - 55 and 50 - 55 at germination time (days)of G_0 - G_5, respectively.

Table 4.4: Hot Water Extract (HWE) (L^o/kg) of the Malt

Malting Conditions	S_{30}	S_{36}	S_{42}
G_0K_{45}	100.61±0.57[a]	110.40±0.56[a]	112.30±0.42[b]
K_{50}	106.50±0.71[a]	108.60±0.57[a]	109.50±0.71[a]
K_{55}	111.51±0.69[a]	114.25±0.07[a]	110.03±0.00[a]
G_1K_{45}	128.40±0.57[b]	124.45±0.49[a]	143.95±0.07[a]
K_{50}	149.85±0.21[a]	127.01±0.14[b]	159.85±0.21[a]
K_{55}	111.10±0.14[b]	116.51±0.64[a]	163.45±0.64[a]
G_2K_{45}	138.30±0.14[a]	131.90±0.14[a]	211.20±0.14[b]
K_{50}	219.53±0.66[a]	117.45±0.64[a]	211.80±0.14[a]
K_{55}	138.40±0.28[a]	138.90±0.14[a]	218.15±0.07[a]
G_3K_{45}	230.50±0.71[a]	182.40±0.57[a]	224.16±0.06[a]
K_{50}	230.00±0.00[a]	205.05±0.07[a]	222.06±0.08[a]
K_{55}	164.50±0.14[b]	223.50±0.14[a]	224.45±0.49[b]
G_4K_{45}	243.25±0.78[a]	229.00±1.41[a]	240.16±0.06[a]
K_{50}	242.20±0.57[a]	237.40±0.28[a]	250.05±0.07[a]
K_{55}	221.40±0.28[a]	238.60±0.00[b]	230.30±0.14[a]
G_5K_{45}	260.70±0.42[a]	214.60±0.00[a]	224.56±0.79[a]
K_{50}	239.50±0.71[a]	227.40±0.28[a]	219.84±0.94[a]
K55	210.45±0.50[a]	228.50±0.14[a]	216.69±2.14[a]

Key: $S_{30,}$ S_{36}, S_{42} = Steeping period of 30, 36 and 42 hours; $G_{0,}$ G_1, G_2, G_3, G_4 and G_5 = Germination period (days); of day zero (0), 1, 2, 3, 4 and 5, respectively; K_{45}, K_{50} and K_{55} = Kilning temperature (^{o}C) of 45, 50 and 55, respectively.

4.5 Free Alpha Amino Nitrogen (FAN) (mg/l) of the Malt

Table 4.5 showed FAN development levels of the malt at different malting conditions. The FAN levels increased with longer period of steeping and germination, reaching its maximum at 4^{th} day of germination and then declined. The highest FAN level was noticed on sample steeped for 42 hours, 4th day of germination and kilned at $50^{o}C$.

The result of analysis of variance (ANOVA) of the free alpha amino nitrogen (FAN) values indicated significant difference ($p < 0.05$) among the parameters [steeping period (hours), germination period (days) and kilning temperature (^{o}C)] on Oba Super 2 (OS2) maize malt.

The LSD and DMRT at 30 h steeping revealed that there was significant difference ($p < 0.05$) between the kilning temperature (^{o}C) of 50 at first day of germination. However, no significant difference ($p > 0.05$) existed among the kilning temperatures (^{o}C) of 45 - 55; 45 and 55; 45 - 55 at germination period (days) of G_0, G_1, and G_2 - G_5, respectively.

At 36 h steeping period, the LSD and DMRT depicted significant difference ($p < 0.05$) among the kilning temperatures (^{o}C) of 50; 45 - 55; 50 at germination period (days) of G_3, G_4 and G_5, respectively, while no significant difference ($p > 0.05$) existed among the kilning cycles of 45 - 55; 45 and 55 at germination days of G_0 - G_2; G_3, and G_5, respectively.

At 42 h steeping period, the LSD and DMRT revealed that there was no significant difference ($p > 0.05$) among the kilning temperatures (^{o}C) of 45 - 50; 45 and55; 55; 45 - 55; 45 and 55; 45 - 55 at germination period (days) of G_0 - G_5, respectively while difference existed significantly ($p < 0.05$) among the kilning temperatures 55; 50; 45 - 50; 50 at germination period (days) of G_0, G_1, G_2, and G_4, respectively.

Table 4.5: Free Alpha Amino Nitrogen (FAN) (mg/l) of the Malt

Malting Conditions	S_{30}	S_{36}	S_{42}
G_0K_{45}	29.25±0.02[b]	28.01±0.01[b]	30.45±0.64[b]
K_{50}	30.49±0.07[b]	29.53±0.10[b]	31.45±0.64[b]
K_{55}	28.81±0.12[b]	28.75±0.21[b]	29.45±0.78[a]
G_1K_{45}	32.15±0.78[b]	30.54±0.76[b]	32.75±0.35[b]
K_{50}	35.14±0.00[a]	32.05±0.78[b]	34.30±0.28[a]
K_{55}	32.86±0.19[b]	31.37±0.66[b]	32.32±0.12[b]
G_2K_{45}	40.89±0.44[b]	38.03±0.04[b]	40.35±0.78[a]
K_{50}	42.89±0.02[b]	41.77±1.46[b]	45.55±0.64[a]
K_{55}	40.72±0.01[b]	38.50±0.57[b]	41.80±0.28[b]
G_3K_{45}	41.78±0.03[b]	41.10±0.14[b]	42.71±0.13[b]
K_{50}	43.75±0.35[b]	43.85±0.07[b]	43.02±0.03[b]
K_{55}	41.90±0.14[b]	41.50±0.71[b]	41.65±0.63[b]
G_4K_{45}	48.25±0.35[b]	45.61±0.71[a]	48.95±0.07[b]
K_{50}	49.50±0.28[b]	49.75±0.21[a]	53.55±0.78[a]
K_{55}	48.60±0.57[b]	45.05±0.06[a]	48.91±0.01[b]
G_5K_{45}	32.33±0.74[b]	32.61±0.66[b]	31.03±0.01[b]
K_{50}	31.58±0.74[b]	32.02±0.02[a]	32.55±0.35[b]
K_{55}	31.30±0.71[b]	32.36±0.65[b]	31.08±0.11[b]

Key: $S_{30,}$ S_{36}, S_{42} = Steeping period of 30, 36 and 42 hours; $G_{0,}$ G_1, G_2, G_3, G_4 and G_5 = Germination period (days); of day zero (0), 1, 2, 3, 4 and 5, respectively; K_{45}, K_{50} and K_{55} = Kilning temperature (^oC) of 45, 50 and 55, respectively.

4.6 Diastatic Power (DP) in Degree Lintner (^{o}L) of the Malt

Table 4.6 showed the results of the diastatic power (DP) measured in degree Lintner (^{o}L). The DP of the maize variety increased equally with longer period of steeping and germination sequences. Also, sample kilned at 50^{o}C gave the highest percentage DP values in all the steep cycles.

The result of analysis of variance (ANOVA) of the diastatic power (DP) values indicated significant difference ($p < 0.05$) among the parameters [steeping period (hours), germination period (days) and kilning temperature (^{o}C)] on Oba Super 2 (OS2) maize malt.

At 30 h steeping, the LSD and DMRT showed that, there was significant difference ($p < 0.05$) among the kilning temperatures (^{o}C) of 50; 45; 50 and 55 at germination period (days) of G_3, G_4 and G_5, respectively, while no significant difference ($p > 0.05$) existed amongs the kilning temperatures (^{o}C) 45 - 55; 45 and 55; 50 and 55; 45 at germination period (days) of G_0 - G_2, G_3, G_4 and G_5, respectively.

The LSD and DMRT at steeping hour 36 revealed that no significant difference ($p > 0.05$) existed among the kilning temperature (^{o}C) of 45 and 50; 50 - 55; 45 - 55; 45 and 55; 55; 45 and 55 at germination period (days) of G_0 - G_5, respectively. However, there was significant difference ($p < 0.05$) among the kilning temperatures (^{o}C) of 50; 45; 50; 45 - 50; 50 at germination period (days) of G_0, G_1, G_3, G_4 and G_5, respectively.

Similarly, at 42 h steeping, LSD and DMRT depicted significant difference ($p < 0.05$) between the kilning temperature (^{o}C) of 55 at first day of germination. However, no significant difference ($p > 0.05$) existed among the kilning temperatures (^{o}C) of 45 - 55; 45 - 50; 45 - 55 at germination period (days) of G_0, G_1, and G_2 - G_5, respectively.

Table 4.6: Diastatic Power (DP) in Degree (^{o}L) of the Malt

Malting Conditions	S_{30}	S_{36}	S_{42}
G_0K_{45}	25.29±0.40^{b}	24.65±0.21^{b}	25.60±0.00^{b}
K_{50}	25.10±0.00^{b}	25.18±0.04^{a}	25.30±0.07^{b}
K_{55}	25.20±0.00^{b}	26.25±0.35^{b}	25.25±0.21^{b}
G_1K_{45}	26.75±0.07^{b}	22.30±0.28^{a}	33.20±0.84^{b}
K_{50}	26.55±0.07^{b}	26.30±0.00^{b}	33.10±0.14^{b}
K_{55}	25.55±0.07^{b}	26.15±0.07^{b}	32.45±0.50^{a}
G_2K_{45}	27.38±0.03^{b}	25.50±0.00^{b}	34.15±0.21^{b}
K_{50}	25.70±0.14^{b}	26.68±0.11^{b}	34.10±0.14^{b}
K_{55}	26.50±0.14^{b}	26.72±0.11^{b}	34.21±0.13^{b}
G_3K_{45}	27.48±0.25^{b}	26.75±0.07^{b}	34.30±0.00^{b}
K_{50}	32.63±0.04^{a}	34.85±0.07^{a}	36.35±0.35^{b}
K_{55}	26.85±0.07^{b}	26.18±0.04^{b}	35.79±0.16^{b}
G_4K_{45}	28.95±0.07^{a}	27.85±0.07^{a}	35.75±0.35^{b}
K_{50}	30.85±0.07^{b}	35.50±0.14^{a}	38.10±0.14^{b}
K_{55}	27.35±0.07^{b}	24.85±0.07^{b}	34.77±0.04^{b}
G_5K_{45}	30.70±0.14^{b}	26.37±0.02^{b}	36.70±0.14^{b}
K_{50}	33.40±0.57^{a}	36.55±0.07^{a}	38.08±0.11^{b}
K_{55}	28.09±0.13^{a}	25.55±0.07^{b}	35.73±0.11^{b}

Key: $S_{30,}$ S_{36}, S_{42} = Steeping period of 30, 36 and 42 hours; $G_{0,}$ G_1, G_2, G_3, G_4 and G_5 = Germination period (days); of day zero (0), 1, 2, 3, 4 and 5, respectively; K_{45}, K_{50} and K_{55} = Kilning temperature (^{o}C) of 45, 50 and 55, respectively.

4.7 Total Soluble Nitrogen (TSN) (%) of the Malt

Table 4.7 showed the result of the total soluble nitrogen of the malted maize. The result in all the cycles displayed gradual decline in total nitrogen content from zero up to 5^{th} day of germination.

The result of analysis of variance (ANOVA) of the total soluble nitrogen (TSN) values indicated significant difference ($p < 0.05$) among the parameters [steeping period (hours), germination period (days) and kilning temperature (^oC)] on Oba Super 2 (OS2) maize malt.

The LSD and DMRT revealed that, there was no significant difference ($p > 0.05$) existed between germination period (days) at zero (G_0) to fifth day (G_5), kilning temperatures of 45 - 55 and at steeping hour 30 (S_{30}) and 36 (S_{36}), respectively.

At steeping period of 42 h, the LSD and DMRT showed that, there was significant difference ($p < 0.05$) between the germination period (days) at first (G_1) and fifth day (G_5), kilned at 55 and 50, respectively. However, no significant difference ($p > 0.05$) existed among the kilning temperatures 45 - 55; 45 - 50; 45 - 55; 45 and 55 at germination time (days) of G_0, G_1, G_2 - G_4, and G_5, respectively.

Table 4.7: Total Soluble Nitrogen (TSN) (%) of the Malt

Malting Conditions	S_{30}	S_{36}	S_{42}
G_0K_{45}	8.20 ± 0.14^b	8.15 ± 0.07^b	8.24 ± 0.06^b
K_{50}	8.31 ± 0.07^b	8.43 ± 0.04^b	8.28 ± 0.11^b
K_{55}	8.18 ± 0.11^b	8.15 ± 0.06^b	8.15 ± 0.04^b
G_1K_{45}	7.44 ± 0.01^b	7.55 ± 0.07^b	7.63 ± 0.04^b
K_{50}	7.42 ± 0.02^b	7.64 ± 0.05^b	7.61 ± 0.01^b
K_{55}	7.35 ± 0.06^b	7.11 ± 0.71^b	7.51 ± 0.08^b
G_2K_{45}	7.19 ± 0.04^b	7.28 ± 0.04^b	7.20 ± 0.07^b
K_{50}	7.19 ± 0.06^b	7.30 ± 0.00^b	7.24 ± 0.06^b
K_{55}	7.16 ± 0.00^b	7.25 ± 0.07^b	7.20 ± 0.04^b
G_3K_{45}	6.35 ± 0.07^b	6.20 ± 0.03^b	6.14 ± 0.03^b
K_{50}	6.55 ± 0.07^b	6.15 ± 0.07^b	6.16 ± 0.03^b
K_{55}	6.45 ± 0.07^b	6.15 ± 0.04^b	6.12 ± 0.02^b
G_4K_{45}	5.40 ± 0.08^b	5.27 ± 0.01^b	5.18 ± 0.04^b
K_{50}	5.58 ± 0.11^b	5.26 ± 0.03^b	5.11 ± 0.01^b
K_{55}	5.48 ± 0.11^b	5.13 ± 0.03^b	5.12 ± 0.01^b
G_5K_{45}	4.22 ± 0.01^b	4.35 ± 0.07^b	4.34 ± 0.06^b
K_{50}	4.23 ± 0.04^b	4.19 ± 0.01^b	4.45 ± 0.04^b
K_{55}	4.13 ± 0.04^b	4.22 ± 0.03^b	4.28 ± 0.03^b

Key: $S_{30,}$ S_{36}, S_{42} = Steeping period of 30, 36 and 42 hours; $G_{0,}$ G_1, G_2, G_3, G_4 and G_5 = Germination period (days); of day zero (0), 1, 2, 3, 4 and 5, respectively; K_{45}, K_{50} and K_{55} = Kilning temperature (^oC) of 45, 50 and 55, respectively.

4.8 Cold Water Soluble Protein (CWS-P) (%) of the Malt

The CWS-P measured in mg/ml is depicted in Table 4.8. It showed gradual increase in this parameter as days of germination progressed. The CWS-P recorded peak on the 3rd day of germination in all the steep cycles kilned at 50^{o}C and then declined.

The result of analysis of variance (ANOVA) of the cold water soluble protein (CWS-P) values indicated significant difference ($p < 0.05$) among the parameters [steeping period (hours), germination period (days) and kilning temperature (^{o}C)] on Oba Super 2 (OS2) maize malt.

The LSD and DMRT at 30 h steeping showed that, there was significant difference (increase) ($p < 0.05$) between the kilning temperature (^{o}C) of 50 at germination period (days) at G_2 and G_3, respectively. However, no significant difference ($p > 0.05$) was recorded among the kilning temperatures 45-55; 45 and 55; 45 - 55 corresponding to germination time; G_0 - G_1, G_2 - G_3 and G_4 - G_5, respectively.

At 36 h steeping, the LSD and DMRT revealed that, there was no significant difference ($p > 0.05$) among the kilning temperatures 45 - 55; 45 and 55; 45 - 55; 45 and 55 corresponding to germination time; G_0 - G_2, G_3, G_4 and G_5, respectively, while significant difference ($p < 0.05$) existed between the kilning temperature of 50 at germination time of G_3 and G_5, respectively.

At steeping period (hour) 42, the LSD and DMRT result revealed that there was a significant difference ($p < 0.05$) between the sample germinated for three (3) days, kilned at 50. However, no significant difference ($p > 0.05$) existed among the sample kilned at 45 - 55; 45 and 55; 45 - 55 corresponding to germination period (days) of G_0 - G_2, G_3, G_4 - G_5, respectively.

Table 4.8: Cold Water Soluble Protein (CWS-P) (%) of the Malt

Malting Conditions	S30	S_{36}	S_{42}
G_0K_{45}	20.75±0.35[b]	19.62±0.54[b]	21.16±1.34[b]
K_{50}	20.56±0.64[b]	20.13±0.11[b]	20.11±0.15[b]
K_{55}	20.70±0.14[b]	20.00±0.07[b]	21.33±0.39[b]
G_1K_{45}	22.93±0.46[b]	21.50±0.71[b]	26.37±0.19[b]
K_{50}	24.65±0.57[b]	23.78±0.31[b]	23.35±0.35[b]
K_{55}	23.34±0.32[b]	22.00±0.57[b]	22.05±0.78[b]
G_2K_{45}	25.10±0.04[b]	23.55±0.07[b]	26.62±0.54[b]
K_{50}	27.95±0.07[a]	21.95±0.07[b]	26.16±0.08[b]
K_{55}	26.50±0.71[b]	22.01±0.33[b]	26.60±0.57[b]
G_3K_{45}	45.95±0.23[b]	44.79±0.72[b]	47.85±0.92[b]
K_{50}	48.83±0.32[a]	47.65±0.92[a]	49.55±0.92[a]
K_{55}	46.34±0.38[b]	44.55±0.50[b]	46.85±0.21[b]
G_4K_{45}	30.09±0.03[b]	30.50±0.35[b]	31.45±0.64[b]
K_{50}	30.13±0.02[b]	30.85±0.21[b]	32.10±0.14[b]
K_{55}	29.58±0.81[b]	30.58±0.46[b]	30.83±0.96[b]
G_5K_{45}	26.61±0.58[b]	23.58±0.45[b]	23.13±0.18[b]
K_{50}	25.93±0.81[b]	26.13±0.18[a]	24.80±0.28[b]
K_{55}	25.00±0.42[b]	22.96±0.06[b]	23.50±0.71[b]

Key: $S_{30,}$ S_{36}, S_{42} = Steeping period of 30, 36 and 42 hours; $G_{0,}$ G_1, G_2, G_3, G_4 and G_5 = Germination period (days); of day zero (0), 1, 2, 3, 4 and 5, respectively; K_{45}, K_{50} and K_{55} = Kilning temperature (^oC) of 45, 50 and 55, respectively.

4.9 Total Non-Protein Nitrogen (TNPN) (%)of the Malt

The result of TNPN depicted in Table 4.9 showed increasing trend as germination progressed up to a maximum on the 4^{th} day and marginally declined.

The result of analysis of variance (ANOVA) of the total non-protein nitrogen (TNPN) values indicate significant difference ($p < 0.05$) among the parameters [steeping period (hours), germination time (days) and kilning temperature (^oC)] on Oba Super 2 (OS2) maize malt.

The LSD and DMRT revealed that, at 30 h steeping, there were significant differences (increases) ($p < 0.05$) among the kilning temperatures (^oC) of 50; 50; 50 and 55 corresponding to germination period (days) of G_1, G_3, G_4, and G_5, respectively. However, no significant difference ($p > 0.05$) existed among the kilning temperatures 45 - 55; 45 and 55; 45 - 55; 45 and 55; 45 - 50 at germination period (days) of G_0, G_1, G_2, G_3, G_4 and G_5, respectively.

At steeping period of 36 h, LSD and DMRT indicated that there was no significant difference ($p > 0.05$) among the kilning temperatures (^oC) of 45 - 55; 45 and 55; 45 - 55; 50 - 55; 45 and 55; 45 - 55 at germination period (days) of G_0 - G_5, respectively. On the other hand, it was observed that significant difference ($p < 0.05$) existed among the kilning temperature of 50; 45; 50 corresponding to germination time G_1, G_3 and G_4, respectively.

The LSD and DMRT at 42 h steeping showed that there was significant difference ($p < 0.05$) between the kilning temperature (^oC) of 50 at germination period(days) G_2, G_3, and G_4, respectively. However, no difference existed significantly ($p > 0.05$) among the kilning temperatures (^oC) of 45 - 55; 45 and 55; 45 - 55 at germination period (days) of G_0 - $G_{1;}$ G_2 – G_4; G_5, respectively.

Table 4.9: Total Non-Protein Nitrogen (TNPN) (%) of the Malt

Malting Conditions	S_{30}	S_{36}	S_{42}
G_0K_{45}	18.15 ± 0.57^{b}	17.70 ± 0.14^{b}	18.45 ± 0.21^{b}
K_{50}	18.50 ± 0.14^{b}	18.10 ± 0.14^{b}	18.70 ± 0.42^{b}
K_{55}	18.20 ± 0.28^{b}	17.22 ± 0.02^{b}	17.90 ± 0.14^{b}
G_1K_{45}	20.30 ± 0.14^{b}	19.55 ± 0.07^{b}	19.50 ± 0.14^{b}
K_{50}	21.98 ± 0.04^{a}	20.95 ± 1.20^{a}	22.45 ± 0.35^{b}
K_{55}	20.70 ± 0.14^{b}	19.50 ± 0.50^{b}	21.64 ± 0.05^{b}
G_2K_{45}	23.65 ± 0.35^{b}	22.30 ± 0.28^{b}	24.40 ± 0.28^{b}
K_{50}	24.15 ± 0.07^{b}	24.55 ± 0.50^{b}	26.08 ± 0.04^{b}
K_{55}	23.40 ± 0.57^{b}	21.95 ± 0.07^{b}	25.10 ± 0.14^{b}
G_3K_{45}	23.30 ± 0.28^{b}	23.70 ± 0.14^{b}	28.38 ± 0.04^{b}
K_{50}	25.40 ± 0.42^{a}	24.95 ± 0.07^{b}	32.10 ± 0.14^{a}
K_{55}	23.85 ± 0.07^{b}	22.50 ± 0.14^{b}	29.24 ± 0.26^{b}
G_4K_{45}	32.11 ± 0.16^{b}	30.59 ± 0.38^{b}	34.50 ± 0.14^{b}
K_{50}	38.68 ± 0.11^{b}	32.33 ± 0.46^{a}	38.50 ± 0.14^{a}
K_{55}	31.50 ± 0.14^{a}	30.08 ± 0.04^{b}	34.83 ± 0.25^{b}
G_5K_{45}	29.50 ± 0.57^{b}	28.05 ± 0.07^{b}	28.15 ± 0.21^{b}
K_{50}	28.83 ± 0.57^{b}	29.50 ± 0.42^{b}	28.34 ± 1.32^{b}
K_{55}	27.90 ± 1.27^{a}	28.23 ± 0.18^{b}	28.63 ± 0.74^{b}

Key: $S_{30,}$ S_{36}, S_{42} = Steeping period of 30, 36 and 42 hours; $G_{0,}$ G_1, G_2, G_3, G_4 and G_5 = Germination period (days); of day zero (0), 1, 2, 3, 4 and 5, respectively; K_{45}, K_{50} and K_{55} = Kilning temperature (^{o}C) of 45, 50 and 55, respectively.

4.10 Protease Activity Development

The result of the crude protease development of the maize malt at different steeping period (hours) and germination period (days), kilned at 50^{o}C is given in Table 4.10. From the result, there were progressive increases in protease developments until after the third day, where the activity gave the maximum values in all the steep cycles. Decrease in the activity was recorded from the fourth day of germination. However, in all the steeping periods, 42 h gave the best protease development and hence was applied for enzyme purification. The maximum protease activities were: G_3S_{30} (0.679 U/ml), G_3S_{36} (0.757 U/ml) and G_3S_{42} (0.834 U/ml). The activities decreased to G_5S_{30} (0.642 U/ml), G_5S_{36} (0.656 U/ml) and G_5S_{42} (0.671 U/ml), respectively on the 5th day of germination.

The result of analysis of variance (ANOVA) of the crude protease development at different steeping period (hours) and germination period (days), kilned at 50^{o}C indicated significant difference ($p < 0.05$) among the parameters [steeping period (hours), germination period (days) and kilning temperature (^{o}C)] on Oba Super 2 (OS2) maize malt.

The LSD and DMRT depicted that, there was no significant difference ($p > 0.05$) in the protease activity developed at sample steeped for 30 h germinated between zero (G_0) to first (G_1) and fourth (G_4) to fifth (G_5) day. However, significant difference ($p < 0.05$) was recorded on second (G_2) and third (G_3) day of germination, respectively.

At steeping period of 36 and 42 h, the LSD and DMRT revealed that, there was significant difference (increase) ($p < 0.05$) in the protease activity developed between zero (G_0) to fifth (G_5) day of germination.

Table 4.10: Crude Protease Activity (U/ml) of the Maize Malt at Different Steeping periods (hours) and Germination period (days), Kilned at 50^{o}C

Malting Conditions	S_{30}	S_{36}	S_{42}
G_0	0.551±0.000^{b}	0.437±0.014^{a}	0.482±0.001^{a}
G_1	0.562±0.001^{b}	0.479±0.001^{a}	0.613±0.001^{a}
G_2	0.614±0.003^{a}	0.496±0.014^{a}	0.794±0.000^{a}
G_3	0.679±0.001^{a}	0.757±0.001^{a}	0.834±0.001^{a}
G_4	0.635±0.007^{b}	0.692±0.001^{a}	0.716±0.001^{a}
G_5	0.642±0.007^{b}	0.656±0.001^{a}	0.671±0.001^{a}

Key: S_{30}, S_{36}, S_{42} = Steeping period of 30, 36 and 42 hours, respectively; G_0, G_1, G_2, G_3, G_4 and G_5 = Germination period (days); of day zero (0), 1, 2, 3, 4 and 5, respectively.

4.11 Purification of the Protease

4.11.1 Elution Profile of Maize Malt Variety, Oba Super 2 (OS2) on Carboxymethyl (CM) Sepharose (fast flow) Ion-exchange Chromatography

Figure 4.1 showed the elution profile of protease from maize malt variety, Oba Super 2 (OS2) on carboxymethyl (CM) Sepharose (fast flow) ion-exchange chromatography (IEC). The elution pattern which showed two major peaks of protease activities between fractions 4 - 11 were pooled and designated Oba Super 2A (OS2A) and fractions 26 - 31, designated Oba Super 2B (OS2B). The linear gradient of 0.5 M NaCl solution was shown from fraction number 14.

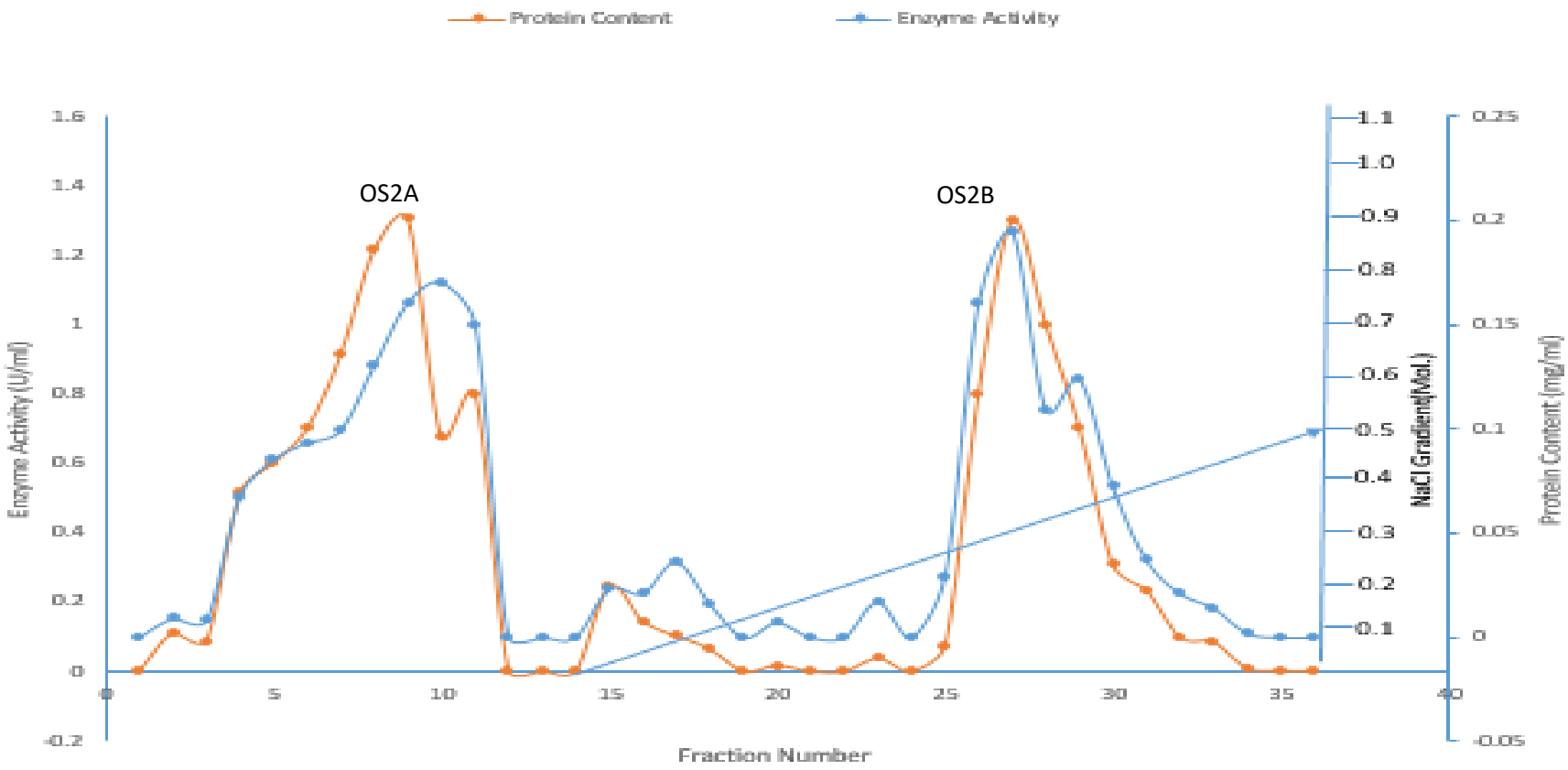

Figure 4.1: Elution Profile of Protease from Maize Malt Variety, Oba Super 2 (OS2) on Carboxymethyl (CM) Sepharose (fast flow) Ion- exchange Chromatography.

4.11.2 Elution Profile of Oba Super 2A (OS2A) Protease on Phenyl Sepharose CL-4B Hydrophobic Interaction Chromatography (HIC)

Figure 4.2 showed the elution profile of OS2A protease on phenyl Sepharose CL-4B hydrophobic interaction chromatography. The blue line represented the protease activity, the red line was protein content, while the ash or grey line indicated the varying concentrations of (3 M, 2 M, 1 M, and 0.5 M) NaCl solution as well as elution buffer. The elution pattern which showed major peaks of protease activities between fractions 26 - 32, were pooled and concentrated further for kinetic studies.

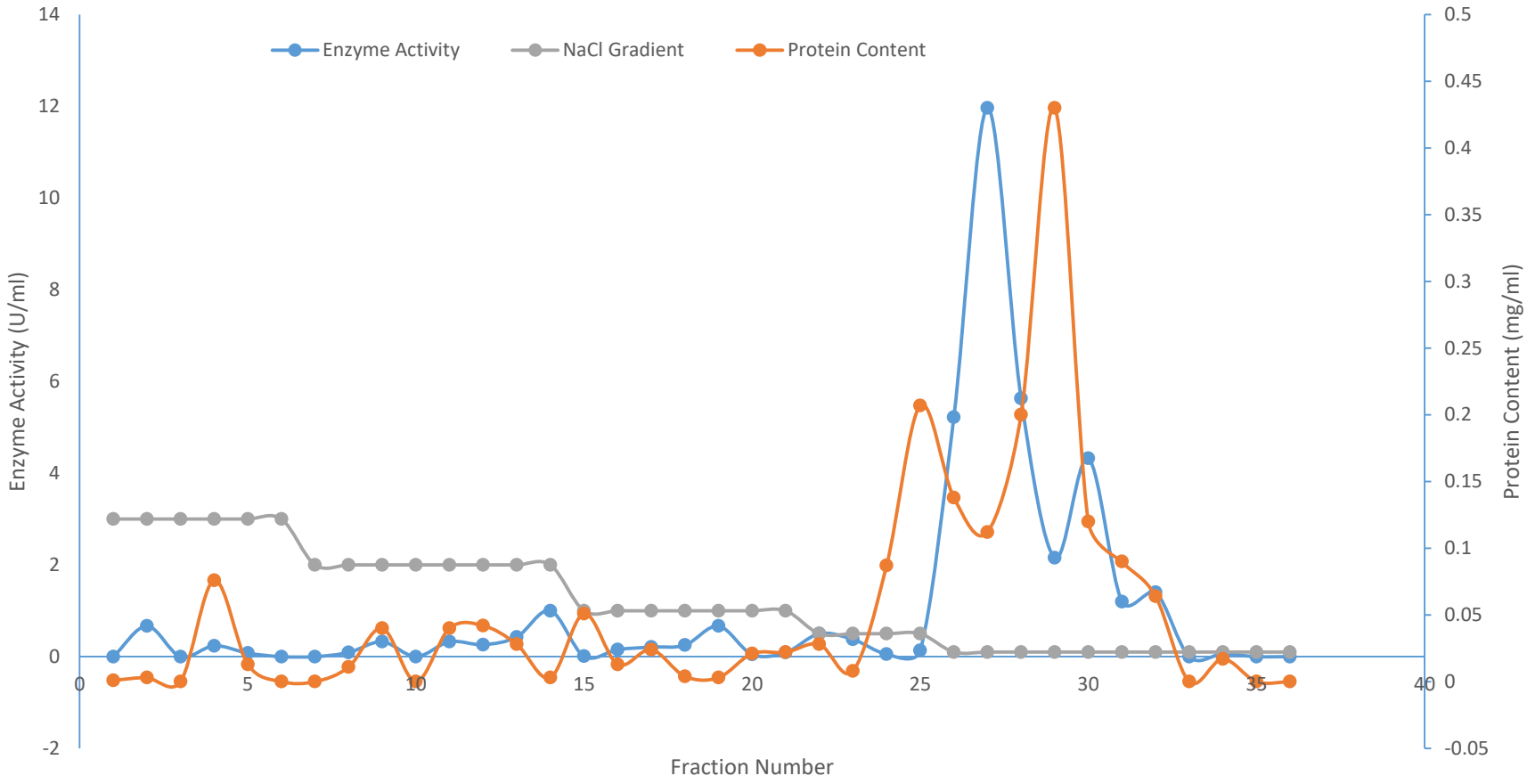

Figure 4.2: Elution Profile of OS2A Protease on Phenyl Sepharose CL-4B Hydrophobic Interaction Chromatography.

4.11.3 Elution Profile of Oba Super 2B (OS2B) Protease on Phenyl Sepharose CL-4B Hydrophobic Interaction Chromatography

Figure 4.3 showed the elution profile of OS2B protease on phenyl Sepharose CL-4B hydrophobic interaction chromatography. The blue line represented the protease activity, the red line was protein content, while the ash or grey line indicated the varying concentrations of (3 M, 2 M, 1 M, and 0.5 M) NaCl solution as well as elution buffer. The elution pattern which showed major peaks of protease activities between fractions 27 - 32, were pooled and concentrated further for kinetic studies.

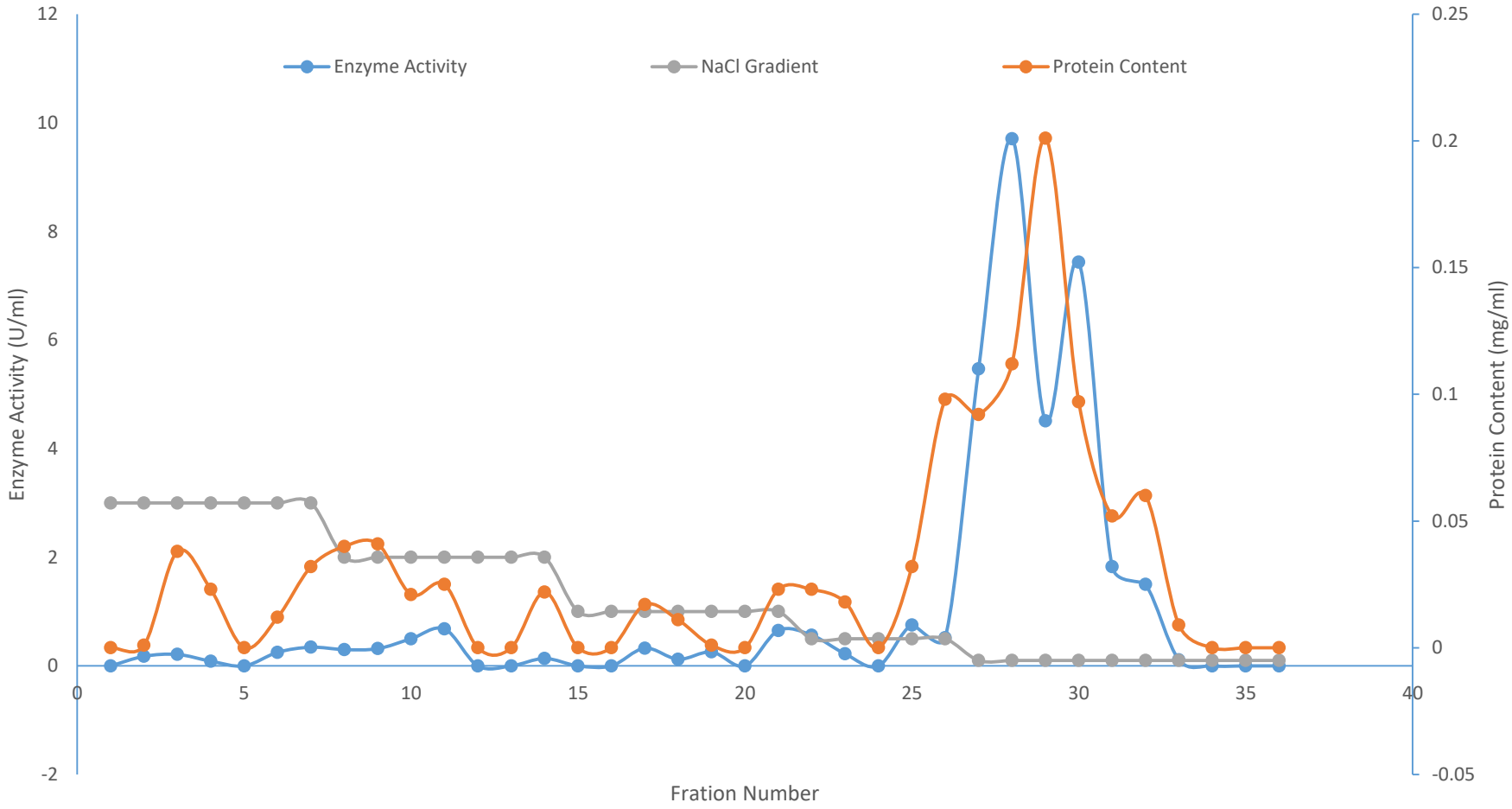

Figure 4.3: Elution Profile of OS2B Protease on Phenyl Sepharose CL-4B Hydrophobic Interaction Chromatography.

4.12 The Partial Purification Summaries of the Maize Malt, OS2A and OS2B Proteases

Table 4.11 and 4.12 gave the summaries of the protease purification of the maize malt proteases. The proteases were partially purified from crude supernatant to obtain a final yield of 3.26% and 4.24%, purification factor of 3.805-fold and 3.932-fold, specific activity 16.21 U/mg and 16.75 U/mg for OS2A and OS2B, respectively. These results were achieved by combination of 70% saturation with ammonium sulphate ($(NH_4)_2SO_4$) at 0^oC which precipitated the enzyme, concentration by overnight dialysis at 4^oC against 5M sucrose, ion- exchange chromatography (IEC) on carboxymethyl (CM) Sepharose fast flow (Figure 4.1) and hydrophobic interaction chromatography (HIC) on phenyl Sepharose CL-4B (Figures 4.2 and 4.3).

Enzyme concentration at every step of purification using 5M sucrose solution proved very successful because their activities were well enhanced.

Table 4.11: Summary of Partial Purification of OS2A Protease

Purification steps	Volume (ml)	Total Activity (Unit)	Total Protein (mg)	Specific Activity (U/mg)	Yield (%)	Purification (fold)
Crude enzyme	250	303.50	71.25	4.26	100	1.00
70% Saturation of Ammonium sulphate, $(NH_4)_2SO_4$	205	210.13	68.06	3.09	69.24	0.725
Ion-exchange chromatography on carboxymethyl Sepharose	20	13.6	6.06	2.24	4.48	0.526
Dialysis against 5M sucrose solution	4.6	3.59	0.62	5.79	1.18	1.359
Hydrophobic interaction chromatography on phenyl Sepharose	10	9.89	0.61	16.21	3.26	3.805

Table 4.12: Summary of Partial Purification of OS2B Protease

Purification steps	Volume (ml)	Total Activity (Unit)	Total Protein (mg)	Specific Activity (U/mg)	Yield (%)	Purification (fold)
Crude enzyme	250	303.50	71.25	4.26	100	1.00
70% Saturation of Ammonium sulphate, $(NH_4)_2SO_4$	205	210.13	68.06	3.09	69.24	0.725
Ion-exchange chromatography on carboxymethyl Sepharose	20	4.16	4.28	0.97	1.37	0.228
Dialysis against 5M sucrose solution	6.5	4.48	1.37	3.27	1.47	0.768
Hydrophobic interaction chromatography on phenyl Sepharose	12	12.86	0.768	16.75	4.24	3.932

4.13 Native Polyacrylamide Gel Electrophoresis (Native PAGE) and Molecular Weight Determination

The result of the native gel electrophoresis is shown in plate 4.1. The average molecular weight of the proteases was estimated to be about 63,124.456 Da (63.1 KDa) as calculated from the migration distance in relation to reference proteins. The OS2A (D2) gave 62,811.93 Da while that of OS2B (D2*) was 63,436.98 Da. The low molecular weight suggested that the enzymes possess short peptide chain and are presumed to be similar.

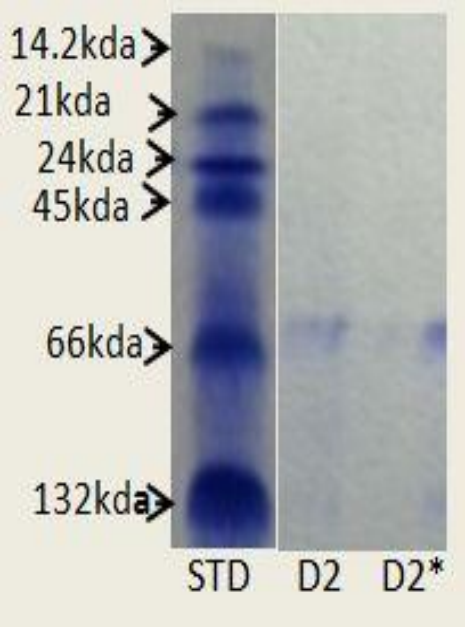

Plate 4.1: Native Polyacrylamide Gel Electrophoresis of Protein, OS2A (D2) and OS2B (D2*).

Native gel electrophoresis of protein samples D2 and D2*, applied on lane D2, 7ug and lane D2* 10ug. Standard proteins on standard lane are; Lysozyme 14,200 Da (14.2 KDa), Soy bean trypsin inhibitor 21,000 Da (21 KDa), Trypsinogen (bovine) 24,000 Da (24 KDa), Egg albumin 45,000 Da (45 KDa), Bovine serum albumin (monomer) 66,000 Da (66 KDa), and Bovine serum albumin (dimer) 132,000 Da (132 KDa). The average molecular weight of the partially purified protease was 63,124.456 Da (63.1 KDa).

4.14 Physicochemical Properties of the Proteases

4.14.1 Effect of pH on the Activity and Stability of the Partially Purified Protease

The studies on the pH effect on the activity and stability were done at the optimal conditions for activity of the proteases. For the OS2A, the optimal activity and stability was at pH 9.0 (Figure 4.4). Protease from OS2B demonstrated optimum activity at 9.0 and maximum stability at pH 8.0 (Figure 4.5). As the pH increased from 3 - 4, there were drop in the stability and increased progressively for both proteases. Protease OS2A and OS2B were more stable at neutral to alkaline pH especially between 7 and 10. Further increase in pH beyond the optimum activity and stability witnessed sharp drop with about 48% and 32%, respectively at pH 11 for protease OS2A and corresponding decrease in the activity and stability with about 49% and 25%, respectively at pH 11 for protease OS2B.

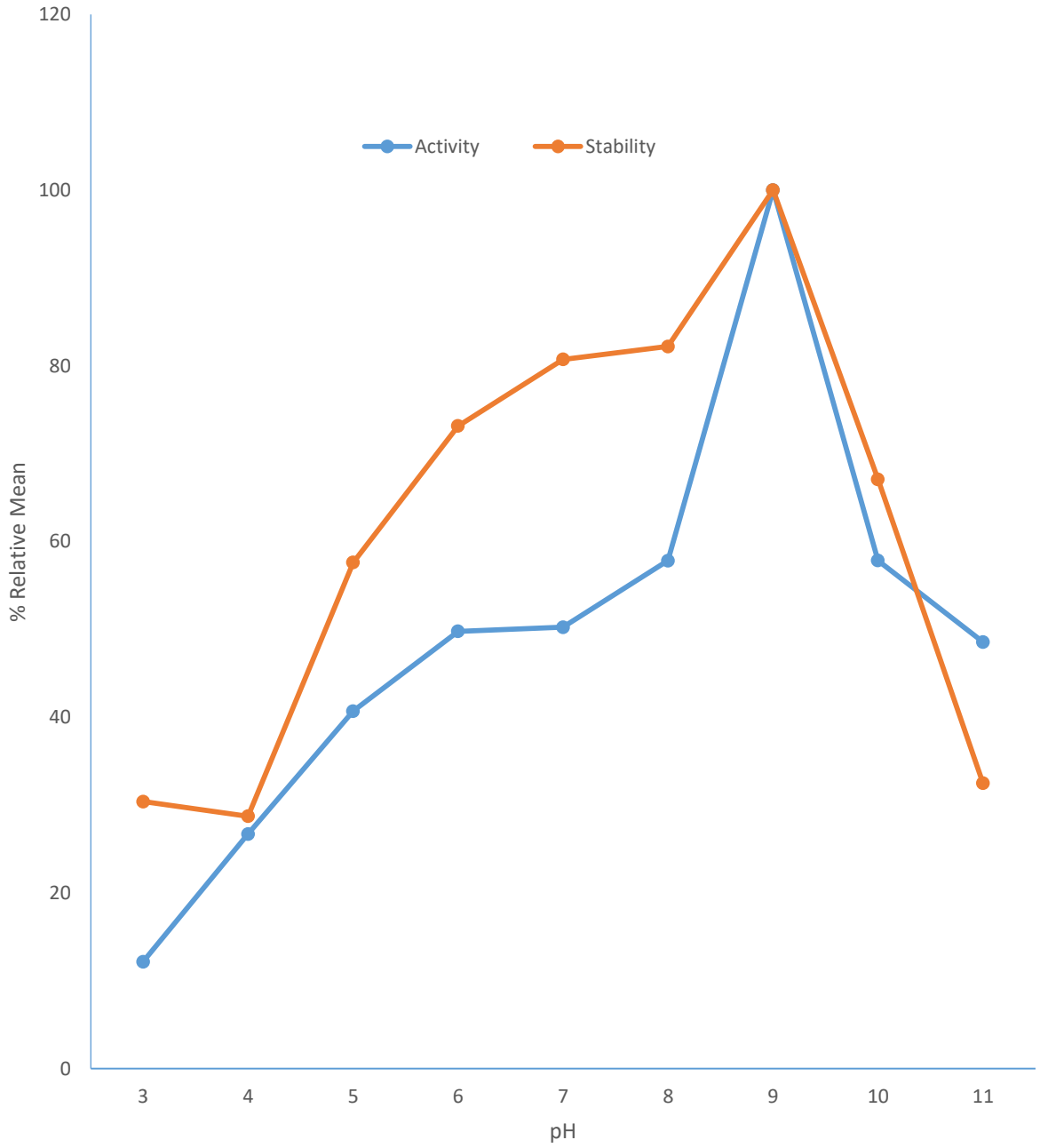

Figure 4.4: Effect of pH on the Activity and Stability of OS2A Protease.

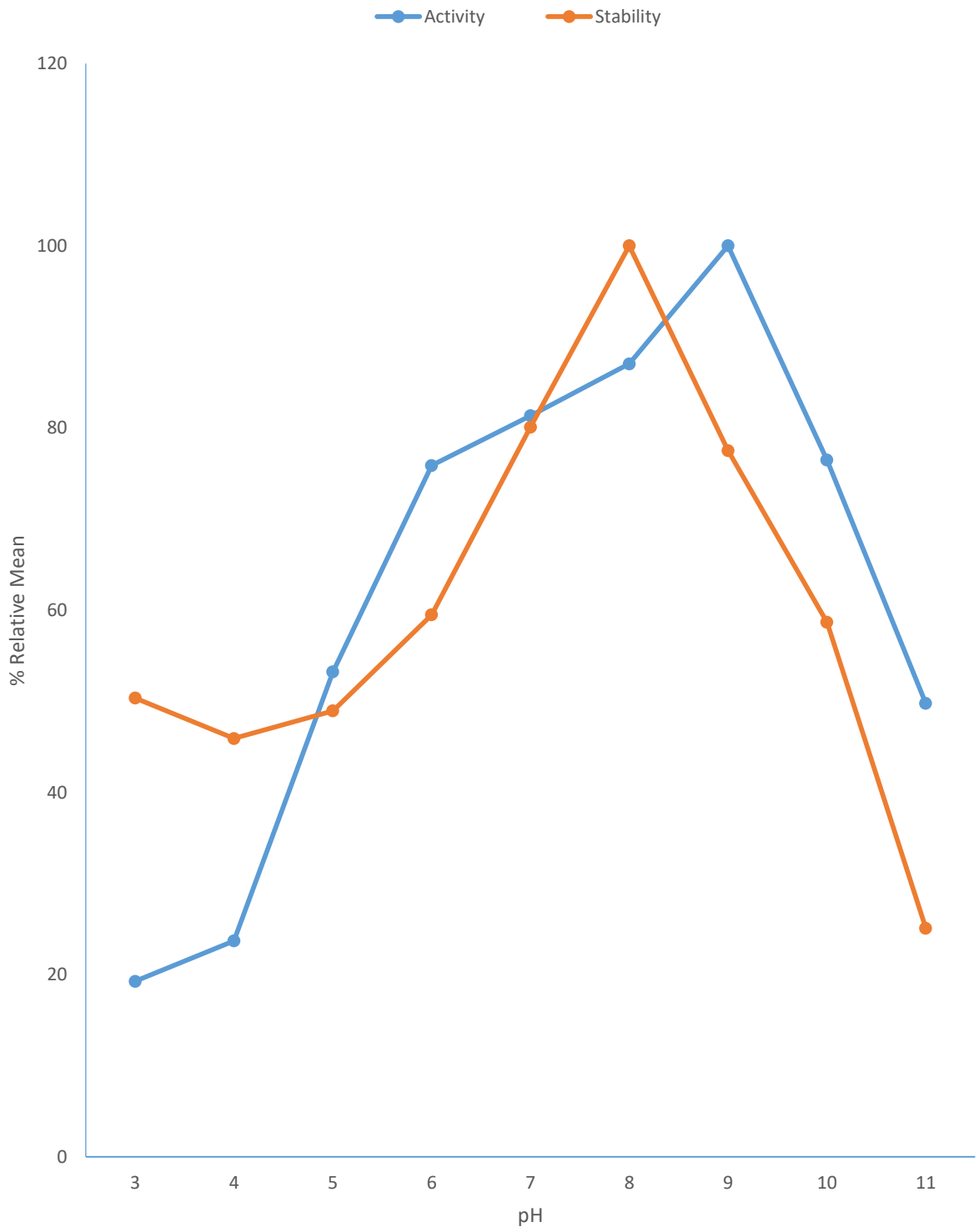

Figure 4.5: Effect of pH on the Activity and Stability of OS2B Protease.

4.14.2 Effect of Temperature on the Activity and Stability of the Proteases

The partially purified enzyme demonstrated optimal temperature activity at 50^{o}C and maximal stability at 60^{o}C (Figure 4.6), but retained about 70% of its original activity after 30 minutes at 60^{o}C for the OS2A. For the OS2B enzyme, the activity was optimum at 50^{o}C (Figure 4.7) and showed maximum stability, also at 50^{o}C. It retained over 70% of its activity after 30 minutes at 60^{o}C. The protease OS2B was slightly more stable to temperature increase with about 68% present in the original enzyme activity at 90^{o}C. On the other hand, OS2A protease exhibited less temperature stability by retaining only 60% after 30 minutes at 90^{o}C.

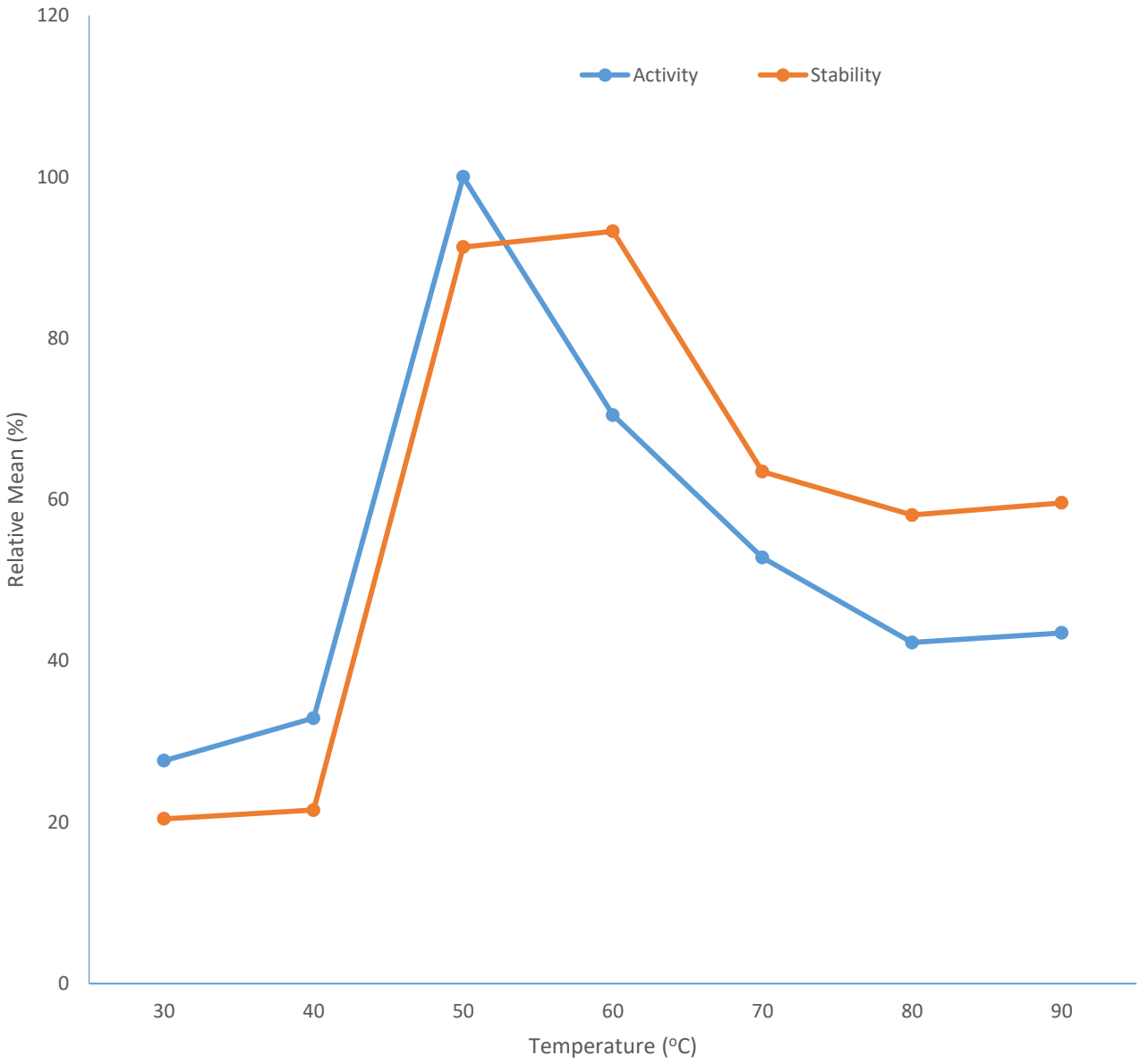

Figure 4.6: Effect of Temperature on the Activity and Stability of OS2A Protease.

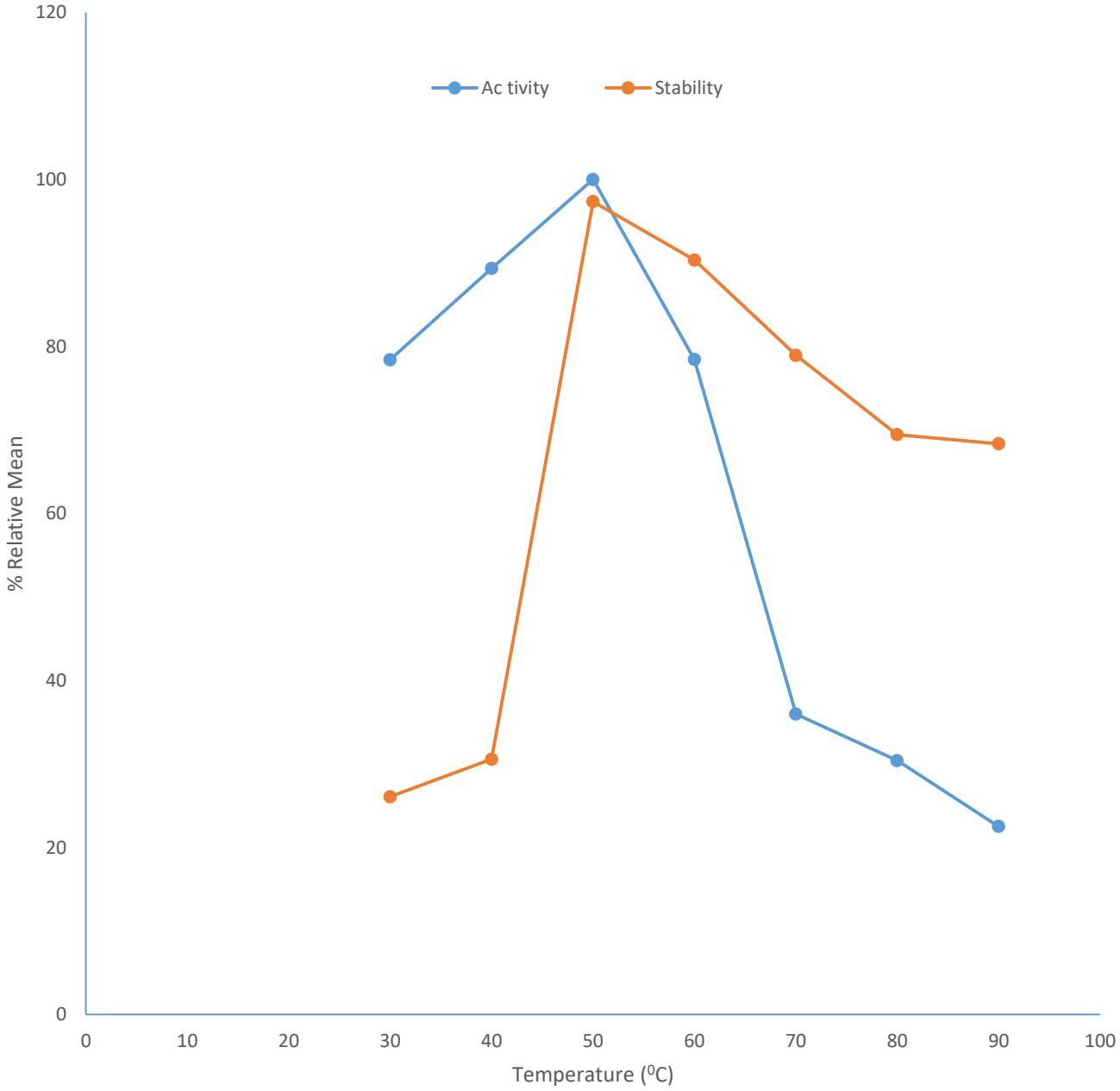

Figure 4.7: Effect of Temperature on the Activity and Stability of OS2B Protease.

4.14.3 Effect of Metal Ions on the Protease Activity

The effect of some cations on the partially purified protease was evaluated and the results are shown in Figure 4.8. The protease of the OS2A was appreciably activated by Cu^{2+}, Ca^{2+}, Mn^{2+} and Ba^{2+} by 89%, 80%, 49%, and 37%, respectively. On the other hand, Hg^{2+} showed only a slight stimulatory effect on the protease. Silver ion (Ag^{+}), Mg^{2+}, Zn^{2+}, Fe^{2+}, and Co^{2+} had inhibitory effect, with Co^{2+} having the least relative activity of 29%. The activity of OS2B was appreciably stimulated by Cu^{2+}, Ca^{2+} and Mn^{2+}, by 62%, 59%, and 50%, respectively (Figure 4.9). There was slight stimulatory effect on the activity with Hg^{2+}, and Ba^{2+} by 18% and 16%, respectively. However, the enzyme was highly inhibited by Ag^{+}, Mg^{2+}, Zn^{2+}, Fe^{2+} at 95%, 70%, 68%, 54% but was slightly inhibited by Co^{2+} with about 24%.

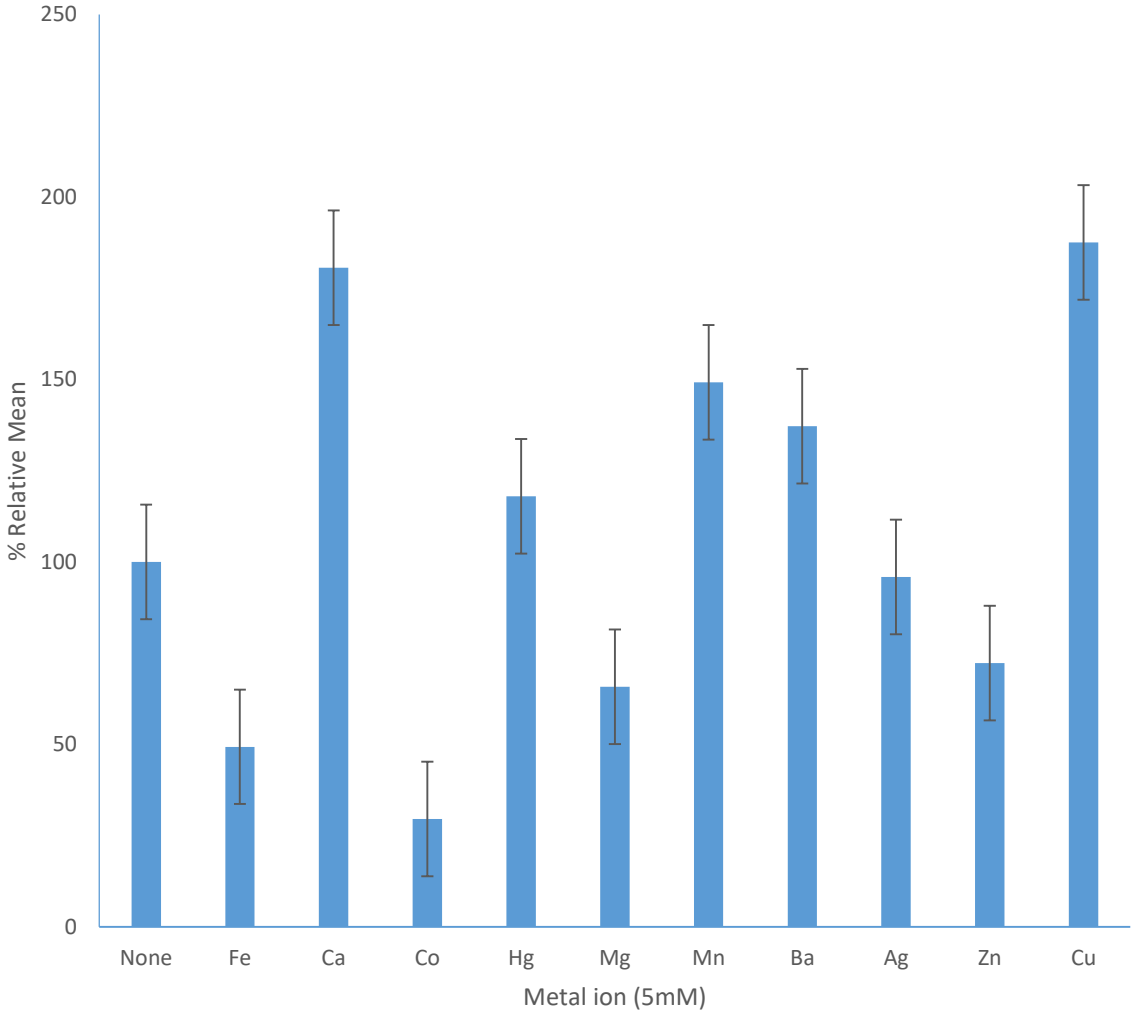

Figure 4.8: Effect of Metal Ions on the Activity of OS2A Protease**.**

The activity of enzyme without metal ion was considered as 100%. The error bars showed the standard deviation (±SD) of duplicates.

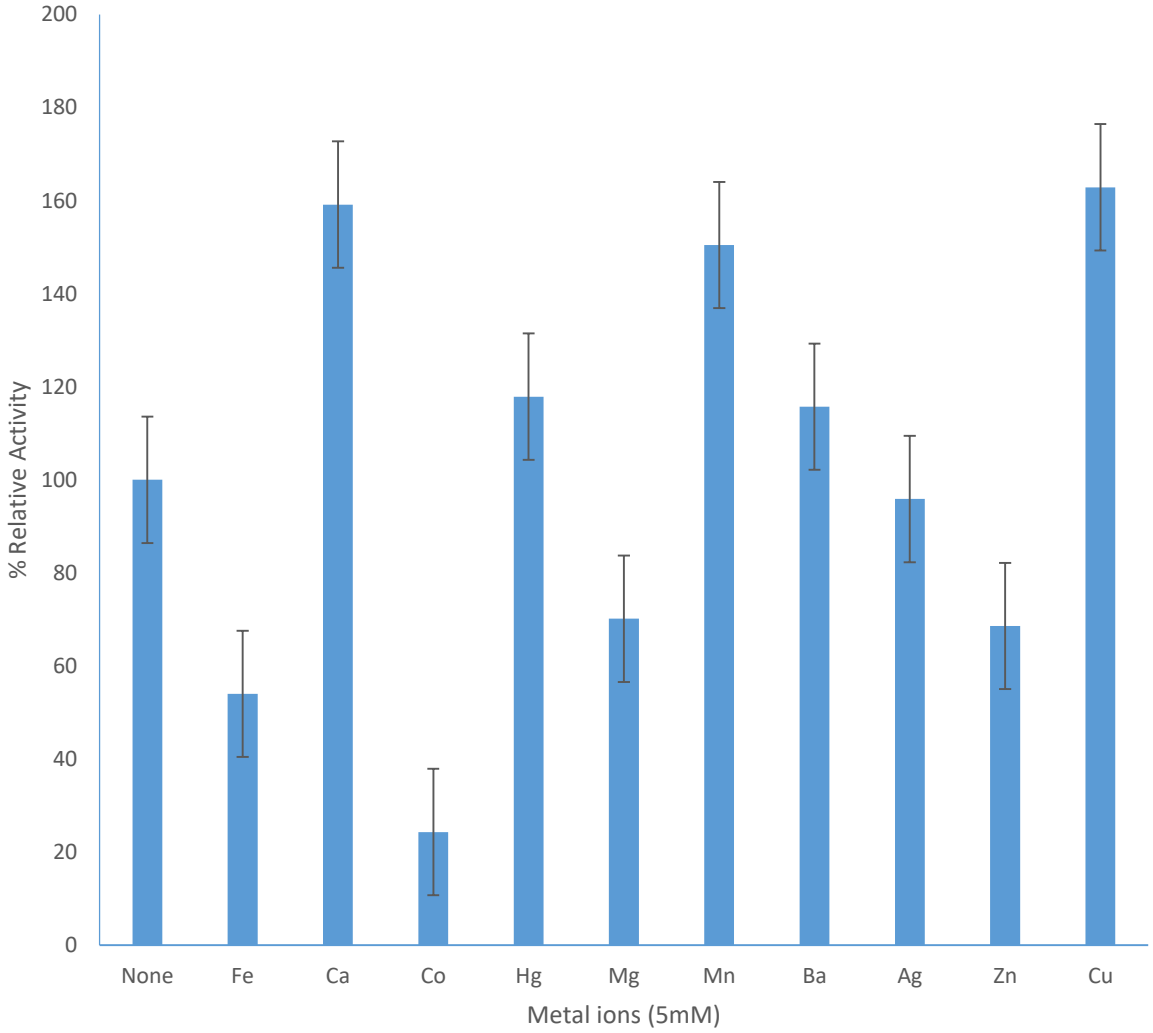

Figure 4.9: Effect of Metal Ions on the Activity of OS2B Protease**.**

The activity of enzyme without metal ion was considered as 100%. The error bars showed the standard deviation (±SD) of duplicates.

4.14.4 Effect of Some Inhibitors on Protease Activity

The effect of some inhibitors on the protease was studied at optimal conditions for enzyme activity. The inhibitors employed were ethylenediaminetetraacetic acid (EDTA), iodoacetic acid (IAA), phenylmethylsulphonylfluoride (PMSF), ethylene bis (oxyethylene nitrilo) tetra acetic acid (EGTA) and para-chloromercuribenzoate (p-CMB). For the OS2A, all the tested inhibitors were inhibitory to the enzyme at varying percentages. The PMSF gave the highest inhibitory activity (60%), followed by EDTA (37%), EGTA (36%), p-CMB (24%) and IAA (21%) (Figure 4.10). Similarly, OS2B protease was greatly inhibited by PMSF at 66% while others showed slight inhibitory effect. The EDTA inhibited the activity (49%), followed by p-CMB (36%), EGTA (26%) and IAA (24%) (Figure 4.11).

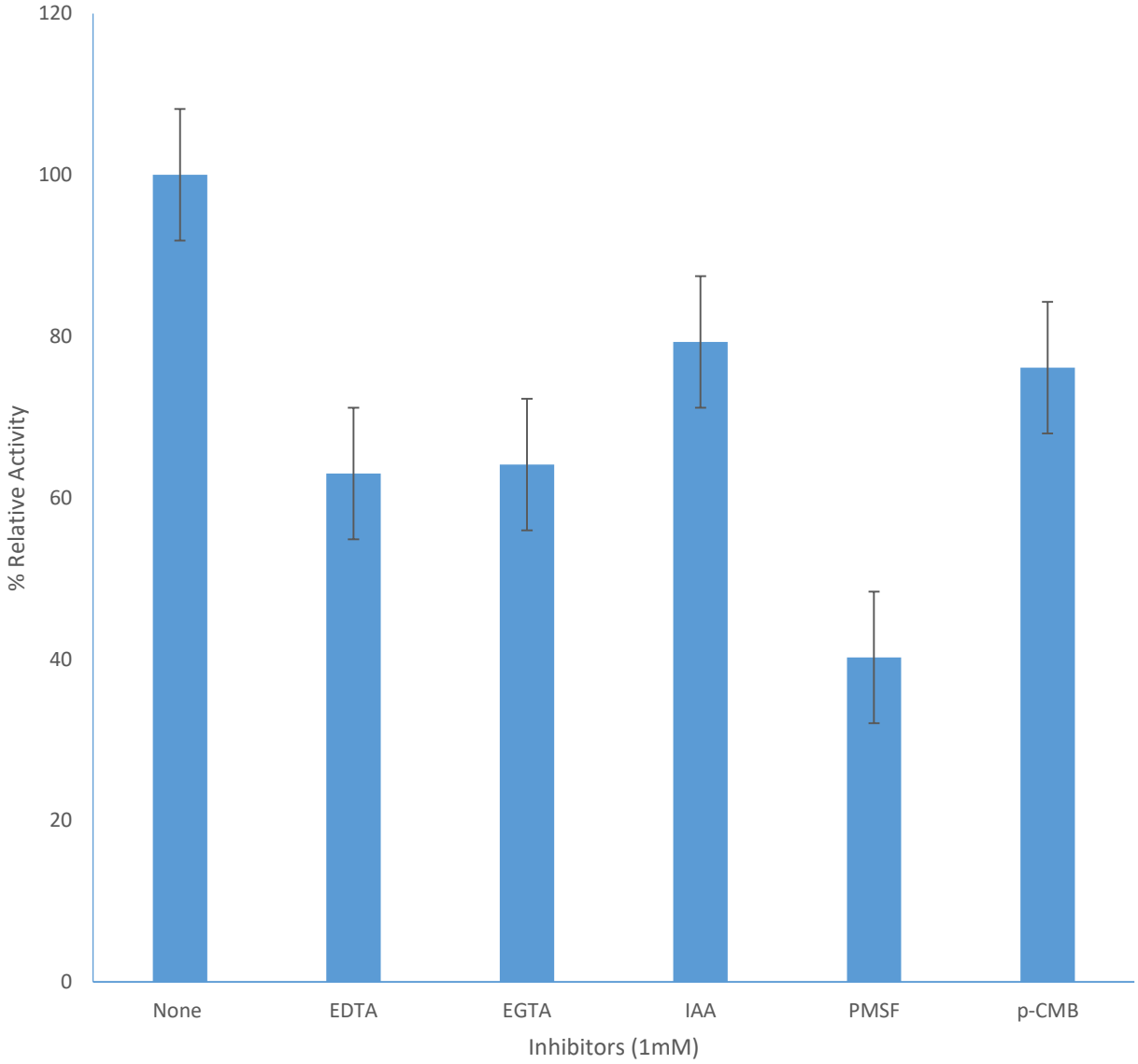

Figure 4.10: Effects of Some Inhibitors on the Activity of OS2A Protease.

The activity of enzyme without inhibitor/modulator was considered as 100%. The error bars showed the standard deviation (±SD) of duplicates.

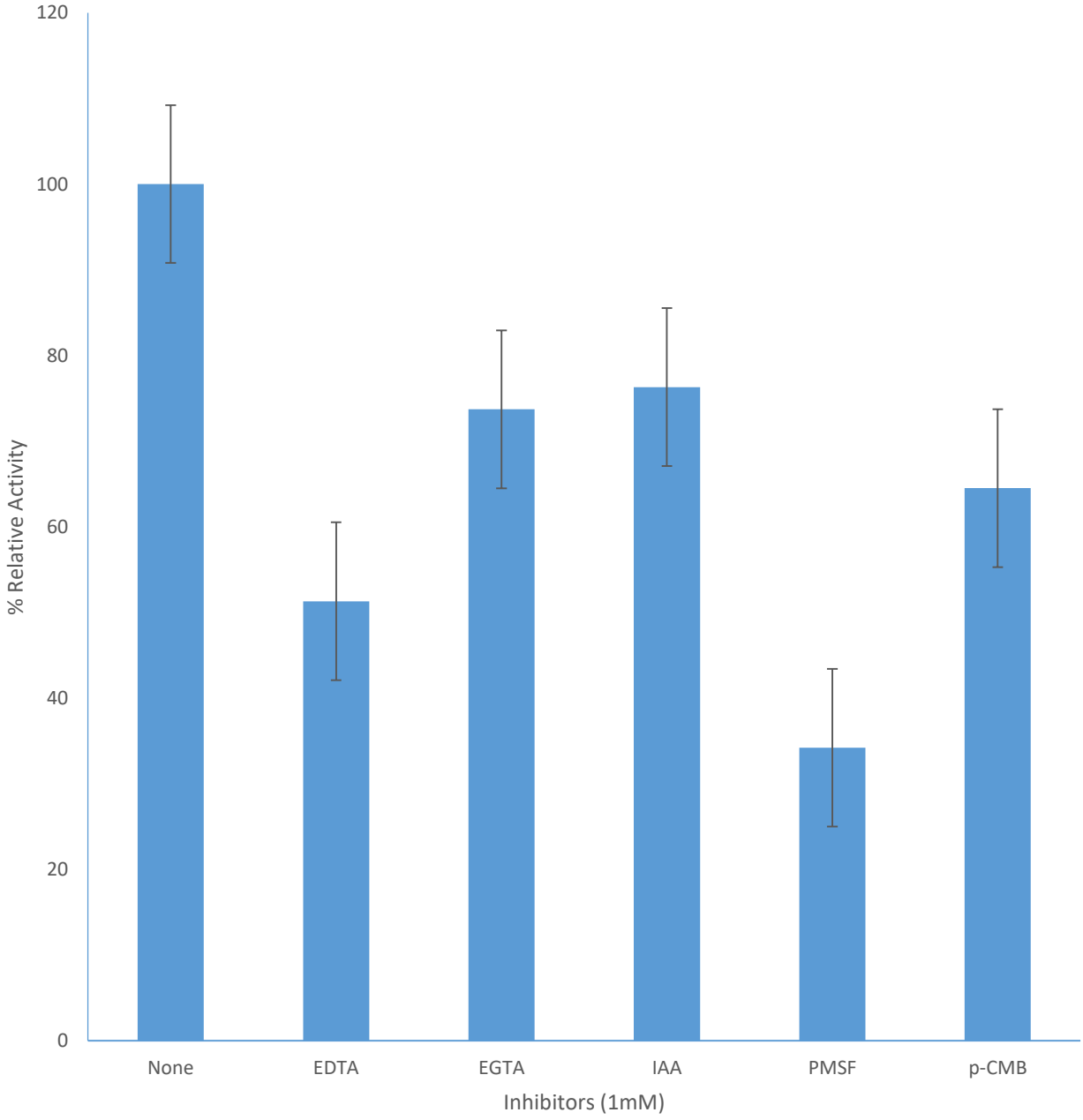

Figure 4.11: Effects of Some Inhibitors on the Activity of OS2B Protease.

The activity of enzyme without inhibitor/modulator was considered as 100%. The error bars showed the standard deviation (±SD) of duplicates.

4.14.5 Effect of Oxidizing Agents on Protease Activity

The impact of oxidizing agents (O.As) on enzyme activity was studied at optimal conditions for enzyme activity. The O.As; hydrogen peroxide, (H_2O_2) and dimethyl sulfoxide (DMSO) were tested at various concentrations of 1.0, 2.0, 3.0, 4.0, 5.0%. The partially purified proteases showed varying degrees of activities and were stable in the presence of these agents. The OS2A protease exhibited compatibility and stability in the presence of O.As studied. It showed an enhanced activity on treatment for 30 minutes with 1.0% H_2O_2 and DMSO, retaining its activity of 107.8 and 117.2%, respectively. As the concentration of O.As increased from 1.0 - 5.0%, there were display of enzyme inhibition (Figure 4.12). Similar trend was exhibited by OS2B protease. The activity of this enzyme was enhanced on treatment for 30 minutes also, with 1.0% H_2O_2 and DMSO, thus retaining 116.7 and 101.80%, of its activities, respectively. The rates of inhibition increases as the concentration of the O.As increased, and thus decreased to 54.7 and 46.6% at 5.0% concentration of H_2O_2 and DMSO, respectively (Figure 4.13).

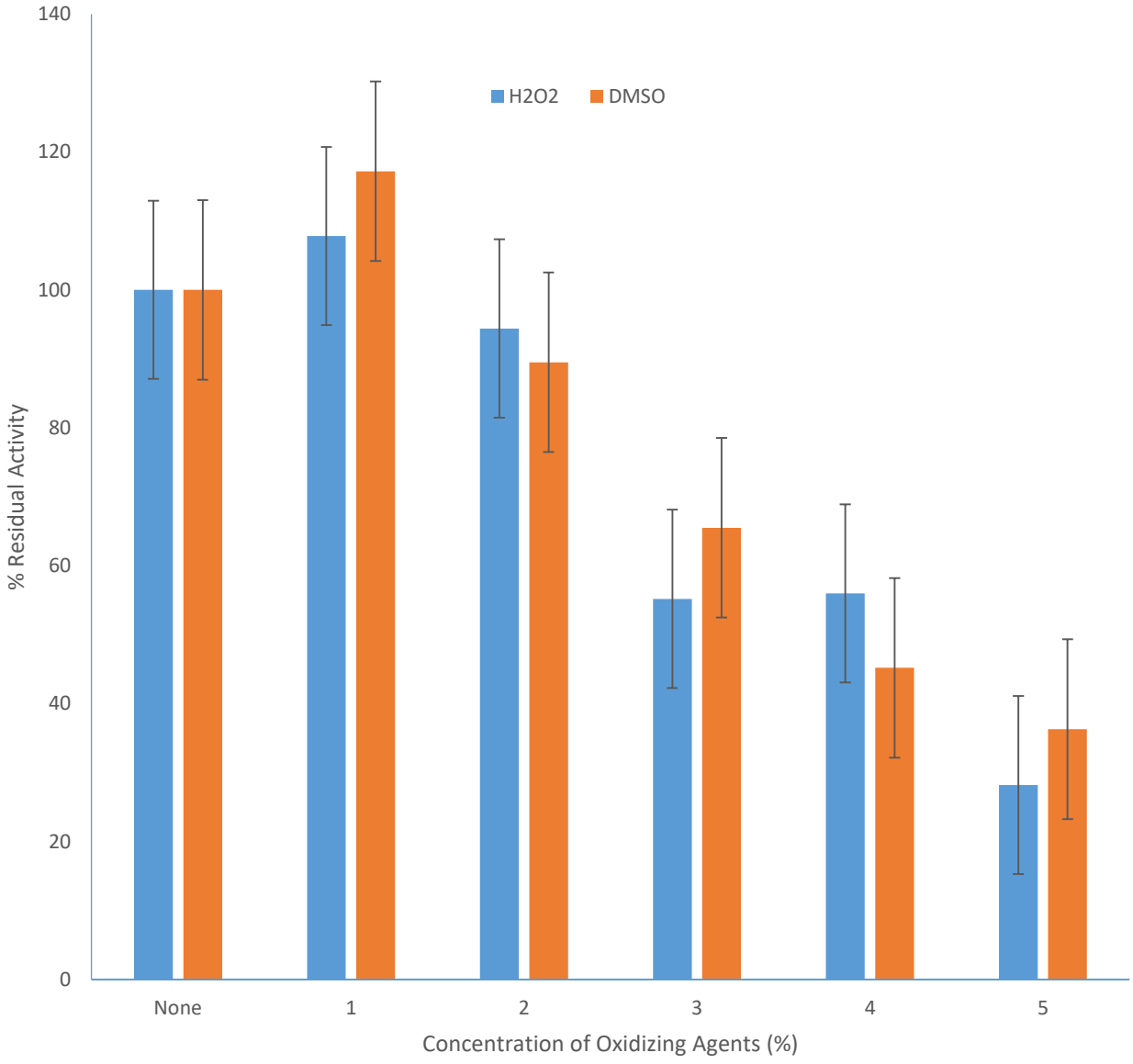

Figure 4.12: Effect of Oxidizing Agents on the Activity of OS2A Protease.

The activity of enzyme without oxidizing agent was considered as 100%. The error bars showed the standard deviation (±SD) of duplicates.

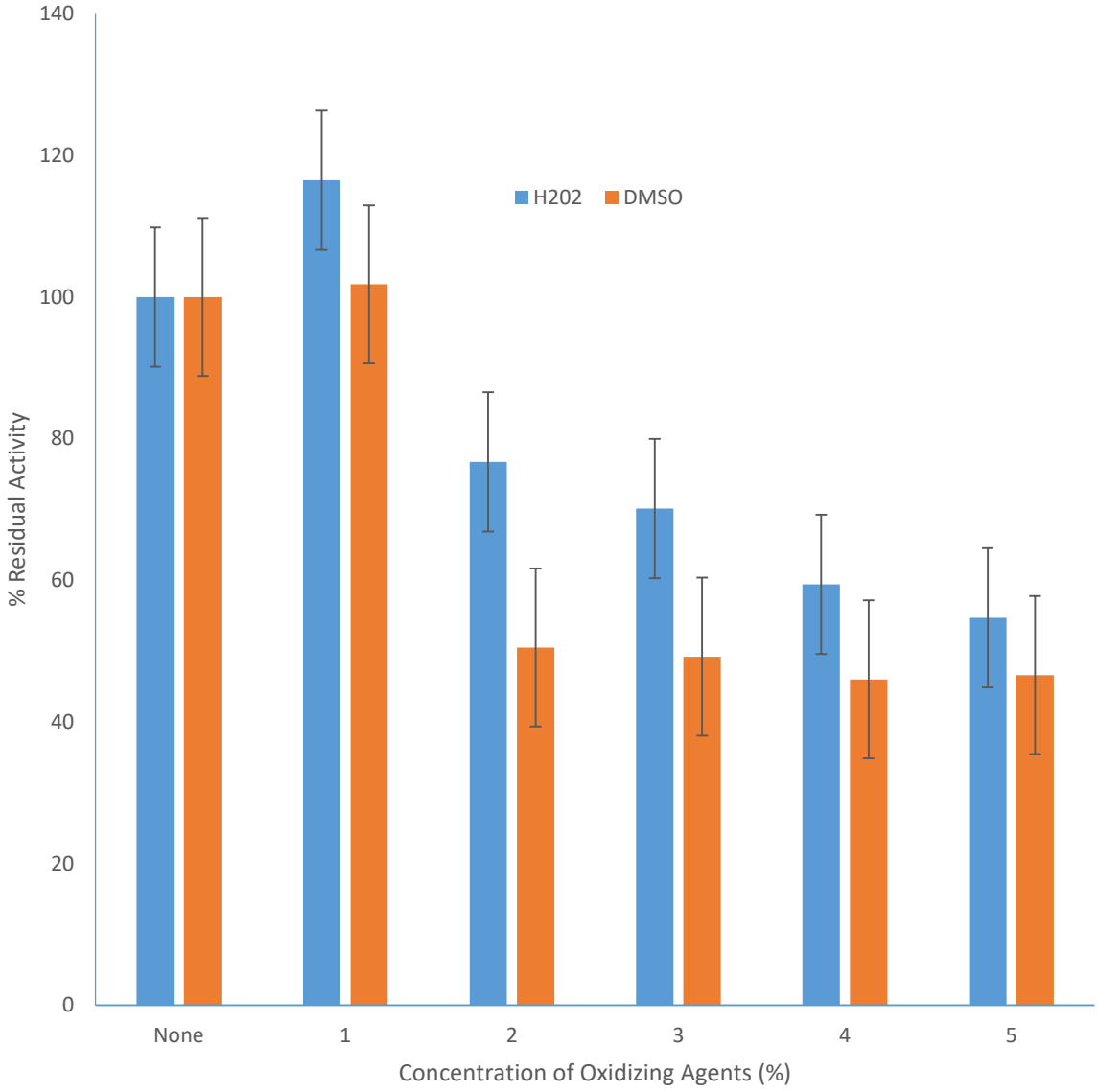

Figure 4.13: Effect of Oxidizing Agents on the Activity of OS2B Protease.

The activity of enzyme without oxidizing agent was considered as 100%. The error bars showed the standard deviation (±SD) of duplicates.

4.14.6 Effect of Reducing Agents on Protease Activity

The effect of some reducing agents (R.As) on the protease was studied at optimal conditions for enzyme activity. The R.As; dithiothreitol (DTT) and 2-mercaptoethanol (2-ME) used were tested at various concentrations of 1.0, 2.0, 3.0, 4.0, and 5.0%. There were varying display of activities and stabilities in the presence of these agents. The OS2A was enhanced in the presence of 1.0% DTT at 117% but the activity decreased gradually with increasing concentration from 1.0 to 5.0%. The enzyme showed decreased activity in the presence of 2-ME after treatment for 30 min with 1.0% of this agent (Figure 4.14). The OS2B protease activity was inhibited in the presence of 1.0% concentration of 2-ME and DTT at 96.3 and 92.7%, respectively. On overall assessment, inhibitory activity increased as the concentrations of DTT and 2-ME increase after incubation for 30 minutes in these agents (Figure 4.15).

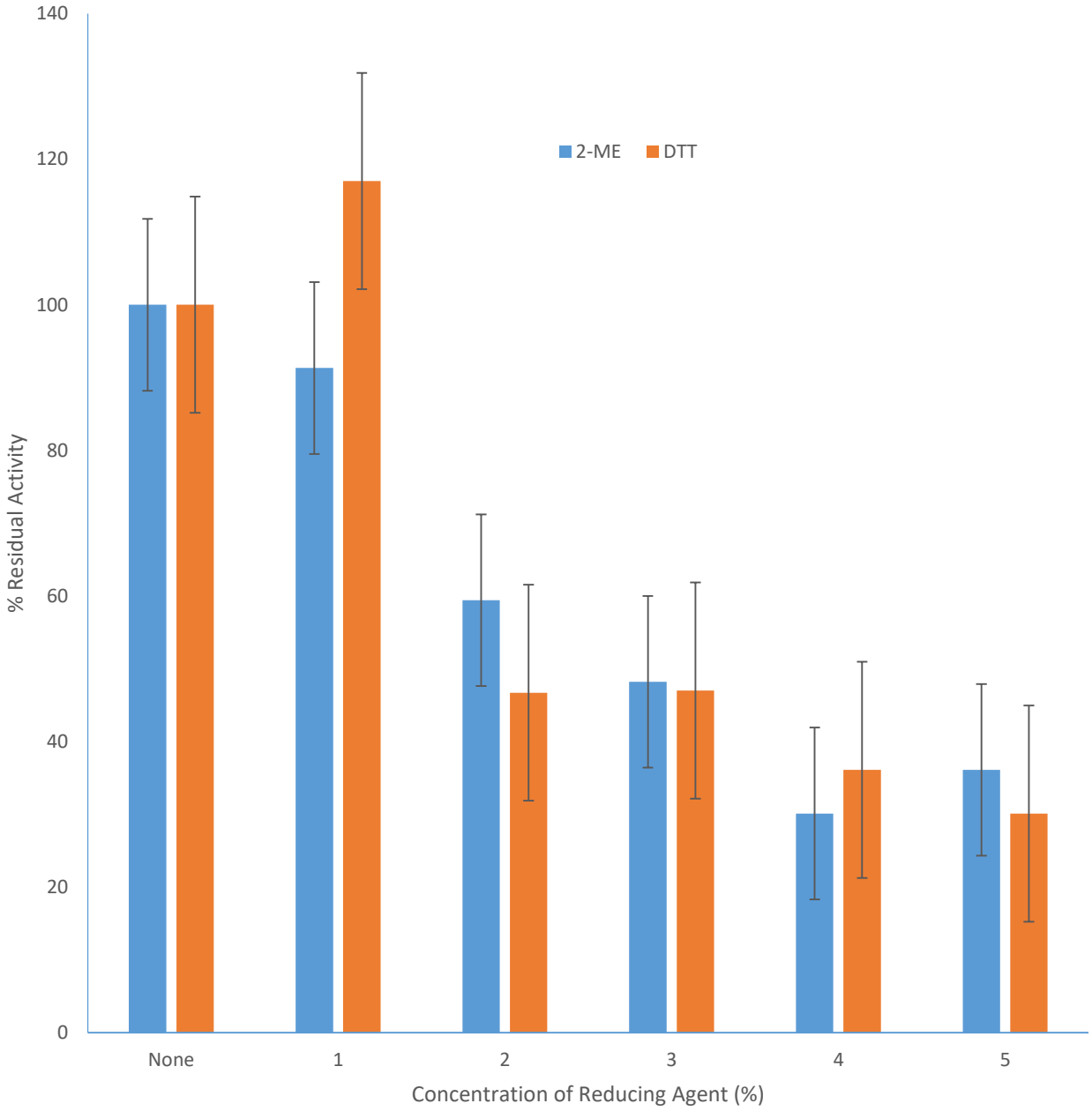

Figure 4.14: Effect of Reducing Agents on the Activity of OS2A Protease.

The activity of enzyme without reducing agent was considered as 100%. The error bars showed the standard deviation (±SD) of duplicates.

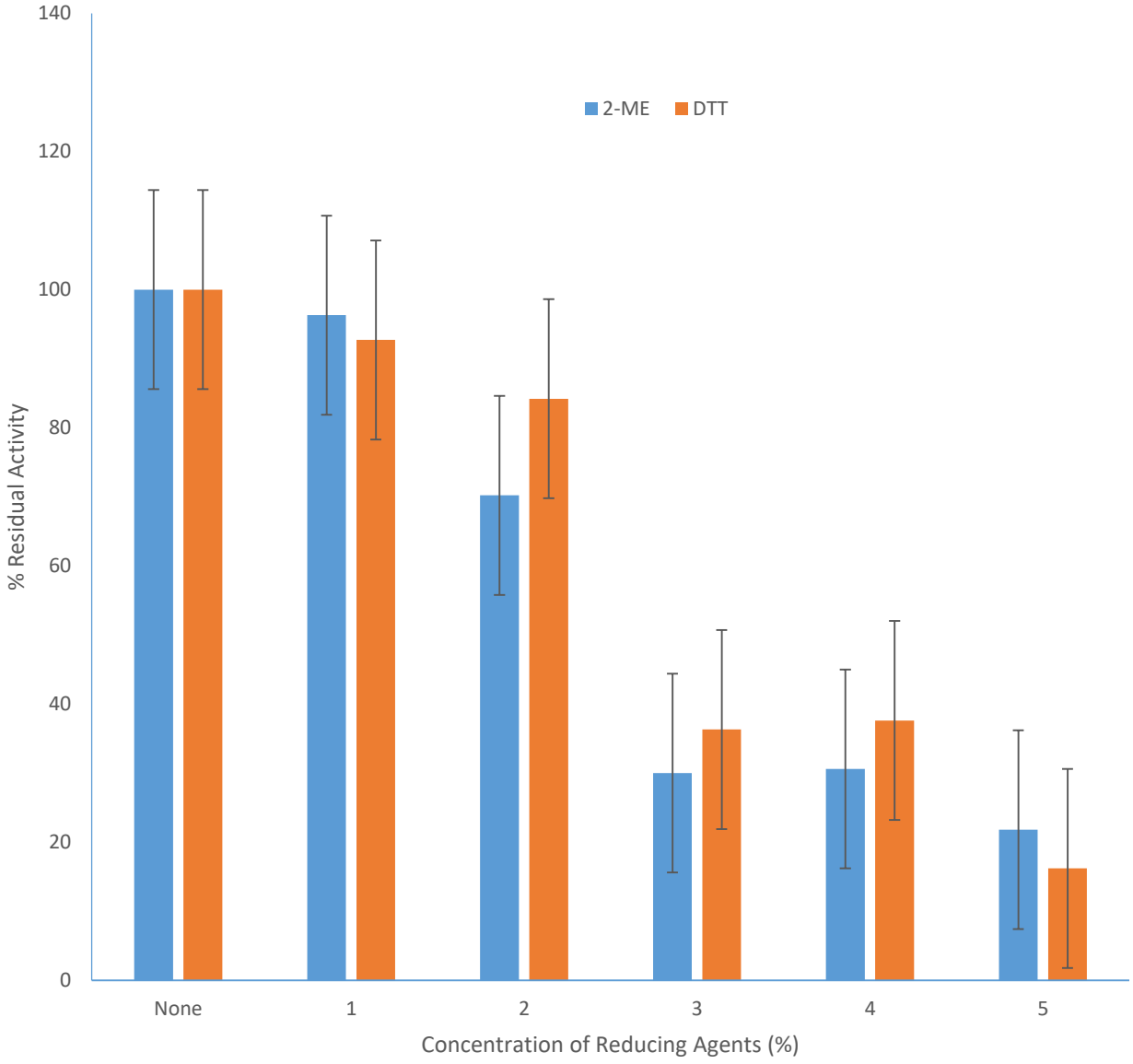

Figure 4.15: Effect of Reducing Agents on the Activity of OS2B Protease.

The activity of enzyme without reducing agent was considered as 100%. The error bars showed the standard deviation (±SD) of duplicates.

4.14.7 Effect of Detergents and Surfactants on Protease Activity

The influence of detergents and surfactants was studied at optimal conditions for enzyme activity. The ionic and non-ionic detergents used were Ariel, Klin, Omo, while the surfactants were sodium dodecyl sulphate (SDS), Triton X-100, Tween-80 and Tween - 20. The partially purified proteases exhibited varying degrees of compatibility and stability in the presence of the tested detergents 1.0% (*w/v*) and surfactants 1.0% (*v/v*). The result revealed that in both proteases (OS2A and OS2B), the surfactants (Triton X-100 and SDS) and detergent (Ariel) appreciably stimulated their activities while Tween - 80, Tween - 20, Klin and Omo were inhibitory. The OS2A protease was stable not only in the presence of non-ionic surfactant like Triton X- 100, at 318.98%, and a strong anionic surfactant (SDS), at 125.46%, but also towards the detergent like Ariel, which retained about 136.02% of its activity after incubation for 30 min (Figure 4.16). The OS2B protease was also stable after incubation for 30 minutes in the presence of 1.0% Triton X- 100 at 216.11% and SDS at 116.07%, whereas, Tween - 80 and 20 were inhibitory. The enzyme was equally compatible and stable on treatment for 30 minutes with 1.0% Ariel, retaining about 118.77% residual activity, whereas, Klin and Omo were inhibitory at 49.97 and 35.36%, respectively (Figure 4.17).

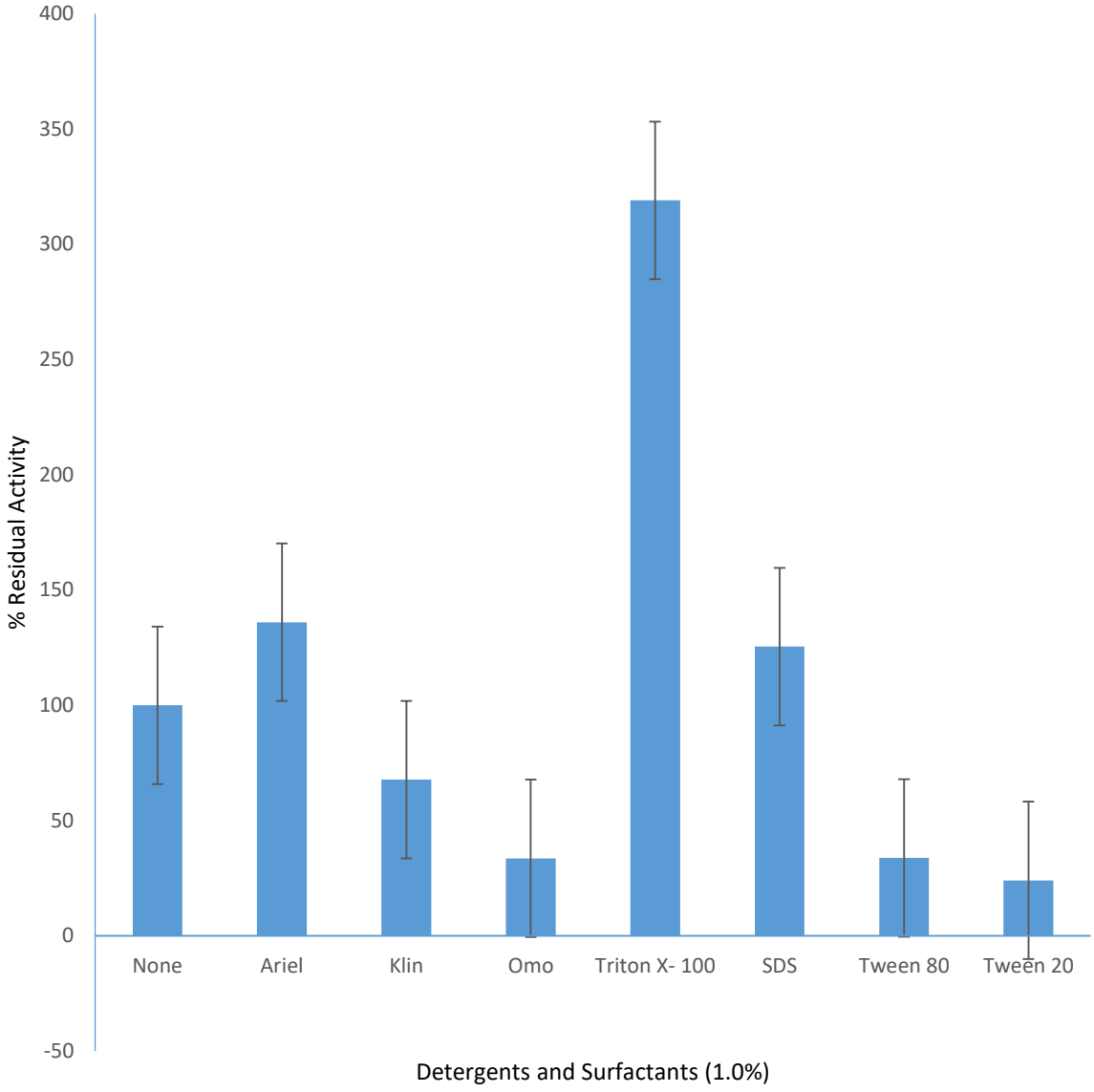

Figure 4.16: Effect of Detergents and Surfactants on the Activity of OS2A Protease.

The activity of enzyme without detergent/surfactant was considered as 100%. The error bars showed the standard deviation (±SD) of duplicates.

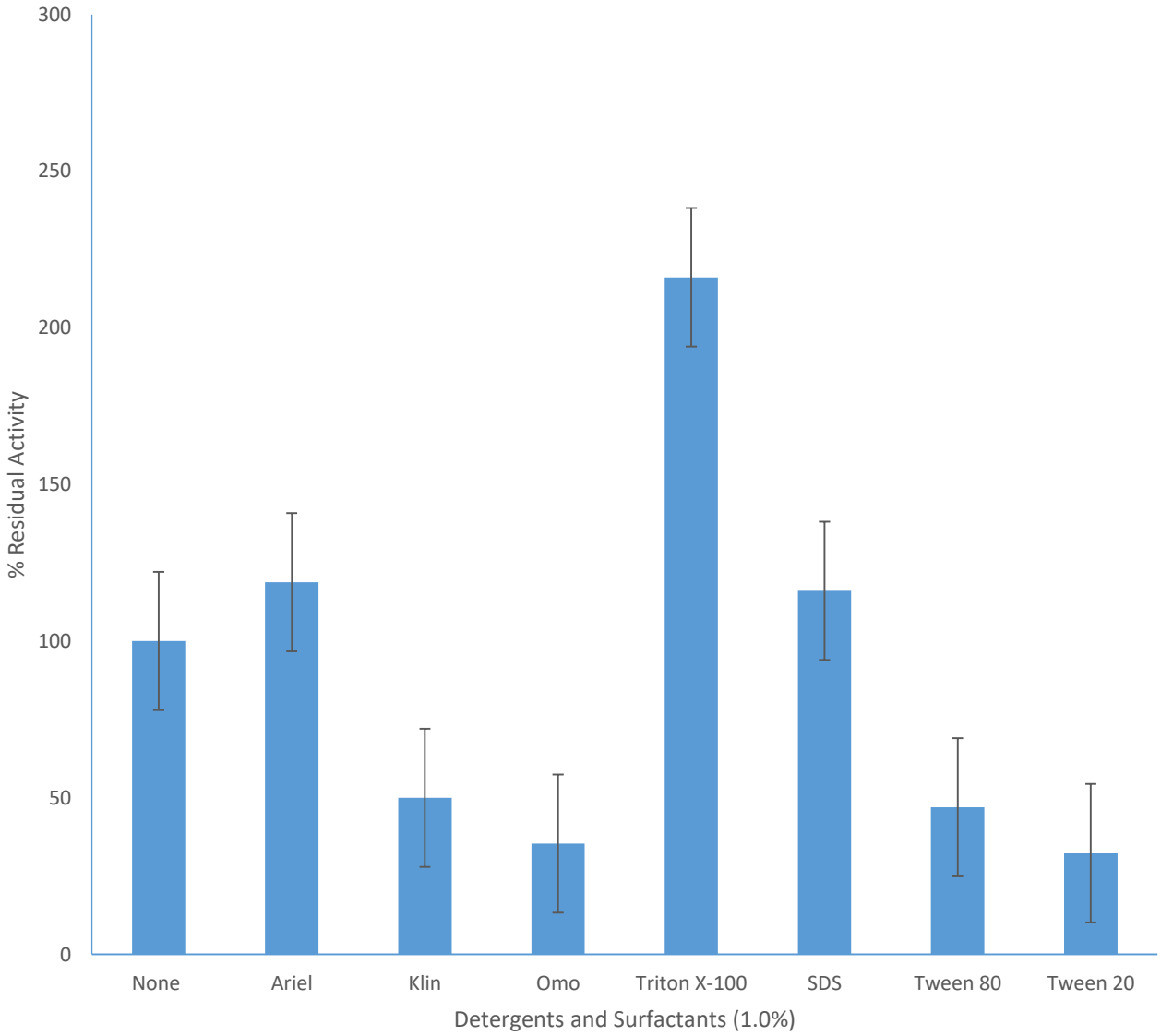

Figure 4.17: Effect of Detergents and Surfactants on the Activity of OS2B Protease.

The activity of enzyme without detergent/surfactant was considered as 100%. The error bars showed the standard deviation (±SD) of duplicates.

4.14.8 De- Staining Effect of the Protease on Blood Stain

The washing performance of the proteases in the presence of detergent (Ariel) are shown in plates 4.2 and 4.3. The detergent incorporated with the partially purified proteases cleansed the blood stained cloth more than that washed with either water or detergent only**.**

A **B** **C**

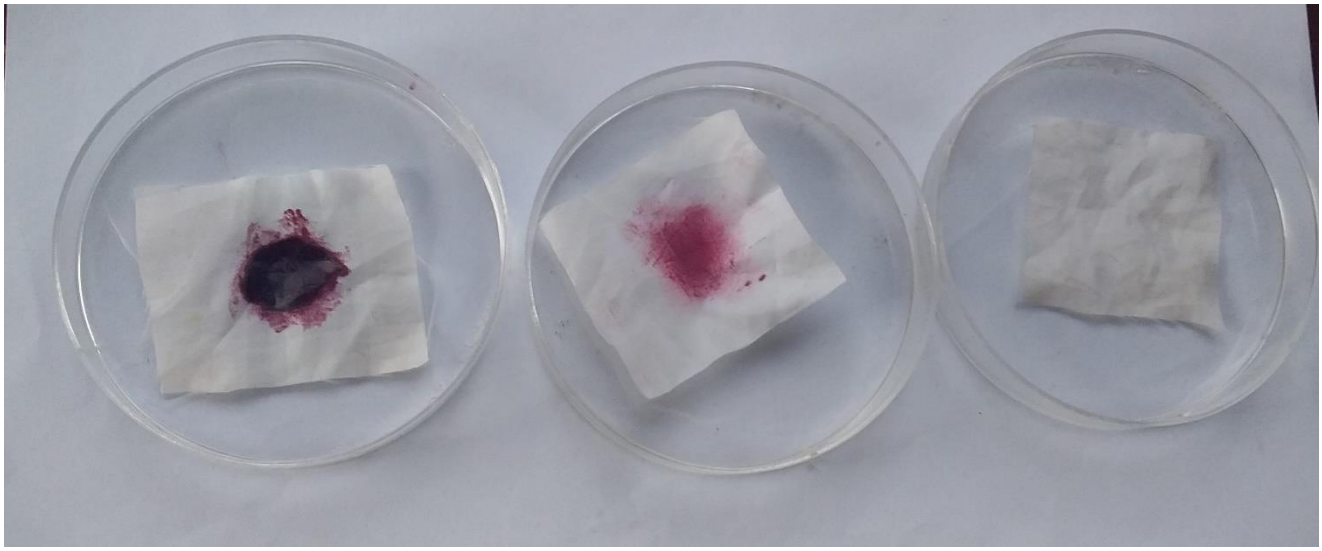

Plate 4.2: Cleansing Effect of OS2A Protease in the Presence of Detergent (Ariel).

A - White Cloth stained with blood and washed with water only

B - Blood stained cloth washed with detergent only

C - Blood stained cloth washed with detergent and OS2A protease.

D **E** **F**

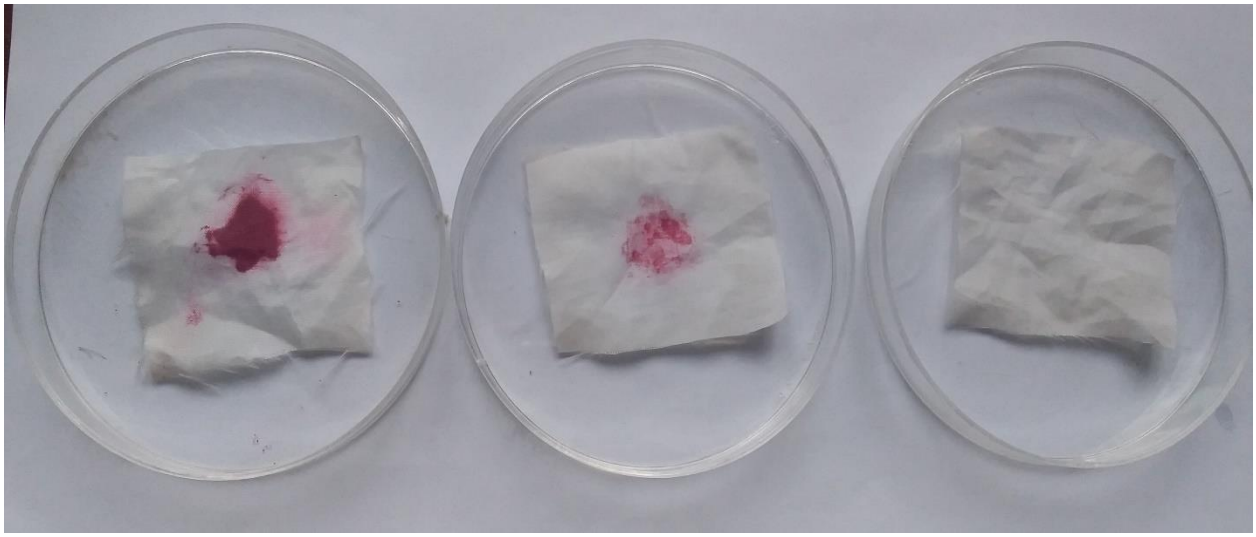

Plate 4.3: Cleansing Effect of OS2B Protease in the Presence of Detergent (Ariel).

D - White Cloth stained with blood and washed with water only

E - Blood stained cloth washed with detergent only

F - Blood stained cloth washed with detergent and OS2B protease.

4.14.9 Relative Rates of Hydrolysis of Various Substrates by the Proteases

The hydrolytic properties of the enzymes were studied at optimal conditions on these substrates; casein, BSA, gelatin and egg albumin (EA). For the OS2A, enzyme hydrolytic activity was highest towards casein at 100% followed by gelatin (85.1%), BSA (41.5%) and egg albumin (39.5%) (Figure 4.18). Similarly, OS2B showed highest preference for casein at 100%, BSA (50%), gelatin (49.4%) and egg albumin (45.6%), but vary from protease OS2A in its higher preference for BSA than gelatin (Figure 4.19).

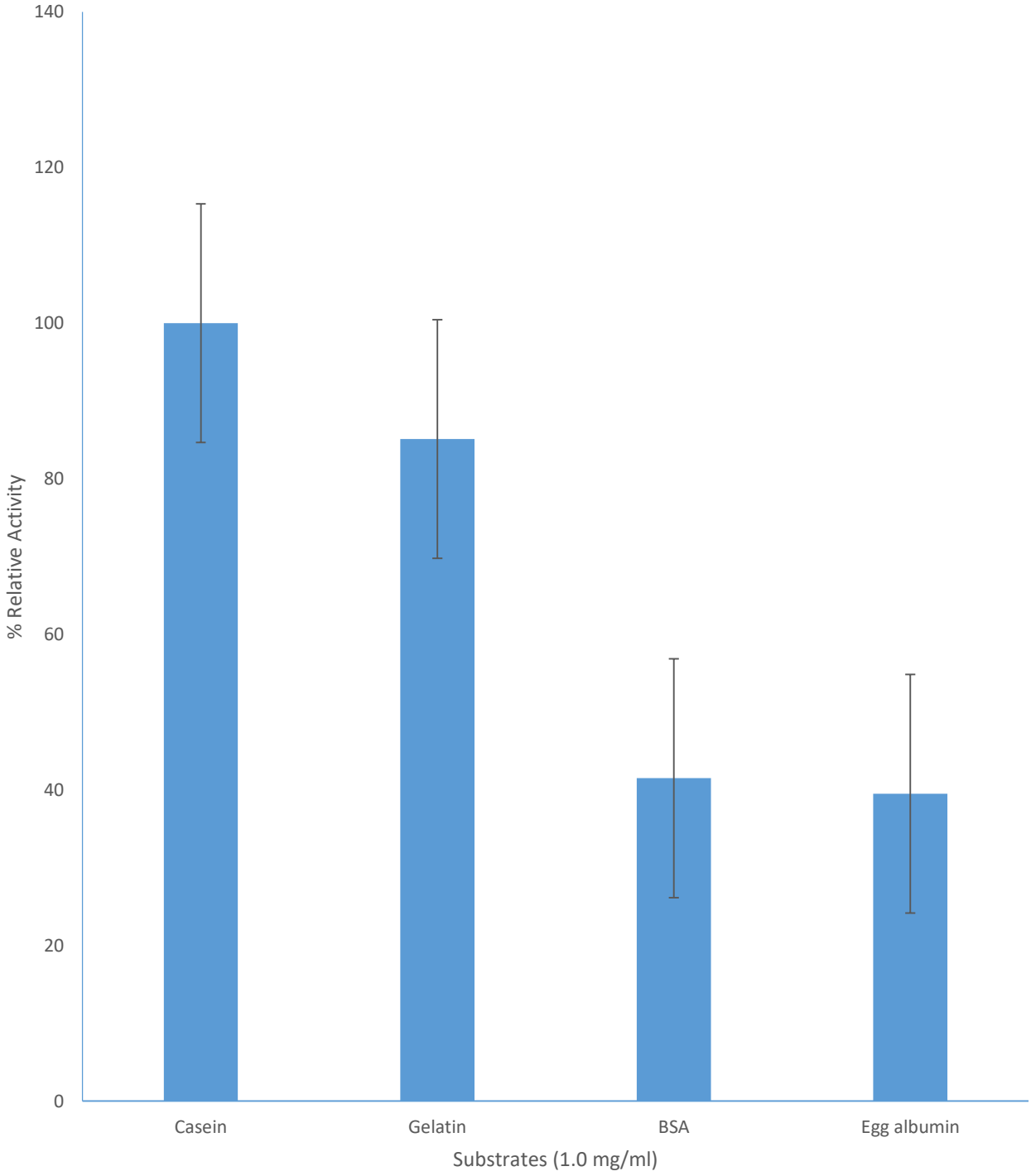

Figure 4.18: Relative Rates of Hydrolysis of Various Substrates by OS2A Protease.

The error bars showed the standard deviation (±SD) of duplicates.

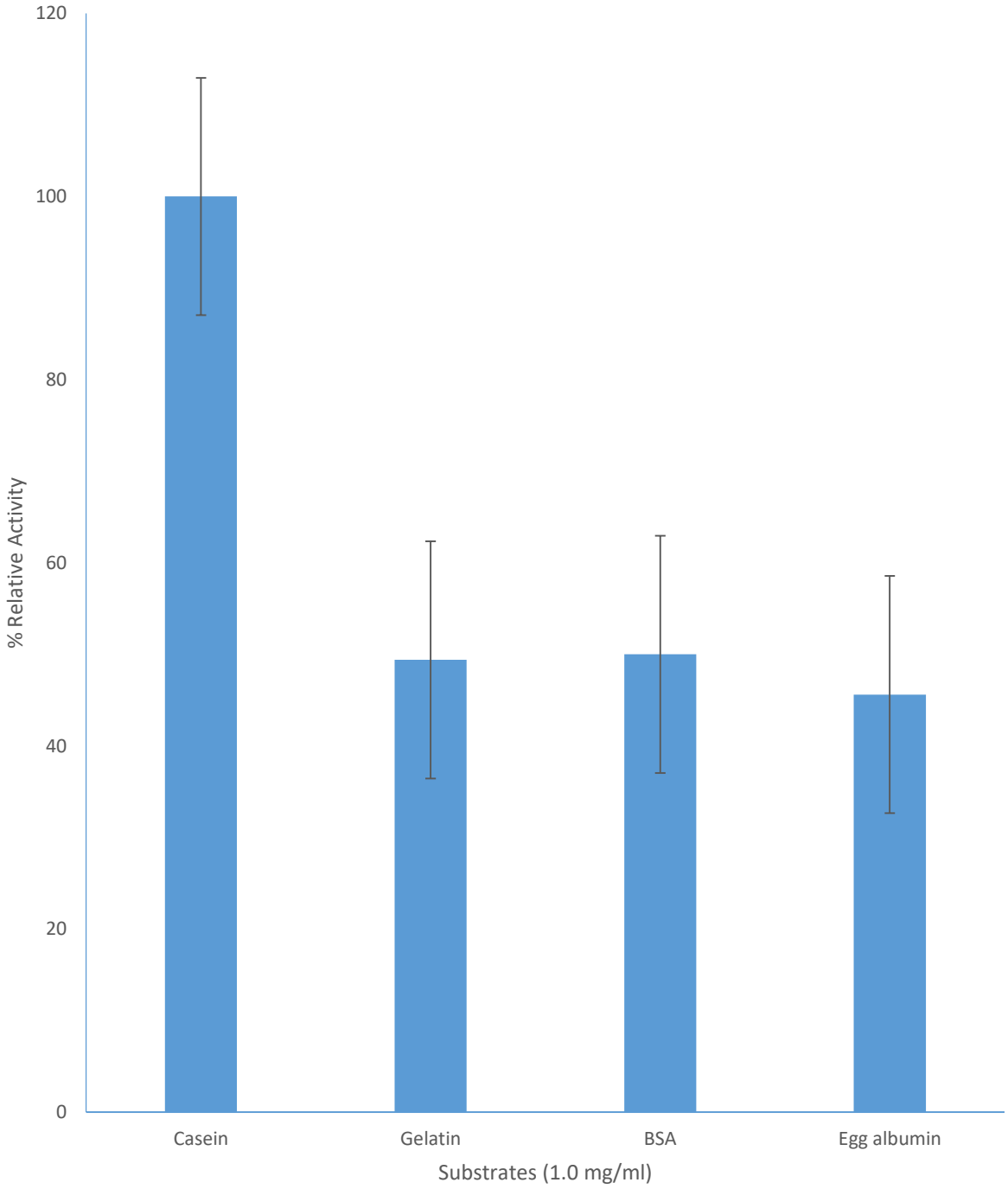

Figure 4.19: Relative Rates of Hydrolysis of Various Substrates by OS2B Protease.

The error bars showed the standard deviation (±SD) of duplicates.

4.14.10 Effect of Substrate Concentrations on Protease Activity

The results of the effect of varying concentrations of the substrates (casein, BSA, gelatin and egg albumin) on the activity of the partially purified enzymes are shown in Figures 4.20 and 4.21. The protease activities obeyed a typical Michaeli's - Menten's type kinetics, being hyperbolic in all the substrates studied.

The kinetic parameters (*Km and Vmax*) of the proteases for the hydrolysis of the substrates were obtained from the double reciprocal of the data according to Lineweaver and Burk (1934). The results of the Michaeli's - Menten's constant (*Km*) and maximum attainable velocity (*Vmax*) shown in Tables 4.13 and 4.14 were determined from the Lineweaver-Burk plot,1/V vs 1/[S], illustrated in Figures 4.22 and 4.23.

The enzymes differed in their affinity for the different substrates. Protease OS2A had highest affinity for gelatin (*Km* 0.022 mg/ml) while protease OS2B demonstrated highest affinity for casein (*Km* 0.077 mg/ml).

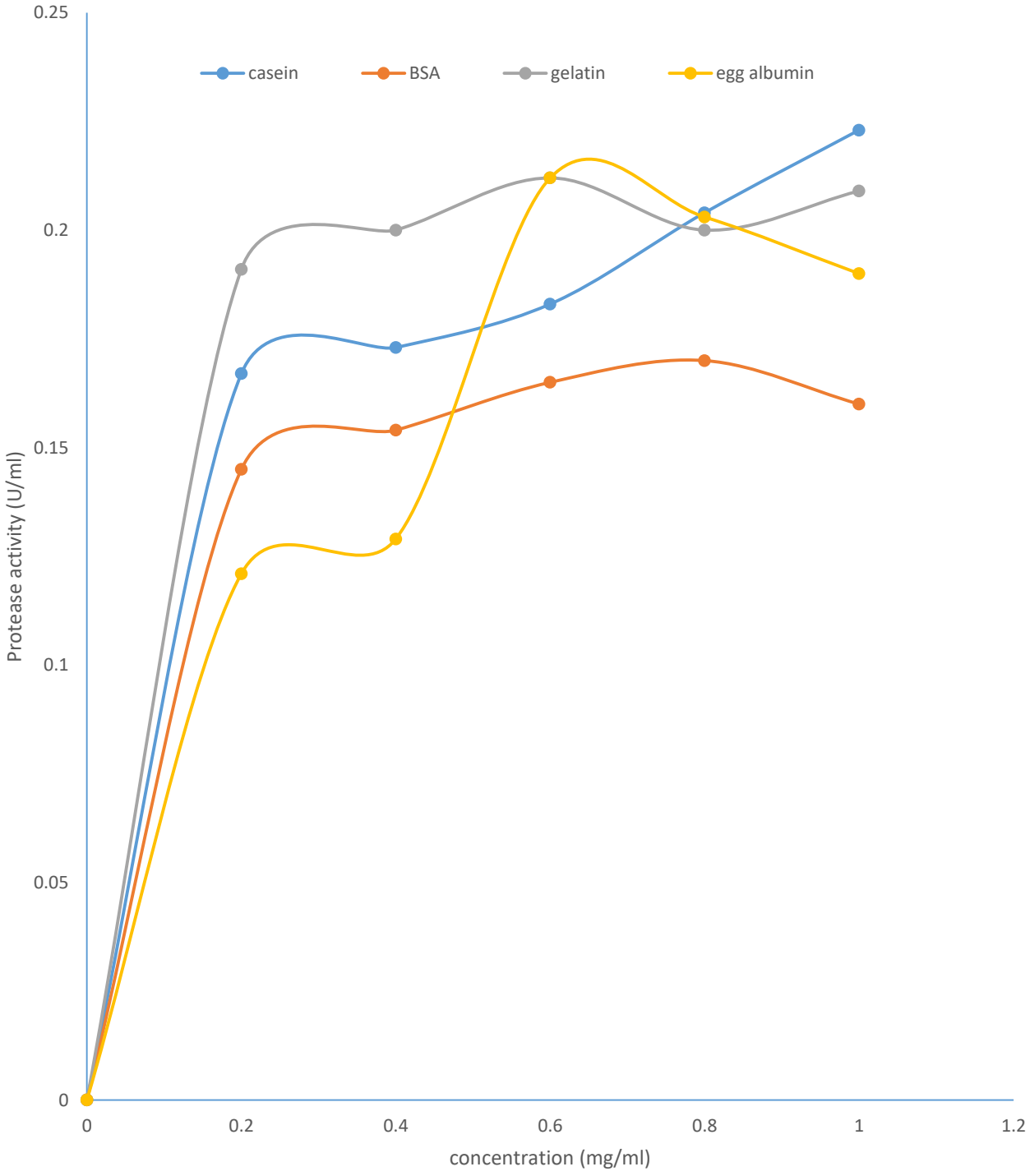

Figure 4.20: Effects of Substrate Concentrations on the Activity of OS2A Protease.

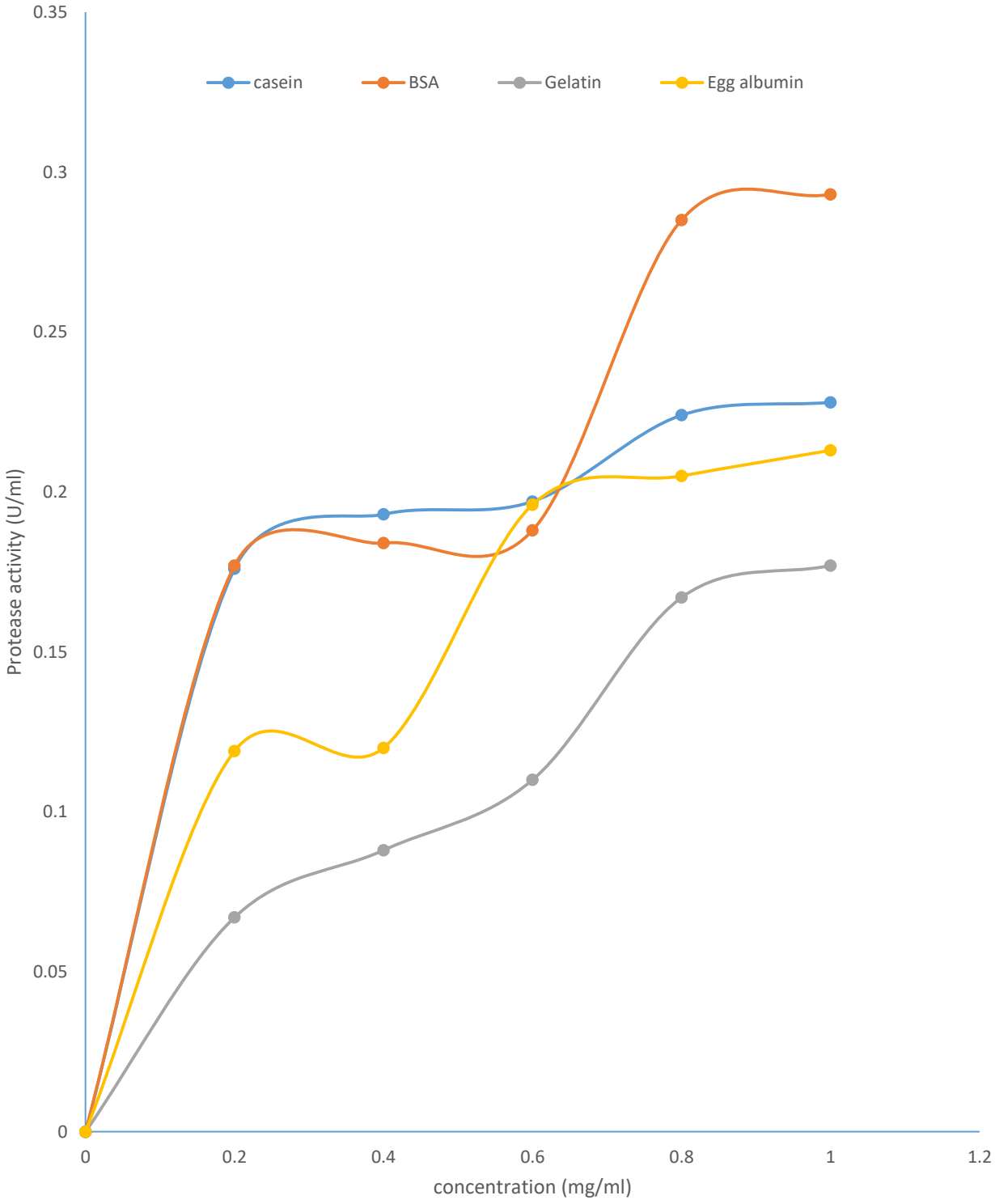

Figure 4.21: Effects of Substrate Concentrations on the Activity of OS2B Protease.

Table 4.13: Kinetic Parameters of OS2A Protease

Substrates	*Vmax* (mg/ml/min.)	*Km* (mg/ml)
Casein	0.217	0.069
BSA	0.172	0.038
Gelatin	0.213	0.022
EA	0.241	0.215

BSA - Bovine serum albumin

EA - Egg albumin

Table 4.14: Kinetic Parameters of OS2B Protease

Substrates	*Vmax* (mg/ml/min.)	*Km* (mg/ml)
Casein	0.234	0.071
BSA	0.284	0.142
Gelatin	0.240	0.547
EA	0.249	0.247

BSA - Bovine serum albumin

EA - Egg albumin

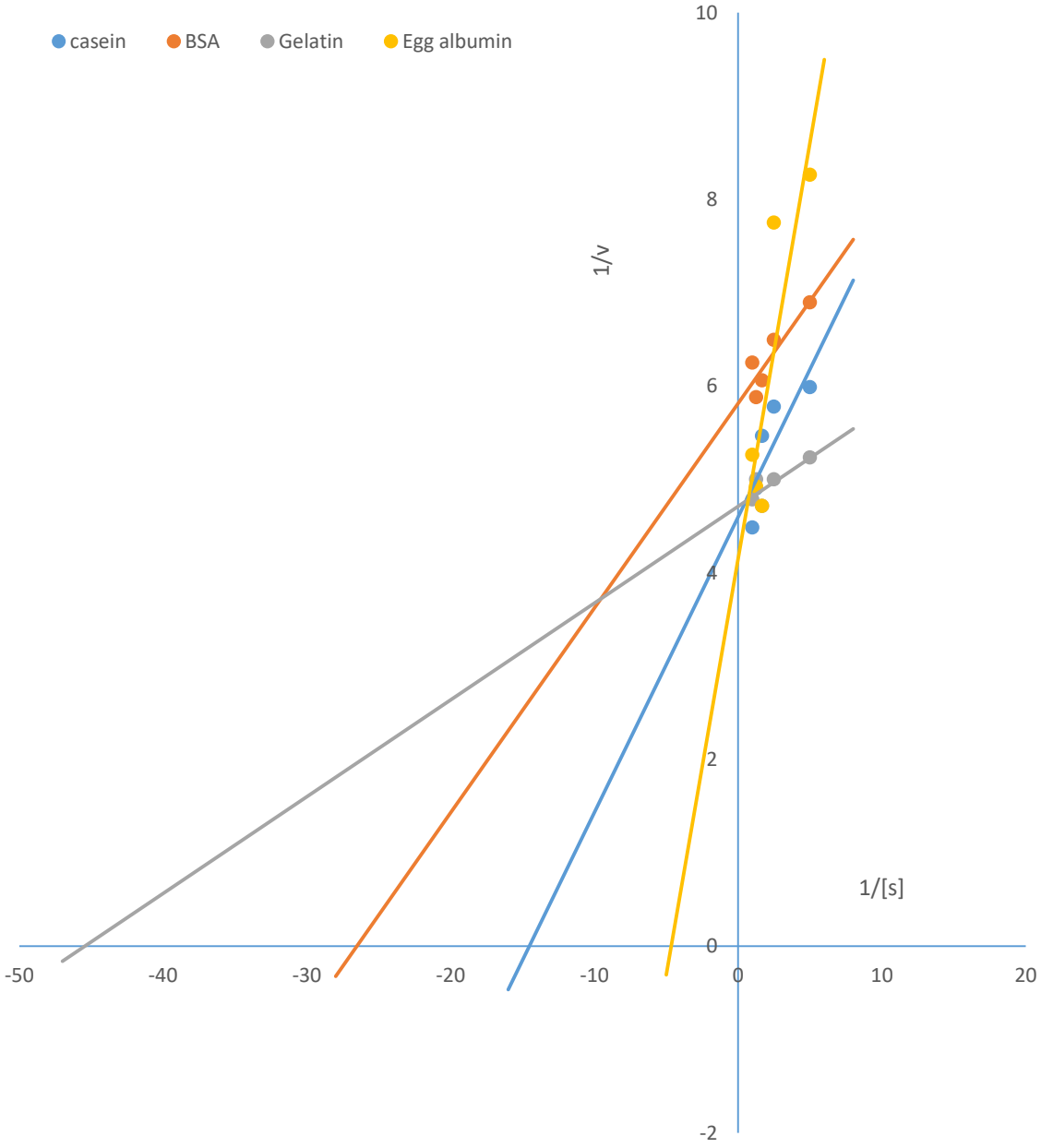

Figure 4.22: Lineweaver-Burk plot of 0S2A Protease for Various Substrates.

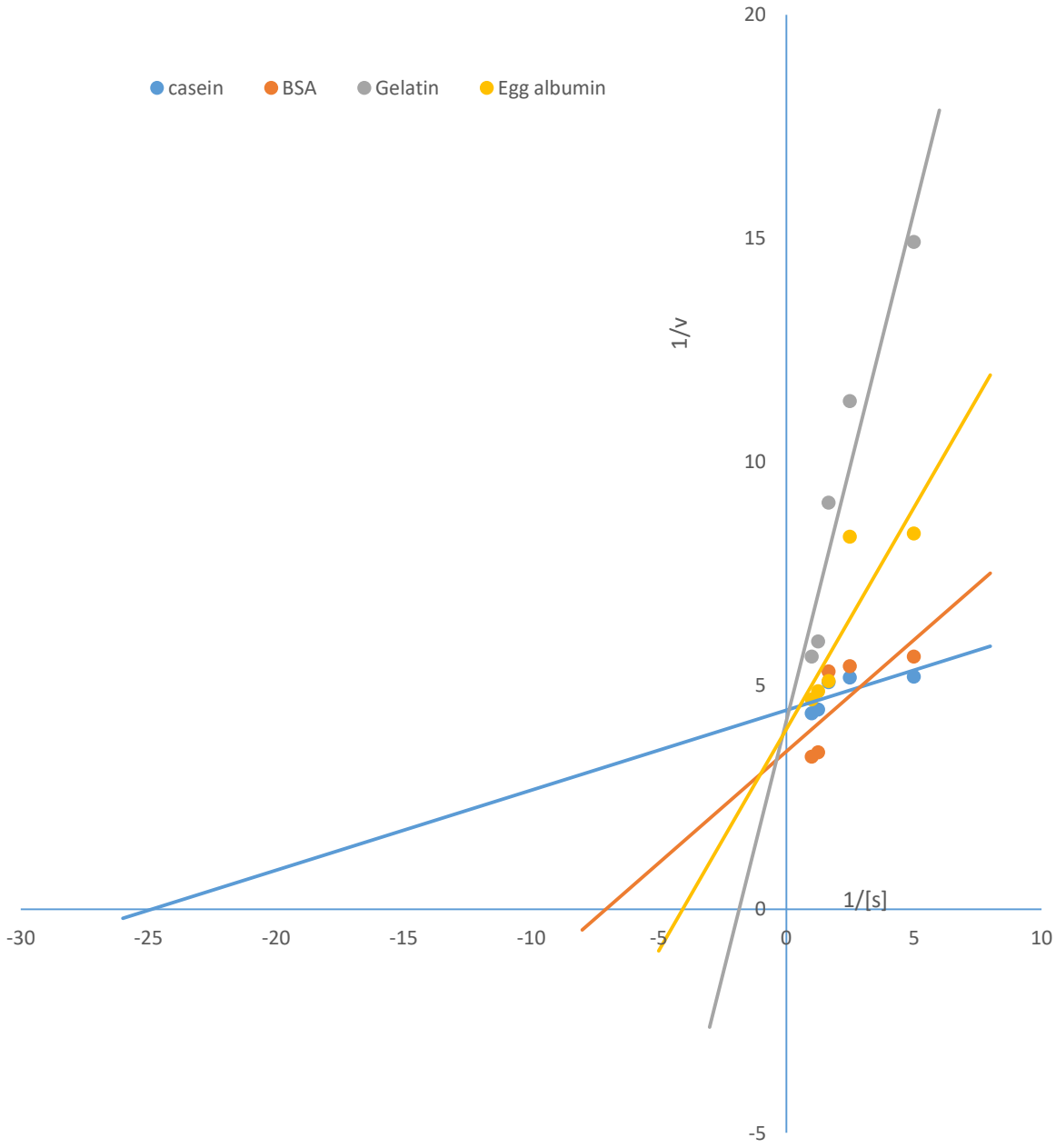

Figure 4.23: Lineweaver-Burk plot of 0S2B Protease for Various Substrates.

4.14.11 Rate of Catalysis (K_{cat}) or Turnover Number

The results of the constant $K_{cat,}$ called the turnover number expressed as K_{cat} = *Vmax* / $[E_T]$ of the proteases are shown in Table 4.15. The turnover number which helps check the efficiency of a given enzyme is calculated from the maximum attainable velocity (*Vmax*) of substrate molecules and the total enzyme activity $[E_T]$. The OS2A protease exhibited varying degrees of substrate molecules conversion to products. The rate of catalysis (K_{cat}) or the maximum number of substrate converted to product was highest with egg albumin at 0.0244 U/mg/min, followed by casein (0.0219), gelatin (0.0215) and BSA (0.0174). Similarly, the rate of catalysis of OS2B protease were; BSA (0.0221), egg albumin (0.0194), gelatin (0.0187) and casein (0.0182). Comparing these results, the OS2A exhibited higher K_{cat} values as against OS2B and these values help to determine the enzyme efficiency. High specificities enhance the conversion of the substrate molecules to useful product.

Table 4.15: Rate of Catalysis (K_{cat}) of the Proteases

Substrates	Kcat (U/mg/ml)	
	OS2A	OS2B
Casein	0.0219	0.0182
BSA	0.0174	0.0221
Gelatin	0.0215	0.0187
EA	0.0244	0.0194

BSA - Bovine serum albumin

EA - Egg albumin

4.14.12 Enzyme or Catalytic Efficiency (Ee)

The results of the protease catalytic efficiency (Ee) expressed as the K_{cat} / *Km* is depicted in Table 4.16. The OS2A protease was more efficient than OS2B protease as shown by their higher catalytic efficient values with gelatin at 0.977 U/ml/min, BSA (0.458), casein (0.317) and egg albumin (0.114). Meanwhile, that of OS2B protease catalytic efficiency values were; casein (0.256), BSA (0.156), egg albumin (0.079), and gelatin (0.034), respectively. The catalytic efficiency gives insight as to the usefulness of this enzyme for industrial application. Since OS2A was more efficient than OS2B, the stability is guaranteed and so present good quality for industrial use.

Table 4.16: Enzyme Efficiency (Ee) of the Proteases

Substrates	Ee (U/ml/min.)	
	OS2A	OS2B
Casein	0.317	0.256
BSA	0.458	0.156
Gelatin	0.977	0.034
EA	0.114	0.079

BSA - Bovine serum albumin

EA - Egg albumin

CHAPTER FIVE

DISCUSSION

5.0 Discussion

Grain Analyses

The results in Table 4.1 showed some physicochemical properties of the unmalted Oba Super 2 (OS2) maize variety. It gave much information which are relevant for determining the suitability of the maize grain for malting and brewing. The 1000 corn weight obtained for OS2 of 280 g was good. In barley, the 1000 kernel weight is a measure of the size of the grains and therefore a reflection of their extract potential (Abiodun, 2002). Hence, the OS2 maize variety had larger 1000-corn value than Farz 23 yellow maize (Iwouno and Odibo, 2015), while sorghum SK5912 had correspondingly lower value (Archibong *et al*., 2015).

The moisture content of 11.5% obtained in this study was lower than the value reported for maize Farz 23 yellow at 12.8% and Farz 34 white at 13.2% (Iwouno and Odibo, 2015), but higher than in Finger millet at 7.67% (Banusha and Vasantharuba, 2013). However, sorghum SSV98001 gave correspondingly higher value of 14.4% when compared with OS2 maize cultivar (Archibong *et al*., 2015).

The germinative properties are useful in selecting grains for malting. In brewing industries, it is necessary to carry out a test on the viability of the grains by making germination counts. If germination falls below 65%, the grain is not viable enough to malt, because diastatic enzymes are activated only during germination (Gomez *et al*., 1997). The determination of the germinative percentage in barley for instance, is to ensure good

germination of the grains to be malted. Germination energy enables maltsters to detect dormancy in barley and it was adopted for the study of maize to detect any sign of dormancy.

The germination capacity is a measure of percentage living grains in the sample. The 0.75% (*v/v*) H_2O_2 technique is regarded as the method of measuring living corn in the sample. The germination energy and capacity recorded in this study were about the same as several varieties of sorghum studied by Abiodun (2002). The germination energy and capacity for the grain in this study fell within the range at 94% and 98%, respectively. These values are at variance with the findings of Iwouno and Odibo (2015) for maize, Farz 23 yellow at 92% and Farz 34 white at 96% as well as Iwuagwu and Izuagbe (2004) for millet at 97%.

Maltsters avoid water sensitive grains or have to adjust the steeping regime to overcome the condition. The value obtained for water sensitivity indicated that this variety was not water sensitive with about 87% achieved. Similar observation was noted by Eneche (2009) for maize grain. However, Archibong *et al*. (2015) achieved 98 and 93% water sensitivity for sorghum SSV98001 and SSV98002, respectively. Germination increased the water absorption capacity of the sample, in line with the work of Gernah *et al*. (2011). The increase observed in this study might be as a result of the production of compounds having good water holding capacity such as soluble sugars.

The broken kernel obtained in this study was 0.82% and this value fell within the range of some workers. Iwouno and Odibo. (2015) reported 1.1% and 0.9%, for two Nigerian maize varieties (Farz 23 yellow and Farz 34 white), respectively. The value we obtained is an indication that it contained tolerable levels of broken kernels. Broken kernels are major sources of microbial infection during malting of grains.

The fat content (ether extract) of 4.20% obtained in this study is in line with some studies. The fat content of 3.70 and 4.14% had been reported for maize Farz 34 white and 23

yellow cultivars, respectively (Iwouno and Odibo, 2015), while the result contrasts with 1.42% obtained for Finger millet (Banusha and Vasantharuba, 2013). Information about fat content is very vital in brewing. The low fat value of the grain is good because high level of lipid can destroy foaming potentials of beer. They can accelerate staling. In brewing, lipid content is important with regard to foam and beer oxidative stability both of which are adversely affected by high levels of lipids (Briggs *et al*., 2004). Whole grain has a considerably higher fat content (approx. 4.4%) (Watson, 2003). Brou *et al*. (2013) reported increase in foam stability with high protein content while characterizing complementary food from maize, millet, beans and soybeans. Higher protein stability for native proteins has equally been reported by the same authors. The increase in foaming stability was as a result of bioavailability of inherent proteins bound by anti-nutritional factors such as phytin. Since this grain variety has comparatively low fat value, it lends itself as promising candidate for use in producing brewer's grits.

The protein content of the grain under study stood at 9.06%. This value was similar to that of maize varieties- Farz 23 yellow at 9.0% and Farz 34 white at 8.65% (Iwouno and Odibo, 2015) but at variance with millet at 10.66% (Banusha Vasantharuba, 2013). The protein content of this grain was higher compared with the generally accepted value for cereals; about 7.5% (Malomo and Alamu, 2013) and the findings of Iwuagwu and Izuagbe (2004) for Nigeria millet, *Pennisetum typhoideum* which was about 8.40%. Grains with high protein contents are not recommended due to problem of haze. It may be inferred that this variety has been specially bred for high protein content since it is used for food. Oba Super 2 used in this study is a high quality protein maize (QPM), having about 70% more essential amino acids (lysine and tryptophan) (Hasjim *et al*.,2009; Giwa and Ikujeniola, 2009), and more polyunsaturated essential fatty acids, reported to have been in suboptimal amount in normal maize varieties (Prasama *et al*., 2001).

Malt Analyses

Malting Loss (ML)

The analysis of the effect of steeping time (sequences) and germination periods on the malting loss (ML) of the malt is shown in Table 4.2. Steeping the grain increases the water content of kernel and also activates enzymes stored in the endosperm (Cozzolion *et al*., 2014), thus prompting these enzymes for action. Germination facilitates the conversion of endosperm and enzymes synthesis in the grain for proper modification (Feyzipour and Ghaboos, 2010; Archibong *et al*., 2009). It is interesting to note that the malting loss through the roots and shoots growths increased progressively with longer days of germination period at different steeping times. The malting losses (ML) were slightly higher in the 42h steep cycle but the increases in all the cycles were consistent.

The result of analysis of variance (ANOVA) of the effects of steeping period (hour) and germination time (days) on the ML of the grain indicated significant difference ($p < 0.05$) between the parameters [steeping period (hours) and germination time (days)] on Oba Super 2 (OS2) maize malt.

Malting loss is a key aspect of malting and should be minimized for economic viability of the malt (Eneje *et al*., 2007). The malting loss can be attributed to higher green malt metabolic activity. At the onset of germination, high protein leads to high rate of germination and root growth would be faster, ML would be greater, while the extract yield would be lower. Conversely, malting leads to reduction of protein, disulfide bond and proteolytic degradations. However, it could be concluded that the effect of the steeping periods made no significant difference ($p > 0.05$) on the malting loss, but rather day of germination. It is entirely due to respiration and so no significant loss occurred during

steeping. ML increased with germination time of these malt quality parameters - DP, FAN, CWE and HWE. Indeed, we realized that the effects of longer steeping time contributed significantly (increase) to the high extract yields and DP values, both indications of malt quality. The higher ML is attributed to higher green malt metabolic activity (Pelembe *et al*., 2004). Similar losses were reported for proso millet (Martin *et al*., 2010) and for sorghum (Irakoze *et al*., 2010). Ogu *et al.* (2004) observed that the relationship between high malting loss with low total nitrogen content is associated with the peculiar modification and proteolytic pattern in sorghum malt. Such report could be attributed to the translocation of more nitrogen materials to its roots and shoots during malting. Malting loss reflects dry matter losses through respiration, rootlet and shoot growths (Badau *et al*., 2006a).

Information concerning the optimum seedling growth period for barley lies between 5 to 6 days (Ghasemi *et al*., 2012). Studies on sorghum show that growth optimum is between 4 and 5 days (Ukwuru, 2010). Such information seems not to be generally available for maize. So, series of malting experimental variables were put to trials to determine the best conditions on the maize variety as regards protease development. Maize malt employed in this study was derived from grains at different steeping cycles and allowed to germinate from zero (0) to five (5) days and analyzed.

Cold Water Extract (CWE)

Table 4.3 showed the CWE of the maize malt at different steeping cycles and germination days. Cold water extract is a general reference for water soluble materials formed during malting and easily extracted with cold water, including readily available sugars and amino acids located in the malt endosperm. It measures only water extract by preventing enzyme action with dilute ammonia solution (Ghasemi *et al*., 2012).

It was observed that the CWE values increased with longer steeping time and germination days. These parameters peaked on 4th day of germination and declined. The results showed that at 30, 36 and 42 h steeping, CWE values ranged from 26.99-43.25; 26.08-44.56; 26.37-50.85%, respectively between day zero to day 4 (G_0 – G_4) germination. The result of analysis of variance (ANOVA) of the cold water extract (CWE) of the malt indicated significant difference ($p < 0.05$) among the parameters [steeping period (hours), germination time (days) and kilning temperature (^oC)] on Oba Super 2 (OS2) maize malt.

Cold water extract is an important malting quality parameter for assessing good malt. CWE is a useful malt property that predicts the degree of modification achieved during malting, especially as it concerns the soluble nitrogen content of malt (Ogu *et al*., 2006). In barley, the CWE value of 17-20% had been established and accepted as a good indicator of well modified malt. The effect of steeping and germination time of barley malt had been reported by Ghasemi *et al*. (2012). They observed that as germination prolonged, malting yield and total nitrogen content decreased while CWE yield increased, reaching maximum at 36h steeping and 7days germination. The CWE value for maize malt and some other tropical cereals are yet to be established. Reports from sorghum and millet malt have shown that the values ranging from 30-40% have been recorded and these values proved good brewing qualities (Ogu *et al*., 2006; Ogbonna, 2007). High CWE values show high rate of production of starch degrading enzymes. Badua *et al*. (2006a) reported that increase in germination time affected the CWE and HWE in pearl millet malt and sorghum.

The maximum CWE values of 43.25, 44.56 and 51.87% obtained after 4th day of germination at 30, 36, and 42 hours, respectively were within the range as previously reported by Ogu *et al*. (2006) for sorghum varieties. However, our findings are higher than the established and accepted CWE value of 17-20% for barley (Ghasemi *et al*., 2012). The increase in the values could be attributed to proper manipulation of the malting parameters;

different steeping sequences, air rest, good watering regime and kilning temperatures employed in the course of this work.

Hot Water Extract (HWE)

The HWE (Table 4.4) showed increasing trends with the increase in germination period. It is an analytical measure of the quality of dissolved solid or soluble materials from the malt when some hydrolytic enzymes have acted optimally. It is one of the key attributes considered when determining the malting quality and performance of brewery grains. It is said to be the major determinant of wort quality. Report had shown that malt quality is determined by the nature of sugars produced during the malting process. Glucose and maltose were found to be the main sugars in the HWE (Jacob, 2010) for sorghum variety. Maximum yield of HWE for barley was obtained after 36 h steeping and 7days germination (Ghasemi *et al*., 2012). HWE values of maize and other tropical cereals are lower than that of barley due to a lower diastatic power. This disagrees with the findings of Aloh and Agu (2010) for *Pennisetum maiwa,* and pearl millet.

It is noteworthy that the HWE values of 243.25, 238.60 and 250.05% obtained here at steeping cycles of 30, 36 and 42 h on 4^{th} day of germination, kilned at 45, 55 and 50, respectively, were lower than those other varieties reported elsewhere. Iwouno and Odibo, (2015) had reported HWE values of 300 and 301% at 42 h on 5^{th} day of germination, kilning temperature of 55^{o}C for maize Farz 23 yellow and Farz 34 white varieties. The reason for their high extract values could be as a result of improvement in the malting quality characteristics of these varieties due mainly to their active brewing- related use in the Nigerian brewery industry. Our findings, however, were much higher than sorghum varieties reported by Nnamchi *et al*. (2014) which could be as a result of smaller grain size. Comparatively, higher HWE values obtained in this present study suggest that grain size influences HWE quality, a reinforcement of the fact that bigger grains contain

proportionately less husk and therefore a higher carbohydrate content than smaller ones (Bamforth, 2002; Abiodun, 2002).

The result of analysis of variance (ANOVA) of the hot water extract (HWE) values indicated significant difference ($p < 0.05$) among the parameters [steeping period (hours), germination time (days) and kilning temperature (^oC)] on Oba Super 2 (OS2) maize malt. The HWE values obtained in this study, showed increasing trends with longer period of steeping and germination, thus reaching maximum, on the 4^{th} day of germination and declined on the 5^{th} day. The highest HWE development was noticed on sample steeped for 42 hours, 4^{th} day of germination and kilned at 50^oC. This increase in HWE with longer germination days is an indication of the progress of modification (breakdown of the endosperm reserves, predominantly by amylase and protease enzymes) of the malt during germination and this finding was supported by Aloh and Agu (2010). However, much protease development recorded at 42 h steeping and on the 3rd day of germination in this study was almost similar with the findings of Ghasemi *et al*. (2012) for barley malt.

Free alpha Amino Nitrogen (FAN)

The Free alpha amino nitrogen (FAN) of the malt which is a source of nitrogen for yeast nutrition, is important in opaque beer brewing because malt constitutes only a relatively small proportion of the cereal grist. Since the protein content of brewing grains must first be converted to amino acids and small peptides (denoted as assimilable nitrogen) before it can be utilized by fermenting yeasts for growth, FAN determination is a very important index of brewing grain quality (Ezeogu and Okolo, 1996).

In this study, FAN level increased with corresponding increase in germination time from zero to day 4 and declined on day 5 (Table 4.5). The results showed that at 30 and 36 h steeping, the FAN levels increased between 28 – 49 mg/L while at 42 h, the trend was

between 29 – 53 mg/L corresponding to germination time (days) of G_0 – G_4. This trend had been found to be true for sorghum and pearl millet (Badau *et al*., 2006a). Free alpha amino nitrogen levels vary in relation to the sugar content of the fermenting wort (Badau *et al*., 2005a).

The result of analysis of variance (ANOVA) of the free alpha amino nitrogen (FAN) values indicated significant difference ($p < 0.05$) among the parameters [steeping period (hours), germination time (days) and kilning temperature (^oC)] on Oba Super 2 (OS2) maize malt.

The FAN levels obtained in the current work were all at levels of < 100, showing that they have low FAN levels. Their levels are at variance with the recommended FAN level necessary for satisfactory yeast growth and fermentation, which has been put at between 100 and 150 mg/L (Ezeogu and Okolo, 1996). The FAN levels obtained in this work, differed significantly ($p<0.05$) from those reported previously by Ogu *et al*. (2006) on sorghum and maize varieties.

The production of endopeptidase enzymes is associated with the production of high FAN levels. This agrees with finding of Pelembe *et al*. (2004) for pearl millet. FAN levels increased with watering regime but unlike DP, germination at high watering regime gave continuously high FAN values. This observation was noted in the course of this research. The increase in FAN level could be due to the fact that high watering favours roots and shoots growth which are very rich in FAN. However, Eneje *et al*. (2007) noted that high malting loss due to excessive loss via roots and shoots may likely cause loss in FAN, which further corroborate with our findings in this work. Ogbonna *et al*. (2003) had already reported that during malting, carboxypeptidase enzymes hydrolyze proteins to free alpha amino nitrogen (FAN) which are utilized by germinating embryo for anabolic function in addition to being essential nutrients for yeast growth and fermentation.

Diastatic Power (DP)

The work on diastastic power (DP) of millet by Pelembe *et al*. (2004) corraborated with this result in this study. They also showed that DP increased with longer germination time. The DP measured in degree Lintner (^{o}L) is not meaningful when malt is rich in β-amylase, but tropical cereal malts are however low in β- amylase when compared with barley malt. The same authors reported that pearl millet with high watering regime gave an initial high level of DP, which later declined over long periods of germination. Germination medium moisture gave high DP value than with high and low watering regime after 5 days of germination. Similar observations were recorded for the decline in the level of DP with high watering regime which may be attributed to high malt moisture content of green malt which causes proportional enzyme denaturation when the malt is dried even at relatively low drying temperature (Egwin and Oluyede, 2006).

The present report of 42 hours steeping regime which gave higher protease activity as compared with 30 and 36 hours (Table 4.6) agreed with previous workers (Evan and Monday, 2009). In this report, DP of the maize variety increased significantly ($p < 0.05$) with longer period of steeping and germination sequences, however, sample kilned at 50^{o}C gave the highest percentage DP values in all the steep cycles. The G_0 hour of germination recorded the lowest diastatic power of 25.10, 25.17 and 25.30 ^{o}L, respectively for 30, 36, and 42 h steeping cycles but gradually increased with increase in germination time. The maximum DP values of 33.40, 36.55 and 38.07 ^{o}L recorded on 5th day, kilned at 50^{o}C showed enhanced development of hydrolytic enzymes (amylase and proteases) resulting from proper modification of the malt products that are more nutritious than the unmalted grain. However, sample steeped at 42 h gave the highest DP value.

The result of analysis of variance (ANOVA) of the diastatic power (DP) values indicated significant difference ($p < 0.05$) among the parameters [steeping period (hours), germination time (days) and kilning temperature (^oC)] of the maize malt.

Research had shown that low kilning schedules produced malts with greater DP and α-amylase activity. Okrah (2008) had reported kilning temperature of 50^oC, while Owusu-Mensah, (2009) in his studies gave the maximum kilning temperature to be between 40-45^oC. Report had shown that there is poor modification of the grain when there is no adequate moisture. However, high watering regime and different steeping cycles as well as the grain size (Agu *et al*., 2007) employed in this work had contributed maximally in the increase of malt produced in terms of brewing quality characteristics such as DP (amylase activity) and free amino nitrogen (free amino acids and short peptides). These findings corroborated with the earlier reports of Okrah (2008) and Owusu-Mensah (2009) on low kilning schedules.

Taylor and Dewar (2001) had earlier reported increase in brewing quality characteristics resulting from increased steeping periods. In general, steep treatment has been reported to increase enzyme activities. There is significant increase in α- amylase activity when wheat (*Triticum aestivum*) grains were hydrated for longer periods (40 hours) than 24 and 16 hours and germinated from zero to 6 days (Mohammed *et al*., 2009). When compared with barley, lower amylase levels were observed in maize, millet, rice, wheat and sorghum after 48 hours of sprouting. Similar observation was noted by Eneje *et al*. (2004) and Pelembe *et al*. (2004) in millet varieties. Increase in DP when maize, acha, rice and sorghum were germinated for 180h had been reported (Evan and Monday, 2009). In contrast, the levels of proteases were comparable in all the cereals with the exception of wheat which notably had the highest value. During malting, hard endosperm is enzymatically converted into friable malt and alpha (α-) amylase which plays a crucial role in this respect. Hydrolytic

enzymes (amylase and proteases) were enhanced, resulting in products more nutritious than the unmalted grain (Akoma *et al*., 2002).

Total Soluble Nitrogen (TSN)

The total soluble nitrogen (TSN) of maize malt depicted marked decrease as the germination progressed from zero to fifth day of germination (Table 4.7). This trend may not be unconnected with the enhancement of proteolytic activities arising from the degradation of protein materials during malting. Similar observation was noted by Malomo and Alamu (2013) for sorghum who reported marked decrease in protein content as modification progressed especially after four to five days probably as a result of more roots growth as germination prolonged.

The results had shown that at zero day, the TSN values of 8.20, 8.15 and 8.15% were recorded and gradually decreased as germination prolonged up to 5^{th} day, and then gave values of; 4.13, 4.19 and 4.28%, respectively for 30, 36, and 42 h steeping periods. This decrease in total soluble nitrogen in this study could be attributed to the degradation of protein to supply the embryo with amino acid. The residual total nitrogen in the raw maize, through the malt decreased gradually up to fifth day.

The result of analysis of variance (ANOVA) of the total soluble nitrogen (TSN) values indicated significant difference ($p < 0.05$) among the parameters [steeping period (hours), germination time (days) and kilning temperature (^oC)] of the maize malt.

These findings agreed with the reports of Malomo and Alamu, (2013) for sorghum who noted decrease in protein content as germination prolonged. These decreased values were difference significantly at $p < 0.05$, recorded on the 5^{th} day of germination at various kilning schedules except on 42 h, kilned at 50^oC which showed no significant difference ($p >$

0.05) between the germination time could have emanated from manipulation of malting parameters for progressive modification.

Cold Water Soluble Protein (CWS-P)

Cold water soluble protein (CWS-P) or nitrogen measures the level of proteolytic activity during malting. It comprises higher molecular weight products of the proteinase during germination (Ogbonna *et al*., 2003). In general, protein composition is crucial with respect to the malting and brewing processes (Bobalova *et al*., 2008; Malomo and Alamu, 2013). Water soluble proteins play vital role in the formation and stability of beer foam (Cizakova *et al*., 2006). Reports had shown that some proteins survived through the multiple steps of malting and brewing processes to end up in beer. Among these proteins, lipid transfer proteins are relatively abundance in this group (Leisegang and Stahl, 2005; Bobalova *et al*., 2008). Proteins are transformed in different manner and variable degrees in the course of malting, mashing and fermentation processes (Osman *et al*., 2003). The water soluble proteins which are resistant to proteolysis and heat coagulations are believed to have passed through these processing steps either intact or modified into final beer

In this study, the CWS-P increased progressively, reaching maximum at day 3 and marginally declined on the day 4 (Table 4.8). The CWS-P values of 48.83, 47.65 and 49.55% were recorded on the 3rd day of germination, at various steeping schedules (30, 36, and 42 h) and kilned at 50^{o}C. The result of analysis of variance (ANOVA) of the cold water soluble proteins (CWS-P) values indicated significant difference ($p < 0.05$) among the parameters [steeping period (hours), germination time (days) and kilning temperature (^{o}C)] of the maize malt.

The increase in CWS-P during malting is as a result of breakdown of the water insoluble hordein component of the reserve protein and release of bound (latent) protein

(Osman *et al*., 2003). It is pertinent to note that these maximum CWS-P values on the 3rd day kilned at 50°C for all the steeping regimes were highly significant at $p < 0.05$. The findings agreed with the previous workers who stated that as modification progressed, the CWS-P increases due to breakdown of water insoluble hordein component of the reserve protein and release of bound protein which contributed in the formation and stability of beer foam (Osman *et al*., 2003; Cizakova *et al*., 2006).

Total Non-Protein Nitrogen (TNPN)

The total non-protein nitrogen (TNPN) consists predominantly of products of storage protein hydrolysis which comprises amino acids and peptide containing 2 to 70 amino acid (Okolo and Ezeogu, 1996). Resting grains contain a reasonable amount of TNPN (Ogbonna *et al*., 2003). According to Okolo and Ezeogu (1996), TNPN of malt just like FAN represents the balance of the rate of TNPN released and the TNPN utilized by the new tissue in germinating grains for plant structure and enzyme synthesis.

The result of analysis of variance (ANOVA) of the total non-protein nitrogen (TNPN) values indicated significant difference ($p < 0.05$) among the parameters [steeping period (hours), germination time (days) and kilning temperature (°C)] of the maize malt. However, samples steeped at 30, 36, and 42 h gave maximum TNPN values of 33.68, 32.33 and 38.50, respectively on the 4th day of germination, kilned at 50°C. The general increase in TNPN value recorded on the 4th day of germination which are statistically difference ($p < 0.05$) may be as a result of translocation of the products of storage protein degradation, while the decrease thereafter to 27.90, 28.05 and 28.15, at kilning temperatures (°C); 55 and 45, respectively may have reflected the synthesis of the non-protein nitrogen into protein during seed germination and exhibited no significant different at $p > 0.05$.

Proteolytic Enzyme Development

The development pattern of the protease activity of the maize malt showed that at early stage of the germination, the enzyme had developed (Table 4.10). The activity was low at zero day but increased as germination progressed, reaching maximum on 3rd day and then declined from 4th day. Malomo and Alamu, 2013 reported increase in protease activity as germination advanced from zero to 5th day for sorghum varieties. The increase in protease activity values from the zero day of germination up to 3rd day and subsequent decline, in the present work, were consistent with the changes in protein content and protease activity of the maize malt.

The proteolytic enzyme in this study was developed by employing the independent variables including; steeping period (hour), germination time (day) and kilning temperatures (^{o}C), while the dependent variables studied were; CWE, HWE, FAN, DP, CWS-P, TSN and TNPN.

The result of analysis of variance (ANOVA) of the protease activity of the malt at various steeping schedules (30, 36, and 42 h), germination time (G_0-G_5) and kilned at 50^{o}C showed significant difference ($p < 0.05$) among the parameters studied. It was noted that there was progressive increase in protease development until after the third day, where the activity gave the maximum values in all the steep cycles. Decrease in the activity was recorded from the fourth day of germination. However, in all the steeping periods, 42 h, on the 3rd day germination, kilned at 50^{o}C gave the best protease development of 0.834 U/ml and hence was applied for enzyme purification. The maximum development of protease activities at G_3 S_{30} (0.679), G_3 S_{36} (0.757) and G_3 S_{42} (0.834 U/ml), respectively indicated significant difference ($p < 0.05$). The activities decreased to G_5 S_{30} (0.642), G_5 S_{36} (0.656) and G_5 S_{42} (0.671 U/ml), respectively at the 5th day of germination.

Crude Enzyme Purification

The crude enzyme was purified by a combination of precipitation with 70% saturated ammonium sulphate, $(NH_4)_2SO_4$ overnight at 4^oC, ion- exchange chromatography on CM Sepharose, dialysis at 4^oC against 5M sucrose solution overnight and hydrophobic interaction chromatography on phenyl Sepharose CL-4B gel. The homogeneity of the partially purified proteases were confirmed by the appearance of single protein bands on native polyacrylamide gel electrophoresis (PAGE).

Proteolytic Enzyme Elution Profile

The crude enzyme after extraction, was fractionated with 70% saturated ammonium sulphate, $(NH_4)_2SO_4$ which precipitated the enzyme and then eluted on ion-exchange chromatography. Fractionation using ammonium sulphate is one the oldest and most commonly used method for enzymes in which neutral salts such as ammonium sulphate are used at high concentrations. Enzymes having high molecular weight usually precipitate at lower ammonium saturation (25-35%) and vice versa. The enzyme in this study precipitated at higher ammonium sulphate saturation (70%) and was presumed to have lower molecular weight.

The crude enzyme was first eluted on ion-exchange chromatography on carboxymethyl (CM) Sepharose. Figure 4.1 showed the elution profile of maize malt protease on ion-exchange chromatography (IEC). The elution pattern which showed two major peaks of protease activities between fractions 4-11 were pooled and designated Oba Super 2A (OS2A) while fractions 26-31, designated Oba Super 2B (OS2B) were concentrated separately at 4^oC against 5M sucrose solution overnight. A plot of fraction number against protein content (mg/ml) and enzyme activity (U/ml) as well as linear gradient of 0.5M NaCl solution was equally indicated.

Figure 4.2 showed the elution profile of maize malt (OS2A) protease on phenyl Sepharose CL-4B hydrophobic interaction chromatography. The elution pattern which showed major peaks of protease activities between fractions 26-32, were pooled and dialyzed at 4^{o}C against 5M sucrose solution overnight.

Figure 4.3 showed the elution profile of maize malt (OS2B) protease on phenyl Sepharose CL-4B hydrophobic interaction chromatography. The elution pattern which showed major peaks of protease activities between fractions 27-32, were pooled and concentrated.

The homogeneity of the partially purified proteases were confirmed by the appearance of single protein bands on native polyacrylamide gel electrophoresis (PAGE). The OS2A (D2) gave 62,811.93 Da, while that of OS2B (D2*) gave 63,436.98 Da. The average molecular weight of the partially purified alkaline (serine) proteases was estimated to be about 63,124.45 Da (63.1 KDa) as calculated from the migration distance in relation to reference proteins used in this study (Plate 4.1). The low molecular weight suggests that the enzymes possess short peptide chain and are presumed to be similar.

The molecular weight of 63.1 KDa in this study was close to 54 KDa of MRP (proteinase I) obtained from *Zea mays* L. malt using ammonium sulphate precipitation, ion-exchange on CM-cellulose, DEAE Sephadex and gel filtration on Sephadex G-100 (Goodfellow et al., 1993). The value also compared with 59 KDa of RSIP obtained from *Zea mays* L. using ion-exchange on Sepharose Q, gel- filtration on Sephacryl S-200, chromatofocusing on Mono-P, hydrophobic interaction chromatography on phenyl-Superose and sodium dodecyl sulphate (SDS) PAGE (Shannon and Wallace, 1979) and 62 KDa serine protease (RSIP) also obtained from *Zea mays* L., using gel filtration (James *et al*., 1996). The finding was also comparable with the 61 KDa serine protease isolated from the fruits of *Melothria japonica* (Thunb.) Maxim, using gel filtration on Sephacryl S-300 (Uchikoba *et*

al., 2001). However, our finding was at variance with 110 KDa serine protease obtained from wheat (Triticum aestivum cv. Pro INTA Isla Verde), using ammonium sulphate precipitation, gel-filtration on Sephadex G-25, and ion- exchange chromatography on diethylaminoethyl (DEAE)-Sepharose/Q-Sepharose/Sephacryl S-200-HR (Robert *et al*., 2003). Similarly, Chen *et al*. 2004 obtained 82 KDa serine protease (IBSP82) purified from sweet potato (Ipomoea batatas [L.] Lam) using ammonium sulphate precipitation, gel-filtration on Superdex 75/ Superdex 200 HR, and tricine-sodium SDS-PAGE.

The summary of the partial purification of protease from OS2A is as depicted (Table 4.11). The enzyme was partially purified 3.805-fold to give a 3.26% yield relative to the total activity in the crude extract and a final specific activity of 16.21 U/mg proteins. Similarly, that of OS2B was partially purified 3.932- fold to give a 4.24% yield relative to the total activity in the crude extract and a final specific activity of 16.75 U/mg protein (Table 4.12). The values recorded in these partially purified enzymes were at variance with 8.4-fold purification and 13.4% yield on a cysteine proteinase from sorghum malt variety, SK 5912 (Ogbonna *et al*., 2004). Similar observation was noted for purified protease from sorghum malt variety, KSV8-11 which was purified 5.0-fold to give a 1.4% yield relative to the total activity (Ogbonna *et al*., 2003). Kusuma et al. (2016) purified protease isolated from *Bacillus cereus* NC77 6.13-fold purity to give a 75.42% yield relative to the total activity of 2.64 U/mg.

Kinetic Properties of the Partially Purified Proteases

Effect of pH on the Activity and Stability of the Proteases

The effect of pH on the activity and stability was studied at the optimal conditions of activity of the proteases. For the OS2A, the optimal activity and stability was at pH 9.0 (Figure 4.4). Protease from OS2B demonstrated optimum activity at 9.0 and maximum

stability at pH 8.0 (Figure 4.5). As the pH increased from 3 - 4, there were drop in the stability and increased progressively for both proteases. Protease OS2A and OS2B were more stable at neutral to alkaline pH especially between 7 and 10. Further increase in pH beyond the optimal activity and stability witnessed sharp drop with about 48% and 32%, respectively at pH 11 for protease OS2A and corresponding decrease in the activity and stability with about 49% and 25%, respectively at pH 11 for protease OS2B.

These results are in conformity with finding of Antao and Malcata (2005) who stated that most plant serine proteases showed optimum pH range of 7-11. The optimum pH of maize proteases in the present study suggests that they acted at neutral to alkaline pH and are still active over these ranges of pH values. Our findings agree with most plant proteases. Ekpa *et al*. (2010) reported optimum pH of 8 and 9 for the leaves and peel of banana proteases, respectively. Optimum pH reported for latex protease from *Euphorbia species* was between 6 and 8 (Khan *et al*., 2008), while Cucumisin- like protease had an optimum pH range of 8-10 (Patel *et al*., 2007).

In contrast to our findings, lower optimal pH values had been reported by some workers on proteases of plants origin. The optimum pH of 4 had been reported for wheat malt protease (Fahmy and Fahmy, 2005); pH 5 for sorghum malt (Ogbonna *et al*., 2003); pH 5.5 for horse gram (Rajeswari *et al*., 2009), pH 7.5 for mushroom (Zhang et al., 2010), pH 7.9 for *Euphorbia mili* (Yadav *et al*., 2006); pH range of 6-9 with optimum activity at pH 8 (Ademola and Malomo, 2017) and pH 8 for sweet potato root (Chen *et al*., 2004). In barley, and germinated barley, aspartic and cysteine proteases were found to be active under acidic conditions, while serine proteases and metalloproteases were more active at pH near 7 and pH ≥7 respectively (Jones, 2005).

Our findings revealed the alkaline nature of these proteases and as such their suitability for applications in alkaline environments especially in detergents, brewing and

allied industries. A lot of research works have been documented, describing proteases with broad pH activities and stabilities. An alkaline serine protease from Triticum aestivum cv. Pro INTA Isla verde was observed to be active in the range of 8 – 10 (Robert *et al*., 2003). Also, Bogacheva *et al*. (2001) had reported extraction of plantagolisin, a subtilisin-like collagenase from leaves of common plantain (Plantago maior). The alkaline protease was active between neutral to alkaline pH (7 – 10). Similarly, an alkaline protease from the sprouts of Pleioblastus hindsii Nakai was equally observed to be active in a broad pH range of 6.5 – 10.5 (Arima *et al*., 2000). An alkaline protease from *B. licheniformis* RSP-09-37 was equally observed to be active in a broad pH range of 4-12. This enzyme was 100% active at pH 10.0 and exhibited 14, 28 and 40% residual protease activity at pH 4.0, 5.0 and 12.0, respectively (Sareen and Mishra, 2008).

Since these partially purified enzymes were stable at neutral to alkaline pH, they are essential for utilization in a range of commercial applications especially in removal of protein hazes in brewing industry, meat tenderization, fur and leather industries.

Effect of Temperature on the Activity and Stability of the Proteases

The OS2A protease demonstrated optimum activity at 50^{o}C and maximum stability at 60^{o}C but retained about 70% of its original activity after 30mins at 60^{o}C.The enzyme exhibited less temperature stability of about 60% at 90^{o}C (Figure 4.6). Similarly, for OS2B protease, the activity was optimum at 50^{o}C and showed maximum stability at 50^{o}C, but retained over 70% of its activity after 30min at 60^{o}C (Figure 4.7). The OS2B protease was slightly more stable to temperature increase with about 68% present in the original activity at 90^{o}C, when compared with OS2A.

Our findings indicated that the proteases are stable at considerably high temperature and this suggests that they may be useful in food industry such as brewing and baking that

utilize protease at elevated temperature of about 50^{o}C to 60^{o}C. The optimum temperature of 50^{o}C for these proteases, agree with those obtained for most alkaline proteases, for example, senescent leaves of an invasive weed *Lantana camara* which had an optimal temperatures 50-60^{o}C (Gaur and Wadhwa, 2008). It equally correlated well with 40- 50^{o}C optimal recorded for banana leaves and peels protease (Ekpa *et al*., 2010). The optimum temperature of activity in this study is equally similar to that of sulphydryl or cysteine protease from sorghum malt variety, KVS8-11 which had an optimum temperature of 50^{o}C (Ogbonna *et al*., 2003). An optimum temperature of 50^{o}C was also reported for protease from *H. vulgare* L., SEP-1 (Fontanini and Jones, 2002). In contrast, the optimum temperature varies somewhat from those obtained from *Citrus sinensis* fruit peel which had an optimum temperature of 40- 45^{o}C (Ademola and Malomo, 2017). An optimum temperature of 45^{o}C was also reported for protease from *Allium cepa* (Ndidi and Nzelibe, 2012) and 40^{o}C for fermenting locust beans and melon seed (Evans *et al*., 2009). Serine protease from *Triticum aestivum*. cv. Pro INTA Isla verde had optimum temperature of 60^{o}C (Robert *et al*., 2003) and 80^{o}C for Ara 12 from *A. thaliana* (Hamilton *et al*., 2003).

In terms of stability to high temperature, the enzymes retained more than 70% activity in the temperature ranges of 50-60^{o}C. The broad optimal temperature range for maximum protease activity and stability reveals the thermostable nature of the partially purified protease. This thermal stability is desirable for most biotechnological applications of proteins. Thermal stability increases the efficiency of proteins and is one of the vital features for their exploitation at commercial scales (Pandhare *et al*., 2002).

The activity of alkaline proteases at broad temperature ranges is a desirable characteristic for their application in detergent formulations. Also, our findings indicate that the proteases are stable at considerably high temperature of 50 - 60^{o}C and this suggest that they may be useful in food industry such as brewing and baking. These industries utilize

protease at elevated temperature. Sareen and Mishra (2008) reported a thermoalkaline protease from *B. licheniformis* which was active at temperature ranges of 30-90^{o}C and had the maximum activity at 50^{o}C. Abusham *et al*. (2009) equally reported an alkaline protease from *B. subtilis* strain Rand with 100% stability in the temperature range of 35-55^{o}C. Ademola and Malomo. (2017) reported retention of more than 80% activity in temperature of 25 – 70^{o}C by protease from *Cittus sinensis* fruit peel.

Effect of Some Metal Ions on the Protease Activities

Certain chemical agents tend to stimulate or inhibit the activities of biocatalysts such as enzymes in biological systems. Most malt enzymes are known to be metal ion dependent, namely divalent ions like Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+} (Pandey *et al*., 2004; Sivaramakrishnan *et al*., 2006). The stimulatory effects of some of these metal ions are in conformity with the earlier work by some researchers. For example, the stimulatory effects of Cu^{2+} is in agreement with acid protease from sorghum (Ogbonna *et al*., 2004) and that of alkaline protease from Bacillus Gs-3 reported by George-Okafor and Odibo, (2011). On the effects of metal ions (Figure 4.8), the protease activity of OS2A was increased by Cu^{2+}, Ca^{2+}, Mn^{2+}, and Ba^{2+}, whereas Hg^{2+} showed only a slight stimulatory effect. Silver ion (Ag^{+}), Mg^{2+}, Zn^{2+}, Fe^{2+} and Co^{2+} had inhibitory effect on the enzyme, with Co^{2+} having the most effect at 29%.

The protease activity of the OS2B was appreciably stimulated by Cu^{2+}, Ca^{2+} and Mn^{2+}. There was slight stimulation with Hg^{2+} and Ba^{2+} by 18% and 16%, respectively. Magnesium ion (Mg^{2+}), Ag^{+}, Zn^{2+}, Fe^{2+} and Co^{2+} had inhibitory effect on the enzyme activity (Figure 4.9). Our findings showed that there is a striking difference in the preference of the divalent ions or otherwise by these partially purified proteases. Both enzymes seem to have

preference for Cu^{2+}, Ca^{2+} and Mn^{2+} for the enhancement of the proteases. Among the metal ions, Cu^{2+} seems to have the most stimulatory effect for both enzymes. Ogbonna *et al*. (2003) and George-Okafor and Odibo (2011) had reported the stimulatory effect on Cu^{2+} on the protease activity and these reports seem to be in conformity with our findings. However, our result is at variance with the reports of Adinarayana *et al*. (2003), where Cu^{2+} was shown to have no appreciable effect on the enzyme activity. Mercury is known for its effect on –SH group on enzyme. The non-inhibition of the proteases by Hg^{2+} suggests the absence of –SH in the active site of the proteases.

The partially purified proteases showed considerable activities at high concentration of various metal ions. Proteases having stability in the presence of metal ions are usually suitable in leather processes, sewage treatments etc. In addition to their stability towards temperature, pH, and metal ions, enzyme used in detergent formulation must be active in the presence of surfactants, oxidizing agents and other detergent additives (Anwar and Saleemuddin, 2000).

Effect of Some Inhibitors on Protease Activities

The inhibition of enzyme activity by specific small molecules and ion is important because it serves as a major control mechanism in biological systems (Schilling and Overall, 2007). In proteolytic enzymes, the knowledge of inhibitors gives the clearest evidence to the type of catalytic site, which in turn forms the basis for the classification of the enzymes (Jones, 2005; Buller and Townsend, 2013).

The partially purified maize malt proteases from OS2A and OS2B were inhibited by all the modulators tested, but PMSF was found to have the greatest inhibitory effect of 60% on the OS2A protease activity (Figure 4.10) and 66% on the OS2B protease activity (Figure 4.11). All other modulators demonstrated varying degrees of inhibition on the protease

activities. The fact that the enzymes were inhibited by PMSF, a serine inhibitor, indicates their requirement of serine for their catalysis. It may be concluded that both are serine proteases because PMSF caused a decrease in their activities. PMSF sulphonates the essential serine residue in the active site of the proteases, thus resulting in extensive loss of enzyme activity (Fontanini and Jones, 2002). Also, PMSF binds covalently to the enzyme, and the complex can be viewed by x-ray crystallography and can therefore be used as a chemical label to identify an essential active site of serine in an enzyme (Kunamneni *et al*., 2003). The effect of PMSF inhibition on the partially purified proteases is comparable to the results of Chen *et al*. (2004) and Robert *et al*. (2003) which indicated that the proteases were completely inhibited by PMSF. The inhibitory effect of 49% showed by EDTA (metal chelator) on the protease activity (OS2B) could mean that it is a metalloprotease but not a sulphydryl or cysteine (thiol) protease. The cysteine indicators- p-CMB and IAA (thiol or SH- blocking reagents) contributed minimal inhibitory effect of ≤ 36% and ≤ 25% for OS2A and OS2B, respectively. This finding above was also supported by Harshada and Geeta (2015), Dubey *et al*. (2010) and Silva *et al*. (2016) who suggested that EDTA and PMSF inhibited enzyme activity at various extents.

Effect of Oxidizing Agents (O. As) on Protease Activities

The partially purified proteases showed varying degrees of activities and were stable towards oxidizing agents. The OS2A protease exhibited compatibility and stability in the presence of O. As studied. It showed an enhanced activity on treatment for 30minutes with 1.0% H_2O_2 and DMSO, retaining its activity of 107.8% and 117.2%, respectively. Inhibitions of enzyme activities were equally noticed as the concentration of the oxidizing agents increased from 1.0- 5.0% (Figure 4.12). Similar trends were exhibited by OS2B protease. The activity of this enzyme was enhanced on treatment for 30 minutes also, with 1.0% H_2O_2 and DMSO. The enzyme retained its activity of 116.7% and 101.80%, respectively. The rates of

inhibition increases as the concentration of the O. As increased, and thus decreased to 54.7 and 46.6% for H_2O_2 and DMSO, respectively (Figure 4.13). Since DMSO is an oxidant and also organic solvent, protease stability in the presence of this reagent makes it essential for synthesis reaction, in particular peptide synthesis useful for pharmaceutical products. Proteases that are stable against oxidants (like DMSO and H_2O_2 studied) have been the common ingredients in modern bleach- based detergent formulations. In addition to the stability towards temperature, pH, metal ions, enzyme in detergent formulation must be active in the presence of surfactants, organic solvents and oxidants. Accordingly, alkaline serine protease from maize malt we studied exhibited such features. Oberoi *et al*. (2001) reported on the stability of alkaline protease from *Bacillus sp* RGR-14 towards oxidants which indicated a 40% loss in enzyme activity with 1.0 % H_2O_2.

Effect of Reducing Agents (R. As) on Protease Activities

The enzyme displayed varying compatibility and stability towards the reducing agents- dithiothreitol (DTT) and 2-mercaptoethanol (2-ME) studied. The OS2A was enhanced in the presence of 1.0% DTT at 117%, whereas, 2-ME showed decrease in the activity, loosing almost 9.0% of its activity on treatment for 30 minutes with 1.0% of this agent. On overall reaction, the activity decreased gradually with increased concentration from 1.0 to 5.0% (Figure 4.14). There was display of inhibition shown by OS2B at 96.3 and 92.7% for 2-ME and DTT, respectively in the presence of 1.0% concentration. The inhibitory activity increased as the concentrations of DTT and 2-ME increase after incubation for 30 minutes in these agents (Figure 4.15). The decreased activities of both enzymes in the presence of these agents present them as useful additives in detergent industry. Our findings agree with Sankeerthana *et al.* (2013) who reported that chelating agents such as EDTA, DTNB and 2-ME caused a considerable decrease in protease activity by *A. niger*. The high

activity of the proteases in the presence of 1.0% DTT is very useful for use as detergent additive since most of the detergents contain these agents as major component.

Effect of Detergents and Surfactants on Protease Activities

The partially purified enzymes exhibited varying stability and compatibility in the presence of tested detergents 1.0% (w/v) and surfactants 1.0% (v/v). Enzyme activity and stability in the presence of some commercial detergents give an insight to their exploit in detergent industry. A good protease is expected to be stable in the presence of these detergents (Devi *et al*., 2008). The results revealed that in both enzymes, some surfactants (Triton X-100 and SDS) appreciably stimulated their activities while others like Tween- 80 and Tween-20 were inhibitory. The partially purified OS2A protease was stable not only in the presence of non-ionic surfactant like Triton X- 100 which retained its activity of 318.98%, and a strong anionic surfactant (SDS), retaining 125.46% after incubation for 30 minutes, but also towards the detergent like Ariel, which retained about 136.02 activity. The other detergents (Klin and Omo) tested exhibited inhibitory effects on the enzyme activity (Figure 4.16). The OS2B was stable after incubation for 30 minutes in the presence of 1.0% Triton X- 100 at 216.11% and SDS at 116.07%, whereas, Tween -80 and 20 were inhibitory (Figure 4.17). The enzyme was equally compatible and stable on treatment for 30 minutes with 1.0% Ariel, retaining about 118.77% residual activity. Other detergents-Klin and Omo were inhibitory at 49.97% and 35.36%, respectively. The retention of the activity in the presence of Ariel at 1.0 % when compared with the control agrees with other researchers.

Our findings agree with that of Madan *et al*. (2002) who reported retention of 84.5% of activity in the presence of vim and more than 40% in the presence of nirma super and wheel. The differences in the retention of the activity and also loss of activity by Klin and Omo shown by these detergents could have emanated from their formulations. In addition to enzyme as additive in these detergents, other active ingredients could contribute to their

varying inhibitory effect. It had been shown that some detergents contain EDTA, a metal ion chelator. The high activity of this protease in the presence of chelating agent is very useful for its use as detergent additive since most of the detergents contain chelating agents as major component (Sanatan *et al*., 2013). The varying stimulatory and inhibitory effects of surfactants could be attributed to their composition (Gupta et al., 2002).

The Cleansing Performance of the Partially Purified Proteases

The washing performance of the enzymes was shown in plates 4.2 and 4.3. Visual examination of various pieces indicated that detergent incorporated with the partially purified enzyme (Flask C) for OS2A and (Flask F) for OS2B protease, cleansed the blood stained cloth more than that washed with detergent only**.** The protease showed excellent stability and compatibility in the presence of detergent (Ariel) used. The blood stain cleansing power showed that the enzyme improved the washing capability of the detergent. We observed that proteins were initially removed from the clothing material surfaces with either by components of the detergents or by water alone. However, when the enzymes were added with the detergent, a complete removal of the stain was observed. The supplementation of these enzymes preparations in detergent significantly improves the cleansing performance and complete removal of the stain after incubation at 50^{o}C, within 15 minutes. Our finding shows the effectiveness of proteases in proteinaceous stain removal and as such their consideration as potential candidates for use as cleansing additives in detergents and textile industries. Report on the usefulness of protease from *Aspergillus flavus* in the facilitation of blood stains removal from cotton cloth both in the presence and absence of detergents, had been documented (Choubey *et al*., 2016). Adejuwon and Olutiola, (2005) had reported blood de-staining property of protease from tomato fruits infested by Fusarium oxysporum. Also, pectinolytic and proteolytic enzymes isolated from deteriorated grapes had been incorporated into formulation of detergents for cleansing stains (Ajayi *et al*., 2014).

The Relative Rates of Hydrolysis of Various Substrates

Protease activity was measured using various substrates including; casein, bovine serum albumin (BSA), gelatin and egg albumin (EA). The OS2A and OS2B proteases hydrolyzed effectively both plant and animal protein substrates, hence their potential application as hydrolase in industry. The two enzymes hydrolyzed in all the tested substrates (Figures 4.18 and 4.19). Casein (100%) was found to be the best substrate for both proteases, with the least proteolytic activity observed by egg albumin, and also for both enzymes at 39.5% and 45.6% for OS2A and OS2B, respectively. Protease OS2A varies from OS2B in its higher preference for gelatin (85%) substrates than the BSA (50%).

The important feature of protease is their ability to discriminate among competing substrates and utility of these enzymes often depends on their substrate specificity (Shanker *et al*., 2011). The substrate specificities profiles of the proteases indicated that the enzymes have wide range of hydrolytic activities on various protein substrates and this quality is a great potential in biotechnological applications. The highest hydrolytic activity exhibited in the presence of casein in this study agreed with some studies. It was reported that protease from seeds of *Holarrhena antidysentrica* exhibited higher activity towards casein than BSA and gelatin (Khan *et al*., 2008). Jaouadi *et al*. (2008) reported that protease from *Bacillus pumilus* exhibited broad substrate specificity of 100% with casein, gelatin (95%), while azocasein and BSA (52%). Similar finding was reported by Qi *et al*. (2007) for cysteine-like protease from *Stichopus japonicas* with the following order of affinity at: casein ≥ BSA ≥ gelatin. However, our finding was at variance with report of Ire *et al*. (2011) who reported that protease from *Aspergillus carbonarius* exhibited broad substrate specificity with 100% activity with casein and BSA and 300% with gelatin. Ademola and Malomo (2017) equally reported that proteases from *Citrus sinensis* fruit peel demonstrated highest substrate affinity and hydrolysis with gelatin (125% relative activity).

The Kinetic Parameters of the Proteases at Different Concentrations of Substrates

The kinetics of the two proteases determined at different concentrations of substrates (casein, BSA, gelatin and EA) obeyed a typical Michaeli's-Menten's type kinetics by being hyperbolic. The kinetic parameters (*Km and Vmax*) determined from the double reciprocal or Lineweaver-Burk plots, 1/V vs 1/[S] (Figures 4.22 and 4.23) reported the lowest *Km* (0.022 mg/ml) and highest *Vmax* (0.213mg/ml/min) for gelatin by OS2A protease (Table 4.13). It suggested that enzyme possessed highest affinity and degradability potential for the substrates. However, the affinities for other substrates were also high as shown from their low *Km* values obtained. Similarly, the lowest *Km* (0.071mg/ml) and highest *Vmax* (0.234mg/ml/min) values recorded for OS2B protease in the presence of casein further suggest highest affinity for this substrate than in gelatin, BSA and EA (Table 4.14). Ability to degrade various substrates together with other properties is desired for their various industrial applications; including detergent, pharmaceutical, food and biotechnology industry.

Our findings were similar with the reports of other workers (Ademola and Malomo, 2017) for gelatin. Fahmy *et al*. (2004) also recorded highest affinity for gelatin compared to hemoglobin and followed by casein. Our findings disagreed with the reports by other workers with cysteine protease purified from *Allium cepa*. The least *Km* was reported for casein followed by gelatin and hemoglobin (Ndidi and Nzelibe, 2012). Our findings were equally at variance with the other researchers with protease from *Beauveria sp* who reported *Km* of 5.1 mg/ml with casein as substrate (Shankar *et al*., 2011). Also, a *Km* value of 0.18mg/ml had been reported for Bacillus sp Gs-3 protease (George-Okafor and Odibo, 2011) and sorghum malt varieties KSV8-11 (Ogbonna *et al*., 2003). Devi *et al*. (2008) reported a *Km* of 0.8mg/ml with casein as substrate for protease from *A. niger* while Ogbonna *et al*. (2004) recorded a *Km* of 0.33mg/ml for sorghum malt variety SK 5912. The results obtained from the kinetic studies also confirmed their broad substrate specificity.

The Turnover Number or Constant (K_{cat}) of the Partially Purified Proteases

The constant K_{cat}, called the turnover number, is often applied to enzyme-catalyzed reactions; obtained from the general expression V_{max} = K_{cat} [$E_{0\ or\ ET}$]. It represents the maximum number of substrate molecules converted to products per molecule enzyme per unit time. The OS2A exhibited varying degrees of substrate molecules conversion to products. The rate of catalysis was highest with egg albumin at 0.0244 U/mg/min, followed by casein (0.0219), gelatin (0.0215) and BSA (0.0174). Similarly, the rate of catalysis with OS2B were; BSA (0.0221), egg albumin (0.0194), gelatin (0.0187) and casein (0.0182) (Table 4.15). Comparing these results, the OS2A exhibited higher K_{cat} values as against OS2B and these values helped to determine the enzyme efficiency.

High specificities enhanced the conversion of the substrate molecules to useful product. Since the partially purified proteases demonstrated excellent substrate hydrolysis, and hence specificities, the higher Kcat values for OS2A make them better utilized than OS2B for calculation of enzyme efficiency.

The Catalytic Efficiency (Ee) of the Partially Purified Proteases

The enzyme or catalytic efficiency (Ee) expressed as Kcat / *Km* gives insight as to the usefulness of such enzyme for industrial application. Our findings showed that both enzymes exhibited high catalytic efficiency. However, protease OS2A was more efficient than OS2B as shown by their higher catalytic values (Table 4.16), and so presents better quality for industrial use. Knowledge of enzyme efficiency helps an enzymologist to guide the manufacturer of such enzyme on the stability and as such their usefulness in industry.

5.1 CONCLUSION

This study showed that Nigerian maize variety, Oba Super 2 (OS2) exhibited excellent malting properties for brewing. Proper manipulations of experimental variables

(independent) during malting enhanced protease development. The information obtained from this study had equally indicated that maize malt can serve as a source of alkaline (serine) protease.

The partially purified alkaline (serine) proteases from this malt extract were sucessfully characterized and enzymes exhibited high proteolytic activities with respect to; high optimal temperatures, thermal stability at alkaline pH, enhanced affinity to substrates, broad substrate specificity, good compatibility and stability with some detergents and surfactants, as well as organic solvent and oxidizing agents. The enzymes equally showed high cleansing power when incorporated into a locally made detergent.

Alkaline (serine) proteases are highly significant in current industrial sectors and this provides opportunity to further explore the suitability of this enzyme in relevant industries, especially in brewing, pharmaceutical, detergent and laundry. On overall assessment, the partially purified proteases presented promising application for industrial utilization.

5.2 RECOMMENDATIONS

In this study on Oba Super 2 Maize Variety, we recommend that:

1. Locally grown maize grains should be used as replacement for other imported grains for brewing purposes and proteolytic enzyme production due to economic considerations and availabilities.

2. Government should support local farmers for massive production of this Oba Super 2 maize which had been proven to be of great potentials as raw material for brewing.

3. Findings in this study should be acted upon soonest. And so, to achieve this, there should be collaborations among the stakeholders (the school, brewing and enzyme industries) for onward use of these findings.

4. Further studies should be carried out to explore the suitability of this enzyme in other relevant industries, especially in brewing, pharmaceutical, detergent and sewage treatment.

5. An important aspect of genetic engineering is the production of hybrid maize grains with excellent malting qualities for brewing and improved protease production. Therefore, application of genetic engineering can improve protein content of maize and it becomes a biological tool to broaden the usefulness of this variety in brewing and enzyme industry. The achievement of such fit will not only reduce the production cost and capital outlay, but increase the income of commercial farmers.

6. Finally, utilization of agro wastes, such as maize bran, wheat bran, citrus peel etc., which are however, inexpensive and widely available should be explored as attractive cheaper substrates for protease production.

5.3 CONTRIBUTIONS TO RESEARCH AND BODY OF KNOWLEDGE

Contributions from this study are as follows:

1. Important knowledge gap filled: In this study on Oba Super 2 Maize variety, we have established to the best of my knowledge, the malting protocols that hold promising prospects for optimal extract yield for brewing purposes. Maize malt of this local cultivar has been proven to have good potentials as brewing malt.

2. Good exploits of this local variety: This locally available and inexpensive food crop has been exploited for protease production, since the protease has not been characterized in this particular maize variety selected.

3. Establishment of some optimal values: Based on the kinetic studies of these partially purified proteases, we have established some critical or optimal values of their operating variables such as temperature, pH, effectors and inhibitors. Indeed, we have shown that this variety could be an alternative and reliable source of protease with high activity and stability

under various conditions for biotechnological and industrial uses. Thus, having such product will help reduce over-dependence on imported ones from technologically advanced nations.

4. Substantiation of previous findings on proteolytic activities of tropical grains: This study substantiated previous findings (theoretically) on the proteolytic activities of some tropical grains, in particular Oba Super 2 Maize. Evidences had shown that it could be used as plant derived protease hitherto unkown.

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Appendix 1

Analysis of variance (ANOVA) of the effects of different steeping (S) period (hours) and germination (G) period (days) on malting loss.

```
GET
FILE='C:\Users\AGBO\Documents\Effects of steeping time (hour) and germination time (days)sav'.
DATASET NAME DataSet1 WINDOW=FRONT.
```

```
ONEWAY HOUR30 HOUR36 HOUR42 BY Days
/STATISTICS DESCRIPTIVES
/MISSING ANALYSIS
/POSTHOC=DUNCAN LSD ALPHA (0.05).
```

Oneway

[DataSet1] C:\Users\AGBO\Documents\Effects of steeping time (hour) and germination time (days)sav

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
HOUR30	malting loss at zero day germination	2	7.10	.000	.000	7.10	7.10	7	7
	malting loss at firstday germination	2	10.25	.212	.150	8.34	12.16	10	10
	malting loss at secod day germination	2	14.20	.141	.100	12.93	15.47	14	14
	malting loss at third day germination	2	16.67	.035	.025	16.36	16.99	17	17
	malting loss at fourth day germination	2	19.58	.106	.075	18.62	20.53	20	20
	malting loss at fifth day germination	2	21.12	1.442	1.020	8.16	34.08	20	22
	Total	12	14.82	5.182	1.496	11.53	18.11	7	22
HOUR36	malting loss at zero day germination	2	8.10	.000	.000	8.10	8.10	8	8
	malting loss at firstday germination	2	10.05	.071	.050	9.41	10.69	10	10
	malting loss at secod day germination	2	14.80	.283	.200	12.26	17.34	15	15
	malting loss at third day germination	2	16.70	.424	.300	12.89	20.51	16	17
	malting loss at fourth day germination	2	20.12	.170	.120	18.60	21.64	20	20
	malting loss at fifth day germination	2	21.10	.071	.050	20.46	21.74	21	21
	Total	12	15.15	5.018	1.449	11.96	18.33	8	21
HOUR42	malting loss at zero day germination	2	8.90	.141	.100	7.63	10.17	9	9
	malting loss at firstday germination	2	10.50	.141	.100	9.23	11.77	10	11
	malting loss at secod day germination	2	14.40	.141	.100	13.13	15.67	14	15
	malting loss at third day germination	2	17.80	.283	.200	15.26	20.34	18	18
	malting loss at fourth day germination	2	21.90	.141	.100	20.63	23.17	22	22
	malting loss at fifth day germination	2	23.00	1.414	1.000	10.29	35.71	22	24
	Total	12	16.08	5.581	1.611	12.54	19.63	9	24

ANOVA

		Sum of Squares	Df	Mean Square	F	Sig.
HOUR30	Between Groups	293.218	5	58.643	163.027	.000
	Within Groups	2.158	6	.360		
	Total	295.376	11			
HOUR36	Between Groups	276.681	5	55.336	1111.171	.000
	Within Groups	.299	6	.050		
	Total	276.980	11			
HOUR42	Between Groups	340.457	5	68.091	189.143	.000

	Within Groups	2.160	6	.360		
	Total	342.617	11			

Appendix 2

Analysis of variance (ANOVA) of the effects of different steeping (S) period (hour), germination (G) period (days) and kilning (K) temperature (^{0}C) on cold water extact.

```
GET DATA /TYPE=XLSX
  /FILE='C:\Users\AGBO\Desktop\COLD WATER EXTACT (CWE) SPSS 1.xlsx'
  /SHEET=name 'Sheet1'
  /CELLRANGE=full
  /READNAMES=on
  /ASSUMEDSTRWIDTH=32767.
EXECUTE.
DATASET NAME DataSet1 WINDOW=FRONT.
ONEWAY Hour30 Hour36 Hour42 BY V1
  /STATISTICS DESCRIPTIVES
  /MISSING ANALYSIS
  /POSTHOC=DUNCAN LSD ALPHA (0.05)
```

Oneway

```
[DataSet1]C:/Users/AGBO/DOCUMENTS/DATA OF CWE (TABLE 4.3)..sav
```

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Hour30	germination at zero day kilned at 45 degrees	2	26.9950	.00707	.00500	26.9315	27.0585	26.99	27.00
	germination at zero day kilned at 50 degrees	2	28.5750	.10607	.07500	27.6220	29.5280	28.50	28.65
	germination at zero day kilned at 55 degrees	2	27.1900	.15556	.11000	25.7923	28.5877	27.08	27.30
	germination at first day kilned at 45 degrees	2	37.5300	.07071	.05000	36.8947	38.1653	37.48	37.58
	germination at first day kilned at 50 degrees	2	36.6850	.02121	.01500	36.4944	36.8756	36.67	36.70
	germination at first day kilned at 55 degrees	2	37.4500	.21213	.15000	35.5441	39.3559	37.30	37.60
	germination at second day kilned at 45 degrees	2	40.9000	.07071	.05000	40.2647	41.5353	40.85	40.95
	germination at second day kilned at 50 degrees	2	41.8500	.07071	.05000	41.2147	42.4853	41.80	41.90
	germination at second day kilned at 55 degrees	2	41.6500	.07071	.05000	41.0147	42.2853	41.60	41.70
	germination at third day kilned at 45 degrees	2	41.4500	.21213	.15000	39.5441	43.3559	41.30	41.60
	germination at third day kilned at 50 degrees	2	42.5000	.28284	.20000	39.9588	45.0412	42.30	42.70
	germination at third day kilned at 55 degrees	2	42.1850	.41719	.29500	38.4367	45.9333	41.89	42.48
	germination at fourth day kilned at 45 degrees	2	41.8550	.10607	.07500	40.9020	42.8080	41.78	41.93
	germination at fourth day kilned at 50 degrees	2	43.0350	2.59508	1.83500	19.7191	66.3509	41.20	44.87
	germination at fourth day kilned at 55 degrees	2	43.2500	1.06066	.75000	33.7203	52.7797	42.50	44.00
	germination at fifth day kilned at 45 degrees	2	42.3650	.23335	.16500	40.2685	44.4615	42.20	42.53
	germination at fifth day kilned at 50 degrees	2	41.9500	.70711	.50000	35.5969	48.3031	41.45	42.45
	germination at fifth day kilned at 55 degrees	2	43.1350	1.18087	.83500	32.5253	53.7447	42.30	43.97
	Total	36	38.9194	5.52378	.92063	37.0505	40.7884	26.99	44.87
Hour36	germination at zero day kilned at 45 degrees	2	26.0800	.02828	.02000	25.8259	26.3341	26.06	26.10
	germination at zero day kilned at 50 degrees	2	29.7500	.07071	.05000	29.1147	30.3853	29.70	29.80

	germination at zero day kilned at 55 degrees	2	28.8450	.20506	.14500	27.0026	30.6874	28.70	28.99
	germination at first day kilned at 45 degrees	2	36.8550	.07778	.05500	36.1562	37.5538	36.80	36.91
	germination at first day kilned at 50 degrees	2	36.0700	.04243	.03000	35.6888	36.4512	36.04	36.10
	germination at first day kilned at 55 degrees	2	36.8800	.49497	.35000	32.4328	41.3272	36.53	37.23
	germination at second day kilned at 45 degrees	2	36.5000	.14142	.10000	35.2294	37.7706	36.40	36.60
	germination at second day kilned at 50 degrees	2	38.8000	.14142	.10000	37.5294	40.0706	38.70	38.90
	germination at second day kilned at 55 degrees	2	38.9850	.02121	.01500	38.7944	39.1756	38.97	39.00
	germination at third day kilned at 45 degrees	2	40.5150	.12021	.08500	39.4350	41.5950	40.43	40.60
	germination at third day kilned at 50 degrees	2	43.1400	1.49907	1.06000	29.6714	56.6086	42.08	44.20
	germination at third day kilned at 55 degrees	2	43.3500	.21213	.15000	41.4441	45.2559	43.20	43.50
	germination at fourth day kilned at 45 degrees	2	43.5150	.16263	.11500	42.0538	44.9762	43.40	43.63
	germination at fourth day kilned at 50 degrees	2	44.4250	.03536	.02500	44.1073	44.7427	44.40	44.45
	germination at fourth day kilned at 55 degrees	2	44.5650	.07778	.05500	43.8662	45.2638	44.51	44.62
	germination at fifth day kilned at 45 degrees	2	41.3250	.03536	.02500	41.0073	41.6427	41.30	41.35
	germination at fifth day kilned at 50 degrees	2	41.8200	.01414	.01000	41.6929	41.9471	41.81	41.83
	germination at fifth day kilned at 55 degrees	2	42.1700	.04243	.03000	41.7888	42.5512	42.14	42.20
	Total	36	38.5328	5.44870	.90812	36.6892	40.3764	26.06	44.62
Hour42	germination at zero day kilned at 45 degrees	2	26.3650	.04950	.03500	25.9203	26.8097	26.33	26.40
	germination at zero day kilned at 50 degrees	2	28.5000	.14142	.10000	27.2294	29.7706	28.40	28.60
	germination at zero day kilned at 55 degrees	2	28.1800	.07071	.05000	27.5447	28.8153	28.13	28.23
	germination at first day kilned at 45 degrees	2	37.5550	.06364	.04500	36.9832	38.1268	37.51	37.60
	germination at first day kilned at 50 degrees	2	38.6300	.04243	.03000	38.2488	39.0112	38.60	38.66
	germination at first day kilned at 55 degrees	2	38.7000	.14142	.10000	37.4294	39.9706	38.60	38.80
	germination at second day kilned at 45 degrees	2	43.4000	.14142	.10000	42.1294	44.6706	43.30	43.50
	germination at second day kilned at 50 degrees	2	46.6100	.01414	.01000	46.4829	46.7371	46.60	46.62
	germination at second day kilned at 55 degrees	2	42.0750	.03536	.02500	41.7573	42.3927	42.05	42.10
	germination at third day kilned at 45 degrees	2	47.8100	.01414	.01000	47.6829	47.9371	47.80	47.82
	germination at third day kilned at 50 degrees	2	47.8000	.14142	.10000	46.5294	49.0706	47.70	47.90
	germination at third day kilned at 55 degrees	2	46.6700	.04243	.03000	46.2888	47.0512	46.64	46.70
	germination at fourth day kilned at 45 degrees	2	51.8650	.04950	.03500	51.4203	52.3097	51.83	51.90
	germination at fourth day kilned at 50 degrees	2	49.9350	.04950	.03500	49.4903	50.3797	49.90	49.97

	germination at fourth day kilned at 55 degrees	2	50.8500	.07071	.05000	50.2147	51.4853	50.80	50.90
	germination at fifth day kilned at 45 degrees	2	50.5650	.04950	.03500	50.1203	51.0097	50.53	50.60
	germination at fifth day kilned at 50 degrees	2	49.6100	.15556	.11000	48.2123	51.0077	49.50	49.72
	germination at fifth day kilned at 55 degrees	2	49.2600	.05657	.04000	48.7518	49.7682	49.22	49.30
	Total	36	43.0211	8.19759	1.36626	40.2474	45.7948	26.33	51.90

ANOVA

		Sum of Squares	Df	Mean Square	F	Sig.
S30	Between Groups	1057.706	17	62.218	109.586	.000
	Within Groups	10.220	18	.568		
	Total	1067.926	35			
S36	Between Groups	1036.407	17	60.965	408.735	.000
	Within Groups	2.685	18	.149		
	Total	1039.092	35			
S42	Between Groups	2351.878	17	138.346	18243.400	.000
	Within Groups	.136	18	.008		
	Total	2352.015	35			

Appendix 3

Analysis of variance (ANOVA) of the effects of different steeping (S) period (hour), germination(G) period (days) and kilning (K) temperature (^{o}C) on hot water extact.

```
GET
  FILE='C:\Users\AGBO\Documents\DATA OF HWE (TABLE 4.4)sav'.
DATASET NAME DataSet1 WINDOW=FRONT.
ONEWAY HOUR30 HOUR36 HOUR42 BY Days
  /STATISTICS DESCRIPTIVES
  /MISSING ANALYSIS
  /POSTHOC=DUNCAN LSD ALPHA (0.05).
```

Oneway

```
[DataSet1] C:\Users\AGBO\Documents\DATA OF HWE (TABLE 4.4)sav
```

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
HOUR30	germination at zero day kilned at 45 degrees	2	100.61	.573	.405	95.46	105.75	100	101
	germination at zero day kilned at 50 degrees	2	106.50	.707	.500	100.15	112.85	106	107
	germination at zero day kilned at 55 degrees	2	111.51	.693	.490	105.28	117.74	111	112
	germination at first day kilned at 45 degrees	2	128.40	.566	.400	123.32	133.48	128	129
	germination at first day kilned at 50 degrees	2	149.85	.212	.150	147.94	151.76	150	150
	germination at first day kilned at 55 degrees	2	111.10	.141	.100	109.83	112.37	111	111
	germination at second day kilned at 45 degrees	2	138.30	.141	.100	137.03	139.57	138	138
	germination at second day kilned at 50 degrees	2	219.53	.665	.470	213.56	225.50	219	220
	germination at second day kilned at 55 degrees	2	138.40	.283	.200	135.86	140.94	138	139
	germination at third day kilned at 45 degrees	2	230.50	.707	.500	224.15	236.85	230	231
	germination at third day kilned at 50 degrees	2	230.00	.000	.000	230.00	230.00	230	230
	germination at third day kilned at 55 degrees	2	164.50	.141	.100	163.23	165.77	164	165
	germination at fourth day kilned at 45 degrees	2	263.25	.778	.550	256.26	270.24	263	264
	germination at fourth day kilned at 50 degrees	2	242.20	.566	.400	237.12	247.28	242	243
	germination at fourth day kilned at 55 degrees	2	221.40	.283	.200	218.86	223.94	221	222
	germination at fifth day kilned at 45 degrees	2	260.70	.424	.300	256.89	264.51	260	261

	germination at fifth day kilned at 50 degrees	2	239.50	.707	.500	233.15	245.85	239	240
	germination at fifth day kilned at 55 degrees	2	210.45	.502	.355	205.93	214.96	210	211
	Total	36	181.48	57.814	9.636	161.92	201.04	100	264
HOUR36	germination at zero day kilned at 45 degrees	2	110.40	.566	.400	105.32	115.48	110	111
	germination at zero day kilned at 50 degrees	2	108.60	.566	.400	103.52	113.68	108	109
	germination at zero day kilned at 55 degrees	2	114.25	.071	.050	113.61	114.89	114	114
	germination at first day kilned at 45 degrees	2	124.45	.495	.350	120.00	128.90	124	125
	germination at first day kilned at 50 degrees	2	127.01	.014	.010	126.88	127.14	127	127
	germination at first day kilned at 55 degrees	2	116.51	.693	.490	110.28	122.74	116	117
	germination at second day kilned at 45 degrees	2	131.90	.141	.100	130.63	133.17	132	132
	germination at second day kilned at 50 degrees	2	117.45	.636	.450	111.73	123.17	117	118
	germination at second day kilned at 55 degrees	2	138.90	.141	.100	137.63	140.17	139	139
	germination at third day kilned at 45 degrees	2	182.40	.566	.400	177.32	187.48	182	183
	germination at third day kilned at 50 degrees	2	205.05	.071	.050	204.41	205.69	205	205
	germination at third day kilned at 55 degrees	2	223.50	.141	.100	222.23	224.77	223	224
	germination at fourth day kilned at 45 degrees	2	229.00	1.414	1.000	216.29	241.71	228	230
	germination at fourth day kilned at 50 degrees	2	237.40	.283	.200	234.86	239.94	237	238
	germination at fourth day kilned at 55 degrees	2	238.60	.000	.000	238.60	238.60	239	239
	germination at fifth day kilned at 45 degrees	2	214.60	.000	.000	214.60	214.60	215	215
	germination at fifth day kilned at 50 degrees	2	227.40	.283	.200	224.86	229.94	227	228
	germination at fifth day kilned at 555 degrees	2	228.50	.141	.100	227.23	229.77	228	229
	Total	36	170.88	52.407	8.734	153.15	188.62	108	239
HOUR42	germination at zero day kilned at 45 degrees	2	112.30	.424	.300	108.49	116.11	112	113
	germination at zero day kilned at 50 degrees	2	109.50	.707	.500	103.15	115.85	109	110

	germination at zero day kilned at 55 degrees	2	110.03	.000	.000	110.03	110.03	110	110
	germination at first day kilned at 45 degrees	2	143.95	.071	.050	143.31	144.59	144	144
	germination at first day kilned at 50 degrees	2	159.85	.212	.150	157.94	161.76	160	160
	germination at first day kilned at 55 degrees	2	163.45	.636	.450	157.73	169.17	163	164
	germination at second day kilned at 45 degrees	2	211.20	.000	.000	211.20	211.20	211	211
	germination at second day kilned at 50 degrees	2	211.80	.141	.100	210.53	213.07	212	212
	germination at second day kilned at 55 degrees	2	218.14	.078	.055	217.45	218.84	218	218
	germination at third day kilned at 45 degrees	2	224.16	.064	.045	223.58	224.73	224	224
	germination at third day kilned at 50 degrees	2	222.06	.085	.060	221.30	222.82	222	222
	germination at third day kilned at 55 degrees	2	224.45	.495	.350	220.00	228.90	224	225
	germination at fourth day kilned at 45 degrees	2	240.16	.057	.040	239.65	240.67	240	240
	germination at fourth day kilned at 50 degrees	2	250.05	.071	.050	249.41	250.69	250	250
	germination at fourth day kilned at 55 degrees	2	230.30	.141	.100	229.03	231.57	230	230
	germination at fifth day kilned at 45 degrees	2	224.56	.792	.560	217.44	231.68	224	225
	germination at fifth day kilned at 50 degrees	2	219.83	.940	.665	211.39	228.28	219	221
	germination at fifth day kilned at 555 degrees	2	216.69	2.143	1.515	197.44	235.93	215	218
	Total	36	194.03	46.599	7.766	178.26	209.79	109	250

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
HOUR30	Between Groups	116982.969	17	6881.351	26398.483	.000
	Within Groups	4.692	18	.261		
	Total	116987.662	35			
HOUR36	Between Groups	96121.900	17	5654.229	23448.560	.000
	Within Groups	4.340	18	.241		
	Total	96126.240	35			

HOUR42	Between Groups	75992.350	17	4470.138	10660.672	.000
	Within Groups	7.548	18	.419		
	Total	75999.897	35			

Appendix 4

Analysis of variance (ANOVA) of the effects of different steeping (S) period (hour), germination (G) period (days) and kilning (K) temperature (^{o}C) on free alpha amino nitrogen.

```
FILE='C:\Users\AGBO\Documents\ DATA OF FAN.sav'.
        DATASET NAME DataSet1 WINDOW=FRONT.
        ONEWAY Hour30 Hour36 Hour42 BY Days
        /STATISTICS DESCRIPTIVES
        /MISSING ANALYSIS
        /POSTHOC=DUNCAN LSD ALPHA (0.05).
```

Oneway

[DataSet1] C:\Users\AGBO\Documents\ DATA OF FAN.sav

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Hour30	germination at zero day kilned at 45 degrees	2	29.25	.021	.015	29.05	29.44	29	29
	rmination at zero day kilned at 50 degrees	2	30.49	.700	.495	24.21	36.78	30	31
	germination at zero day kilned at 55 degrees	2	28.81	.120	.085	27.73	29.90	29	29
	germination at first day kilned at 45 degrees	2	32.15	.778	.550	25.16	39.14	32	33
	germination at first day kilned at 50 degrees	2	35.14	.007	.005	35.08	35.21	35	35
	germination at first day kilned at 55 degrees	2	32.86	.191	.135	31.15	34.58	33	33
	germination at second day kilned at 45 degrees	2	40.89	.438	.310	36.95	44.83	41	41
	germination at second day kilned at 50 degrees	2	42.89	.021	.015	42.69	43.08	43	43
	germination at second day kilned at 55 degrees	2	40.72	.014	.010	40.59	40.85	41	41
	germination at thirth day kilned at 45 degrees	2	41.78	.028	.020	41.53	42.03	42	42
	germination at thirth day kilned at 50 degrees	2	43.75	.354	.250	40.57	46.93	44	44
	germination at thirth day kilned at 55 degrees	2	41.90	.141	.100	40.63	43.17	42	42
	germination at fourth day kilned at 45 degrees	2	48.25	.354	.250	45.07	51.43	48	49
	germination at fourth day kilned at 50 degrees	2	49.50	.283	.200	46.96	52.04	49	50
	germination at fourth day kilned at 55 degrees	2	48.60	.566	.400	43.52	53.68	48	49
	germination at fifth day kilned at 45 degrees	2	32.33	.742	.525	25.65	39.00	32	33
	germination at fifth day kilned at 50 degrees	2	31.58	.735	.520	24.97	38.19	31	32
	germination at fifth day kilned at 55 degrees	2	31.30	.707	.500	24.95	37.65	31	32
	Total	36	37.90	7.001	1.167	35.53	40.27	29	50
Hour36	germination at zero day kilned at 45 degrees	2	28.01	.014	.010	27.88	28.14	28	28
	germination at zero day kilned at 50 degrees	2	29.53	.099	.070	28.64	30.42	29	30
	germination at zero day kilned at 55 degrees	2	28.75	.212	.150	26.84	30.66	29	29
	germination at first day kilned at 45 degrees	2	30.54	.764	.540	23.68	37.40	30	31
	germination at first day kilned at 50 degrees	2	32.05	.778	.550	25.06	39.04	32	33

	germination at first day kilned at 55 degrees	2	31.37	.658	.465	25.46	37.27	31	32
	germination at second day kilned at 45 degrees	2	38.03	.035	.025	37.71	38.34	38	38
	germination at second day kilned at 50 degrees	2	41.77	1.457	1.030	28.68	54.86	41	43
	germination at second day kilned at 55 degrees	2	38.50	.566	.400	33.42	43.58	38	39
	germination at thirth day kilned at 45 degrees	2	41.10	.141	.100	39.83	42.37	41	41
	germination at thirth day kilned at 50 degrees	2	43.85	.071	.050	43.21	44.49	44	44
	germination at thirth day kilned at 55 degrees	2	41.50	.707	.500	35.15	47.85	41	42
	germination at fourth day kilned at 45 degrees	2	45.61	.714	.505	39.19	52.02	45	46
	germination at fourth day kilned at 50 degrees	2	49.75	.212	.150	47.84	51.66	50	50
	germination at fourth day kilned at 55 degrees	2	47.05	.064	.045	46.47	47.62	47	47
	germination at fifth day kilned at 45 degrees	2	32.61	.658	.465	26.71	38.52	32	33
	germination at fifth day kilned at 50 degrees	2	34.02	.021	.015	33.82	34.21	34	34
	germination at fifth day kilned at 55 degrees	2	32.36	.651	.460	26.52	38.20	32	33
	Total	36	37.02	6.774	1.129	34.73	39.31	28	50
Hour42	germination at zero day kilned at 45 degrees	2	30.45	.636	.450	24.73	36.17	30	31
	germination at zero day kilned at 50 degrees	2	31.45	.636	.450	25.73	37.17	31	32
	germination at zero day kilned at 55 degrees	2	29.45	.778	.550	22.46	36.44	29	30
	germination at first day kilned at 45 degrees	2	32.75	.354	.250	29.57	35.93	33	33
	germination at first day kilned at 50 degrees	2	34.30	.283	.200	31.76	36.84	34	35
	germination at first day kilned at 55 degrees	2	32.32	.120	.085	31.23	33.40	32	32
	germination at second day kilned at 45 degrees	2	40.35	.778	.550	33.36	47.34	40	41
	germination at second day kilned at 50 degrees	2	45.55	.636	.450	39.83	51.27	45	46
	germination at second day kilned at 55 degrees	2	41.80	.283	.200	39.26	44.34	42	42
	germination at thirth day kilned at 45 degrees	2	42.71	.134	.095	41.50	43.91	43	43
	germination at thirth day kilned at 50 degrees	2	43.02	.028	.020	42.77	43.27	43	43
	germination at thirth day kilned at 55 degrees	2	41.65	.629	.445	35.99	47.30	41	42
	germination at fourth day kilned at 45 degrees	2	48.95	.071	.050	48.31	49.59	49	49
	germination at fourth day kilned at 50 degrees	2	53.55	.778	.550	46.56	60.54	53	54
	germination at fourth day kilned at 55 degrees	2	48.91	.014	.010	48.78	49.04	49	49
	germination at fifth day kilned at 45 degrees	2	31.03	.014	.010	30.90	31.16	31	31
	germination at fifth day kilned at 50 degrees	2	32.55	.354	.250	29.37	35.73	32	33
	germination at fifth day kilned at 55 degrees	2	31.08	.106	.075	30.12	32.03	31	31
	Total	36	38.44	7.520	1.253	35.89	40.98	29	54

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Hour30	Between Groups	1712.118	17	100.713	503.264	.000
	Within Groups	3.602	18	.200		
	Total	1715.720	35			
Hour36	Between Groups	1599.755	17	94.103	279.565	.000
	Within Groups	6.059	18	.337		
	Total	1605.814	35			
Hour42	Between Groups	1975.433	17	116.202	538.249	.000
	Within Groups	3.886	18	.216		
	Total	1979.319	35			

Appendix 5

Analysis of variance (ANOVA) of the effects of different (S) steeping period (hour), germination (G) period (days) and kilning (K) temperature (^{o}C) on the diastatic power.

```
GET DATA /TYPE=XLSX
 /FILE='C:\Users\AGBO\Desktop\D P.xlsx'
          /SHEET=name 'Sheet1'
          /CELLRANGE=full
          /READNAMES=on
          /ASSUMEDSTRWIDTH=32767.
        EXECUTE.
        DATASET NAME DataSet1 WINDOW=FRONT.
        ONEWAY Hour30 Hour36 Hour42 BY V1
          /STATISTICS DESCRIPTIVES
          /MISSING ANALYSIS
          /POSTHOC=DUNCAN LSD ALPHA (0.05).
```

Oneway

[DataSet1]C: /AGBO/DOCUMENTS/DATA OF DP (TABLE 4.6)..sav.

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Hour30	germination at zero day kilned at 45 degrees	2	25.2850	.40305	.28500	21.6637	28.9063	25.00	25.57
	germination at zero day kilned at 50 degrees	2	25.1000	.00000	.00000	25.1000	25.1000	25.10	25.10
	germination at zero day kilned at 55 degrees	2	25.2000	.00000	.00000	25.2000	25.2000	25.20	25.20
	germination at first day kilned at 45 degrees	2	26.7500	.07071	.05000	26.1147	27.3853	26.70	26.80
	germination at first day kilned at 50 degrees	2	26.5500	.07071	.05000	25.9147	27.1853	26.50	26.60
	germination at first day kilned at 55 degrees	2	25.5500	.07071	.05000	24.9147	26.1853	25.50	25.60
	germination at second day kilned at 45 degrees	2	27.3800	.02828	.02000	27.1259	27.6341	27.36	27.40
	germination at second day kilned at 50 degrees	2	25.7000	.14142	.10000	24.4294	26.9706	25.60	25.80
	germination at second day kilned at 55 degrees	2	26.5000	.14142	.10000	25.2294	27.7706	26.40	26.60
	germination at third day kilned at 45 degrees	2	27.4750	.24749	.17500	25.2514	29.6986	27.30	27.65
	germination at third day kilned at 50 degrees	2	32.6250	.03536	.02500	32.3073	32.9427	32.60	32.65
	germination at third day kilned at 55 degrees	2	26.8500	.07071	.05000	26.2147	27.4853	26.80	26.90
	germination at fourth day kilned at 45 degrees	2	28.9500	.07071	.05000	28.3147	29.5853	28.90	29.00
	germination at fourth day kilned at 50 degrees	2	30.8500	.07071	.05000	30.2147	31.4853	30.80	30.90
	germination at fourth day kilned at 55 degrees	2	27.3500	.07071	.05000	26.7147	27.9853	27.30	27.40
	germination at fifth day kilned at 45 degrees	2	30.7000	.14142	.10000	29.4294	31.9706	30.60	30.80
	germination at fifth day kilned at 50 degrees	2	33.4000	.56569	.40000	28.3175	38.4825	33.00	33.80
	germination at fifth day kilned at 55 degrees	2	28.0900	.12728	.09000	26.9464	29.2336	28.00	28.18
	Total	36	27.7947	2.50345	.41724	26.9477	28.6418	25.00	33.80

Hour36	germination at zero day kilned at 45 degrees	2	24.6500	.21213	.15000	22.7441	26.5559	24.50	24.80
	germination at zero day kilned at 50 degrees	2	25.1750	.03536	.02500	24.8573	25.4927	25.15	25.20
	germination at zero day kilned at 55 degrees	2	26.2500	.35355	.25000	23.0734	29.4266	26.00	26.50
	germination at first day kilned at 45 degrees	2	22.3000	.28284	.20000	19.7588	24.8412	22.10	22.50
	germination at first day kilned at 50 degrees	2	26.3000	.00000	.00000	26.3000	26.3000	26.30	26.30
	germination at first day kilned at 55 degrees	2	26.1500	.07071	.05000	25.5147	26.7853	26.10	26.20
	germination at second day kilned at 45 degrees	2	25.5000	.00000	.00000	25.5000	25.5000	25.50	25.50
	germination at second day kilned at 50 degrees	2	26.6750	.10607	.07500	25.7220	27.6280	26.60	26.75
	germination at second day kilned at 55 degrees	2	24.7200	.11314	.08000	23.7035	25.7365	24.64	24.80
	germination at third day kilned at 45 degrees	2	26.7500	.07071	.05000	26.1147	27.3853	26.70	26.80
	germination at third day kilned at 50 degrees	2	34.8500	.07071	.05000	34.2147	35.4853	34.80	34.90
	germination at third day kilned at 55 degrees	2	26.1750	.03536	.02500	25.8573	26.4927	26.15	26.20
	germination at fourth day kilned at 45 degrees	2	27.8500	.07071	.05000	27.2147	28.4853	27.80	27.90
	germination at fourth day kilned at 50 degrees	2	35.5000	.14142	.10000	34.2294	36.7706	35.40	35.60
	germination at fourth day kilned at 55 degrees	2	24.8500	.07071	.05000	24.2147	25.4853	24.80	24.90
	germination at fifth day kilned at 45 degrees	2	26.3650	.02121	.01500	26.1744	26.5556	26.35	26.38
	germination at fifth day kilned at 50 degrees	2	36.5500	.07071	.05000	35.9147	37.1853	36.50	36.60
	germination at fifth day kilned at 55 degrees	2	25.5500	.07071	.05000	24.9147	26.1853	25.50	25.60
	Total	36	27.3422	3.94244	.65707	26.0083	28.6762	22.10	36.60
Hour42	germination at zero day kilned at 45 degrees	2	25.6000	.00000	.00000	25.6000	25.6000	25.60	25.60
	germination at zero day kilned at 50 degrees	2	25.3000	.07071	.05000	24.6647	25.9353	25.25	25.35
	germination at zero day kilned at 55 degrees	2	25.2500	.21213	.15000	23.3441	27.1559	25.10	25.40
	germination at first day kilned at 45 degrees	2	33.2000	.84853	.60000	25.5763	40.8237	32.60	33.80
	germination at first day kilned at 50 degrees	2	33.1000	.14142	.10000	31.8294	34.3706	33.00	33.20
	germination at first day kilned at 55 degrees	2	32.4500	.49497	.35000	28.0028	36.8972	32.10	32.80
	germination at second day kilned at 45 degrees	2	34.1500	.21213	.15000	32.2441	36.0559	34.00	34.30
	germination at second day kilned at 50 degrees	2	34.1000	.14142	.10000	32.8294	35.3706	34.00	34.20
	germination at second day kilned at 55 degrees	2	34.2050	.13435	.09500	32.9979	35.4121	34.11	34.30
	germination at third day kilned at 45 degrees	2	34.3000	.00000	.00000	34.3000	34.3000	34.30	34.30
	germination at third day kilned at 50 degrees	2	36.3500	.35355	.25000	33.1734	39.5266	36.10	36.60
	germination at third day kilned at 55 degrees	2	35.7850	.16263	.11500	34.3238	37.2462	35.67	35.90
	germination at fourth day kilned at 45 degrees	2	35.7500	.35355	.25000	32.5734	38.9266	35.50	36.00
	germination at fourth day kilned at 50 degrees	2	38.1000	.14142	.10000	36.8294	39.3706	38.00	38.20
	germination at fourth day kilned at 55 degrees	2	34.7700	.04243	.03000	34.3888	35.1512	34.74	34.80

	germination at fifth day kilned at 45 degrees	2	36.7000	.14142	.10000	35.4294	37.9706	36.60	36.80
	germination at fifth day kilned at 50 degrees	2	38.0750	.10607	.07500	37.1220	39.0280	38.00	38.15
	germination at fifth day kilned at 55 degrees	2	35.7250	.10607	.07500	34.7720	36.6780	35.65	35.80
	Total	36	33.4950	3.99295	.66549	32.1440	34.8460	25.10	38.20

ANOVA

		Sum of Squares	Df	Mean Square	F	Sig.
S30	Between Groups	218.698	17	12.865	352.481	.000
	Within Groups	.657	18	.036		
	Total	219.355	35			
S36	Between Groups	543.666	17	31.980	1733.874	.000
	Within Groups	.332	18	.018		
	Total	543.998	35			
S42	Between Groups	556.569	17	32.739	403.968	.000
	Within Groups	1.459	18	.081		
	Total	558.028	35			

Appendix 6

Analysis of variance (ANOVA) of the effects of different steeping (S) period (hour), germination (G) period (days) and kilning (K) temperature (^{o}C) on the total soluble nitrogen.

```
GET
  FILE='C:\Users\AGBO\Documents\DATA OF TSN (TABLE 4.7).sav'.
DATASET NAME DataSet1 WINDOW=FRONT.
ONEWAY HOUR30 HOUR36 HOUR42 BY Days
  /STATISTICS DESCRIPTIVES
  /MISSING ANALYSIS
  /POSTHOC=DUNCAN LSD ALPHA (0.05).
```

Oneway

```
[DataSet1] C:\Users\AGBO\Documents\DATA OF TSN (TABLE 4.7).sav
```

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
HOUR30	Germination at zero day kilned as at 45 degrees	2	8.20	.141	.100	6.93	9.47	8	8
	Germination at zero day kilned as at 50 degrees	2	8.31	.071	.050	7.67	8.95	8	8
	Germination at zero day kilned as at 55 degrees	2	8.18	.106	.075	7.23	9.14	8	8
	Germination at first day kilned as at 45 degrees	2	7.44	.014	.010	7.31	7.57	7	7
	Germination at first day kilned as at 50 degrees	2	7.42	.021	.015	7.22	7.61	7	7
	Germination at first day kilned as at 55 degrees	2	7.35	.057	.040	6.84	7.86	7	7
	Germination at second day kilned as at 45 degrees	2	7.19	.042	.030	6.81	7.57	7	7
	Germination at second day kilned as at 50 degrees	2	7.19	.064	.045	6.61	7.76	7	7
	Germination at second day kilned as at 55 degrees	2	7.16	.007	.005	7.09	7.22	7	7
	Germination at third day kilned as at 45 degrees	2	6.35	.071	.050	5.71	6.99	6	6
	Germination at third day kilned as at 50 degrees	2	6.55	.071	.050	5.91	7.19	7	7
	Germination at third day kilned as at 55 degrees	2	6.45	.071	.050	5.81	7.09	6	7
	Germination at fourth day kilned as at 45 degrees	2	5.40	.078	.055	4.71	6.10	5	5
	Germination at fourth day kilned as at 50 degrees	2	5.58	.106	.075	4.62	6.53	6	6
	Germination at fourth day kilned as at 55 degrees	2	5.48	.106	.075	4.52	6.43	5	6
	Germination at fifth day kilned as at 45 degrees	2	4.22	.014	.010	4.09	4.35	4	4
	Germination at fifth day kilned as at 50 degrees	2	4.23	.042	.030	3.85	4.61	4	4
	Germination at fifth day kilned as at 55 degrees	2	4.13	.035	.025	3.81	4.44	4	4
	Total	36	6.49	1.352	.225	6.03	6.95	4	8

HOUR36	Germination at zero day kilned as at 45 degrees	2	8.15	.071	.050	7.51	8.79	8	8
	Germination at zero day kilned as at 50 degrees	2	8.43	.035	.025	8.11	8.74	8	8
	Germination at zero day kilned as at 55 degrees	2	8.15	.057	.040	7.64	8.66	8	8
	Germination at first day kilned as at 45 degrees	2	7.55	.071	.050	6.91	8.19	8	8
	Germination at first day kilned as at 50 degrees	2	7.64	.049	.035	7.19	8.08	8	8
	Germination at first day kilned as at 55 degrees	2	7.11	.714	.505	.69	13.52	7	8
	Germination at second day kilned as at 45 degrees	2	7.28	.035	.025	6.96	7.59	7	7
	Germination at second day kilned as at 50 degrees	2	7.30	.000	.000	7.30	7.30	7	7
	Germination at second day kilned as at 55 degrees	2	7.25	.071	.050	6.61	7.89	7	7
	Germination at third day kilned as at 45 degrees	2	6.20	.028	.020	5.95	6.45	6	6
	Germination at third day kilned as at 50 degrees	2	6.15	.071	.050	5.51	6.79	6	6
	Germination at third day kilned as at 55 degrees	2	6.15	.042	.030	5.77	6.53	6	6
	Germination at fourth day kilned as at 45 degrees	2	5.27	.014	.010	5.14	5.40	5	5
	Germination at fourth day kilned as at 50 degrees	2	5.26	.028	.020	5.01	5.51	5	5
	Germination at fourth day kilned as at 55 degrees	2	5.13	.028	.020	4.88	5.38	5	5
	Germination at fifth day kilned as at 45 degrees	2	4.35	.071	.050	3.71	4.99	4	4
	Germination at fifth day kilned as at 50 degrees	2	4.19	.014	.010	4.06	4.32	4	4
	Germination at fifth day kilned as at 55 degrees	2	4.22	.028	.020	3.97	4.47	4	4
	Total	36	6.43	1.400	.233	5.96	6.90	4	8
HOUR42	Germination at zero day kilned as at 45 degrees	2	8.24	.057	.040	7.73	8.75	8	8
	Germination at zero day kilned as at 50 degrees	2	8.28	.113	.080	7.26	9.30	8	8
	Germination at zero day kilned as at 55 degrees	2	8.15	.035	.025	7.83	8.46	8	8
	Germination at first day kilned as at 45 degrees	2	7.63	.042	.030	7.25	8.01	8	8
	Germination at first day kilned as at 50 degrees	2	7.61	.014	.010	7.48	7.74	8	8
	Germination at first day kilned as at 55 degrees	2	7.51	.078	.055	6.81	8.20	7	8
	Germination at second day kilned as at 45 degrees	2	7.20	.071	.050	6.56	7.84	7	7
	Germination at second day kilned as at 50 degrees	2	7.24	.057	.040	6.73	7.75	7	7
	Germination at second day kilned as at 55 degrees	2	7.20	.035	.025	6.88	7.51	7	7
	Germination at third day kilned as at 45 degrees	2	6.14	.028	.020	5.89	6.39	6	6
	Germination at third day kilned as at 50 degrees	2	6.16	.028	.020	5.91	6.41	6	6
	Germination at third day kilned as at 55 degrees	2	6.12	.021	.015	5.92	6.31	6	6
	Germination at fourth day kilned as at 45 degrees	2	5.18	.035	.025	4.86	5.49	5	5
	Germination at fourth day kilned as at 50 degrees	2	5.11	.014	.010	4.98	5.24	5	5
	Germination at fourth day kilned as at 55 degrees	2	5.12	.014	.010	4.99	5.25	5	5

Germination at fifth day kilned as at 45 degrees	2	4.34	.057	.040	3.83	4.85	4	4
Germination at fifth day kilned as at 50 degrees	2	4.45	.042	.030	4.07	4.83	4	4
Germination at fifth day kilned as at 55 degrees	2	4.28	.028	.020	4.03	4.53	4	4
Total	36	6.44	1.387	.231	5.97	6.91	4	8

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
HOUR30	Between Groups	63.891	17	3.758	728.984	.000
	Within Groups	.093	18	.005		
	Total	63.984	35			
HOUR36	Between Groups	68.089	17	4.005	131.415	.000
	Within Groups	.549	18	.030		
	Total	68.638	35			
HOUR42	Between Groups	67.321	17	3.960	1610.882	.000
	Within Groups	.044	18	.002		
	Total	67.366	35			

Appendix 7

Analysis of variance (ANOVA) of the effects of different steeping (S) period (hour), germination (G) period (days) and kilning (K) temperature (^{o}C) on the cold water soluble protein.

```
GET DATA /TYPE=XLSX
  /FILE='C:\Users\AGBO\Desktop\ DATA OF CWS - P.xlsx'
  /SHEET=name 'Sheet1'
  /CELLRANGE=full
  /READNAMES=on
  /ASSUMEDSTRWIDTH=32767.
EXECUTE.
DATASET NAME DataSet1 WINDOW=FRONT.
ONEWAY Hour30 Hour36 Hour42 BY V1
  /STATISTICS DESCRIPTIVES
  /MISSING ANALYSIS
  /POSTHOC=DUNCAN LSD ALPHA (0.05).
```

Oneway

```
[DataSet1]C: /Users/AGBO/DOCUMENTS/DATA OF CWS-P (TABLE 4.8)..sav.
```

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Hour30	germination at zero day kilned at 45 degrees	2	20.7500	.35355	.25000	17.5734	23.9266	20.50	21.00
	germination at zero day kilned at 50 degrees	2	20.5650	.64347	.45500	14.7837	26.3463	20.11	21.02
	germination at zero day kilned at 55 degrees	2	20.7000	.14142	.10000	19.4294	21.9706	20.60	20.80
	germination at first day kilned at 45 degrees	2	22.9250	.45962	.32500	18.7955	27.0545	22.60	23.25
	germination at first day kilned at 50 degrees	2	24.6500	.56569	.40000	19.5675	29.7325	24.25	25.05
	germination at first day kilned at 55 degrees	2	23.3350	.31820	.22500	20.4761	26.1939	23.11	23.56
	germination at second day kilned at 45 degrees	2	25.1000	.04243	.03000	24.7188	25.4812	25.07	25.13
	germination at second day kilned at 50 degrees	2	27.9500	.07071	.05000	27.3147	28.5853	27.90	28.00
	germination at second day kilned at 55 degrees	2	26.5000	.70711	.50000	20.1469	32.8531	26.00	27.00
	germination at third day kilned at 45 degrees	2	34.4450	3.30219	2.33500	4.7760	64.1140	32.11	36.78
	germination at third day kilned at 50 degrees	2	36.3250	3.85373	2.72500	1.7006	70.9494	33.60	39.05
	germination at third day kilned at 55 degrees	2	34.3350	2.45366	1.73500	12.2897	56.3803	32.60	36.07
	germination at fourth day kilned at 45 degrees	2	28.5900	.67882	.48000	22.4910	34.6890	28.11	29.07
	germination at fourth day kilned at 50 degrees	2	28.6350	.72832	.51500	22.0913	35.1787	28.12	29.15
	germination at fourth day kilned at 55 degrees	2	28.5750	.60104	.42500	23.1749	33.9751	28.15	29.00
	germination at fifth day kilned at 45 degrees	2	27.1100	1.28693	.91000	15.5474	38.6726	26.20	28.02
	germination at fifth day kilned at 50 degrees	2	25.9250	.81317	.57500	18.6189	33.2311	25.35	26.50
	germination at fifth day kilned at 55 degrees	2	25.0000	.42426	.30000	21.1881	28.8119	24.70	25.30
	Total	36	26.7453	4.70267	.78378	25.1541	28.3364	20.11	39.05

Hour36	germination at zero day kilned at 45 degrees	2	19.6150	.54447	.38500	14.7231	24.5069	19.23	20.00
	germination at zero day kilned at 50 degrees	2	20.1250	.10607	.07500	19.1720	21.0780	20.05	20.20
	germination at zero day kilned at 55 degrees	2	19.9900	.05657	.04000	19.4818	20.4982	19.95	20.03
	germination at first day kilned at 45 degrees	2	21.5000	.70711	.50000	15.1469	27.8531	21.00	22.00
	germination at first day kilned at 50 degrees	2	23.7800	.31113	.22000	20.9846	26.5754	23.56	24.00
	germination at first day kilned at 55 degrees	2	22.0000	.56569	.40000	16.9175	27.0825	21.60	22.40
	germination at second day kilned at 45 degrees	2	23.5500	.07071	.05000	22.9147	24.1853	23.50	23.60
	germination at second day kilned at 50 degrees	2	21.4500	.77782	.55000	14.4616	28.4384	20.90	22.00
	germination at second day kilned at 55 degrees	2	22.0100	.32527	.23000	19.0876	24.9324	21.78	22.24
	germination at third day kilned at 45 degrees	2	31.7900	3.52139	2.49000	.1516	63.4284	29.30	34.28
	germination at third day kilned at 50 degrees	2	32.1500	1.62635	1.15000	17.5379	46.7621	31.00	33.30
	germination at third day kilned at 55 degrees	2	33.0500	2.61630	1.85000	9.5435	56.5565	31.20	34.90
	germination at fourth day kilned at 45 degrees	2	25.1750	.10607	.07500	24.2220	26.1280	25.10	25.25
	germination at fourth day kilned at 50 degrees	2	28.0500	.07071	.05000	27.4147	28.6853	28.00	28.10
	germination at fourth day kilned at 55 degrees	2	27.0750	.24749	.17500	24.8514	29.2986	26.90	27.25
	germination at fifth day kilned at 45 degrees	2	24.0800	.25456	.18000	21.7929	26.3671	23.90	24.26
	germination at fifth day kilned at 50 degrees	2	26.1250	.17678	.12500	24.5367	27.7133	26.00	26.25
	germination at fifth day kilned at 55 degrees	2	22.9550	.06364	.04500	22.3832	23.5268	22.91	23.00
	Total	36	24.6928	4.25284	.70881	23.2538	26.1317	19.23	34.90
Hour42	germination at zero day kilned at 45 degrees	2	21.1550	1.33643	.94500	9.1476	33.1624	20.21	22.10
	germination at zero day kilned at 50 degrees	2	20.1050	.14849	.10500	18.7708	21.4392	20.00	20.21
	germination at zero day kilned at 55 degrees	2	21.3250	.38891	.27500	17.8308	24.8192	21.05	21.60
	germination at first day kilned at 45 degrees	2	26.3650	.19092	.13500	24.6497	28.0803	26.23	26.50
	germination at first day kilned at 50 degrees	2	23.3500	.35355	.25000	20.1734	26.5266	23.10	23.60
	germination at first day kilned at 55 degrees	2	22.0500	.77782	.55000	15.0616	29.0384	21.50	22.60
	germination at second day kilned at 45 degrees	2	25.6150	.86974	.61500	17.8007	33.4293	25.00	26.23
	germination at second day kilned at 50 degrees	2	26.1550	.07778	.05500	25.4562	26.8538	26.10	26.21
	germination at second day kilned at 55 degrees	2	26.1000	1.27279	.90000	14.6644	37.5356	25.20	27.00
	germination at third day kilned at 45 degrees	2	34.3500	.21213	.15000	32.4441	36.2559	34.20	34.50
	germination at third day kilned at 50 degrees	2	39.5500	.91924	.65000	31.2910	47.8090	38.90	40.20
	germination at third day kilned at 55 degrees	2	38.3000	2.40416	1.70000	16.6995	59.9005	36.60	40.00
	germination at fourth day kilned at 45 degrees	2	27.4500	.77782	.55000	20.4616	34.4384	26.90	28.00
	germination at fourth day kilned at 50 degrees	2	27.6000	.56569	.40000	22.5175	32.6825	27.20	28.00
	germination at fourth day kilned at 55 degrees	2	25.6000	.56569	.40000	20.5175	30.6825	25.20	26.00

germination at fifth day kilned at 45 degrees	2	23.1300	.18385	.13000	21.4782	24.7818	23.00	23.26
germination at fifth day kilned at 50 degrees	2	24.8000	.28284	.20000	22.2588	27.3412	24.60	25.00
germination at fifth day kilned at 55 degrees	2	25.0000	1.41421	1.00000	12.2938	37.7062	24.00	26.00
Total	36	26.5556	5.47800	.91300	24.7021	28.4090	20.00	40.20

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
S30	Between Groups	736.704	17	43.336	20.899	.000
	Within Groups	37.324	18	2.074		
	Total	774.029	35			
S36	Between Groups	609.021	17	35.825	26.856	.000
	Within Groups	24.011	18	1.334		
	Total	633.032	35			
S42	Between Groups	1035.160	17	60.892	72.408	.000
	Within Groups	15.137	18	.841		
	Total	1050.297	35			

Appendix 8

Analysis of variance (ANOVA) of the effects of different steeping (S) period (hour), germination (G) period (days) and kilning (K) temperature (^{o}C) on total non-protein nitrogen.

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  /SHEET=name 'Sheet1'
  /CELLRANGE=full
  /READNAMES=on
  /ASSUMEDSTRWIDTH=32767.
EXECUTE.
DATASET NAME DataSet1 WINDOW=FRONT.
ONEWAY Hour30 Hour36 Hour42 BY V1
  /STATISTICS DESCRIPTIVES
  /MISSING ANALYSIS
  /POSTHOC=DUNCAN LSD ALPHA (0.05).
```

Oneway

```
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```

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Hour30	germination at zero day kilned at 45 degrees	2	18.15	.071	.050	17.51	18.79	18	18
	germination at zero day kilned at 50 degrees	2	18.50	.141	.100	17.23	19.77	18	19
	germination at zero day kilned at 55 degrees	2	18.20	.283	.200	15.66	20.74	18	18
	germination at first day kilned at 45 degrees	2	20.30	.141	.100	19.03	21.57	20	20
	germination at first day kilned at 50 degrees	2	21.98	.035	.025	21.66	22.29	22	22
	germination at first day kilned at 55 degrees	2	20.70	.141	.100	19.43	21.97	21	21
	germination at second day kilned at 45 degrees	2	23.65	.354	.250	20.47	26.83	23	24
	germination at second day kilned at 50 degrees	2	24.15	.071	.050	23.51	24.79	24	24
	germination at second day kilned at 55 degrees	2	23.40	.566	.400	18.32	28.48	23	24
	germination at third day kilned at 45 degrees	2	24.30	.283	.200	21.76	26.84	24	25
	germination at third day kilned at 50 degrees	2	25.40	.424	.300	21.59	29.21	25	26
	germination at third day kilned at 55 degrees	2	23.85	.071	.050	23.21	24.49	24	24
	germination at fourth day kilned at 45 degrees	2	32.11	.163	.115	30.65	33.58	32	32
	germination at fourth day kilned at 55 degrees	2	33.68	.106	.075	32.72	34.63	34	34
	germination at fourth day kilned at 55 degrees	2	31.50	.141	.100	30.23	32.77	31	32
	germination at fifth day kilned at 45 degrees	2	29.50	.566	.400	24.42	34.58	29	30
	germination at fifth day kilned at 50 degrees	2	28.83	.573	.405	23.68	33.97	28	29

	germination at fifth day kilned at 55 degrees	2	27.90	1.273	.900	16.46	39.34	27	29
	Total	36	24.78	4.807	.801	23.16	26.41	18	34
houir36	germination at zero day kilned at 45 degrees	2	17.70	.141	.100	16.43	18.97	18	18
	germination at zero day kilned at 50 degrees	2	18.10	.141	.100	16.83	19.37	18	18
	germination at zero day kilned at 55 degrees	2	17.22	.021	.015	17.02	17.41	17	17
	germination at first day kilned at 45 degrees	2	19.55	.071	.050	18.91	20.19	20	20
	germination at first day kilned at 50 degrees	2	20.95	1.202	.850	10.15	31.75	20	22
	germination at first day kilned at 55 degrees	2	19.50	.495	.350	15.05	23.95	19	20
	germination at second day kilned at 45 degrees	2	22.30	.283	.200	19.76	24.84	22	23
	germination at second day kilned at 50 degrees	2	24.55	.495	.350	20.10	29.00	24	25
	germination at second day kilned at 55 degrees	2	21.95	.071	.050	21.31	22.59	22	22
	germination at third day kilned at 45 degrees	2	23.70	.141	.100	22.43	24.97	24	24
	germination at third day kilned at 50 degrees	2	24.95	.071	.050	24.31	25.59	25	25
	germination at third day kilned at 55 degrees	2	22.50	.141	.100	21.23	23.77	22	23
	germination at fourth day kilned at 45 degrees	2	30.59	.375	.265	27.22	33.95	30	31
	germination at fourth day kilned at 55 degrees	2	32.33	.460	.325	28.20	36.45	32	33
	germination at fourth day kilned at 55 degrees	2	30.08	.035	.025	29.76	30.39	30	30
	germination at fifth day kilned at 45 degrees	2	28.05	.071	.050	27.41	28.69	28	28
	germination at fifth day kilned at 50 degrees	2	29.50	.424	.300	25.69	33.31	29	30
	germination at fifth day kilned at 55 degrees	2	28.23	.177	.125	26.64	29.81	28	28
	Total	36	23.98	4.757	.793	22.38	25.59	17	33
hour42	germination at zero day kilned at 45 degrees	2	18.45	.212	.150	16.54	20.36	18	19
	germination at zero day kilned at 50 degrees	2	18.70	.424	.300	14.89	22.51	18	19
	germination at zero day kilned at 55 degrees	2	17.90	.141	.100	16.63	19.17	18	18
	germination at first day kilned at 45 degrees	2	19.50	.141	.100	18.23	20.77	19	20
	germination at first day kilned at 50 degrees	2	22.45	.354	.250	19.27	25.63	22	23
	germination at first day kilned at 55 degrees	2	21.64	.049	.035	21.19	22.08	22	22
	germination at second day kilned at 45 degrees	2	24.40	.283	.200	21.86	26.94	24	25
	germination at second day kilned at 50 degrees	2	26.08	.035	.025	25.76	26.39	26	26
	germination at second day kilned at 55 degrees	2	25.10	.141	.100	23.83	26.37	25	25
	germination at third day kilned at 45 degrees	2	28.38	.035	.025	28.06	28.69	28	28
	germination at third day kilned at 50 degrees	2	32.10	.141	.100	30.83	33.37	32	32
	germination at third day kilned at 55 degrees	2	29.24	.262	.185	26.88	31.59	29	29
	germination at fourth day kilned at 45 degrees	2	34.50	.141	.100	33.23	35.77	34	35

	germination at fourth day kilned at 55 degrees	2	38.50	.141	.100	37.23	39.77	38	39
	germination at fourth day kilned at 55 degrees	2	34.83	.247	.175	32.60	37.05	35	35
	germination at fifth day kilned at 45 degrees	2	28.15	.212	.150	26.24	30.06	28	28
	germination at fifth day kilned at 50 degrees	2	28.34	1.322	.935	16.45	40.22	27	29
	germination at fifth day kilned at 55 degrees	2	28.63	.742	.525	21.95	35.30	28	29
	Total	36	26.49	5.986	.998	24.47	28.52	18	39

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
hour30	Between Groups	805.582	17	47.387	267.640	.000
	Within Groups	3.187	18	.177		
	Total	808.769	35			
houir36	Between Groups	789.202	17	46.424	311.841	.000
	Within Groups	2.680	18	.149		
	Total	791.882	35			
hour42	Between Groups	1250.979	17	73.587	437.244	.000
	Within Groups	3.029	18	.168		
	Total	1254.008	35			

Appendix 9

Analysis of variance (ANOVA) of the crude protease activity (U/ml) of the maize malt at different steeping (S) and germination (G) regimes, kilned at 50^{O}C.

```
ONEWAY hour30 hour36 hour42 BY Days
  /STATISTICS DESCRIPTIVES
  /MISSING ANALYSIS
  /POSTHOC=DUNCAN LSD ALPHA (0.05).
```

Oneway

```
[DataSet0] C:/AGBO/DOCUMENTS/DATA OF CRUDE PROTEASE (TABLE 4.10)..sav
```

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
hour30	Enzyme activity at zero day steeping and germination time	2	.55	.000	.000	.55	.55	1	1
	Enzyme activity at first day steeping and germination time	2	.56	.001	.001	.55	.57	1	1
	Enzyme activity at second day steeping and germination time	2	.61	.003	.002	.59	.64	1	1
	Enzyme activity at third day steeping and germination time	2	.68	.001	.001	.67	.69	1	1
	Enzyme activity at fourth day steeping and germination time	2	.64	.007	.005	.57	.70	1	1
	Enzyme activity at fifth day steeping and germination time	2	.64	.007	.005	.58	.71	1	1
	Total	12	.61	.047	.014	.58	.64	1	1
hour36	Enzyme activity at zero day steeping and germination time	2	.44	.014	.010	.31	.56	0	0
	Enzyme activity at first day steeping and germination time	2	.48	.001	.001	.47	.49	0	0
	Enzyme activity at second day steeping and germination time	2	.50	.001	.001	.48	.51	0	0
	Enzyme activity at third day steeping and germination time	2	.76	.001	.001	.75	.76	1	1
	Enzyme activity at fourth day steeping and germination time	2	.69	.001	.001	.69	.70	1	1
	Enzyme activity at fifth day steeping and germination time	2	.66	.001	.001	.65	.66	1	1
	Total	12	.59	.126	.036	.51	.67	0	1
hour42	Enzyme activity at zero day steeping and germination time	2	.48	.001	.001	.48	.49	0	0
	Enzyme activity at first day steeping and germination time	2	.61	.001	.001	.61	.62	1	1
	Enzyme activity at second day steeping and germination time	2	.79	.000	.000	.79	.79	1	1
	Enzyme activity at third day steeping and germination time	2	.83	.001	.001	.83	.84	1	1
	Enzyme activity at fourth day steeping and germination time	2	.72	.001	.001	.70	.73	1	1
	Enzyme activity at fifth day steeping and germination time	2	.67	.001	.001	.66	.68	1	1
	Total	12	.68	.122	.035	.61	.76	0	1

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
hour30	Between Groups	.025	5	.005	266.995	.000
	Within Groups	.000	6	.000		
	Total	.025	11			
hour36	Between Groups	.175	5	.035	1027.841	.000
	Within Groups	.000	6	.000		
	Total	.175	11			
hour42	Between Groups	.164	5	.033	49063.400	.000
	Within Groups	.000	6	.000		
	Total	.164	11			

Appendix 10

Ammonium sulphate precipitation profile.

1.0 Set up nine different tubes.

2.0 Pipette 10 ml of the crude enzyme into each of the tubes.

3.0 Measure out 1.06, 1.64, 2.26, 2.91, 3.61, 4.36, 5.16, and 6.03 grammes of ammonium sulphate salt into each of the tubes provided.

4.0 Mix gently to achieve homogeneity (each mixture correspond to 20, 30, 40, 50, 60, 70, 80, and 90% ammonium sulphate saturations, respectively).

5.0 Allow the mixture to stand for 30hr at 4^{o}C.

6.0 Centrifuge the mixture, decant the supernatants into nine different tubes.

7.0 Re-dissolve the pellet with equal volume of your working buffer.

8.0 Assay for the enzyme activity in both pellet and supernatant.

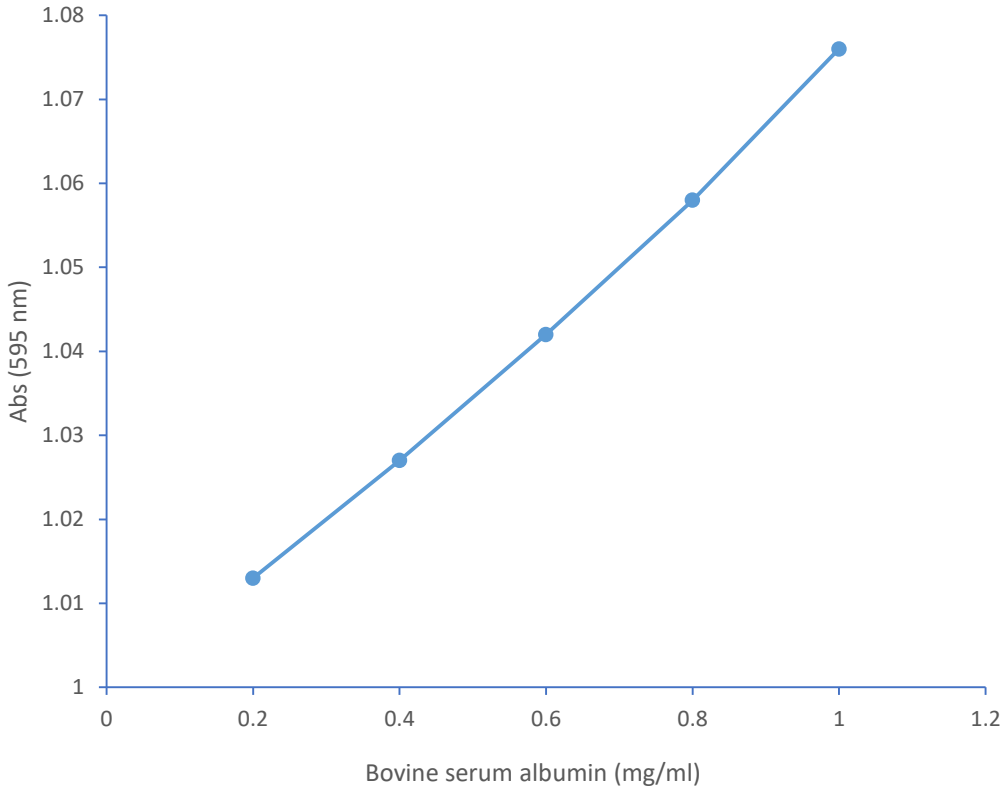
9.0 Plot the graph of enzyme activity against percentage saturation; use that to obtain the ammonium sulphate saturation suitable to precipitate protein with the highest enzyme activity.

10. Use the saturation that precipitated protein with highest activity to mass precipitate your protein of interest and repeat step 4-8.

11. Scale up the grammes of the salt to correspond with volume of your crude protein.

Appendix 11

Standard Curve for Protein Estimation by Bradford method.



Appendix 12

Procedure for the Calculation of Purification Summary.

Total activity (U) = Unit/ml × Volume of fraction

Total protein (mg) = Mg/ml × Volume of fraction

Specific activity (Unit/mg protein) = $\frac{\text{Total Activity/ Unit}}{\text{Total protein/ Mg}}$

Yield (%) for crude = 100%, but in subsequent steps, the yield is calculated as thus;

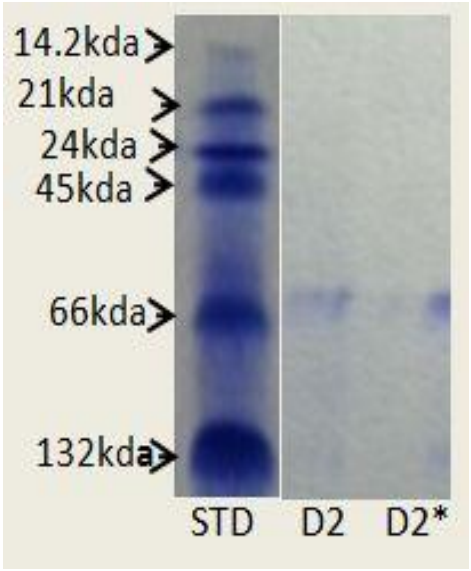
$$\frac{\text{Total activity of step}}{\text{Total activity of the crude}} \times \frac{100}{1}$$

Purification- The purification of the crude enzyme is usually one (1), then subsequent purification values for other purification steps are obtained as follows;

$$\frac{\text{Specific activity of step}}{\text{Specific activity of crude enzyme.}}$$

Appendix 13

Native Polyacrylamide gel electrophoresis (Native PAGE) of protein samples OS2A (D2) and OS2B (D2*).



Native gel electrophoresis of protein samples OS2A (D2) and OS2B (D2*). Applied on lane D2, 7ug and lane D2* 10ug.

Standard proteins on standard lane are;

Lysozyme 14,200 Da

Soy bean trypsin inhibitor 21,000 Da

Trypsinogen (bovine) 24,000 Da

Egg albumin 45,000 Da

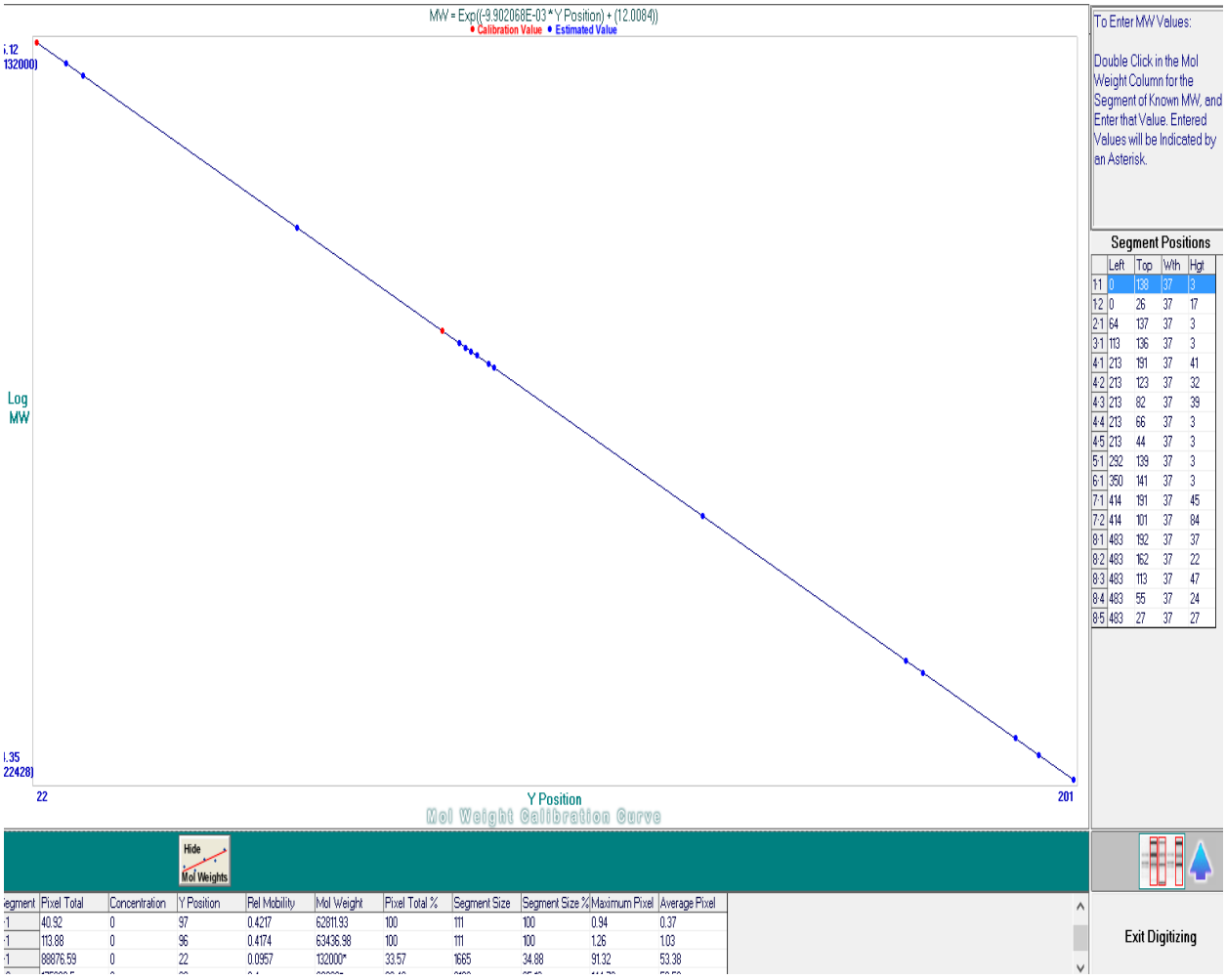
Bovine serum albumin (monomer) 66,000 Da

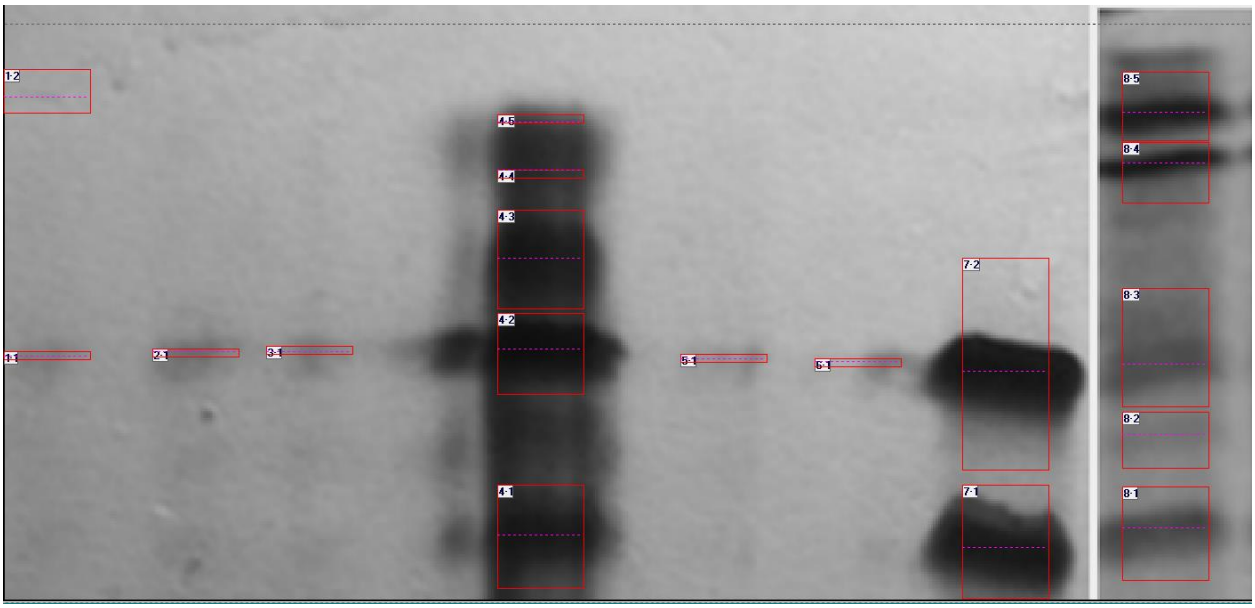
Bovine serum albumin (dimer) 132,000 Da

The average molecular weight of the protein is 63,124 Da = 63.1 K Da.

Molecular weight was estimated using UNSCAN-IT software version 6.1(Silk Scientific).

The data below can be disregarded. It is the software analysis data.

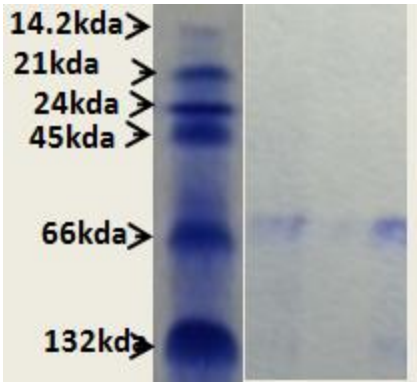




The samples are in lane 5.1 and 6.1. This analysis gives more confident results and average molecular weight is 63,124 Da= 63.1 K Da.

Segment	Pixel Total		Y Position	Rel Mobility	Mol Weight
1·1	16.33	98	0.4261	62193.03	
1·2	1148.14		201	0.8739	22428.44
2·1	17.36	100	0.4348	60973.46	
3·1	31.23	100	0.4348	60973.46	
4·1	28761.11		27	0.1174	125623.6
4·2	23953.24		101	0.4391	60372.68
4·3	20693.1		137	0.5957	42269.35
4·4	120.82	172	0.7478	29888.98	
4·5	65.91	191	0.8304	24763	
5·1	40.92	97	0.4217	62811.93	
6·1	113.88	96	0.4174	63436.98	
7·1	88876.59		22	0.0957	132000*
7·2	175838.5		92	0.4	66000*
8·1	42156.03		30	0.1304	121946.6
8·2	5612.41		67	0.2913	84538.5
8·3	38502.18		95	0.413	64068.27
8·4	23907.34		175	0.7609	29014.15
8·5	30718.35		195	0.8478	23801.35

Another attempt using different image



Segment	Pixel Tota	Y Position	Rel Mobili	Mol Weight
1·1	51512.88	33	0	132000*
1·2	20827.46	95	0	66000*
1·3	7398.18	159	0	45000*
1·4	7302.78	174	0	24000*
1·5	8739.5	196	0	21000*
1·6	-64.56	222	0	14200*
2·1	5420.98	102	0	62774.19
3·1	3166.17	100	0	64240.42
	Average molecular weight			63507.31

Appendix 14

Ninhydrin Colour Reagent, Diluent and Standard for Ninhydrin Assay

Ninhydrin colour reagent: To prepare ninhydrin colour reagent, dissolve in distilled water:100 g disodium hydrogen phosphate ($Na_2HP0_4 \cdot 12H_20$); 60 g potassium dihydrogen phosphate ($KHP0_4$); 5 g ninhydrin (Merck cat. 6762); 3 g fructose (Merck cat. 5323) and make up to 1 litre. This colour reagent will keep for 2 weeks if stored at 0 - 4 °C in an amber or foil-covered bottle.

Diluent: Dissolve 2 g potassium iodate ($KI0_3$, Merck cat. 5051) in 600 ml distilled water and add 400 ml 96% ethanol.

Standard for ninhydrin assay: Dissolve 107.2 mg glycine (Merck cat. 4201) in 100 ml distilled water in a volumetric flask. This is a stock solution and will keep for 1 week if stored at 0-4 °C. For each set of analyses carried out, 2 ml of this stock solution is diluted to 100 ml with distilled water in a volumetric flask.