UNIVERSITY OF NAIROBI

ENZYME HYDROLYSIS OF CHICKEN FEATHERS AND CHARACTERIZATION OF THE METABOLITES

BY

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DECLARATION

I declare that this thesis is my original work and has not been submitted elsewhere for examination, award of a degree or publication. Where other people's work or my own work has been used, this has properly been acknowledged and referenced in accordance with the University of Nairobi's requirements.

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DEDICATION

This work is dedicated to my Parents, Edward & Ruth Mwakazi, My grandmother Hawe Salama & Ambang'o and my siblings Kabibi, Wakio, Wughanga & Chief.

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ABSTRACT

Chicken feathers are largely produced as waste from domestic and poultry processing industries. Waste feathers are discarded into the environment without any effort to recycle. Current methods of feather disposal have proved harmful as they contribute to environmental pollution. Feathers comprise of protein, and they can be utilized as an alternative source of animal feed protein.

In an effort to reduce environmental concerns caused by the discarding of feather waste, this study evaluated the efficacy of alkaline protease from bacteria isolated in Kenya's extreme environment for the hydrolysis of feathers. Factors affecting enzyme hydrolysis such as enzyme concentration, pH, and temperature, were investigated and optimized. Feather hydrolysate was characterized using Fourier Transmission Infra-Red (FTIR), High-Performance Liquid chromatography (HPLC) and Gas Chromatography mass Spectroscopy (GC-MS). Proximate analysis was investigated to determine the nutritional attributes of the feather hydrolysate.

The findings from the study revealed that optimum conditions for enzymatic hydrolysis were 80˚C and pH of 13 using undiluted crude alkaline protease. The kinetics of the reaction showed that it was zero-order with respect to the concentration of the reactants, and the Michaelis-Menten constant value determined was 0.0362×10-7 µM. FTIR characterization showed clear peaks at 3267.41 cm-1, 1543.33 cm-1, 1242.16 cm-1, 1665.64 cm-1 that were attributed to protein functional groups in Amide A, Amide I, Amide II, Amide III respectively. GC-MS results showed the presence of Vitamin E and Octadecenamide. The amino acid analysis showed that glycine and glutamic acid were high in % molar composition with 11.3% and 9.6% respectively, while the lowest was histidine with 0.5%. Proximate analysis of the hydrolysate revealed that crude protein content was 73.3%, fibre content 0.315%, moisture content 6.6%, carbohydrate content 19.8%, and ash content 6.76%.

The study showed that crude alkaline protease effectively hydrolysed feathers waste and can be used to recycle feathers and reduction of environmental pollution. The findings show that feathers hydrolysate is nutritious and can be utilized as an alternate and sustainable source of protein in animal feeds fortification

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LIST OF ABBREVIATIONS

AA	Amino Acid
ATR	Attenuated Total Reflectance
EAA	Essential Amino acids
F&C	Folin & Ciocalteu's reagent
FID	Flame Ionization Detector
FTIR	Fourier Transformance Infra-Red
GC-MS	Gas Chromatography Mass Spectroscopy
GDP	Gross Domestic Product
Hcl	Hydrochloric Acid
HIV	Human Immuno Deficiency Virus
HPLC	High Performance Liquid Chromatography
LC-MS	Liquid Chromatography Mass Spectroscopy
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
PH	Potential of hydrogen
PM	Particulate Matter
TCA	Tetra Chloro Acetic acid
UV	Ultraviolet

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

Kenya in recent years has seen growth of Poultry farming attributed to increased demand for chicken meat in preference to other meats present in the market. The year 2014 saw the commercial production of both egg and chicken meat in Turkey reach amounts of 1,942,000 tonnes. This resulted to 430,244 tonnes of meat being exported at a value of 699.6 million (Prasanthi *et al.*, 2016) . Estimates of 31 million birds are found in Kenya which comprises of 75% indigenous, 22% broilers and 1% layers. Indigenous chicken produce almost 55% of the total meat and 47% of the total egg production in the country (Kingori *et al.*, 2010). The other poultry that make up 2% of the population include geese, ducks, guinea fowls, turkeys and quails. 25% of the gross domestic product (GDP) is contributed by agriculture in Kenya alone. Poultry makes significant contribution to the agricultural sector therefore playing a major role in Kenya's economy (Kingori, 2010)

The population awareness to better health and diseases like cancer, obesity, diabetes attributed to bad eating habits plays a significant role in the growth of consumption of white chicken meat (Dudyński *et al.*, 2012) .Such mindfulness has resulted to chicken meat becoming the preferred alternative to red meat. Poultry farming require less investment resources and emits less greenhouse gases as compared to larger livestock farming like sheep, goat and cattle keeping. Chicken meat provides a more affordable source of protein and healthier choice as compared to the goat meat because it contains less amount of fat as compared to red meat. Chicken white meat is also affordable as compared to fish and other expensive sources of protein in the market. Poultry farming has been easy to practise because you require small space to rear, have faster growth rate as compared to livestock farming thereby getting better returns in shorter periods of time. Poultry farming has also helped the people who have been affected by Human Immuno

Deficiency Virus by providing income to their families because it does not require hard manual labour (Kingori *et al.*, 2010). The government policies have supported poultry farming through promotion of self-employment especially in marginalised communities such as the Maasai who have welcomed this practise. In such communities the men are given the task to take care of the large livestock such as cows and the women are tasked with rearing the small animals like chickens. Such moves have involved the marginalised women in decision making on their households as they contribute to the family income. The Kenyan population earns a living through agriculture is estimated to be about 75% where poultry farming is among them (Kingori *et al.*, 2010). About 90% of the rural homes in Kenya practise poultry farming, this includes both the free range chicken and the (broilers and layers) exotic chicken (Kingori *et al.*, 2010) .

Growth in poultry farming has resulted in high production of feathers by poultry processing industries after the slaughtering of chickens. Consequently, large quantities of chicken feathers are produced globally as waste from numerous poultry processing industries. Every year an estimate of 24 billion chickens are slaughtered in total (Fellahi *et al.*, 2014). A chicken is estimated to produce 125 grams of feathers; this is so because chicken feathers are light in weight (Prasanthi *et al.*, 2016). Feathers comprise 8 to 10% of the overall chicken weight (Gupta *et al.*, 2011). The demand for more chicken meat has resulted to an increase in feather waste from poultry processing industries. Worldwide 400 million chickens are processed weekly which results to 3000 tonnes of feathers produced in a week worldwide (Brandelli, 2008). From the 24 billion chickens that are processed yearly we have to discard about 8.5 billion tonnes of feather that has been produced (Tesfaye *et al.*, 2017). Presently there are no uses of feathers and this can be a great resource if utilised to its full potential. Feathers having no use are discarded into the environment without the consideration of the pollution they cause. Feathers are considered pollutants because it takes about two years to completely decompose

(Fellahi *et al.*, 2014). Traditional methods of feather disposal include burying in landfills, burning, and disposal in main sewer lines. Burying in landfills renders the land useless to more productive use such as farming. This also leads to growth of pathogens leading to transmission of diseases. During the dry seasons the landfills harbour rodents and birds that carry diseases. Landfills also inevitably lead to the congestion of people in the cities due to lack of space. The congestion of people combined with poor feather disposal where rodents harbour in the landfills, provides conducive environment for the transmission of diseases since they crossover to domestic houses. Diseases related to poor feather disposal include growth pathogens such as mycoplasmas and even the case of HPA H5N1 virus reported in 2006 in Nigeria where 46 humans were infected with 282 fatalities (Fasina *et al.*, 2011) owing to poor feather disposal methods. Disposal on main sewer lines during the rainy season causes flooding; the feathers are carried in main sewer lines leads to blockage of the drainage system leading to spread of diseases such as cholera and dysentery as the main sewer lines are used for disposal of human waste. Environmental pollution caused by feather pollution occurs when they are burnt. Burning leads to emission of carbon monoxide emission and sulphur dioxide gas being emitted into the environment (Tesfaye *et al.*, 2017). Feathers contain disulphide bonds and the method of burning causes air pollution by producing sulphur dioxide gases into the atmosphere, and further pollute the soil and water sources (Prasanthi *et al.*, 2016). A study done by the North Carolina department of environment and natural resources reported burning of feathers emitted much higher levels of carbon dioxide as compared to the levels of carbon monoxide emitted from a coal plant (Fellahi *et al.*, 2014). Production of greenhouse gases through burning and decay leads to the gases leaching to water sources. This affects the growth of aquatic life and acidity in soil leading to use of harmful pesticides to stabilise the pH which further contributes to the cycle of pollution (Wisuthiphaet *et al.*, 2016). Globally the poultry industry is facing the problem of disposing an estimated 8 billion tonnes of feather waste in an environmentally friendly way.

Feathers comprise of around 90% protein content and can be sought after as a substitute animal protein source (Onifade *et al.*, 1998). Keratin is an insoluble structural protein mainly present in feathers and is known for its recalcitrance properties. Keratin is a complex protein insoluble in both water and organic solvents (Poovendran *et al.*, 2017). Keratin found in feathers is made up of several amino acids that include proline, methionine, cysteine, threonine, lysine, serine among others (Dudyński *et al.*, 2012). Disulphide bonds and hydrogen bonds present in keratin are formed by crosslinking of the amino acids that are present. Hence, this brings forth to formation of a very tough and complex structure with good thermal properties (Mazotto *et al.*, 2013). Keratin can be hydrolysed into high nutritional protein animal feed that is digestible. Increased interest in finding alternative sources of protein for animal feed is on the increase. Presently there is competition of food between man and animal. In Kenya the *Rastrineobola argentea* (Omena or Dagaa) and maize are used as fish and chicken feed respectively. Dagga has been used as a protein source in the production of fish feeds as a replacement for protein source for human consumption (Kingori *et al.*, 2010). The high demand of "Omena" has led to inadequate supplies to meet human population and animal requirements, often leading to overfishing. This has resulted to shortage of food especially in developing countries. Every year, Kenya as a country is faced with drought as a result of various factors such as delayed or insufficient rainfall, often contributing to food shortage and competition of food between man and animal.

Presently the methods of feather hydrolysis include acid, hydrothermal and alkaline hydrolysis. Products of hydrolysate using these methods results in feeds with poor nutritional value due to extreme processing conditions that result in denaturing of amino acids already present in feathers (Brandelli *et al.*, 2015). Chemical treatments include acid and alkaline hydrolysis. The strong acids and bases are used to achieve bond breakage of the disulphide bonds making the proteins soluble. Chemical hydrolysis entails harsh conditions of high temperature and high

pressure which results in environmental pollution. Bromine, potassium cyanide and sodium sulphide solutions used acted as reducing and oxidising agents in chemical feather hydrolysis .They are toxic to the environment because of the presence of sulphur groups leading to products of low nutritional value as the process is detrimental to amino acids (Mazotto *et al.*, 2013).

Hydrothermal hydrolysis is done under conditions of high pressure and temperature. The ideal conditions would be short reaction time, but this is not the case in this method. Physical treatments include thermal treatments by either autoclaving, using a pressure cooker and steam cooking. The result of thermal treatments is increased solubility of feathers due to breaking of bonds as a result of high temperature and resulting in changing the structure. But these methods consume high energy and the products of hydrolysis are of low nutritional value with poor digestibility (Brandelli *et al.*, 2015). Amino acid present in the final product of hydrolysis determines the nutritional value. The high prices of feeds is the most serious problem facing the poultry industry (Yegani, 2012). Protein feeds for animals includes soya-beans, legumes, forages, canola meal, bone meal and fish meal. Fish meal can be utilised in small quantities of about 5% if these quantities are exceeded the poultry meal and eggs will have a fishy flavour (Jacob, 2018). Considering the above extreme feather processing conditions, high energy and other inputs required, the final products sold are costly and possess low nutritional value. Because of these main challenges faced in the poultry industry alternative sources of feed sources have to be developed while curbing the waste management problem. The global feed production of animals in the year 2008 was 680 million tonnes of which around 150-170 million tonnes were equated to protein consumption (Vladau *et al.*, 2008). There is a huge demand for animal protein which has led to competition between man and animal feeds. Alternative sources of protein for animal consumption have to be provided whilst maintaining the nutritive value, quality, affordability and accessibility to the market (Vaclavik & Christian,

2014). Utilisation of feather waste for animal feeds solves two problems with one single action because feather is rich in protein and the action will eliminate environmental pollution (Swetlana & Jain, 2010).

Enzyme hydrolysis of feathers may provide a cheaper and environmentally friendly way of feather recycling. There is increasing interest in use of enzymes such as *Bacillus licheniformis* for various applications (Orts *et al.*, 2018). Over the past decade, there has been increased effort by different researchers to develop suitable processes based on microbial enzymes to breakdown feather waste into palatable proteins and amino acids (Gupta *et al.*, 2013). Goodrich and Lee (1993) stated the original use of a keratinase (*Bacillus licheniformis*) to hydrolyze feathers, a process that increased the total amino acid digestibility of raw feather from 30% to 66% and concomitantly increased commercial feather meal content from 77% to 99%. Therefore, new technologies such as enzyme hydrolysis of chicken feathers may provide a cheaper, more efficient and environmentally friendly way of feather processing. The focus of this study will be to recycle feathers that will address the waste management problems of disposal of chicken features and also assess their potential for use in animal feeds formulations.

1.2 Objectives

1.2.1 Main objective

To determine the efficacy of enzymatic catalysis in hydrolysis of chicken feathers and characterization of the value addition products obtained.

1.2.2 Specific objective

1. To determine the optimum process operating conditions for the hydrolysis of chicken feathers.
2. To characterize feather hydrolysate using FTIR and GC-MS
3. To determine the amino acid composition of the hydrolysed feathers using HPLC.

4. To determine proximate composition, nutritional attributes and mineral composition of hydrolysed feathers.

1.3 Statement of the problem

Increase in the number of chickens reared in poultry farms has brought about increase in waste; nonetheless, there are no proper channels present for handling feather waste. Conservation of the environment, recycling and providing a cleaner and better tomorrow for the future generation are some of the major concerns that are being addressed. Feathers are considered pollutants because it takes roughly two years for the feather to decompose (Fellahi *et al.*, 2014). Current methods of disposal include burying in landfills, burning and disposal in main sewer lines. Burying in landfills leads to growth of rodents that transmit diseases to humans as they crossover to domestic houses. The emission and formation of odors of greenhouse for example methane and sulphur dioxide are an effect of degradation of substances contained in the feathers biomass (Pawel *et al.*, 2016). The practice of burning feathers also results to emission of carbon monoxide emission and sulphur dioxide gas emitted into the environment. The emissions of greenhouse gas may lead to climate change concerns, acid rain, and particulate matter and public health respiratory concerns. Furthermore, non-volatile decomposing products can penetrate alongside water into the soil and contaminate groundwater (Staroń *et al.*, 2011).

Increased interest in finding other sources of protein for animal feed is on the rise. Feathers contain 90% of protein and this is a good substitute for protein source (Fellahi *et al.*, 2014). Present feeds in the market are very expensive and of low nutritive value. This is a result of the chemical and hydrothermal methods used in feather hydrolysis. The methods consume a lot of heat and temperature while denaturing the amino acids. The farmer does not get value for money as products sold are low in nutritional value. Enzyme hydrolysis provides an environmentally friendly process while producing products of high nutritional value.

1.4 Justification and significance

The aim of this research was to produce an environmentally friendly way of obtaining value addition products from chicken feather wastes. Enzyme hydrolysis is a bio-conversion process that is low in cost compared to chemical processes, environmentally friendly and the products obtained are of higher nutritional value. The research sought to address the current menace of environmental pollution posed by lack of proper solid waste management systems for handling large quantities of feathers. The process is anticipated to eliminate the menace posed by rodents that transmit diseases, air pollution through burning and blockage by disposal into the main sewer lines. Enzyme hydrolysis of chicken feathers will create products that are rich as protein meals and increase value addition of the product chain. The research outcomes are estimated to contribute to Kenya's industrial process and realization of Kenya's big four agenda: increase employment opportunities, value addition and overall the country's economic growth.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Environmental pollution caused by feathers

Modern poultry processing industries are faced with the challenge of disposing feathers in an economically and environmentally friendly way. This is due to stringent measures placed by waste management bodies on disposal as well as finding disposal sites. Previous research has shown that USA poultry industry is estimated to have 8 billion broilers produced annually that yield 3 billion tonnes of feather waste (Zhang *et al.*, 2014). The year 2014 saw Europe's poultry industry produce 3.1 million tonnes of feather waste .South Africa annual production of feather waste is more than 28,000 tonnes (Tesfaye *et al.*, 2017). Australia annual feather waste production of feathers waste from poultry plants is estimated at 86,000 to 111,000 tonnes. It has been reported that in eastern Europe an average farm size of farm produces about 7 tons of chicken feathers a day and nationally, 77,000 tons per year of feather waste is produced(Pawel *et al.*, 2014). Feathers have been termed as pollutant due to the fact, process of decomposition results in emission of greenhouse and toxic gases leading to air pollution and global warming.

The negative environmental effects that have been associated with feather waste arise from the way they are disposed in the environment. Methods practised in the disposal of feather waste include burying in landfills, burning, and disposal in main sewer lines (Tesfaye *et al.*, 2017). An inadequate disposal method of this bio-waste causes health problems for example bad odour, flies, rodents near poultry farms, land scape degradation, environmental damage and transmission of diseases (Cheong *et al.*, 2017).The accumulation of feathers and their slow deterioration form of sulphurous compounds that give off extremely unpleasant smell and act as a source of disease causing pathogens. The rodents and birds transmit diseases that they carry to human since most of them cross over to domestic households**.** Figure 1 shows a picture

of disposed waste feathers in one of the dump sites at a chicken slaughterhouse in Nairobi, Kenya.

Figure 1: A picture showing environmental pollution caused by feather waste disposal

Other negative effects feathers have had on the environment are the consequences associated with disposing them in main sewer lines and rivers. The disposal in main sewer lines and rivers gives rise to blockage of the drainage system as well as the rivers. This leads to bursting of the drainage waste pipes and the stagnation of water in the river leads to growth of pathogens and breeding grounds of mosquitos that cause malaria and other diseases. Burning is one of methods practised to dispose feather but this has had its negative effects as it contributes to air pollution. Feathers contain disulphide bonds, when burnt they emit sulphur dioxide and carbon monoxide (Tesfaye *et al.*, 2017). Sulphur dioxide produced is then converted to acid rain causing corrosion in roofs as well as changing the soil and water pH.

There have been several efforts used to manage feather pollution in the environment. Both traditional and modern methods have been used to curb feather pollution by utilising them in different forms. Traditionally in many African communities the feathers have been used as

decorations for hats and costumes during traditional dances. For example in India feathers have been used as medicine for snake bites, infertility and coughs (Bird, 2018) .Feathers were used as droppers for ear drops or used in testing the temperature of boiling oil. If the tip of the feather burns, then the oil is ready. Similarly feathers found use also in education and communication as a tool in writing. Educated people in the society would dip the tip of the feather in ink and use it to write to convey different messages. The traditional methods utilised feathers but in very small quantities, African cities such as Nairobi are faced with the huge amounts of feathers that still end up in the environment. Currently, feathers are utilised in making pillow stuffing, diapers, upholstery padding and paper.

Feathers have also been recycled to a feathery meal by mechanical practises where they are chopped to small sizes and fed to animals. However, efforts to use feather as animal feeds is unattainable because the feathery meal is indigestible in this form. The animal uptake of protein in the digestive system is in its constituent form of amino acids but not in raw keratin present in feathers that has not be broken down. Animal lack keratinase that break down keratin structure present in feathers. In the efforts to reduce environmental pollution and adding value to feathers recycling of feathers from slaughterhouses using enzyme hydrolysis provides an excellent solution. Feathers contain Keratin which is difficult to break down, owing to the presence of highly cross-linked protein matrix stabilized by hydrogen bonds as well as hydrophobic interactions (Brandelli, 2007). Further, cross-linking of the polypeptide chains resulting from presence disulfide bonds offers resistance to bio-degradation using proteolytic enzymes such as papain and trypsin (Brandelli, 2008). Current methods employed in recycling of feathers to feather meal use harsh chemicals which include acid and alkaline hydrolysis as well as thermal treatments to break down the keratin structure (Kornilłowicz-Kowalska & Bohacz, 2011) .

2.2 Chemical hydrolysis

Chemical hydrolysis has been used for a very long time to convert feathers to a feather meal. Acid and alkaline hydrolysis is the common practise used in chemical treatments as they can break down the keratin structure. Strong acids and bases accompanied with conditions of high temperature and pressure are used to achieve bond breakage of the disulphide bonds making the proteins soluble. The method produces animal feeds of low nutritional value because of the harsh conditions and reagents used that denature the amino acids present (Pawel *et al.*, 2014).

2.2.1 Alkaline hydrolysis

Alkaline hydrolysis involves use of chemicals such as sodium hydroxide and other strong bases. Alkaline hydrolysis is very slow leading to process of hydrolysis slowing down. Bromine permanganate and hydrogen oxide have been used in this method as an oxidising agents to break down the keratin structure but they resulted in the reaction being slow (Cheong *et al.*, 2017). In contrast Potassium cyanide, sodium sulphide and thioglycolic acid solution have been used in alkaline hydrolysis as reducing agents and the reaction is fast (Cheong *et al.*, 2017) .Though as a result of sulphites and thiols groups present, this reagent is toxic to the environment and difficult to handle (Cheong *et al.*, 2017). The main disadvantage of alkaline hydrolysis is a longer reaction time and huge consumption of power. High concentrations of the alkaline reagents result in low production of amino acid making the animal feeds to be of low nutritional value.

2.2.2 Acid hydrolysis

This process involves the hydrolysis of feathers using sulphuric acid accompanied with conditions of high temperature and pressure. Acid hydrolysis is not very specific which tends to break any bond resulting even in the breakage of peptide bonds to small chain peptides and constituent amino acids. Tryptophan, cysteine, serine and threonine amino acids are destroyed in acid hydrolysis whereas asparagine and glutamine are converted to their acidic form of

aspartic acid and glutamic acid (Cheong *et al.*, 2017). Products of acid hydrolysis must be neutralised to a neutral pH. Neutralisation leads to formation of salts and interferes with functional groups of food systems. Alkali hydrolysis feathery meal is of low nutritional value and because of neutralisation process it further leads to creation of secondary pollutants. Reagents used for acid hydrolysis pollute the environment by causing soil acidity and lowering pH when they leach into water sources resulting to death of aquatic organisms.

2.3 Mechanical treatment

The methods employed in this treatment to recycle feathers to a feathery meal are quite mechanical. This method involves cutting and grinding feather in small particles however the meal ends up being indigestible and the nutrients are not absorbed in the animal body. The uptake of protein is its ability to be hydrolysed to constituent amino acids plus be utilised in the body. Keratinase can breakdown the keratin structure to its constituent amino acids. These enzymes lack in most animals most animals utilise protein in their constituent from of amino acids. Conversion of feathers to a feather meal by use of milling and grinding produces feathery meal that is of low nutritional value, as their main aim is to decrease the particle size of the feathers. This method is inefficient in turning feathers to feather meal as they have not been broken down to constituent amino acids that be absorbed in the animal body. These methods also consume high energy and its implementation in an industrial process could be expensive.

2.4 Hydrothermal treatment

Hydrolysis by hydrothermal treatment is done under conditions of high pressure and temperature. Physical treatments include thermal treatments by either autoclaving, using a pressure cooker and steam cooking. Hydrothermal treatment method consumes high energy and the high temperature resulting in denaturing of the proteins. Essential amino acids for instance tryptophan and lysine are denatured in hydrothermal treatments results to feeds or products of hydrolysis that are of low nutritional value as and products are of poor digestibility

(Tiwary, 2012). Presence of Amino acids contained in the final product of hydrolysis determines the nutritional value. Because of the high temperature and pressure in the process of hydrolysis this translates to the feeds being expensive and of low nutritional value.

2.5 Use of feathers as fertilizer

Feathers contain 90% protein that constitutes nitrogen which can be used as a nitrogen rich fertiliser (Tesfaye *et al.*, 2017). Natural degradation of feather waste in the environment takes more than 2 years (Dudyński *et al.*, 2012). This concept is unattainable because it's not time efficient. The plants require the nitrogen as they grow and slow decomposition of feathers to release nutrients makes them inferior source of fertilizer. The other disadvantage of utilisation of feather as fertilizer is the fact that feather does not only contain nitrogen but also carbon, sulphur groups (Cheong *et al.*, 2017). When the feathers decompose into the environment, greenhouse gases are emitted such as carbon monoxide, hydrogen sulphide, ammonia, methane and sulphur dioxide brings about air pollution to the surroundings environment (Maciel *et al.*, 2017).

Utilisation of feather as fertilizers, recycling through chemical and mechanical treatment presents a way of breaking down the keratin structure but they each have their own drawbacks. Products such as animal feeds are expensive, low in nutritional value and the processes contribute to environmental pollution. Bioconversion of feathers into a hydrolysate using an enzyme may offer a valuable solution to this problem.

2.6 Protein feeds

Protein is essential to animal growth especially in the development of body tissue, muscle, skin, cartilage beak and feathers. Proteins comprise of amino acids that are classified as essential amino acids and non- essential amino acids. Essential amino acids are very vital in the body and if they are not generated in the right amount for the body to function, they must be supplemented in the food intake. Quantity of amino acids present in a protein animal feed

determine its quality. For animal protein requirements, methionine and lysine are the amino acids that must be present. In most protein feeds, they lack theses amino acids therefore methionine and lysine supplements have to be added thereby creating additional cost in the feeds due to the supplementation (Onifade *et al.,* 1998). The current animal protein feeds in the market are expensive and nutritional quality is poor. Traditional sources of feeds used that are readily available and of good nutritional value such as "Omena or Dagaa" have led to competition of food between man and domestic animals. In most European countries feeding domestic animals with flour or protein concentrate as sources of protein have been forbidden because of mad cow diseases, creutzfeldt Jakob disease and others which pose fatal health risks to animals (Mokrejs *et al.,* 2011). Enzymatic hydrolysis on chicken feathers may provide a cheaper, more efficient and environmentally friendly way of utilizing feather into feather meal. It reduces competition for food between man and animal by providing an alternative source of protein for animal feeds.

2.7 Enzyme hydrolysis

Enzymes are proteins that catalyse only specific reactions. Enzyme act as catalyst in enzyme catalysed reactions by increasing rate of reaction. During reactions, the enzyme is not consumed and returns to its original state after reaction. The application of enzyme to break down keratin in feathers to constitute amino acids for use as feather meal is an environmentally friendly method that is conceptually appropriate. Keratin the protein found in feathers is made up of several amino acids it has presence of disulphide and hydrogen bonds that are formed by crosslinking of the amino acids that are present (Mazotto *et al.,* 2011). This results to a very tough and complex structure with good thermal properties. Keratins structurally are insoluble proteins in both organic solvents and water. In comparison to other soluble proteins, keratin structure is quite stable to be degraded by proteolytic enzymes in particular papain, pepsin and trypsin (Gousterova *et al.,* 2005). Enzyme hydrolysis is preferred because it's specific and

catalyses a specific reaction. Keratinase breaks down the cross-linked keratin structure in feathers. This process is very environment friendly and products of hydrolysis are of higher nutritional value because it reduces loss of amino acids.

2.7.1 Keratinases

Keratinases are proteolytic enzymes capable of degrading the keratin structure by destabilising the disulphide bonds present. Certain micro-organisms have the ability to breakdown keratin waste in spite of their resistance. Keratinases are produced by a wide variation of microorganism specifically fungi (*Aspergillus*), actinomycetes (*Streptomyces pactum,*) and several bacterial species (*Bacillus Licheniformis, B. pumilus*,) (Sahoo *et al.*, 2017). The micro-organisms have been obtained from soils, hot springs, where keratinous material have been deposited (Brandelli *et al.*, 2015). The ability of bacterial isolates to be exploited for feather degradation is in fact determined by letting the isolates to grow on the feather waste medium as the only source of carbon and nitrogen, from this hydrolysis ability is determined. The use of keratinase in feather degradation provides an alternative method of hydrolysis that is non-polluting in comparison to steam pressure-cooking and chemical hydrolysis. Final products of enzyme hydrolysis are of good nutritional value to be utilized as animal feed, polymers and fertilizers (Brandelli *et al.*, 2010). Previous studies done demonstrates that *Bacillus spp*. constitute a main source of keratinolytic enzymes for feather hydrolysis (Brandelli, 2008). The original application of a keratinase enzyme to hydrolyze feathers was first reported: whole feathers were completely soluble by the keratinase from *Bacillus pumilus A1* after 6-h incubation with temperatures ranging from 45°C to 60°C (Fakhfakh-Zouari *et al.*, 2010).

A study carried using *Bacillus licheniformis* demonstrated that the crude enzyme solution from the bacterium had the ability to degrade native feather up to 63% with soluble protein of 440 mg/g of feather (Abdel-Fattah *et al.*, 2018). The optimum operating conditions of the enzyme

were investigated and found to be at pH 8.0 and 65°C. The produced enzyme exhibited stability over a varied pH and a temperature range up to 2 hours.

Keratinases are very stable in high temperatures. At very high temperature when the enzyme is more stable and active the structure of keratin gains flexibility which results in the structure to be more susceptible to protease attack (Brandelli, 2008). An example of a keratinase enzyme isolated from Lake Nakuru that has extreme conditions of salinity and high temperature is *Bacillus agaradhaerens* (*B.agaradhaerens*). The bacterium showed great potential in the hydrolysis of chicken feather waste and its optimal conditions were determined. The optimal conditions of this bacterium were found to be pH 10, Salinity of 4% w/v NaCl and temperatures of 34˚C (Mazotto *et al.*, 2013).

Previous research has shown that feather degrading enzymes have mostly been isolated from poultry waste. Examples we have *Bacillus licheniformis* from untreated soils and natural feathers composting sites and also *Bacillus subtilus* (Synowiecki, 2010). An example of an enzyme isolated from *Bacillus licheniformis* is *Subtilisius of bacillus* Origin (Synowiecki, 2010). The optimum operating conditions of enzyme were pH 8.3 and temperatures of 60˚C. *Bacillus licheniformis* is a Thermophillic bacterium it grows well in high temperature this ability helps in efficient feather degradation.

Different kinds of bacteria isolated from different environment with ability to degrade chicken feather include *Chryseo bacterium sp.kr6*. The bacterium was used in the degradation of feathers and showed great potential (Riffel *et al.*, 2007). *Bacillus licheniformis* ER-15 has also been used to degrade feather waste to a complete feathery meal with optimum parameters of pH8.5 and temperature 50˚C (Riffel *et al.*, 2007). The biochemical characteristics of Keratinases discussed by (Brandelli *et al.*, 2010) is that most Keratinases have high activity at neutral or alkaline extending from pH7 to pH9.5 and are stable at high temperatures. Conversion of keratin waste using Keratinases presents an important added value to the poultry

industry (Brandelli *et al.*, 2010) . The advantage of enzyme hydrolysis is it produces products of high nutritional value.

2.7.2 Factors affecting enzyme hydrolysis

Enzymes being made of protein are affected by pH, Temperature and Incubation time (Brandelli, 2008). All these factors should be optimised during the process of enzyme hydrolysis. Enzymes are stable and efficient at optimum temperatures. Increase in temperature is a linear relationship to rate of reaction. As temperature increase the number of collisions increase because of the kinetic energy gained from the heat. The increased number of collisions led to higher rate of reaction as the substrate and enzyme molecules have a higher probability of colliding creating enzyme substrate complex, leading to increase rate of hydrolysis that directly results to increase in rate of reaction (Gardner *et al.*). Because the Enzyme is made of protein, very high temperatures denature the enzyme. It is of uttermost importance to investigate the optimum temperature.

Numerous effects a pH of a solution is on the activity of enzymes and the structure as well. The pH affects the state of ionisation of acidic and basic amino acids. An acidic amino acid has a carboxyl functional group while the basic amino acid has an amide functional group. Changes in pH of results in deprotonating or protonating an enzyme side group changing its chemical structure. Protonating or deprotonating of either carboxyl or amide terminus affects the protein forces holding the protein together either due to formation of additional bonds or breakage of already existing bonds (Gardner *et al.*, 2019). In the case of bond breakage the substrate is not able to attach itself to the active site and cannot undergo catalysis and the enzyme undergoes denaturing or become inactive(Gardner *et al.*, 2019). In the case of formation of additional bonds, the substrate binds to active site leading to increase in rate of hydrolysis rate of reaction.

Molecular concentration of enzyme affects rate of hydrolysis. High concentrations of enzyme at optimum pH and temperature lead to an Increase in hydrolysis. High enzyme concentration

led to more substrate molecules colliding with enzyme molecules since additional enzyme active sites are present for the substrate to bind. Increased enzyme active sites result to conversion of more desired products in a short period of time thereby increasing rate of reaction.

2.7.3 Kinetics of enzyme hydrolysis

During enzymatic hydrolysis the reactions take place and they are explained by the Michaelis-Menten equation. The Michaelis–Menten model is a simple model of an enzymatic reaction developed by Leonor Michaelis and Maud Menten in 1913 (Gardner *et al.*, 2019). During enzyme reaction the substrate attaches itself to active site of the enzyme forming an enzyme substrate Complex (E×S) the reaction rate co-efficient is given by K_1. As soon as the enzyme substrate complex forms it disappears and breaks down to form the product and the free enzyme . This means that rate of reaction for the enzyme substrate complex is equated to zero (rE×S=0). The enzyme substrate reaction (equation 1) is a reversible reaction with its own reaction rate co-efficient (Dudyński *et al.*, 2012).

$$\mathbf{S+E \xrightarrow{K1} (E\ X\ S)} \quad \textbf{..... Equation 1}$$

Where S is the substrate, E is the enzyme, K_1 is the reaction rate constant for enzyme substrate complex, (E×S) is the enzyme substrate complex and K1 is the reaction rate constant for this reaction.

The substrate reacts with the enzyme to produce enzyme substrate complex

$$\mathbf{(E\ X\ S) \underset{K-2}{\overset{K2}{\rightleftharpoons}} E + P} \quad \textbf{..... Equation 2}$$

Where k_2 is the reaction rate constant formation of free enzyme and substrate and K_{-2} is the reaction rate constant formation of enzyme substrate complex. The enzyme substrate complex is formed as fast as it disappears and dissociates to free enzyme and product. k_2 is the reaction rate constant for this reaction (Coward-Kelly *et al.*, 2006).

The power at the concentration of the substrate is raised to help in identifying which order of reaction. But to calculate the overall rate of reaction we use the Michaelis- Menten equation given below, The Michaelis-Menten equation gives the rate of substrate consumption during the reaction by the enzyme.

$$r_1S=-K_1(E)(S) \quad \text{Equation 3}$$

$$r_2S=K_2\,(E\ X\ S) \quad \text{Equation 4}$$

$$r_3P=K_3(E\ X\ S)\ W \quad \text{Equation 5}$$

The net rate for substrate consumption is

$$-r_s=K_1(E)(S)-K_2\,(E\ X\ S) \quad \text{Equation 6}$$

We will express (E×S) in terms of measured variables. The net rate of formation of the enzyme-substrate complex is given in equation 7:

$$-r(E\ XS) = K1(E)(S)-K2(EXS)-K3\ (EXS)(W) \quad \text{Equation 7}$$

Consequently a reactive intermediate (E×S) reacts virtually as fast as it is formed, the net rate of formation of an active intermediate (e.g. r E×S) is zero,

The rate law for substrate consumption is

$$-rS= K_1K_3(Et)(S)(W))/(K_1(S)+K_2+K_3(W)) \quad \text{Equation 8}$$

2.7.4 Michaelis-Menten equation

The Michaelis constant (K_m) is a parameter in the Michaelis–Menten equation. Km is equal to the substrate concentration where the corresponding reaction rate is $\frac{1}{2}$Vmax.

In the Michaelis–Menten model it is presumed that the third reaction is the rate-limiting step, and the related rate constant k or K_{cat} is the turnover number.

$$K_{cat}= K_3W \quad \text{Equation 9}$$

K_{cat}- is denoted as the turnover number. Turnover number is the number of substrate molecules converted to product at a given time on a single-enzyme molecule when the enzyme is saturated with substrate (i.e., all the active sites on the enzyme are occupied (English *et al.*, 2006).

$$K_M = (K_{cat}+K_2)/K_1 \quad \text{Equation 10}$$

K_M-Michaelis-Menten constant, for simple systems is a measure of affinity of the enzyme for its substrate, so it's also called the affinity constant.

After simplifying the equation, it takes this form, where substrate consumption is given by

$$-rS = (K_{cat}\,(Et)(S))/((S)+K_M) \quad \text{Equation 11}$$

Where:

(S) - Concentration of the substrate.

V_{max} - maximum rate of reaction for a certain enzyme concentration

$$V_{max} = K_{cat}\,(Et) \quad \text{Equation 12}$$

The Michaeli's-Menten takes the form:

$$rS = (V_{max}(S))/((S)+K_M) \quad \text{Equation 13}$$

K_M - Michaeli's-Menten constant.

(S)-Substrate concentration

V_{max}- is the maximum rate of reaction for a particular enzyme concentration.

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2.8 Methods of characterization

2.8.1 High Performance Liquid Chromatography (HPLC) analysis

HPLC is a technique of separation that separates molecules according to their composition and molecular structure. The HPLC works on the principle of retention time where some molecules take a longer period in the chromatograph column than others (Guiochon *et al.*, 2008). The

retention time of a molecule on the column depends on the affinity of molecule to the mobile phase and the stationary phase. An inert gas or liquid is preferred for the mobile phase so that it does not react with the product. Its main role is to carry the sample to the chromatograph column. The mobile phase transports the sample to the stationary phase and if it has higher affinity to the stationary phase it will move slowly and if it has lower affinity it moves quickly through the chromatograph column.

The HPLC is a significant tool in the analysis of composition of foods as well as metabolic studies. HPLC employed in determining the amino acid composition of the feather hydrolysate. Amino acid composition of the hydrolysed feathers seeks to relate the quality of products with the amino acids obtained. For the hydrolysed feathers to serve as a protein source great consideration is given to the amino acids available in the products and at what quantity. The HPLC helped in identification and quantification of the amino acid present in feather hydrolysate thus determining its nutritional value to be used as a protein source (Coward-Kelly *et al.*, 2006).

2.8.2 Fourier Transform Infra-Red (FTIR) analysis

FTIR is used in the analysis of organic compounds and has found it uses also in analysis of Inorganic samples such as polymers, coatings and drugs. The FTIR is used to ascertain the functional groups present in hydrolysed feathers. Functional groups are the portions of an organic molecule that indicate how a molecule will react (Berthomieu & Hienerwadel, 2009). In this research the results from FTIR analysis was used to determine if the feather hydrolysate was composed of amino acids by looking at the functional groups present. FTIR assists in confirming presence of amino acids that the enzyme produced by *Bacillus spp* strain can hydrolyse feathers to constituent amino acids. The FTIR works on the principle that molecules absorb light in the infra-red region of the electromagnetic spectrum. The resulting absorption spectrum from the bond natural vibration frequencies shows the existence of several chemical

bonds and functional groups present (Berthomieu & Hienerwadel, 2009). The FTIR uses Attenuated Total Reflectance (ATR) the purpose of this is so that it can be able to analyse solids, liquids, and even viscous liquids where previously it was only Liquids. Within the ATR set up, the infrared beam is reflected within the ATR crystal. At each reflection the evanescent waves probes a layer of the feather hydrolysate deposited on the crystal within a thickness of about 1µm (Berthomieu & Hienerwadel, 2009). FTIR is useful in identification of molecules because of the presence of functional groups, side chains and crosslinks present in the sample. Each functional group, side chain has its own vibrational frequency in the infra-red range.

2.8.3 Gas Chromatography Mass Spectroscopy (GC-MS) analysis

GC-MS is analytical techniques that combine's gas chromatography and mass spectrometry. Gas chromatography is used in identifying organic compounds (Hussain & Maqbool, 2014). This helps in value addition of the hydrolysed chicken feathers thereby increasing its nutritional value for other uses other than protein animal feeds.

Gas chromatography is a separation analytical technique that is carried out by distributing the sample among two phases that is the stationary phase and the mobile phase. Gas-chromatography principle works when a sample is heated and converted to vapour phase it passes through the stationery phase (Hussain & Maqbool, 2014). The higher affinity of molecules to stationary phases the higher the retention time. The sample is carried through the column by an inert gas either helium or hydrogen. Faster eluded molecules are carried to the mobile phase where they are separated, and they come out through the column to the mass spectrometer. In the mass spectrometer the molecule is fragmented either by electronic ionisation or chemical ionisation to generate charged ions known as radical cation. Fragmentation of each molecule is unique. The mass spectrometer is a detector that measures the mass to charge ratio of charged particles. The gas chromatography separates the sample and the mass spectrometer provides structural information (Hussain & Maqbool, 2014).

2.9 Proximate analysis

In this research the hydrolysed feathers were proposed as an alternative source of protein for animal feeds. Key facets need to be undertaken to establish and to provide a clear indication that the feather hydrolysate can be considered as protein source for animal feeds. This is done by a nutritional evaluation process that seeks to assess the different components of feeds through a process known as proximate analysis. Proximate analysis is the examination of the feed composition extracted and to look at its normal compositional parameters. This method divided nutrients feeds into 6 components that is crude protein, moisture content, carbohydrate content, ash content and fat content. The parameters were investigated by the methods of the Association of Official Analytical Chemists AOAC (1990) .

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Feather collection

The feathers were collected from Kim's poultry processing plants located in Thika Kiambu County. They were washed thoroughly in running water to get rid of all the dirt, blood and flesh remains before drying on the sun for 3 days. The dried feathers were put in storage in porous bags at room temperature all set for experiments.

3.2 Reagents and equipment's

The reagents used in this research were purchased from Pyrex East Africa and were of analytical grade. They include sodium hydroxide NaOH, hydrochloric acid Hcl, trichloroacetic acid (TCA), sodium carbonate, folin & Ciocalteu's reagent (F-C reagent). Equipment used in this research included Ultraviolet Spectrophotometer (UV-VIS Shimadzu UV mini 1240), Auto clave (Tuttanauer steam 121˚C sterilizer), Bio reactor (R'ALF plus duet fermenter, 3.7 L), Centrifuge (Sorvall ST16R), pH meter, Timer, Thermal incubator and oven.

3.3 Enzyme preparation

Crude protease used in this study was obtained from bacterium (*Bacillus cerus strain wwcp1*) isolated from lake Bogoria in the Kenyan Rift valley region (Wanyonyi *et al.*, 2014). The bacterium was re-plated, incubated for 24 hours at 30˚C and used in enzyme production. The crude protease used in the feather hydrolysis was prepared according to the modified procedure by (Wanyonyi *et al.*, 2014). The enzyme was produced using a bio reactor set at 46˚C and pH 10.5 incubated for 36 hours. The media for the bacteria contained 0.23% casein and 0.3% glucose, urea 0.75 g, yeast extract 1.35 g, calcium chloride $CaCl_2$ 0.72 g, 0.01g sodium chloride NaOH and 3.78 g KH_2PO_4 in distilled water. The pH of the bacteria media was adjusted to 10.5 using 1M sodium hydroxide NaOH and 1M hydrochloric acid Hcl using a pH meter, then was placed in the autoclave (Tuttanauer steam 121˚C sterilizer) for 25

minutes. The media was allowed to cool at 25˚C. The media was inoculated with 5% seed bacteria *(Bacillus cereus* wwcp1strain) culture and the required conditions set that is temperature to 42˚C, 120 rpm and the aeration was checked if it was working effectively. After three days the crude enzyme was ready to harvest, it was centrifuged in Sorvall ST16R centrifuge (1500 rpm, 5˚C for 5 minutes). The enzyme was stored in the cold room at temperatures of 4 ˚C to be used for hydrolysis.

3.5 Generation of standard curve

Reagents used in generation of the standard curve are as follows; 1.87 µl of 0.4 M sodium carbonate, 1ml of trichloroacetic acid, tyrosine 0.02g, 375µl folin & ciocalteu's F-C reagent (33.3% v/v) and distilled water. The F-C reagent was prepared by diluting to three parts of water. Different amount of tyrosine with ranges from (15µl - 150µl) were measured in microliters using micro pipettes and placed in falcon tubes with one sample being a blank. Different amounts of water (750µl - 600µl) were added to the tyrosine present in the falcon tubes followed by varying amounts of 0.4M sodium carbonate Na_2CO_3 as shown in the table 1. Folin & Ciocalteu's reagent was added to the contents in the falcon in different amounts according to the table below. The falcons with its contents were set aside in an incubator at 30˚C for a period of 30 minutes for the colour to develop. Readings of the sample were taken using a UV-VIS Shimadzu UV mini 1240 spectrophotometer at 660 nm and recorded. The same method was applied in optimisation of parameter for the Enzyme hydrolysis of chicken feathers. Table 1 shows the reaction scheme for tyrosine standard curve.

Table 1: Tyrosine standard curve

Reagent	Number of standards										
	1	2	3	4	5	6	7	8	9	10	11
Tyrosine Standard solution in µl	0	15	30	45	60	75	90	105	120	135	150
Reducing Tyrosine volume in µl	0	5	10	15	20	25	30	35	40	45	50
Distilled Water in µl	750	735	720	705	690	675	660	645	630	615	660
Sodium carbonate In µl	1.875	1.875	1.875	1.875	1.875	1.875	1.875	1.875	1.875	1.875	1.875
Diluted F-c Reagent In µl	375	375	375	375	375	375	375	375	375	375	375
Total Volume (ml)	3.0	3.0	3.0	3	3	3	3	3	3	3	3
Adjusted Volume (ml)	1	1	1	1	1	1	1	1	1	1	1
Tyrosine Concentration in µM	0	0.0055	0.011	0.0165	0.022	0.0275	0.0330	0.0385	0.044	0.0495	0.055

3.6 Optimization of parameters

The Enzyme was prepared by a known bacterium known as *Bacillus cereus wwcp1 strain* isolated from Lake Bogoria here in Kenya. The optimisation of parameters was carried out to determine the optimal conditions that produce maximum yield and evaluate the efficiency of the enzyme. The parameters that were investigated included pH, temperature, time and enzyme concentration.

3.6.1 PH optimisation

1 g of raw feathers was placed in 100 ml enzyme solution contained in a 500 ml conical flask. The Experiment was carried out in triplicate under the pH ranges of pH 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 13. The feathers together with the enzyme were placed in an oven at temperatures of 45˚C where the pH was adjusted accordingly using 1 M sodium hydroxide and 1 M hydrochloric acid while being stirred periodically. The pH optimisation was carried out side by side with control samples that contained 1g of feathers with respective pH3 to pH13, but the samples were placed in 100 ml of distilled water and all other conditions maintained. An aliquot of 1 ml of sample was taken from the main samples to test for degree of hydrolysis and the rest of the sample was returned in the oven. Periodic aliquots were extracted and tested after every

12 hours until hydrolysis was completed. The 1 ml aliquot was measured using a micro pipette and place in a falcon tube. The sample was allowed to cool then 5 ml of 10% w/v trichloroacetic acid was added to sample to stop the reaction. The trichloroacetic acid coagulates proteins that are in suspension. The sample was kept in an ice bath for 25 minutes and allowed to freeze. The sample was then centrifuged for 7 minutes using a centrifuge. Centrifugation helps in separation of coagulated proteins from the mixture. The centrifuged sample is then transferred to a clean falcon tube where 5 ml of 0.4 M sodium carbonate Na_2CO_3 was added to neutralise TCA. Folin & Ciocalteu's (F-C reagent) was added in 1 ml measurement to the aliquot sample in the falcon tubes. The use of F-C reagent is to develop colour for the presence of tyrosine released to absorb the wavelength placed in the UV-VIS Shimadzu mini 1240 spectrophotometer. The Falcon tubes were placed in an Incubator at a temperature of 30 ˚C for the colour to develop with a time period of 30 minutes. The sample was subjected to UV analysis at a wavelength of 660 nm and the absorbance was measured. The degree of hydrolysis was then investigated with the help of the generated standard curve and a graph is plotted of concentration against time. The amount of tyrosine released corresponds to the degree of hydrolysis because tyrosine is an amino acid.

3.6.2 Temperature optimisation

The optimum temperature was studied in the temperature ranges of 30˚C to 80˚C. At the beginning of the experiment the pH of the sample is set at optimum pH which had been investigated in section 3.6.1. A sample of 1.0g of raw feathers was placed in 100 ml enzyme solution contained in a conical flask and the pH was adjusted to pH 12. An aliquot of 1.0 ml sample was taken to test for degree of hydrolysis. Periodic samples were tested after every 12 hours until hydrolysis was completed. A sample of 1.0ml was measured using a micro pipette and placed in a falcon tube. The sample was allowed to cool then 4.0 ml of 10% w/v TCA (Trichloroacetic acid) added to stop the reaction. The trichloroacetic acid TCA coagulates proteins that are in suspension. The sample was placed in an ice bath for 20 minutes and

allowed to freeze. The sample is then centrifuged for 7 minutes using a centrifuge. Centrifugation helps in separation of coagulated proteins from the mixture. The centrifuged sample was then transferred to a clean falcon tube where 5.0 ml of 0.4 M sodium carbonate Na_2CO_3 was added to neutralise TCA. F-C reagent was added in 1.0 ml measurement to the sample. The Falcon tubes were placed in an incubator at a temperature of 30 ˚C for the colour to develop within 30 minutes. The sample was subjected to UV analysis and the absorbance measured at 660 nm. The degree of hydrolysis was then determined with the aid of the generated standard curve and a graph of concentration against time was plotted.

3.6.3 Optimisation of enzyme concentration

Enzymatic concentration was a parameter that was analysed at different dilute concentration of the enzyme. The previous experiments have enabled us to get the optimum parameters at pH 13 and temperature at 80 ˚C. Therefore, during analysis of enzyme concentration, the set temperature and pH are constant in all samples the only varying factor was enzyme concentration. The enzyme was diluted to various percentage from crude to almost 50%, 25%, 12.5% dilution. 1g of raw feathers was placed in 100 ml enzyme solution contained in a conical flask the pH was adjusted and placed in an oven with the adjusted optimum temperature from above experiments. An aliquot was taken from the sample to test for degree of hydrolysis. Samples were tested after every 12 hours until hydrolysis was complete. 1.0 ml aliquot was drawn from the sample using a micro pipette and placed in a falcon tube. The sample was left to cool at room temperature, and then 4.0 ml of 10% w/v trichloroacetic acid was added to stop the reaction and coagulates proteins. The sample was placed in an ice bath for 20 minutes and allowed to freeze before centrifuging for 7 minutes. Centrifugation helps in separation of coagulated proteins from the mixture. The centrifuged sample was then transferred to a clean falcon tube where 5.0 ml of sodium carbonate Na_2CO_3 was added to neutralise TCA which is an acid. F-C reagent was added in 1.0 ml measurement to the sample. The use of F-C reagent is to develop colour for the presence of tyrosine released to absorb the wavelength placed in

the UV. The falcon tubes were placed in an incubator at a temperature of 30˚ C for the colour to develop with a time period of 30 minutes. The sample was subjected to UV analysis and the absorbance measured. With this range the enzyme concentration was determined while determining degree of hydrolysis using UV absorbance and a graph is plotted to determine the optimum enzyme concentration against time.

3.6.4 Preparation of feather hydrolysate

1kg of feathers was weighed using an analytical balance, placed in a 2 litres conical flask and 1liter of freshly prepared crude alkaline protease added. The contents of the conical flask were transferred to an oven maintained at optimum temperature and pH with periodic stirring. After hydrolysis, the hydrolysate was sterilized by autoclaving for 45 minutes, cooled and then filtered using a sieve to remove any residue. The dried hydrolysed feathers were placed in a fridge at -80 ˚C and then transferred to the freeze drier to dry the sample. The solid dried sample was stored in airtight containers in a freezer to avoid contamination.

3.7 Characterization of hydrolyzed feathers using spectroscopic techniques and proximate analysis.

3.7.1 Characterisation of feather hydrolysate using Fourier transform infra-red (FTIR)

The samples consist of 1.0g powder feather hydrolysate which was kept in measuring cells created by two IR transparent windows disjointed by a path length smaller than 10µm.The samples were taken to FTIR Machine, where the machine was cleaned with absolute ethanol to avoid contamination. The sample was placed in the sample area and the analysis was done which produces a spectrum of the functional groups present. Each spectrum had an average of 4 scans, carried out at a resolution of 4 cm^{-1} in the range of 4000-400cm^{-1}. The analysis was done with a UV-VIS Shimadzu UV mini 1240 FTIR.

3.7.2 Characterisation of feather hydrolysate using gas chromatography–mass spectrometer (GC-MS)

The above mentioned processed involved soaking 1g hydrolysed feathers in hexane for 24 Hours while shaking vigorously after every two hours. A flame ionisation detector (FID) detector attached with gas chromatography was employed to analyse the feather hydrolysate. The organic phase was transferred to the vials using a clean syringe and taken to the GC-MS system Agilent 6890 series for analysis. The samples were injected using a split ratio 100:1 into a fused silicate GC column Cp sil 8CB low bleed (30mm ×25 mm×0.25µm) coupled with a Cp sil 5C low bleed/MS (30mm×0.25mm×0.25µm) column with helium as the carrier gas (Hussain, 2014 #185} . The system was mechanised with a flame ionization detector. The original oven temperature was 120˚C and a temperature program of 8˚C per min began at injection and continued to 3 minutes. The temperature for both the injector port and detector were set at 250˚C.The mass spectrometer data was acquired in flame ionization mode at 70eV.The mass spectra of unknown compounds were determined by use of an inbuilt library search at 99% matching (Hussain & Maqbool, 2014).

3.7.3 Determination of amino acid composition using high performance liquid chromatography (HPLC)

Samples were weighed and placed in about 1.0 ml of 6N HCl along with the internal standard and hydrolysed at 100˚C for 22 hours. An aliquot, usually 10 ul or 20 ul of the hydrolysate is dried then brought up in 250 ul 0.4N borate buffers; One microliter of this diluent is injected. Chromatography separation was performed in rapid resolution HT column (3.0mm×50mm, 1.8 micron) at 16˚C.The eluent gradient was 0.6mL/min flow rate, 6% B for 3 minutes then elution from 6% to 4% in 7 minutes kept for 3.5 minutes from 14% to 19.5%B in 2 Minutes (Bartolomeo, 2006).The composition of the solvents followed: phase A, 25mM acetate buffer (pH 5.8) with 0.02 % of sodium azide: mobile phase B100% acetonitrile. The injection volume was 2µl.The target compounds were identified according to their retention times of their

corresponding standards. Quantification was performed by means of the calibration curves of the corresponding standards, which went through the same process of derivatisation as the sample for the quantification of proline, ethanolamine and serine (Pawel *et al.*, 2014). The matrix-matched calibration curve was needed. Internal standard method established on the areas of the peaks derivatives was used.

3.8 Proximate analysis of hydrolyzed feathers

Proximate analysis of the feather hydrolysate is significant in detailing its nutrition value and the part it plays in meeting an animal's nutrient requirement. For hydrolysed chicken feathers the components that were determined include total ash content, fat content, crude protein content, carbohydrate content, fibre content and moisture content. Methods used in compositional analysis were consistent with the ones acclaimed by the association of official analytical chemist AOAC (1990).

3.8.1 Reagents and materials used to determine proximate analysis

Reagents and materials used include methyl orange indicator, concentrated sulphuric acid, 0.1N hydrochloric acid solution, Nessler reagent, burette 50 ml, conical flask ,digestion and distillation units, kjedahl catalyst tablets, 40% sodium hydroxide solution, pipette, 0.1 N sodium hydroxide solution, glass wool, muffle furnace operated at 550˚C , hotplate, Buchner funnel, 25 ml pipette, 600 ml beakers, suction filtering flask, porcelain dishes, 2.04 N sulphuric acids, 1.73N potassium hydroxide solution, desiccator, rotary evaporator, desiccator, flat bottomed flask of 250ml, soxhlet extractors, condensers, petroleum ether (60-80˚C) and extraction thimbles.

3.8.2 Determination of moisture content

A sample of 5g of hydrolysed chicken feathers sample was weighed. Weight of sample together with the dishes was recorded before drying the sample. The feathers in the dishes were placed in an oven that was kept at a constant temperature of 105˚C. The feathers were dried for a few

hours cooled and weighed. The contents were weighed after the experiment and the percentage moisture present was determined by the following calculations. Moisture was determined by weighing the hydrolysed feathers and dish before drying and weight of dish and hydrolysed feathers after drying divided by weigh of hydrolysed feathers and multiplied by 100%.

%Moisture content = $(W_I-W_A)/W_B$ x 100%t

W_I-weight of hydrolysed feathers and dish before drying

W_A- weight of hydrolysed feathers and dish after drying

W_B- weight of hydrolysed feathers

3.8.3 Determination of ash content

Ash content was investigated by burning solid hydrolysed feathers at very high temperatures of about 400-600 ˚C at stipulated time of four hours. 5 g of hydrolysed feathers was weighed and then taken to the oven to be burned. Weight of hydrolysed feathers and crucible before burning was recorded and weight of ash and crucible was also weighed and divided by weight of hydrolysed feathers and multiplied by 100%.

%Ash = $(W_C-W_D)/W_B$ x 100%

W_C-weight of ash and crucible

W_D- weight of crucible

W_B-weight of hydrolysed feathers

3.8.4 Determination of crude protein using Kjedahl method

Protein determination assists in knowing if the feather hydrolysate is a potential protein source. Crude protein was carried out by Kjeldahl method , association of analytical chemist (AOAC) method 920 where the factor N 6.25 was used to convert nitrogen into crude protein (AOAC, 1990). Procedure was done in triplicate where one was a blank. 5.0 g of dried hydrolysed feather was weighed using an analytical balance. Hydrolysed feathers were placed in a Kjedahl flask and boiled using sulphuric acid in a fume cupboard the heat was increased until a clear

solution was obtained. Solution was cooled and distilled water was added to 3/4 full the flask. Phenolphthalein indicator was added to all three flasks including the blank. 400 ml of conical flasks that contain 50 ml of 0.1N hydrochloric acid were placed in the distillation unit. 40% of the sodium hydroxide solution was added into the solution. Solution was distilled until the solution could no longer react with the Nessler reagent (AOAC, 1990). Back titration was performed with 0.1N sodium hydroxide solutions and titre volume was recorded. Crude protein was calculated by following method.

Crude protein = (FA-FB*constant (0.875))/FC x 100%

FA-blank

FB-feather hydrolysate sample titre volume

FC- feather hydrolysate weight

3.8.5 Crude fibre determination

4.0 grams of hydrolysed feathers was weighed and placed into the 600ml beaker and transferred to a fume chamber. 25 ml of the 2.04 N sulphuric acid and 100ml of boiling distilled water was added to the feathers. The volume was made up to 200 ml with the boiling distilled water and its maintained while the solution was boiling for a period of thirty minutes AOAC (1990). The solution was cooled, filtered using a Buchner funnel and glass wool. The remaining residues together with the glass wool are transferred back to the beaker. The volume was increased to 200 ml and maintained for 30 minutes on a hot plate. The residue was washed 3 times with ethanol measurements and is placed in the air oven to dry. The measurement were taken after drying and taken to the furnace where it's ignited at temperatures of 550˚C for 5 hours, the desiccator was cooled and measurements taken AOAC (1990). The crude fibre was calculated by following formula

Crude fibre% = (FD-FE)/FG x 100%

FD- (crucible + glass wool+ feather hydrolysate sample) weight

FE- (crucible + wool) weight

FG- sample weight

3.8.6 Determination of crude fat content

Analysis of crude fat content present in feather hydrolysate was extracted by means of the Soxhlet apparatus and amount of fat was determined gravimetrically AOAC (1990). A sample of 5.0 grams of hydrolysed feathers were weighed on an extraction thimble. The feather hydrolysate was covered with cotton wool and placed the thimble in the Soxhlet extractor. Place a flat-bottomed flask with 200 ml of petroleum ether on a heating mantle and connected to the Soxhlet extractor. The extraction was done for a period of 8 hours. The solvent is evaporated using a rotary evaporator. The residue of the feather hydrolysate is dried at 105 ˚C in an oven followed by cooling. After cooling the flask with the oil is weighed and recorded. The crude fat content is calculated by the following formula.

% Crude fat= (FO-FW)/FS x 100%

FO- (flask + oil) weight

FW-flask weight

FS-sample weight

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Tyrosine standard curve

The tyrosine standard curve was used to determine the concentration of a substance in an unknown sample by relating the unknown to a set of standard samples of known concentration. Tyrosine was used because it develops colour in presence of the F-C reagent is which can absorb the wavelength when placed in the UV. In this research different amount of tyrosine concentrations ranging from (15 µl to150 µl) were measured and absorbance determined at a maximum wavelength of 660nm and the results are summarised in figure 2.

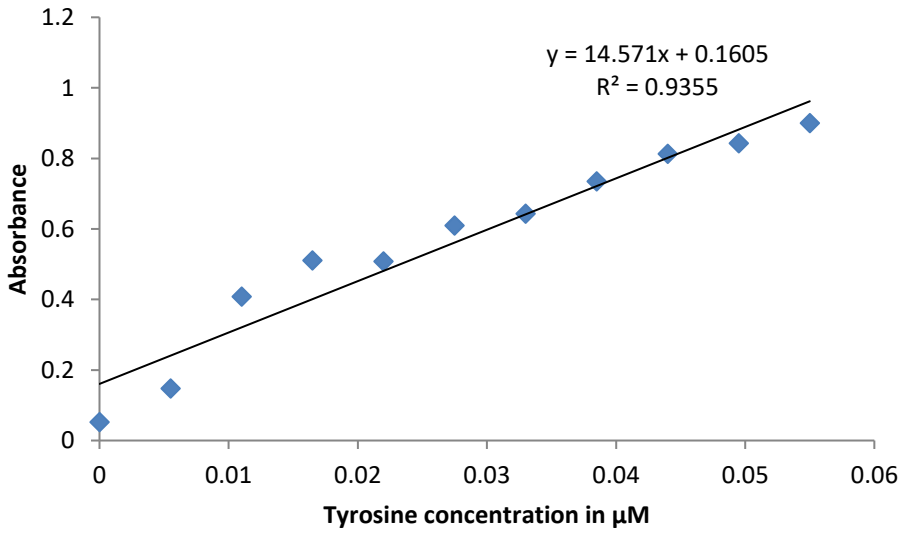


Figure 2: Tyrosine standard calibration curve

In subsequent experiments the standard curve was used to find the tyrosine concentration in the unknown feather hydrolysate.

4.1.1 Optimization of pH

pH is amongst the critical parameters that affects enzymatic reactions. The optimum pH ensures that the enzyme works efficiently in producing desired products in the shortest time possible. For example, a change in pH can protonate and deprotonate a side group thereby changing its chemical features. Protonation or deprotonating R-groups happens on the carbonyl

terminus or from the amino terminus affecting the forces holding the protein together (Gardner *et al.*, 2019). The effect of pH was investigated from pH3 to pH13 to determine the optimum pH and the results summarised in figure 3.

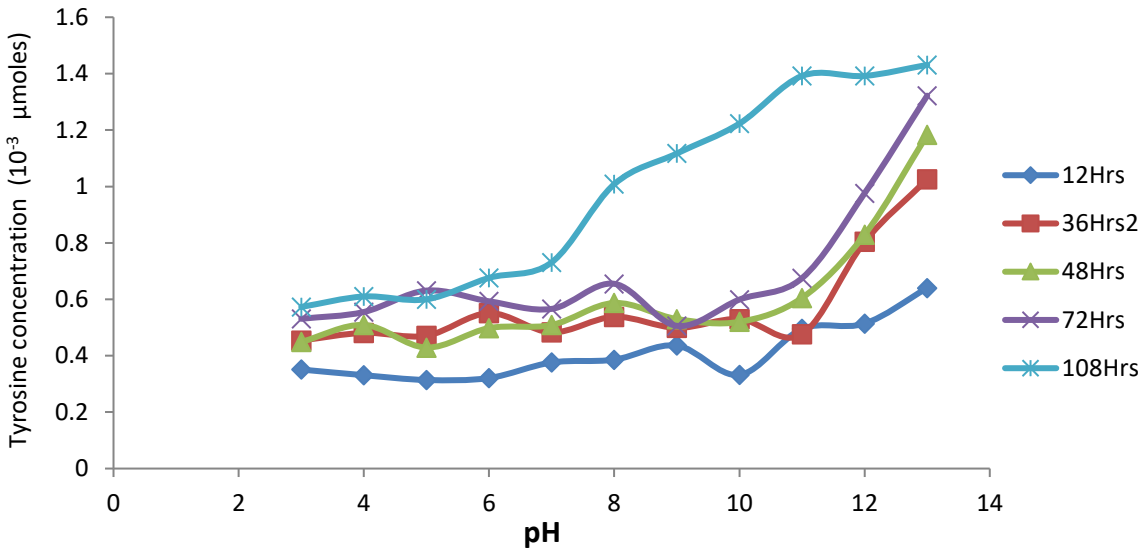


Figure 3: Effect of pH on enzymatic feather hydrolysis

Generally, it was observed that increase in pH led to an increase in the rate of feather hydrolysis. This trend was similar for all pH tested at different time. It was further observed that at pH13, highest amount of tyrosine was realized in the shortest time implying that it was the optimal pH for crude protease extracted from *Bacillus cerus strain wwcp1*. This can be attributed to the extreme environment where the bacterium was isolated**.** The enzyme in this study works efficiently at high pH because the bacterium that produces the enzyme was isolated from Lake Bogoria which is an alkaline, hot and saltwater lake. The Optimum pH corresponds by a study done by Abdel-Fattah *et al.* (2018) where *Bacillus licheniformis* was used to degrade feathers showed increase in pH increased enzyme activity and the enzyme showed stability with alkaline pH in the ranges between pH (7-9). Similarly results by Abdel-Fattah *et al.* (2018) was concluded that most keratinases work best at neutral or alkaline pH.

4.1.2 Effect of temperature on enzymatic feather hydrolysis

Temperature is a significant parameter in enzymatic reactions. Enzymes are proteins that are easily denatured at very high temperature. Investigating optimum temperature gives an insight how temperature affects enzyme activity and the best temperature the enzyme can work efficiently without being denatured. In the optimisation of temperature, the experiment was done in triplicate at 25˚C, 60˚C and 80˚C at a predetermined pH12. The influence of temperature on the hydrolysis of the feather waste is presented in figure 4.

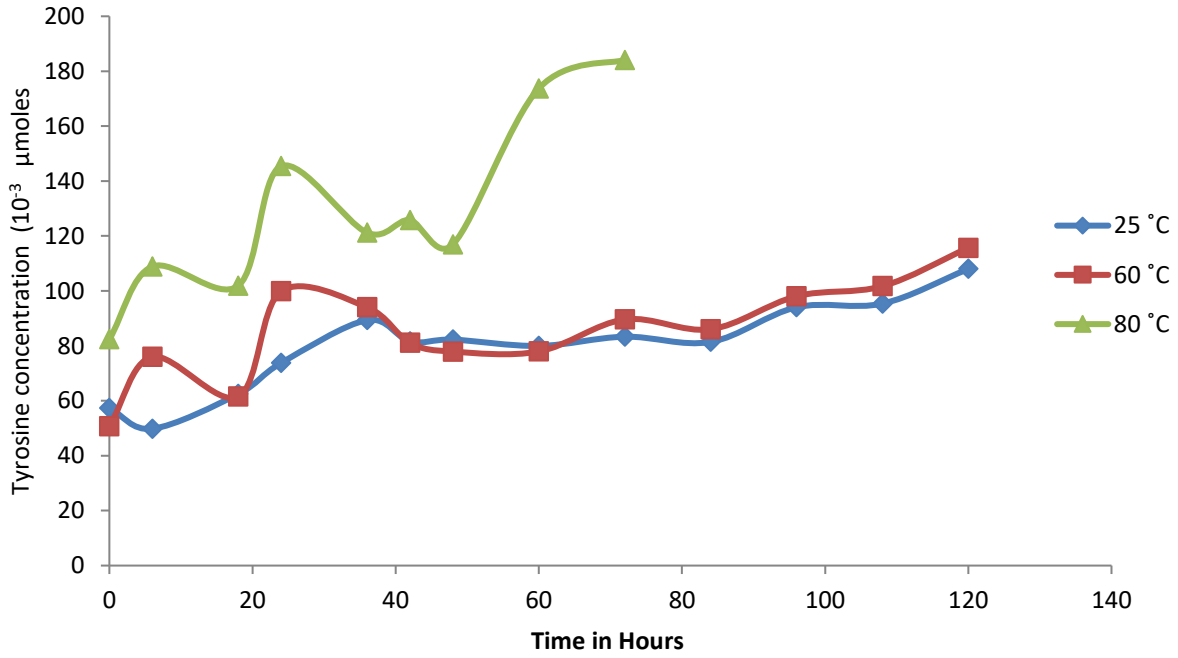


Figure 4: Effect of temperature on enzymatic feather hydrolysis

The results show that feather hydrolysis increased with increase in temperature. For example, at 72 hours there was an increase in the amount of tyrosine released when the temperatures were increased from 25˚C to 80˚C. It was noted that at 80 ˚C feather hydrolysis was complete within 72 hours while at room temperature the hydrolysis proceeded slowly and was incomplete even after 120 hours. This observation can be explained in the sense that with increased temperature increases molecular collisions as a result of gained kinetic energy therefore molecules are colliding faster and reacting faster than when temperatures are lower. The preferred optimum temperature in this study is 80˚C. The results correspond to a study by

Abdel-Fattah *et al.* (2018) where increase in temperature correlated to increase in enzyme activity. In the study feather keratin was degraded by *Bacillus licheniformis* and the optimum temperature for this enzyme was in the range of (50-60) ˚C.

4.1.3 Optimisation of enzyme concentration

Enzyme concentration is an important parameter to investigate as the results assist in determining order of reactions. The tests were carried out over varying concentrations of undiluted crude enzyme depicted by 100%, 50%, 25% and 12.5% with predetermined pH13 and a temperature of 80˚C. The analysis was carried out in triplicate and the absorbance readings were taken using a UV-VIS machine. Figure 5 shows the effects of enzyme concentration on the rate of hydrolysis for feather waste.

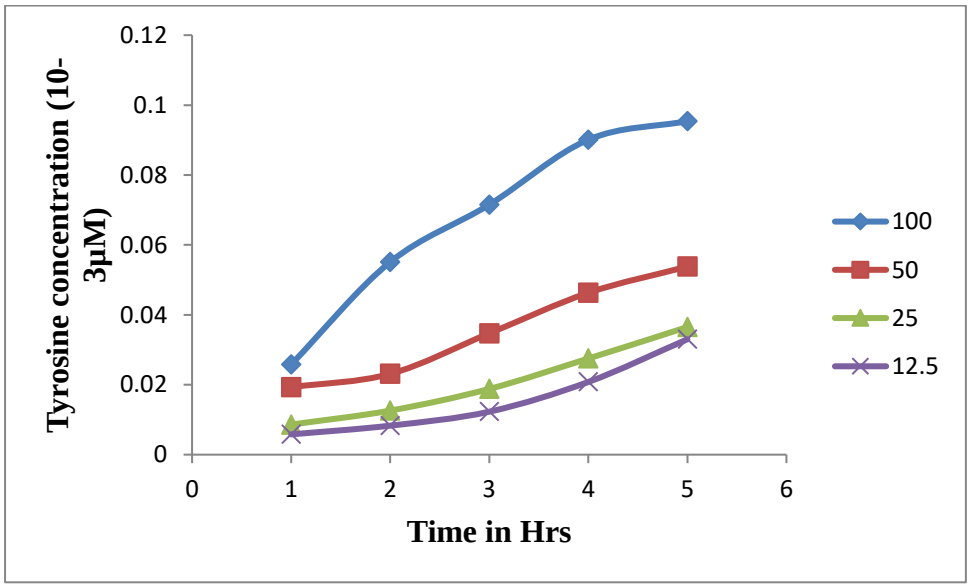


Figure 5: Effect of enzyme concentration on enzymatic feather hydrolysis

The results showed that with increased enzyme concentration there was an increase in rate of feather hydrolysis. From the graph we can see comparing the various concentrations at 24 hours, higher amounts of tyrosine were released at undiluted crude enzyme concentration implying that it is the optimum enzyme concentration. It was also observed that the reaction at undiluted crude enzyme concentrations stops at 48 hours while at 12.5% concentration hydrolysis continued past 120 hours. The observation in this study is related to an increase in

enzyme concentration leads to more enzyme active sites therefore the substrates were able to bind to the enzyme producing more desired products compared to where low concentrations of enzymes were present (Gardner *et al.*, 2019).This results to a linear relationship where enzyme activity is directly proportional to enzyme concentration.

4.2 Determine order of the reaction and K_M

The order of reaction was obtained by plotting values of tyrosine released at optimum enzyme concentration of undiluted crude enzyme against time. The K_M was determined using the Michaelis Menten plot and the Lineweaver burke plot.

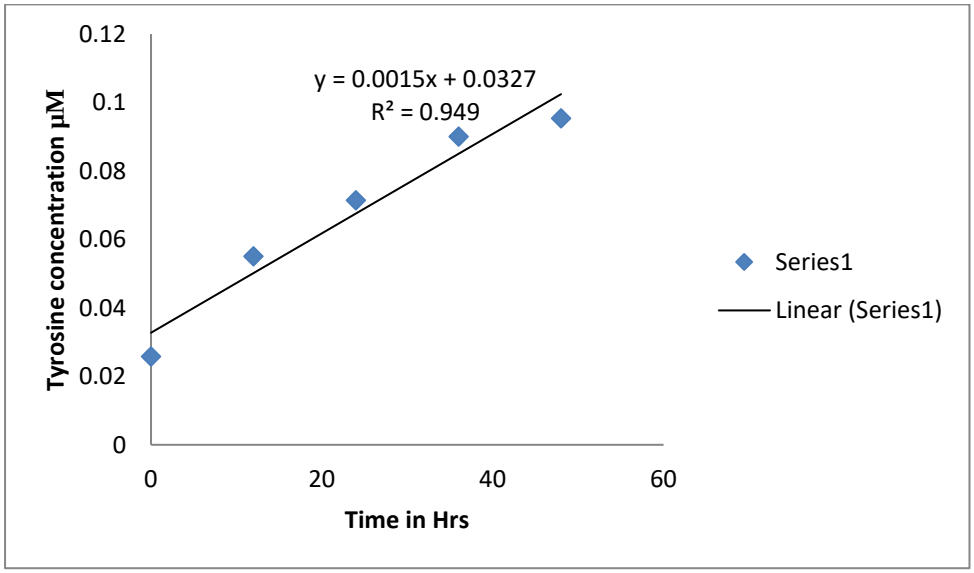


Figure 6: Order of reaction for enzymatic feathers hydrolysis

The graph showed an increase in the amount of tyrosine released, the product, increased as time progressed. The graph demonstrates the rate of reaction is independent of reaction concentrations concluding it's a zero-order reaction with a slope of K 0.0015 obtained from the graph and R^2 =0.0949.

4.3 A plot of Michaelis Menten constant (KM)

The simplest model where rate of reaction is a function of substrate concentration is known as the Michaelis Menten equation. The equation is applicable where increase in rate of reaction

increases with substrate concentration. However, increase in rate of reaction reaches a maximum velocity Vmax where addition of more substrate does not increase rate of reaction because the enzyme is already saturated. For the determination of the K_M value for this study, the substrate concentrations were obtained from the standard curve and the curve generated in the figure 7 of rate vs substrate concentration.

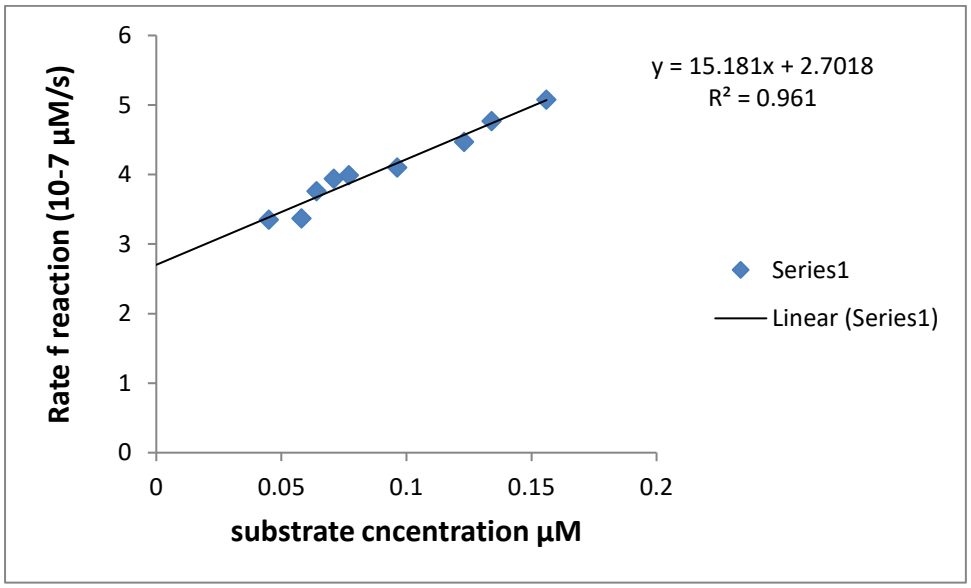


Figure 7: Michaelis-Menten plot of enzymatic hydrolysis of chicken feathers

KM -Michaelis-Menten constant, for simple systems is a measure of the attraction of the enzyme for its substrate, so it's also called the affinity constant. The graph in figure 7 observed increased in rate of reaction to increase in rate of reaction until a maximum velocity was reached where the enzyme was saturated at 0.16 µM concentration.To obtain the value of the K_M a linear plot where the inverse of substrate concentration and inverse of rate of reaction are plotted to obtain values of the Km. The plot is known as the lineweaver burke $1/vo = \frac{1}{Vmax} + \frac{Km}{Vmax} \times \frac{1}{(S)}$. The y intercept $\frac{1}{Vmax}$ and the slope is $\frac{Km}{Vmax}$. The plot is $\frac{1}{Vo}$ against $\frac{1}{S}$ the values for K_M and V_{max} are obtained from the graph presented in figure 8.

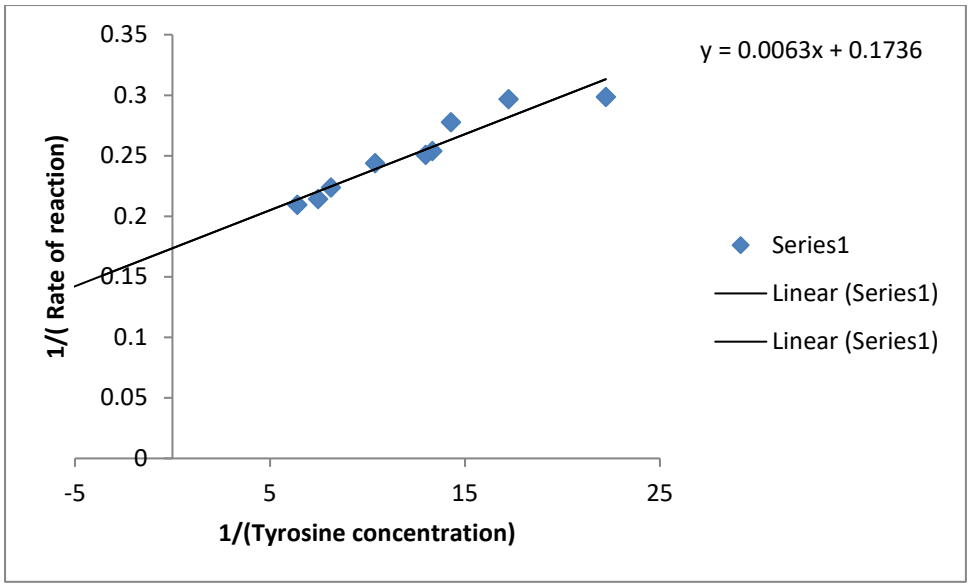


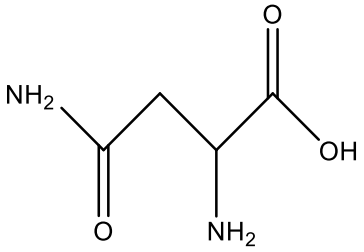
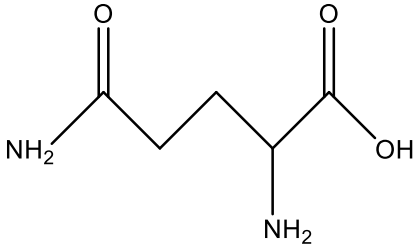
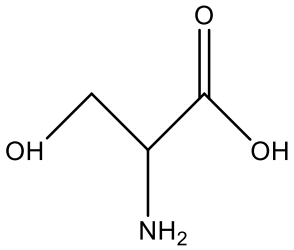
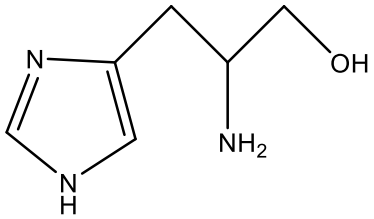
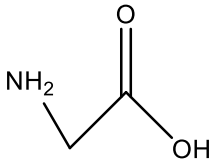
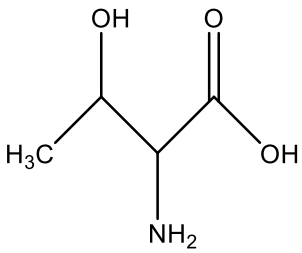
Figure 8: Lineweaver burke plot of enzymatic hydrolysis of chicken feathers

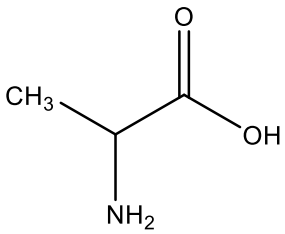
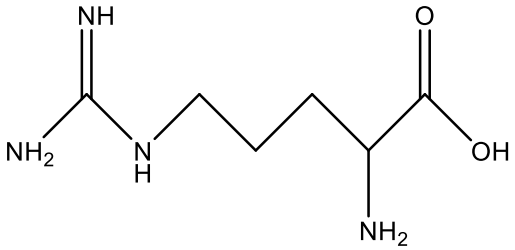
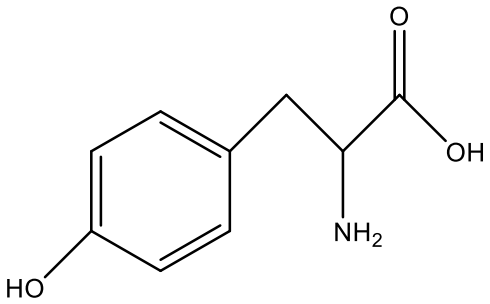
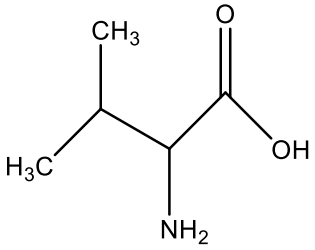
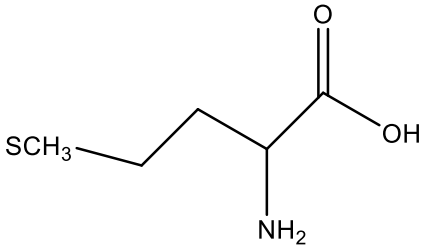
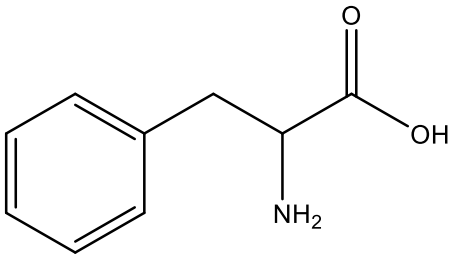
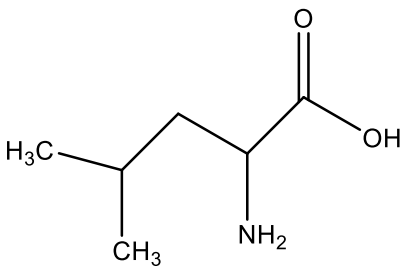
The y intercept 0.1736 in the line weaver burke plot is equal to ½ V_{max}. Therefore, V_{max} is equivalent to 5.76×10^{-7}µM.The slope 0.0063 is equivalent to $\frac{Km}{Vmax}$ since the value of V_{max} is known. The Michaelis- Menten constant (K_M) for this study was calculated and found to be 0.0362×10^{-7}µM.

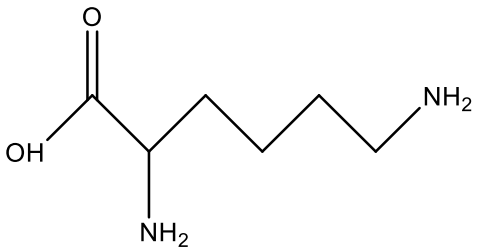
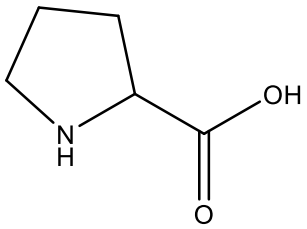
4.4 Amino acid composition of feather hydrolysate

Amino acid analysis of the hydrolysed feathers seeks to relate the quality of products with the amino acids obtained. The most significant aspect of a potential protein animal feed, from a nutritional perspective, is the presence of essential amino acids (EAA), because they have carbon skeletons that cannot be synthesised by animals, therefore they must be supplemented through the diet. Essential amino acids are significant in the growth of the animal as well or maintenance of metabolic processes. For the hydrolysed feathers to serve as a protein source, great consideration is given to the amino acids present in the products. The analysis was done in triplicate using a HPLC machine and standard amino acids were run and used in identification of unknown amino acids present in the feather hydrolysate. The results of amino composition for this study are displayed in Table 2, 3 and figure 9, 11 and 12.

Table 2 : Retention time of amino acids standards

Amino Acids standards	Retention time in minutes	Structure of Amino acid
Aspartic acid	1.322	
Asparagine		
Glutamic acid	1.507	
Seronine	3.846	
Histidine	4.315	
Glycine	4.397	
Threonine	4.625	

Alanine	5.954	
Arginine	6.602	
Tyrosine	7.625	
Valine	9.391	
Methionine	9.663	
Phenylalanine	11.095	
Isoleucine	11.290	
Leucine	12.007	

Lysine	12.732	
Proline	16.931	

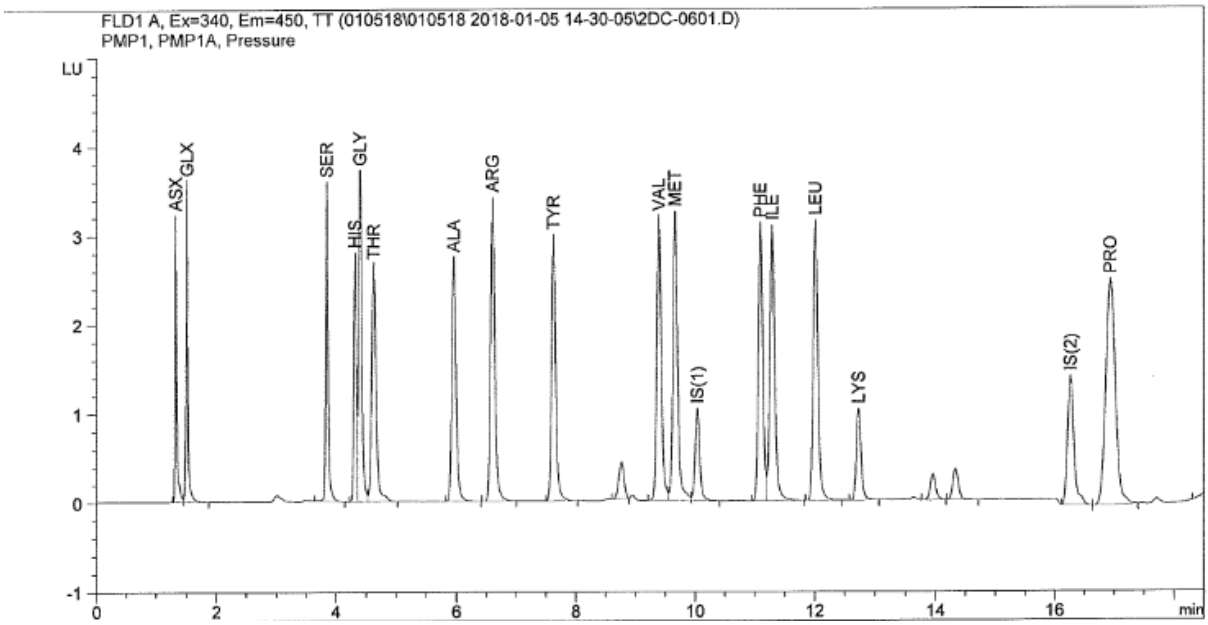


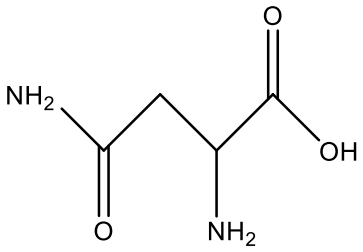
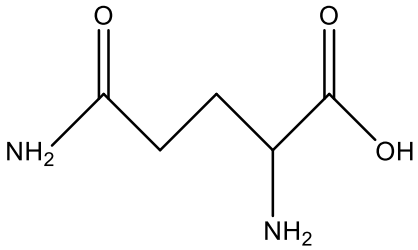
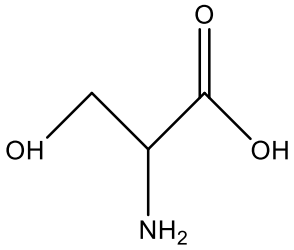
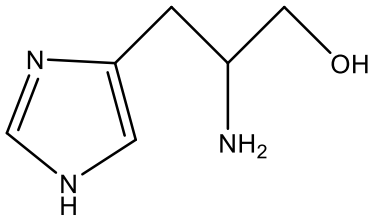
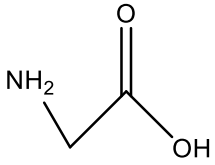
Figure 9: Amino acids standards spectrum

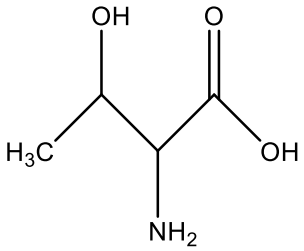
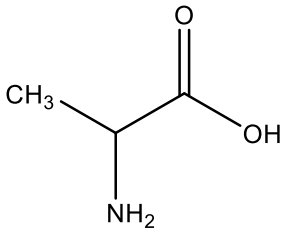
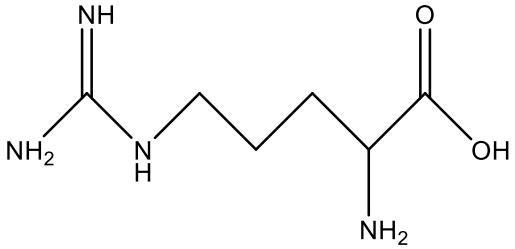
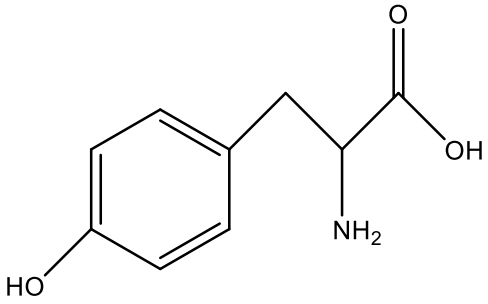
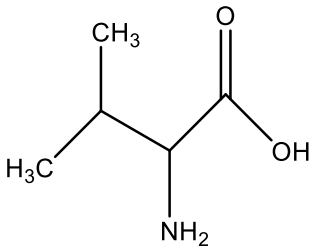
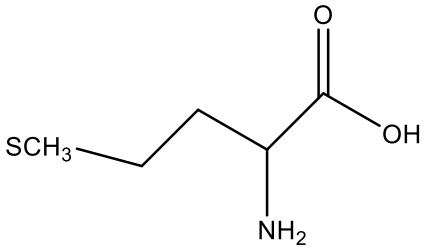
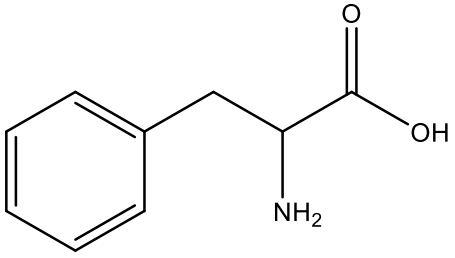
The standards were run at retention time of 16 minutes as seen in figure 9. At a retention time of 4- and 6-minutes presence of serine and alanine are observed in the chromatogram in figure 9 respectively. Standard amino acids were run against the samples and they were to correlate the unknown amino acids that were present in the feather hydrolysate.

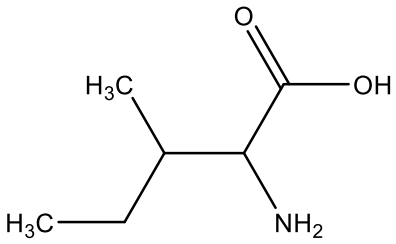
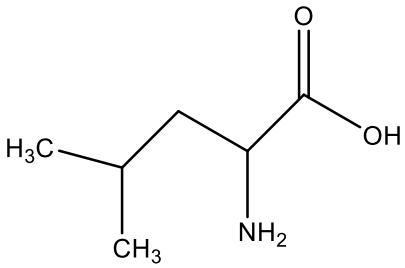
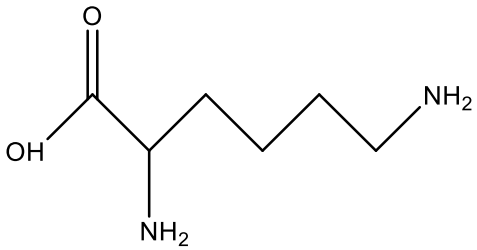
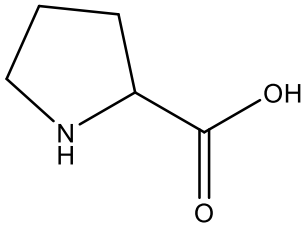
4.5 Comparative analysis of amino acid composition

Comparative analysis of the average amino acid composition for white feathers and black chicken feathers is presented in table 3. The feather hydrolysate amino acid results are validated by the chromatograms in figure 10 and 11. The height of the peak corresponds to quantity of amino acid.

Table 3: Amino acid composition of black and white feathers

Amino acids	Black feathers mg/g	White feathers mg/g	Structure of Amino acids
Aspartic acid	45.12	40.69	
Glutamic acid	58.13	51.68	
Seronine	52.79	48.98	
Histidine	1.52	2.26	
Glycine	127.12	106.84	

Threonine	10.98	15.28	
Alanine	63.33	55.11	
Arginine	20.04	27.13	
Tyrosine	6.56	8.88	
Valine	53.96	45.69	
Methionine	2.20	2.50	
Phenyl Alanine	25.73	22.61	

Isoleucine	32.47	28.44	
Leucine	55.27	47.57	
Lysine	4.84	7.78	
Proline	51.81	69.00	

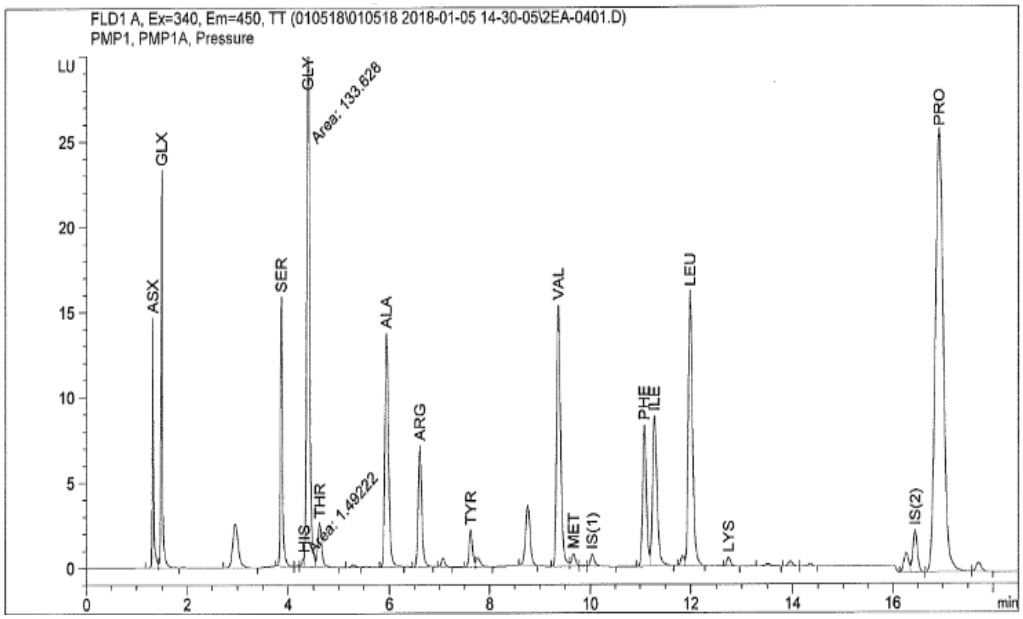


Figure 10: HPLC Spectrum of amino acid composition of black feathers

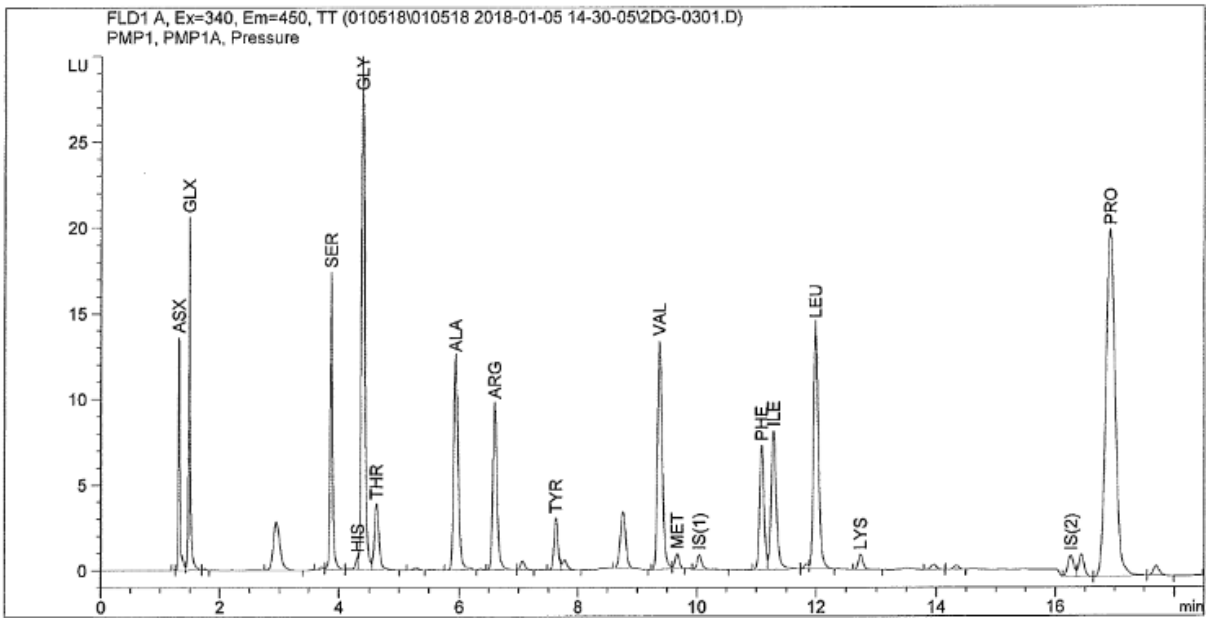


Figure 11: HPLC spectrum of amino acid composition for white feathers

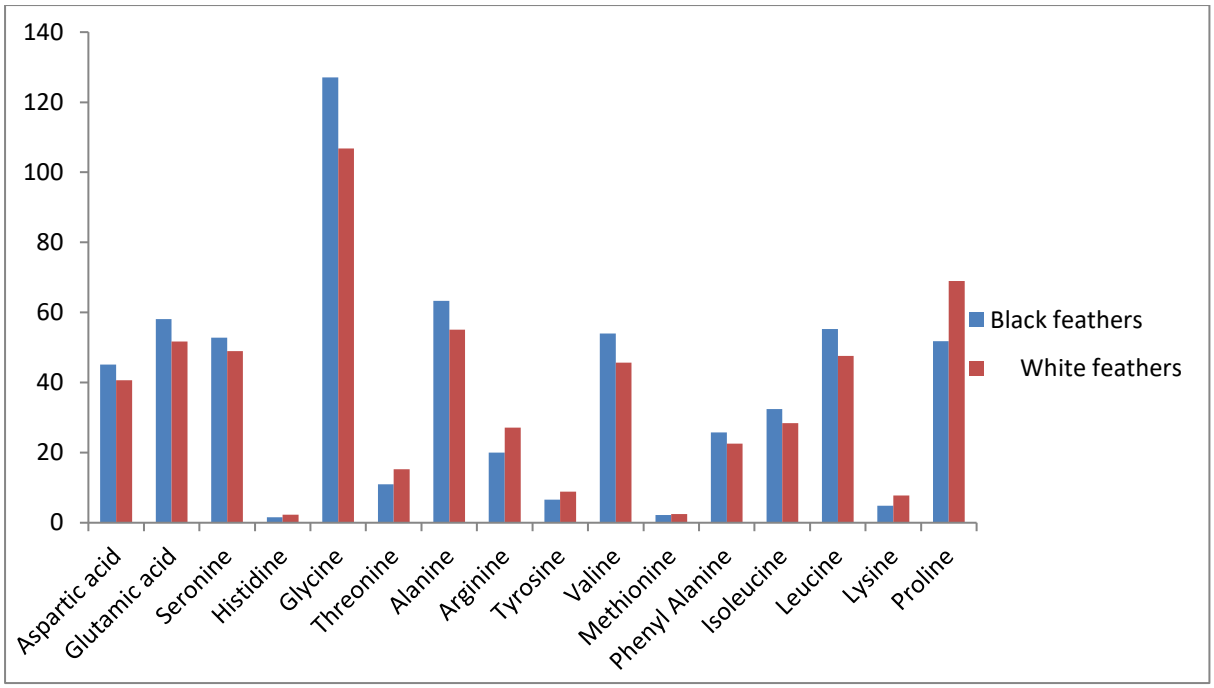


Figure 12: Comparative analysis of amino acids of black and white feathers

The results show the presence of the essential amino acids because feather contains 90% protein (Tesfaye *et al.*, 2017). Asparagine and glutamine are converted into aspartic acid and glutamic acid. In this study the highest amino acid present in black feathers as observed were found to be aspartic acid 45.12mg/g, glutamic acid 58.13mg/g, glycine 127.12mg/g, alanine 63.3mg/g, valine 53.96mg/g, phenylalanine 25.73mg/g, isoleucine 32.47mg/g, leucine 55.27 mg/g and seronine 52.79mg/g. Amino acids that were found to be highest in white feathers were observed to be proline 69.00mg/g, lysine 7.78mg/g, methionine 2.50mg/g, tyrosine 8.8mg/g and histidine 2.26 mg/g. Between the two the black feathers is much richer in amino acid composition. Glycine which is found to be highest in both feathers increases fat absorption in animals, immunity in fish, skeletal muscle growth in chicken broilers and weight gain in weaning pigs (Abdel-Fattah *et al.*, 2018). Methionine and lysine found to be present in this feathery meal promote egg production in laying hens as well as reducing abdominal fat in chicken broilers. Methionine plays very important role in metabolic pathways as it is used in protein synthesis in animals. Furthermore it provides a sulphur source for creation of other sulphur comprising biochemical and a methyl donor for methylation reactions (Jia *et al.*, 2016).

Deficiency of methionine in fish diets causes bilateral cataracts. Serine in animal growth is used in protein synthesis as well as synthesis of D-serine a neurotransmitter in the brain .Proline and lysine are essential for optimal growth and health of animals as well as increased food intake and weight gain (Jia *et al.*, 2016).

These findings are different to other studies on amino acid composition of chicken feathers by Tiwary (2012) where the highest concentration was serine and histidine with 72.8 mg/g and 65.9 mg/g respectively while the least being lysine and leucine with concentrations of 14.85mg/g and 17.15 mg/g respectively. The differences in results can be related to the methods used in feather hydrolysis that can denature some amino acids.

Further comparison of amino acid composition was done with soya bean meal ,according to (Mateos-Aparicio *et al.*, 2008). Amino acid analysis of soya bean meal had the following results: leucine 2.3%, lysine 1.77% while the two least amino acids is cysteine 0.54% and methionine 0.48%.In comparison to black feather glycine and arginine are highest with 58.13% and 45.12% molar composition respectively, the lowest amino acid being methionine 2.25% and histidine 1.52% molar composition .The lowest is methionine in both soya bean meal and feathery meal.

The low values of cysteine and methionine in soya bean meal is because the structure of soya bean meal lacks the disulphide bonds present in feathery meal. The low values in leucine and lysine is because in comparison of the crude protein of soya bean and feathery meal, the soya meal contains 47.8% and feathery meal is 75% (Cervantes-Pahm & Stein, 2008).Because of this the most abundant amino acids present in soya bean meal appear to be lower in amounts compared to feathery meal. Other results that correspond to these results are the degradation of feathers using *Bacillus pumilus* where the feathers degraded contained essential amino acids like methionine, tyrosine, valine, lysine .Histidine, threonine, isoleucine Călin *et al.* (2017) and this demonstrated that feathers contains the essential amino acids to be used as a valuable

source of protein. Both types of feathers show the presence essential amino acids, thereby indicating the product is of high nutritional value and can serve as an animal protein source in feed formulation

4.6 GC-MS analysis results

GC-MS has been widely used for identification of metabolites and can provide efficient and reproducible results (Zhang *et al*., 2012). GC-MS analysis of hexane extract of black feather hydrolysate dissolved in hexane was subjected to GC-MS analysis using Agilent 6890 series equipped with chemstation software and the results displayed in figure 13 and 14.

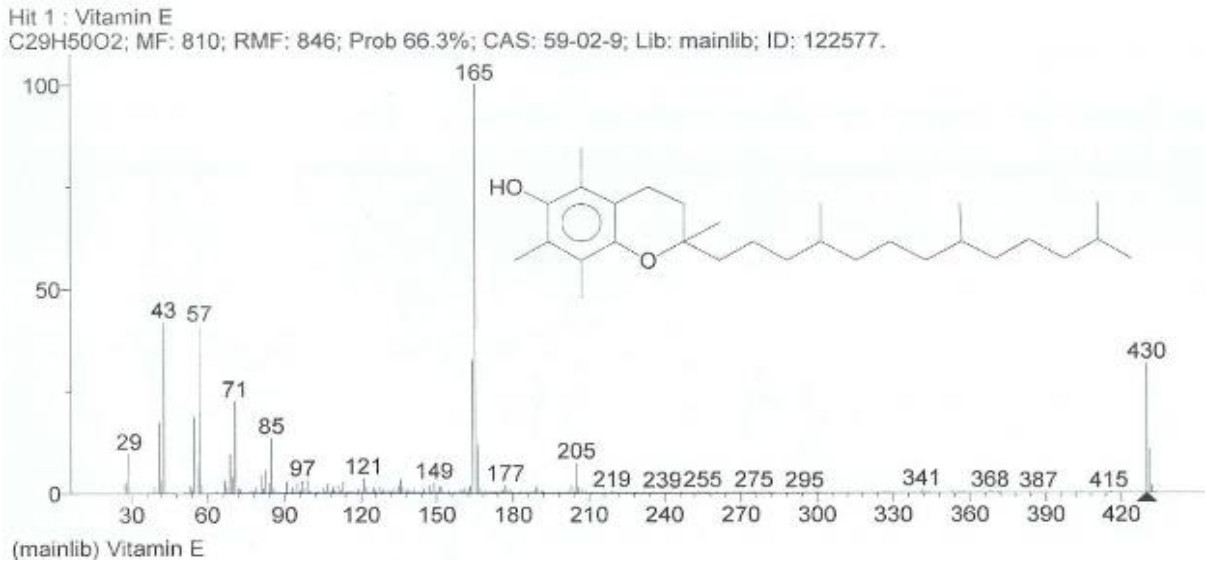


Figure 13: GC-MS spectrum of Vitamin E

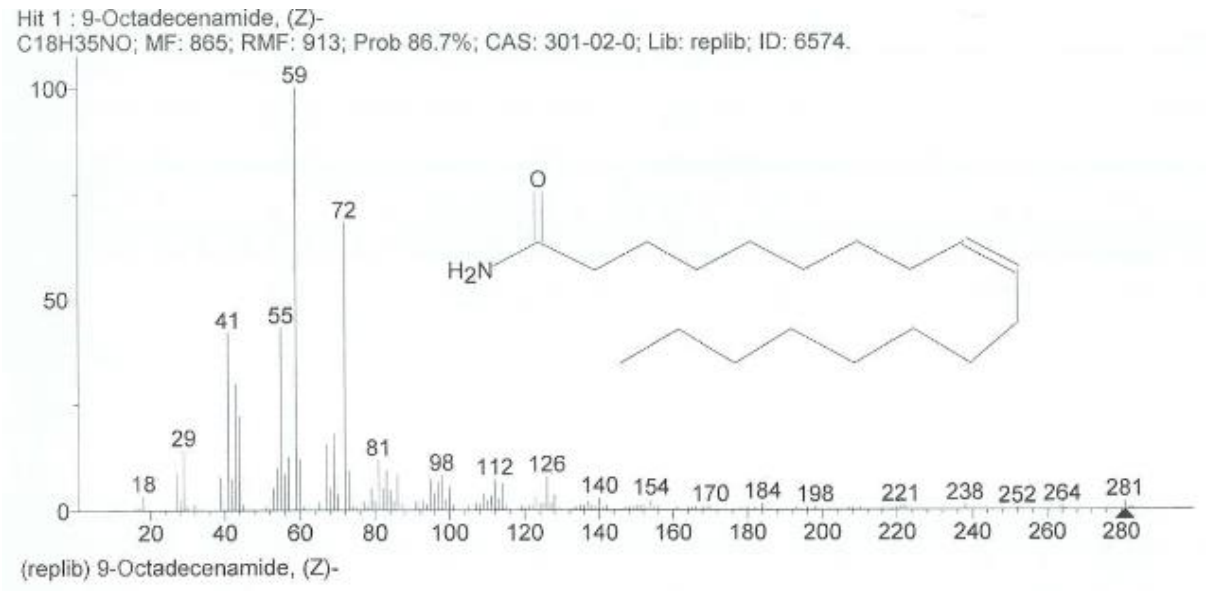


Figure 14: GC-MS spectrum of 9-octadecenamide, (Z)

The GC-Ms results showed presence of *(*2R)-2,5,7,8-Tetramethyl-2-[(4R,8R)-4,8,12 -trimethyltridecycl]-3,4-dihydro-2H-chromen-6-ol also known as vitamin E (MW 430, $C_{29}H_{50}O_2$) at retention time of 25.610 minutes and Octadecenamide (MW 281,$C_{18}H_{35}NO_{2)}$ at a retention time of 18.166 minutes in figure 13. Presence of Vitamin E in hydrolysate may be attributed to the dietary supplements of Vitamin E given to chicken against mortality caused by E coli infection (Tengerdy & Nockels, 1975).

The GC-MS results in figure 14 shows presence of Octadecenamide which is an Oleamide which is an amide of the fatty acid oleic acid. It occurs naturally in the body of animals. It accumulates in the cerebrospinal fluid during sleep deprivation and induces sleep in animals. It is being studied as a possible medical treatment for mood and sleep disorders, and cannabinoid-regulated depression (Cravatt *et al.*, 1996). The results in this study are in comparison by study done on GC-MS analysis of Keratin in feathers that found presence of Octadecenamide in mature feathers (Saitta *et al.*, 2017).

The presence of vitamin E in feather hydrolysate presents an advantage to the feather meal since vitamin E is vital for preventing free radical damage throughout the body, including the brain and nervous system (Lacey *et al.*, 2018). In addition, Octadecenamide can be extracted

and find additional applications in human because it can be used in promoting calm relaxed state and improve sleeping patterns. These two compounds that were found present in hydrolysed feathers can find their application in the various areas mentioned above thereby adding value addition in the product chain.

4.7 Fourier transmittance infra-red (FTIR) analysis results

FTIR is a spectroscopic technique that relies on the principle that each molecule absorbs light in the infra-red region of the electromagnetic spectrum and the absorption matches the bonds present in the molecule. FTIR analysis was done to identify the functional groups present in the feather hydrolysate. The samples were freeze dried and loaded on the FTIR machine and analysis was done on the both black and white feathers. The frequency ranges were measured as wave numbers typically over the range 600-4000cm^{-1}. The results of the FTIR analysis in this study are presented figure 15 and 16.

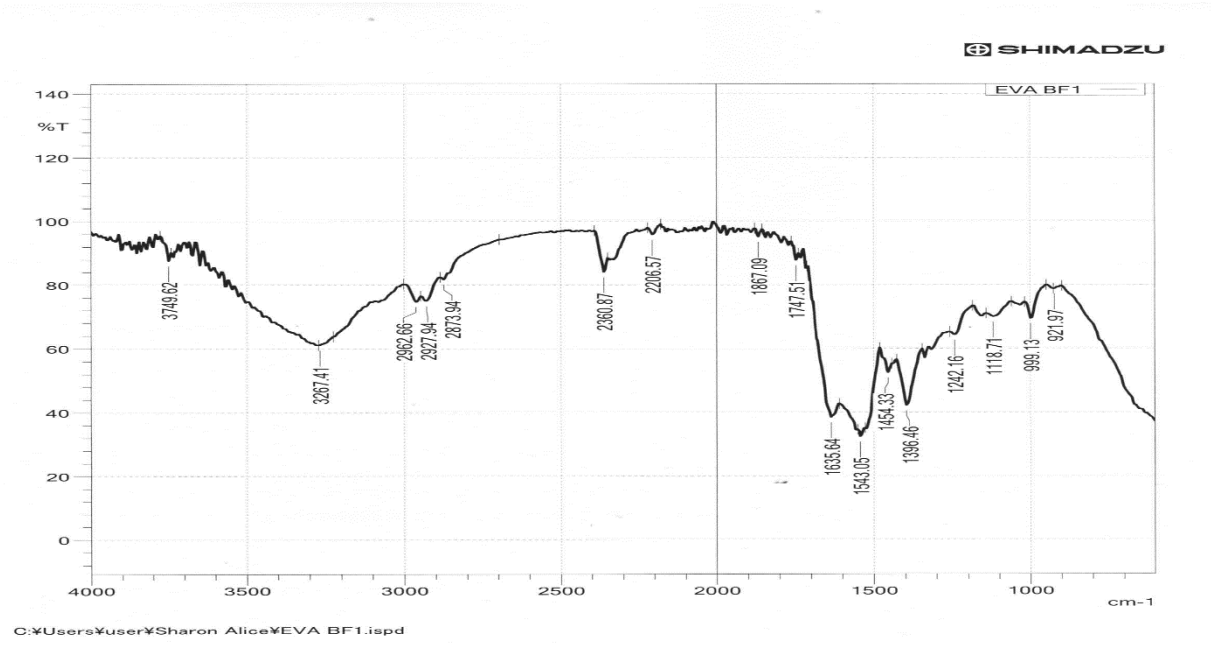


Figure 15: FTIR spectrum of black feathers.

The fundamentals of a polypeptide chain in keratin are known as amide I, II, III. The spectra in figure 15 shows a broad-spectrum peak at 3749.62 cm-^{1} that are attributed to a stretching

free alcohol. The peak at 3267.41 cm^{-1} is accredited to an Amide A. Amide I band is accredited to the stretching of C=O and stretching of C-N which from the spectrum occurs at 1635.64 cm-[1] (Tesfaye *et al.*, 2017).Amide II band is attributed to N-H bending and C-N stretching which can be seen to occur at 1543.33 cm-[1.] . Amide III band is attributed to the peak that occurs at 1242.16 cm 1. The peak at 1867 cm-[1] is attribute to a C=O (carboxylic group), the peak at 2873 cm^{-1} is attributed to C-H stretching alkane. The results present that it was an amino acid present in black feathers due to break down of the protein structure and the disulphide linkages present in feathers .The results in this study correspond to findings by Călin *et al.* (2017); (Saravanan *et al.*, 2013) which indicated the enzyme was able to hydrolyze the keratin structure to constituent amino acids.

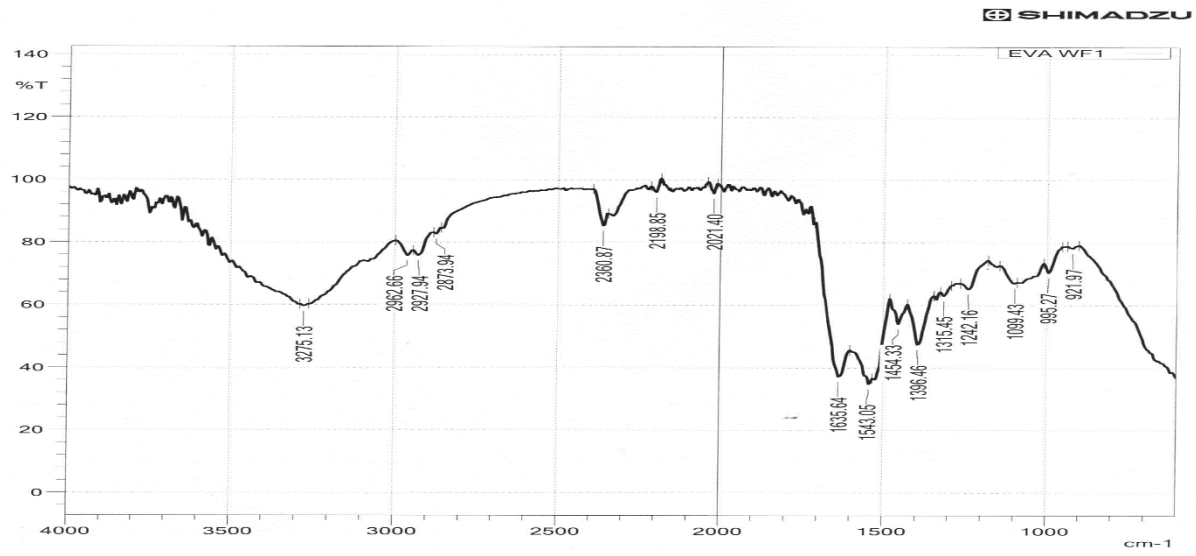


Figure 16: FTIR spectrum of white feathers

The FTIR spectrum of white feathers is shown in figure 16. The fundamentals of a polypeptide chain in keratin are known as amide I, II, III. The characteristic peak at 1635.61 cm^{-1} is related to a C-O which is characteristic of amide I. Figure 16 shows a broad band absorption peak around 1242.16 cm^{-1} that shows the presence of C-N stretch of and C-O bending combination of both are characteristic of an Amide III . Amide is characterized by the C-N stretch at 1542cm-[1.]Carbon dioxide O=C=O stretch at the peak 2360.87 cm^{-1}.There is presence of a carboxylic acid in the O-H stretch at the peak around 1393.46 cm^{-1} .The presence of these

functional groups shows that the structure is an amino acid is present and the functional groups detected show this. This results for white feathers are in comparison to an analysis by Călin *et al.* (2017) and Gupta *et al.* (2011) which indicated the enzyme was able to hydrolyze the keratin structure to constituent amino acids.

4.8 Proximate analysis

Proximate analysis was done to determine the main constituents of feed and it is used to asses if a feed is within its standard compositional parameters. The present study aimed at analysing black and white feather hydrolysate for proximate composition to look for alternative sources of animal feed protein. Total crude protein, lipid, fibre, moisture and carbohydrate content were determined by the methods described by AOAC (1990). The results of this study are presented in Table 4 and figure 17.

Table 4: Proximate analysis of white and black feathers

Parameters	Results(%mm) white feathers	Results (%mm) black feathers
Moisture content	6.6	9.83
Protein content	73.3	71.12
Fat content	0.3	2.51
Fibre content	0.315	0.44
Total ash content	6.76	8.73
Carbohydrate content	19.8	0.4

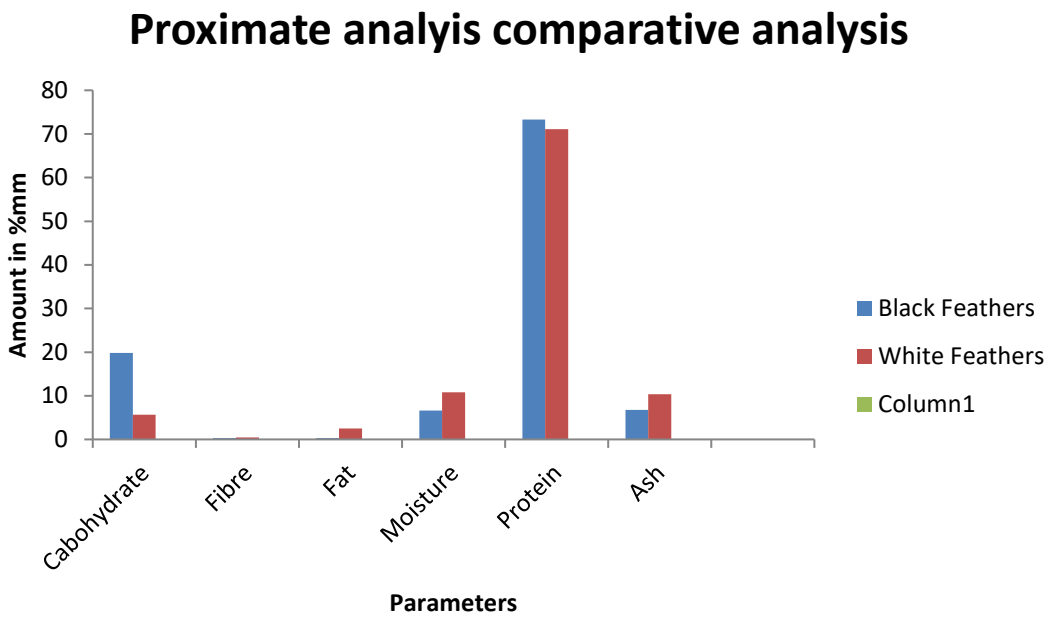


Figure 17: Proximate comparative analysis of black and white feathers

Crude protein is obtained by determining nitrogen content of a feed and multiplying by a factor of 6.25. The factor is a result that proteins comprise of 16% nitrogen. Total crude protein analysis is determined by the Kjedahl method AOAC (1990). Percentage of crude protein in feathery meal plays a key part in promoting growth in animals. In the present study the analysed crude protein content was found to be lowest in white feathers with a percentage of 71.12% while black feathers had the highest amount of crude protein with a percentage of 73.3%. The difference is insignificant and can be attributed to the reagents used as well as human error. The results of this study differ with study by Tesfaye *et al.* (2017) small margins to a research done on chicken feathers where crude protein was found to be slightly higher with a percentage of 82.36% The result of this study thereby confirms that these feathers can be good sources of animal protein and recommended as an alternative source of protein for feed fortification.

Ash content is the percentage of mineral matter which is essential for many processes in the body. Phosphorus and calcium are the main components found in ash content. These minerals are used in building of the animal skeleton. Ash is an impurity that will not burn. Presence of ash in a sample affects the combustion efficiency of the furnaces which result in clinkering.

Higher amounts of ash content also reduce burning and handling capacity. If a sample has low ash content, then it can therefore be considered as a source of fuel. Ash content is the residue left after sample is subjected to combustion in an oven at temperatures of 600 ˚C for about 4 hours. In this study ash content as summarised in table 4 was found to be lowest in white feathers with 6.76% and highest in black feathers with amounts of 8.73%. The difference in amount of ash in white and black feathers is a result of the different procedure used in determining ash content as well as the reagents used. The results of this present study differ with the analysis done by Tesfaye *et al.* (2017) on raw feathers that showed the ash content was 1.46%.This could be attributed to the subject material being raw material while the present study carried out proximate analysis on hydrolysed feathers,

The ability of hydrolysate to absorb moisture from atmosphere affects various parameters of transportation, storage, processing. If the moisture content is high, then it will have a shorter shelf life as compared to a hydrolysate that has low moisture content. Moisture was determined by the loss in weight that took place when the feather hydrolysate was dried to a constant weight in an oven. The results of this study as presented data in table 4 shows higher moisture content in black feathers with amounts 9.83% and lowest amounts of moisture content in white feathers with amounts of 6.6%. The findings of this present study differ with a study by Tesfaye *et al.* (2017) where raw feather were subjected to proximate analysis and determination of moisture content was found to be 12.33%. Required amounts of moisture content in a potential feed are between 8-13%. This therefore means that the feather hydrolysate can be safely stored for long periods of time with no contents deteriorating due to microbial growth caused by excess moisture.

Carbohydrate in animals is used to provide energy as well as producing body heat to keep the animal warm. Carbohydrates comprise of 75% of the animal diet. Animals require higher amounts of carbohydrates in their feeds and this has to supplemented in their diet. In this study

carbohydrate content is higher in white feathers with 19.35% while in black feathers has amounts of 0.4%. The proximate analysis of fat content in black and white feathers varied slightly. The lowest value of fat content was observed in white feathers with amounts of 0.3% and the highest was observed black feathers with amounts of 2.51%. (Table 4, figure 17) presents the findings of this study. The results of this present study slightly differed to research that was done on soya bean by Cervantes-Pahm and Stein (2008). Proximate analysis of soya bean meal which is also used as a protein source in animal feeds presented crude fat content to be 1.5%. Fibre content tell its ability to be used as a fuel, Comparative analysis as presented in Table 4 and graph shows slight variations in fibre content for black and white feathers .Highest amount of fibre content were observed in black feathers with amounts of 0.44% and lowest amounts in white feathers with of 0.315 % amounts. The slight variations could be attributed to human error, laboratory procedure and reagents used.

Soya bean meal has been used as a substitute for animal protein over the year's .According to research that was done on soya bean by Cervantes-Pahm and Stein (2008) proximate of feathery meal was compared to the proximate analysis of soya bean meal which is also used as a protein source in animal feeds. The results showed protein content of (47.8%), moisture 12%, ash 6.4%, and crude fat content 1.5%. Comparing the crude protein of soya bean and feathery meal 47.8% and 73.3 % respectively shows between the two the feathery meal has high content of crude protein. This only means that it has added nutritional value than soya bean and can be used as an alternative source of protein in animal feed.

CHAPTER FIVE

5.1 CONCLUSION

The research findings show that ***bacillus cereus*** can be used in hydrolysis of chicken feathers. The bacteria stain was isolated from Lake Bogoria in Kenya which is a saltwater lake characterized by high pH and presence of hot springs. It is shown that the product obtained from hydrolysis of feather waste can be utilized as a cheap substitute for protein sources in formulation of animal feeds. Proximate Analysis has shown that the feather hydrolysate contains 73.3% of crude protein fibre content 0.315%, moisture content 6.6%, carbohydrate content 19.8% and ash content 6.76%.

The Amino acid analysis that was performed on the feathers indicates that 18 out of the 20 essential amino acids are present in the product obtained. The most abundant amino acid was glycine with 20.7% and 17.8% molar composition in black and white feathers respectively. The least abundant amino acid was histidine with 0.2% molar composition in black feathers and 0.4% molar composition in white feathers. The most important Amino acids used in the growth and body function of animal were also indicated and in high values as compared to soya bean meal. The FTIR characterization showed clear peaks at 3267.41 cm^{-1}, 1543.33 cm^{-1}, 1242.16 cm^{-1}, 1665.64 cm^{-1} that were attributed to protein functional groups in Amide A, Amide I, Amide II, Amide III respectively. The FTIR analysis was carried out to investigate functional groups present in feather hydrolysate; the results confirm presence of protein functional groups in the feather hydrolysate.

Optimisation of conditions was carried out to determine which conditions the enzyme works best. Besides, the thermophillic bacteria used is derived from Lake Bogoria which is a saline lake and it was expected that high temperature and pH conditions may be essential for optimum activity. The keratinase has optimum pH at pH 13 and Optimum temperature at 80˚C and

undiluted crude enzyme concentration was used. The spectroscopic methods by GC-MS showed presence of vitamin E and a fatty acid derivative of oleic acid known as Octadecenamide. Presence of vitamin E in the feathery meal can assist the animal growth in skin structure and development of the nervous system. The presence of the fatty acid can find its application in the medical fields in human in the aid to fight Anxiety, Stress and sleeping disorders.

5.2 RECOMMENDATION

1) Further Research should be carried on feathers from other sources of poultry other than chicken.

2) Different Strains of Enzymes can be investigated on the ability to degrade the feather structure other than using Bacillus Strain.

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APPENDICES

Appendix 1: Effect of pH on hydrolysis of feathers

PH/TIME	3 (µM)	4 (µM)	5(µM)	6(µM)	7(µM)	8(µM)	9(µM)	10(µM)	11(µM)
0HRS	50.691	28.128	43.912	47.150	36.121	45.430	48.263	43.103	48.668
12HRS	35.514	33.491	31.771	32.479	38.044	39.056	44.115	33.592	50.084
18HRS	48.162	50.590	48.971	50.894	49.073	49.680	46.644	50.489	63.238
24HRS	54.840	69.005	71.737	65.363	66.273	79.528	68.297	66.678	79.528
36HRS	45.430	51.501	43.406	50.388	51.501	59.393	53.626	52.614	61.214
48HRS	45.936	48.668	47.656	55.750	48.870	54.435	50.489	53.626	48.162
60HRS	56.965	17.302	58.078	56.054	51.400	15.784	57.369	60.101	65.565
72HRS	63.845	56.155	63.845	60.000	57.268	66.273	51.197	60.607	68.297
84HRS	97.234	69.207	86.307	84.587	84.182	86.610	103.609	79.629	87.521
96HRS	127.184	105.835	135.278	119.696	146.509	136.290	115.953	104.216	128.803
108HRS	57.976	78.921	60.708	68.398	73.963	101.990	113.019	123.744	140.843
120HRS	80.337	89.039	82.159	90.455	86.003	100.371	87.015	100.16	108.870

Appendix 2: Effect of enzyme concentration on hydrolysis of feathers

100%	**0hrs**	**12hrs**	**24hrs**	**36hrs**	**48hrs**
Trial 1	0.746	1.506	1.91	2.303	2.574
Trial 2	0.645	1.442	1.941	2.495	2.577
Trial 3	0.695	1.51	1.928	2.486	2.563
Average	0.695	1.486	1.926	2.428	2.571
Tyrosine concentration(µl)	0.0258	0.0551	0.0715	0.0901	0.0954
50%					
Trial 1	0.505	0.646	0.909	1.228	1.452
Trial 2	0.561	0.618	0.947	1.266	1.401
Trial 3	0.496	0.605	0.946	1.246	1.499
Average	0.520667	0.623	0.934	1.246667	1.450667
Tyrosine concentration(µl)	0.0193	0.0231	0.0347	0.0463	0.0538
25%					
Trial 1	0.239	0.398	0.505	0.719	0.994
Trial 2	0.215	0.3118	0.502	0.723	0.986
Trial 3	0.24	0.3114	0.511	0.783	0.974
Average	0.231333	0.3404	0.506	0.741667	0.984667
Tyrosine concentration(µl)	0.0086	0.0126	0.0188	0.0275	0.0365
12.50%					
Trial 1	0.145	0.225	0.255	0.522	0.909
Trial 2	0.148	0.239	0.314	0.569	0.803
Trial 3	0.158	0.182	0.389	0.528	0.858

Average	0.150333	0.215333	0.319333	0.539667	0.856667
Tyrosine Concentration(µl)	0.0058	0.0083	0.0123	0.0208	0.0330

Appendix 3: Effect of temperature on hydrolysis of feathers

Temperature	Absorbance 24 hours	Tyrosine Concentration(µM)
80˚C	1.437	0.053
30 ˚C	0.987	0.037
Room temperature(22.7˚C)	0.729	0.027
Temperature	Absorbance 48 hours	Tyrosine Concentration(µM)
80 ˚C	1.156	0.043
30 ˚C	0.770	0.029
Room temperature(22.7˚C)	0.813	0.030
Temperature	Absorbance 72 hours	Tyrosine Concentration(µM)
80 ˚C	1.819	0.067
30 ˚C	0.886	0.033
Room temperature(22.7˚C)	0.824	0.031

Appendix 4: Amino acid composition of white feathers weight in (µMoles)

Amino acids	First trial	Second trial	Third trial	Average in µ moles
Aspartic acid	36.36±3.06	45.03±3.07	40.70±0.01	40.69
Glutamic acid	45.68±4.24	57.67±4.24	52.85±0.83	51.68
Seronine	38.92±7.12	59.04±7.11	64.37±10.88	48.98
Histidine	2.06±0.14	2.47±0.15	2.04±0.16	2.26
Glycine	93.30±9.57	120.38±9.57	101.78±3.58	106.84
Threonine	13.53±1.24	17.03±1.23	14.48±0.57	15.28
Alanine	48.47±4.70	61.75±4.70	53.90±0.86	55.11
Arginine	24.14±2.11	30.13±2.12	27.97±0.59	27.13
Tyrosine	7.87±0.71	9.89±0.71	9.25±0.26	8.88
Valine	40.02±4.01	51.37±4.01	47.43±1.23	45.69
Methionine	2.24±0.18	2.76±0.18	2.51±0.01	2.50
Phenylalanine	20.08±1.79	25.13±1.79	23.00±0.27	22.61
Isoleucine	25.01±2.42	31.86±2.42	29.52±0.77	28.44
Leucine	42.17±3.82	52.96±3.81	48.37±0.57	47.57
Lysine	6.97±0.58	8.59±0.57	7.55±0.14	7.78
Proline	70.84±1.30	67.16±1.30	81.33±8.72	69.00

Appendix 5: Amino acid composition of black feathers weight in (µMoles)

Amino acids	First trial	Second trial	Third trial	Average in µmoles
Aspartic acid	44.28±0.59	50.14±3.56	40.94±2.96	40.69
Glutamic acid	57.16±0.69	65.27±5.05	51.96±4.36	51.68
Seronine	39.35±9.50	61.77±6.35	57.25±3.15	48.98
Histidine	1.63±0.07	1.44±0.06	1.49±0.02	2.26
Glycine	118.08±6.34	147.95±14.73	115.32±8.34	106.84
Threonine	10.47±0.36	12.26±0.91	10.20±0.55	15.28
Alanine	59.72±2.55	72.28±6.32	57.98±3.78	55.11
Arginine	19.60±0.311	22.20±1.52	18.33±1.20	27.13
Tyrosine	6.41±0.11	7.28±0.51	5.98±0.40	8.88
Valine	52.78±0.84	58.33±3.09	50.76±2.25	45.69
Methionine	2.25±0.001	2.46±0.15	2.05±0.14	2.50
Phenyl alanine	25.56±0.12	27.75±1.42	23.87±1.31	22.61
Isoleucine	32.16±0.22	35.02±1.80	30.23±1.58	28.44
Leucine	53.80±1.04	60.84±3.94	51.15±2.91	47.57
Lysine	4.74±0.07	5.39±0.39	4.38±0.33	7.78
Proline	48.09±2.63	21.24±21.61	86.10±24.25	69.00

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