UNIVERSITY OF NAIROBI

ENZYMATIC HYDROLYSIS OF FISH WASTE, CHARACTERIZATION OF HYDROLYSATE AND PROXIMATE ANALYSIS

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 requirement

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DEDICATION

To my parents Judy and Cosmas Ochieng, Brother Adrian Ochanyo and companions through many a long night of research and writing.

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ABSTRACT

The fish industry generates a lot of waste which is attributable to the fact that there is an increased demand for fish and that filleting of tilapia fish yields about 37% skin-off fillets. Enzymatic hydrolysis used in this research project affords the prospect to apply rudimentary alkaline protease obtained from extremophile bacteria in processing fish waste. The optimum working conditions of the crude alkaline protease were established to be at 80ºC, pH12 and a crude enzyme concentration of 100%. The Amino acid composition was determined using HPLC while fatty acid analysis using the FAME method using GC equipped with the FID. Proximate analysis was determined in accordance with the standard test methods. The parameters evaluated included moisture, crude protein, ash, carbohydrates and fats. The kinetics of the reaction showed that it was zero order with respect to the concentration of the reactants. Glycine and alanine were determined to be the peak amino acids present in the Fish Protein Hydrolysate, while histidine was the least abundant. Fatty acid composition study disclosed that palmitic acid was most prominent and the least abundant was lauric acid. Proximate analysis of the freeze-dried hydrolysate yielded crude 6 protein, moisture, ash, crude fat, fibre and carbohydrates mean content of 54.2%,10.4 %, 21.8 %, 12.8 %, 0.6 % and 0.2 % respectively. The results demonstrate that the crude enzyme used in this study can be used for fish waste hydrolysis to obtain useful value-added products and can be used effectively in managing the fish waste disposal problem.

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LIST OF ABBREVIATIONS

AA	Amino Acid
FAME	Fatty Acid Methyl Ester
FID	Flame Ionization Detector
FPC	Fish Protein Concentrate
FPH	Fish Protein Hydrolysate
FT-IR	Fourier Transform Infrared
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
OPA	o-phthaldialdehyde
RP-HPLC	Reverse –phase High Performance Liquid Chromatography

CHAPTER ONE

INTRODUCTION

1.1 Background

The fishing industry is an enormous business that encompasses the activities ranging from catching of fish, their processing, culturing and marketing. The industry comprises three sectors, the traditional, commercial and recreational sectors ("Fisheries Department 2014 Stats Bulletin," 2014.). The traditional sector involves people or businesses of indigenous origin carrying out the fishing activities with regards to their traditions whereas recreation dictates that the products obtained are not for sale. Fish farming on the other hand involves the commercial rearing of fish in enclosed spaces. Sometimes this involves cordoning off a section of a larger water body to rear the fish so as not to remove them from their natural habitat.

World fish catches reported in 2014 in inland waters was approximately 11.9 million tones, reflecting a 37% increase in the past decade (FAO, 2016). Further, 16 nations, with yearly internal water catches more than 200 000 tones cumulatively represent 80% of the entire global production. According to (FAO, 2016) for FAO the World individual apparent fish intake has risen from about 9.9 kg to 14.4 kg over a period of thirty years from the 1960's and most recently to 19.7 kg in 2013, and 2014/2015 estimates suggest further growth beyond 20 kg. In addition, the estimated value of the overall sum of fishing vessels globally as at 2014 was approximately 4.6 million. The year 2014 also had fisheries exports from the developing countries exceed the mainstream agricultural exports as the gross exports accounted for US$80 billion and the net was at US$42, (FAO, 2016).

Table 1: Inland waters capture production Source: FAO (2016)

					VARIATION	
COUNTRY	**AVERAGE 2003-2012**	**2013**	**2014**	**AVERAGE (2003-2012) 2014**	**2013-2014**	**2013-2014**
		TONNES		**PERCENTAGE**		**TONNES**
China	2 215 351	2 307 162	2 295 157	3.6	-0 5	-12 005
Myanmar	772 522	1 302 970	1 381 030	78.8	6.0	78 060
India	968 411	1 226 361	1 300 000	34.2	6.0	73 639
Bangladesh	967 401	961 458	995 805	2.9	3.6	34 347
Cambodia	375 375	528 000	505 005	34.5	-4.4	-22 995
Uganda	390 331	419 249	461 196	18.2	10.0	41 947
Indonesia	324 509	413 187	420 190	29.5	1.7	7 003
Nigeria	254 264	339 499	354 466	39.4	4.4	14 967
Tanzania	301 631	315 007	278 993	-9.3	-11.5	-36 074
Egypt	257 006	250 196	236 992	-8.5	-5.3	-13 204
Brazil	243 170	238 553	235 527	-3.1	-1.3	-3 026
Russian Federation	228 563	262 050	224 854	-1.6	-14.2	-37 196
D.R.C	225 557	223 596	220 000	-2.5	-1.6	-3 596
Philipines	168 051	200 974	213 536	27.1	6.3	12 562
Thailand	212 937	210 293	209 800	-1.5	-0.2	-493
Viet Nam	198 677	196 800	208 100	4.7	5.7	11 300
TOTAL 16 MAJOR COUNTRIES	8 111 756	9 395 355	9 540 590	17.6	1.5	145 236
WORLD TOTAL	10 130 510	11 706 049	11 895 881	17.4	1.6	189 832
SHARE 16 MAJOR COUNTRIES (%)	80.1	80.3	80.2			

The commercial sector of the fishing industry is vital because it is associated with income generation and job creation. This is because, worldwide the fishing industry accounts for 16% of the protein consumed by the world's population (FAO Fisheries and Aquaculture Department, 2013). According to the Food and Agriculture Organization, world aquaculture accounted for 62.7 million tonnes of food fish which has an estimated value of $130 billion. The industry has however,

not explored its full potential in terms of revenue generation. This is because only30-35% of the fish (tilapia) is processed for consumption and the rest discarded as waste. Fish waste pose environmental challenge in terms of diseases and pollution. In addition to the menace of waste generation, the processing of only 35% of the food fish symbolizes a direct loss to the farmer or the producer of the food fish.

Kenya has both marine and inland fishing grounds. Fisheries in Kenya have for a long time fallen under the jurisdiction of the national government. It is until recently that the devolved system of government was put in charge of the fisheries, both marine and inland fisheries in Kenya. The Kenyan coastal strip is about 536 Km long and fishing industry is relatively not well developed, since it accounts for a meager 6% of the total fish produced in the country. Inland fishing grounds, especially the Lake Victoria still accounts for principal amount of the 80% of the total catch while the rest of the 14% accounted for by the fish farms (Fisheries Department 2014 Stats Bulletin, 2014).

The production of fish in Kenya has increased over the years prompting the growth of this industry from subsistence and local trade to an industry that accounts for 0.5% of the Kenyan Gross Domestic product from 0.3% in 2003 (Njiru et al., 2008). This also suggests that the fish processing waste has increased. Thus, an increase in the gross domestic product is neutralized by the amount of funds used in taking care of the waste menace.

The increased consumption of fish both locally and worldwide is ascribed to their high protein content. Fish is rich in protein and low in fat. They are also known to provide substantial amounts of vitamins and minerals as well (Routray et al., 2018). The health benefits of ω polyunsaturated fatty acids (PUFA) are innumerable but to put into perspective their usefulness, they have been

shown to improve eye health, aid brain development during pregnancy and early life and lessens the risk factors for heart diseases (Swanson et al., 2012). Health restrictions and religious factors do not affect its consumption as other sources of protein like beef, pork and other meat sources. The increased consumption of fish however, comes with challenges, especially fish production and the waste generation that comes from increased fish production. This results in the production of large deposits of waste which poses a challenge with regards to their management and/or disposal.

The biggest challenge faced by the fishing industry however is the waste that results from fish processing. Whereas the growth of the fisheries industry provides a much needed boost to the economy, the waste produced cannot be ignored. The increased demand locally for processed fish and an equally demanding emerging global market, inevitably results in the production of large amounts of waste from fish processing (Shirahigue et al., 2016). The waste is usually in the form of head, exoskeletons, scales, skin and guts (Rodriguez et al., 2017). Due to their nature, fish waste tend to persist in the environment as they take a relatively long time to degrade. In their freshly dumped state, they tend to possess a foul and pungent smell. In their heaps, they tend to attract rodents which transmit diseases like the Hantavirus Pulmonary syndrome from its bites that may lead to death from fluid in lungs complications (Kruger et al., 2015).

1.1.1 Effects of fish waste on environment

These wastes are usually released into the water bodies while some are dumped as compost and end up in landfills (Routray et al., 2018). Dumping on landfills takes up valuable land around urban areas which would have otherwise been used for more productive activities such as housing, agriculture, schools or recreation sides. The landfills also are subjected to leeching thereby contaminating the groundwater which is likely to be used by humans causing various waterborne

diseases like dysentery and cholera (Chopda & Malek, 2018). However, some of the wastes are disposed of on their industry dumping sites. Their effects on the environment are felt through pollution of air, land and water. When the wastes are disposed into the water bodies, they provide nutrients to aquatic organisms leading to their rapid increase. This in turn rapidly depletes the amount of dissolved oxygen in water resulting to anoxic waters (Crab et al., 2012) affecting the biodiversity of the benthic assemblages.

The Biochemical Oxygen Demand (BOD) is bound to increase as the wastes are organic which require dissolved oxygen to be decomposed by microorganisms. The Chemical Oxygen Demand(COD) signifies the amount of inorganic matter to be oxidized and the fish wastes are rich in protein that contain an amino group thereby increasing the demand for dissolved oxygen for their oxidation. The fish waste from fish processing is sometimes crashed into fine particles. This when dumped in water forms a suspension thus increasing the turbidity of the water. Fish is also known to contain minerals that are water soluble. Upon decomposition and oxidation of both the organic and inorganic waste, the water-soluble minerals are released, increasing the amount of solutes dissolved in the water body. This by extension affects the pH. Consequently, this kind of dumping causes algal blooms and production of toxins detrimental to the health of both humans and aquatic life. Humans are affected with diseases such as cholera, bilharzia and dysentery.

1.1.2 Available methods of fish waste management

Substantial research work has been done on the effective utilization of fish waste by producing a variety of products. Products include biodiesel and biogas as alternative sources of fuel. Chitosan which is a dietetic product, has uses in food packaging, and collagen from fish has uses in the production of cosmetics, enzyme isolation, fertilizers and animal feeds (Arvanitoyannis & Kassaveti, 2008). The fish waste or byproducts as a result of fish processing have been established

to be a source of many bioactive compounds. The fish waste products are nutritious, high in protein and thus through fermentation, make excellent feeds for animals (Rodriguez et al., 2017). It is not only through fermentation that they become feeds for animals. The scales and bones are usually subjected to high temperatures by boiling and further crushed and then fed to both pigs and chickens. The meat that is left on the bones is also used to make fish balls that are later consumed as food. This method, however good, is inefficient as the animals fed on such feeds do not fully benefit from the nutrient content of the waste since they exist in complex compounds that cannot be broken down by the animals.

Besides fishmeal, fertilizers can also be obtained from fish waste materials (Rodriguez et al., 2017). Fish waste fertilizers are made from the remnants of the fish offal. The process of making fertilizers on a smaller scale, involves composting them for three to four weeks and sometimes extended up to four months. The composting process for production of the fish waste fertilizer, involves the combination of the fish waste with other components like seaweed and pine bark or other kinds of sawdust (Illera-Vives et al., 2015). This is done to improve the carbon to nitrogen ratios. This method of waste management is sensible in that it makes use of the waste but it is not fully effective as it only consumes a small amount of it. Furthermore, the compositing process is labor intensive and requires frequent turning of the compost mixture. It also takes a long time to compost and come up with the fertilizer, whose nutrient value is low. The industrial technologies for fertilizer production is slightly advanced in that it employs the use of enzymes, *alcalase* enzyme (Villamil et al., 2017). The process is however energy consuming as it involves mincing of the fish waste and further heating then mixture after hydrolysis.

Chemical hydrolysis is also carried out to produce fertilizers (Rodriguez et al., 2017). This is through acid and base hydrolysis. FPH can be generated through this process and the products are

not limited to fertilizers only. There can be food additives and in the flavoring agents generated from the chemical hydrolysis (Vázquez et al., 2017). However, the downside to this is that the extent of hydrolysis cannot be managed and the products have a salty taste as the neutralizing agents are sodium salts and HCl. Acid hydrolysis is associated with the complete destruction of Tryptophan while the alkali hydrolysis has side reactions, beta elimination reactions producing toxic products. The other waste constituents like skin, scales and cartilages are applicable in the production of gelatin (Chalamaiah et al., 2012).

The skins are also used as a source of leather for accessories such as bags and belts. However, the inadequacy of protein supplements in the market has prompted the exploration of feedstocks derived from fish hydrolysates. The technique currently involved in the recovery of the physiologically and nutritionally active peptide in fish waste is enzymatic hydrolysis. The fats present in fish also have numerous applications in humans. Therefore through the advancement of technology, it has been established that fish oil rich in polyolefin and hydrolysates with applications in various fields of science like nanotechnology in the amino acid mediated synthesis of silver nanoparticles (Rodriguez et al., 2017).

Such interventions can avert the menace of environmental pollution through lack of proper solid waste management systems particularly in developing countries. Further, most technologies available for waste treatment are energy and labor intensive which translates to increased cost. Therefore there is need to develop alternative technologies based on locally available materials that can be used by fish processing industries.

1.2 Statement of the Problem

The large volume of wastes generated during fish processing not only symbolize a direct loss to the producer but also pose environmental pollution and disposal problems.

Current methods of disposal used in Kenya include solid waste buried in landfills, dumping in sewer systems and water bodies, and composting. This contributes to contamination of water bodies, high BOD loading and may lead to algal bloom.

The wastes are protein rich and thus provide a variety of nutrients to the aquatic life (Quinto et al., 2018). The water body where the wastes are discharged may experience robust growth and consequently deplete the amount of dissolved oxygen present in water. The excessive growth of aquatic life also leads to pollution of the water body (Crab et al., 2012).

Dumping on an open compost heap leads to pollution of air as the waste has the smell of rotting flesh as it composts. Landfills do not necessarily prevent the waste from decomposing and leaching through the soil thus altering its composition in terms of pH and mineral content. They are usually persistent in the environment due to their nature. Their smell is pungent in their freshly dumped state and it progresses throughout the decomposition of their fleshy areas. Thus, considering the above threats to the environment, there is need to develop new technologies to handle the disposal of fish wastes and more importantly to obtain useful value addition products.

1.3 Objectives

1.3.1 General objective

To determine the efficacy of locally isolated enzyme in the hydrolysis of different types of fish waste and characterize the resultant metabolite.

1.3.2 Specific objectives

1. To optimize factors affecting enzymatic hydrolysis of fish waste
2. To determine the amino acid composition of the hydrolyzed fish waste and other properties using spectroscopic methods.
3. To characterize recovered oil from the hydrolyzed fish.
4. To determine proximate composition, nutritional attributes and mineral composition of different species of hydrolyzed fish waste.

1.4 Justification and significance

With regards to environmental conservation and preservation, it is prudent that an effective way of fish waste recycling and re-use is established. This will serve to minimize the pollution of soil, air and water sources that may have adverse effects on aquatic and human life. Therefore, an alternative to dumping in water sources, open compost heaps and landfills ought to be established. Literature review indicates that there are relatively very few studies reported on enzymatic hydrolysis of fish waste and subsequent determination of its products for fresh water fish species.

The existence and the continuous growth of the fish processing industry are projected to produce a large amount of bio waste. Currently, an Industry in Kisumu, East African Seafood Limited already produces one hundred to one hundred and fifty kilograms of waste every day (Aura et al.,

2019). The waste also attracts scavengers like birds as shown in figure 1. This research thus aims to provide environmental friendly ways of hydrolyzing of fish wastes and determine the nutrition value of the resultant hydrolysate.

In addition to the aforementioned, the enzymatic hydrolysis of the fish waste provides an alternative clean, ecofriendly and long term sustainable process. In terms of costs with regards to energy, the costs are low and this directly translates less pollution to the environment.

CHAPTER TWO

LITERATURE REVIEW

2.1 Effect of Fish Waste on the Environment.

The fish waste generated in Kenya primarily consists of bones, head, viscera, skin and guts. Water pollution often results from the direct disposal of fish wastes into water bodies without adequate treatment. Aquatic organisms require oxygen for respiration and other physiological processes which increases the demand for dissolved oxygen. The depletion of dissolved oxygen leads to the waters becoming anoxic due to increased BOD and COD which adversely affects aquatic life and may lead to fish kills. The wastes are sometimes converted to gases like carbon (IV)oxide (Crab et al., 2012) that when it is dissolved in water, forms carbonic acid which alters the pH of the water and may affect aquatic life. Algal and bacterial blooms are also bound to increase as these microorganisms feed on the fish waste. Crab *et al.*, (2012) reported that some of the algae arising from the algal bloom may produce toxins that are harmful to marine life. The bacteria may also cause foul smelling waters due to extreme pH and temperature conditions.

The dumping of fish waste on open compost heaps, pose a hazard to the population and subsequently may cause soil pollution, as well as public health concerns. The fish waste is protein rich and consists of fish heads, viscera and fins that also attracts rodents and other animals because it is a source of food. Oyedele *et al.*,(2013) reported that rodents transfer diseases like plague to humans, causing ill health to the population surrounding the dumpsite. The foul smell emanating from the dumpsite due to slow decomposition aided by microorganisms like bacteria also poses air pollution problems.

Figure 1: Effect of fish waste pollution on the environment Source; author, Pap Mbuta, Kisumu dumpsite.

2.2 Current Methods in Fish Waste Management

Whereas landfills are commonly used for waste disposal, they may cause ground water and soil contamination. Besides limiting the use of land, landfills leachates due to decomposition of waste are of concern (Chopda & Malek, 2018). The runoff after rainfall sometimes forms puddles which infiltrate into the ground and mixes with the landfill leachate. Upon reaching the permeable geologic strata, the runoff infiltrates and mixes with the leachate and then percolates to the depths of the water table thus contaminating it.

Therefore, in order to reduce the levels of environmental pollution, there is need to focus on finding alternative uses of fish waste. Fish wastes have been investigated and found to be rich in collagen, especially the fish bones, scales and the skin (Tang et al., 2015). There exists diverse sorts of collagen, from type I to type XIX and the collagen fibre most predominantly found in fish skins, bones and scales is the type I collagen. However, the type II is the most predominant and is the one studied mostly.

The fish wastes also have various bioactive substances, proteins, lipids, polysaccharides and thus through fermentation processes animal feeds can be derived from fish waste products. The bones, scales and fins however, are more persistent in the environment and do not undergo fermentation and therefore require quite some energy to convert them into feeds that can be consumed by animals such as pigs. Usually they are boiled or heated to high temperatures for pretreatment and preparation for additional processing and further crushed to fine particles which are then fed to animals. This process however, consumes a lot of energy and does not consume a substantial amount of waste thus not cost effective. Furthermore the above process does not involve the breakdown of the complex molecules present in the waste into forms that can be made useful by the animals they are fed to.

The fish offal can also be processed into fertilizers through composting and therefore making use of the nutrients present (Rodriguez et al., 2017). The most important process in this method of fish waste management is the carbon and nitrogen ratio adjustments to the prospective fertilizer. The composting of the fish waste with sawdust takes roughly three to four weeks making the process time consuming. Not only does it not consume a large amount of waste for effective waste management, but also is labor intensive as it requires consistent turning for proper aeration.

Chemical hydrolysis process has been developed for hydrolysis of fish waste and the true value of the protein waste studied reported (Mota et al., 2014). Hydrolysis yields hydrolysate products which are simpler compounds like peptides and Amino Acid (AA) generated by the breaking down of complex protein structures (Kristinsson & Rasco, 2000a). Due to the effectiveness of the process of hydrolyzing proteins, various methods have been explored towards its advancement and multiple areas of application have been investigated. Thus hydrolysis of protein has been applied to various processes like management of protein waste, leather treatment and industrial manufacture of various animal feeds and fertilizers (Routray et al., 2018). The most commonly used methods to hydrolyze fish waste include chemical and biochemical techniques.

2.3 Composition of Fish Waste

The fish waste aside containing proteins also contains collagen, gelatin, amino acids, enzymes, minerals and oils. The proteins in fish are in the form of muscle proteins, the light and dark muscle, that are easily digested and highly nutritious. They are of value and offer a better balance of the essential amino acid and are thus extracted through the hydrolysis of the fish waste using an enzyme (Ghaly et al., 2013). The fish muscle protein properties vary from different species and their locations in that fish species in the cold regions are bound to have proteins prone to denaturation by heat while those at the tropic possess relatively high denaturation temperatures and pH. Overall, the fish protein is made up of structural proteins, sarcoplasmic protein and insoluble connecting tissue in the ratios of 70-80%, 20-30% and 2-3% respectively (Ghaly et al., 2013).

The collagen and gelatin exist as hydrolysates of the skin and scales of the fish. The gelatin is usually as a result of partially hydrolyzed collagen or that collagen that has been denatured by heat. They are the same macromolecules with the only difference arising from the extent of hydrolysis.

The collagen and gelatin thus have various applications in the food, cosmetic and biomedical fields like making of ice cream, anti-aging creams and treatment of osteoarthritis, rheumatoid arthritis respectively (Jayathilakan et al., 2012). The properties of the gelatin depends on the sequence of the amino acids and that the amino acids present are non-polar. This gives it advantages over other gelatin sourced from porcelain and other animal sources in that they have enhanced biological activities for instance antioxidant and hypersensitivity properties. The distinctive arrangement of glycine-proline-alanine that repeats itself in its sequence allows the gelatin have an oxidative property (Ghaly et al., 2013).

The amino acid content in fish is relatively high with its composition varying from 16-18 amino acids depending on the species and differences in the environment. The boarfish for example has an amino acid profile as in table 2 (Ojha et al., 2016).

Table 2: Free amino acids profile in mg/g of boarfish sample after 24h of enzymatic hydrolysis compared to control (Ojha et al., 2016)

Amino Acid	Alcalase	Protease	Control	MSD
Cysteic acid	0.42[a]	0.42[a]	0.55[a]	0.193
Asparagine	1.05[a]	0.77[ab]	0.42[b]	0.349
Threonine	1.15[b]	1.97[a]	0.09[c]	0.311
Serine	2.75[a]	2.39[a]	1.04[b]	0.474
Glutamine	8.25[a]	6.63[b]	4.64[c]	1.240
Glycine	1.26[a]	1.49[a]	0.48[b]	0.342
Alanine	3.80[a]	3.22[b]	1.52[c]	0.512
Cysteine	6.90[a]	7.07[a]	4.87[b]	1.438
Valine	4.42 [a]	1.69 [b]	1.30 [b]	0.639
Methionine	0.74 [a]	0.62 [a]	0.92 [a]	1.675
Isoleucine	0.93 [a]	0.87 [a]	0.29 [a]	2.240
Leucine	8.22 [a]	6.51 [b]	4.77 [c]	1.112
Tyrosine	3.59 [a]	3.84 [a]	2.25 [a]	1.951
Phenylalanine	6.75 [a]	6.61 [a]	6.27 [a]	1.930
Histidine	12.28 [ab]	13.25 [a]	10.34 [b]	1.977
Lysine	2.39 [a]	1.98 [a]	0.77 [b]	0.537
Arginine	2.95 [a]	2.19 [b]	1.38 [c]	0.481
Proline	0.14 [b]	0.19 [a]	0.00 [c]	0.00
Total	67.81 [a]	61.72 [a]	41.91[b]	12.884
Taurine	6.07 [b]	5.37 [c]	7.23 [a]	0.217
NH_3	0.19 [a]	0.27 [a]	0.38 [a]	0.236

[abc] Letters followed by the same alphabet within a row are not significantly different (P<0.05)

MSD : Minimum Significant Difference

The fish waste is also rich in oil and the content is dependent on the species and the fat content of the fish. Approximately, 2-30% of the 50-73% fish waste from fish processing has fat potential for human consumption (Mbatia et al., 2010). The fat content of the fish waste is majorly the eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The species from cold waters have a higher percentage of these omega three fats but have similarly been found in tropic water fish. The Nile Perch present in Lake Victoria has been reported to contain substantial amount of omega three polyunsaturated fats (Mbatia et al., 2010). This prompts the research on other species like the tilapia (*Oreochromis niloticus*) on the existence of PUFAs and in their respective quantities.

2.4 Chemical Hydrolysis

These processes are made up of acids, base and chemical extraction hydrolysis and are predominantly used in industrial processes. Prior to development of the above hydrolysis methods, fish processing concentrates was used as a mode of chemical hydrolysis and their chemical process involved a solvent extraction step. The water and oil are separated from the protein elements of the fish using isopropanol solvent. For this process to be effective the entire fish is eviscerated and the whole viscera extracted thrice with azeotropic isopropanol (Kristinsson & Rasco, 2000a).

The fish protein concentrate (FPC) is then extracted at low temperatures of about 20℃-30℃. This process is then further followed by two extractions done at high temperatures for a combined 160 minutes. The physical characteristics of the concentrate obtained is that it is a colorless and odorless with very low lipid concentration (Kristinsson & Rasco, 2000b). In as much as it is an inexpensive way to obtain protein from fish waste, the resultant product of the process is not versatile for use in many forms; in addition, it is not readily dispersible or soluble in food

substrates. Its emulsification properties are also poor and these are attributed to the temperatures used during the extraction process (Kristinsson & Rasco, 2000b).

The poor functionality of this process is however countered by its good foaming ability that occurs over a broad series of pH values therefore suitable for high fat content pelagic fish like sardines. It was further explored that the fish protein concentrate could form a good starting substrate for enzymatic hydrolysis (Ishak & Sarbon, 2018). This was not entirely successful as the initial chemical process included protein breakdown and as a result, a lot of functionality was lost due to excessive protein breakdown but the solubility of nitrogen is improved (Kristinsson & Rasco, 2000b).

2.4.1 Acid Hydrolysis

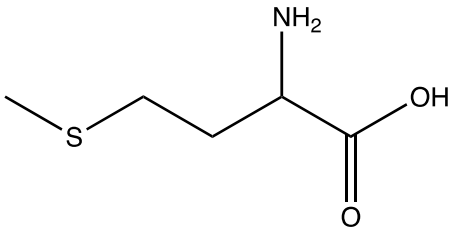
It is the most prevalent mode of hydrolysis used for proteins and most preferred for relatively cheap vegetable protein sources mostly used for flavor and taste enhancement (Kristinsson & Rasco, 2000b). This is usually in the likes of processed meats and soups as an additive to enhance the flavor. For the hydrolysis of fish protein, the acid predominantly used is hydrochloric acid and at certain times sulfuric acid at high temperatures and pressure.

The resultant hydrolysate is neutralized to a neutral pH using sodium salts like sodium carbonate and hydroxide and is highly soluble due to extensive hydrolysis of the protein (Kristinsson & Rasco, 2000b). The formation of sodium chloride may cause the hydrolysate product to be unpalatable in addition to extensive and uncontrollable levels of hydrolysis. Acid hydrolysis has made major contributions to the flavoring and fertilizer industry as a result of its extensive hydrolytic property. It however, remains a difficult process to control as it is also associated with the complete destruction of tryptophan amino acid (Ishak & Sarbon, 2018).

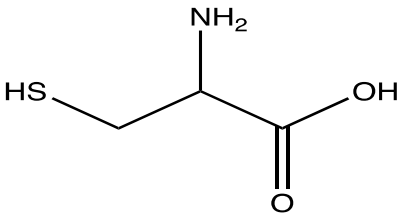
2.4.2 Base/ Alkali Hydrolysis

It involves the use of sodium hydroxide predominantly as the alkali and is not necessarily a popular method for protein hydrolysis as the products are poor functionality and nutritive value. It is usually characterized by high solubility of the collagen fibres (Kristinsson & Rasco, 2000b). In alkali hydrolysis, the starting material is usually preferred to be the fish protein concentrate which is usually marked by the swift cleavage of water soluble peptides. Their degradation follows slowly thereafter.

Base hydrolysis improves the qualities of dispersion and solubility previously not present in the fish protein concentrate as in acid hydrolysis (Kristinsson & Rasco, 2000b). Alkali hydrolysis lack of popularity can be attributed to the occurrence of various toxic reactions during hydrolysis usually initiated by the abstraction of alpha hydrogen from the amino acid. These reactions also comprise racemization of the L- amino acids to produce D-amino acids that are not taken up for use by humans (Kristinsson & Rasco, 2000b). Beta elimination reactions that occur also lead to the breakage of disulfide bonds in the cases of cysteine and methionine resulting in toxic substances. In the event of the presence of an enzyme, the alkaline environment serves to reduce the rate of hydrolysis.



Methionine



Cysteine

2.5 Enzymatic Hydrolysis

An enzyme is easily described as a macromolecule substance that is protein in nature and catalyzes a chemical reaction (Tiwari et al., 2016). They exist as biological catalysts that increases the degree of a chemical reaction without necessarily being consumed or having its structure altered and has the structure of a folded protein (Roy et al., 2014). The folding is into shapes that allows it to have active sites within which the substrate molecules participating in the chemical reaction fit and the importance of the shape is to provide the active site. This is important because in the event that it is destroyed, then enzyme is said to have been denatured (Kingsley & Lill, 2015).

Enzymes catalyze reactions by reducing the activation energy which is the minimum amount of energy required by a chemical reaction in order for the said chemical reaction to take place (Zhang et al., 2017). The working of enzymes is also governed by theories that within which the enzyme catalyzes reactions in accordance with enzyme specificity which means that the enzyme only catalyzes one type of reaction and it is the reason for the lock and key theory as well as the induced fit theory (Rahman et al., 2014). The former theory outlines how an enzyme displays specificity like a lock and key where the active site is only designed to fit only one kind of substrate while the former is more dynamic in that it states that an enzyme can catalyze more than one chemical reaction.

One characteristic of enzymes that makes it suitable for this process is specificity which makes one able to predict the process and the products as well exercising an enhanced control of the reaction process and thus maximizing efficacy. The products resulting from enzymatic hydrolysis are usually simple and thus require not complex and elaborate methods of separation.

Enzymes are usually naturally occurring substances that are protein in nature. They are of different types and classes namely; oxidoreductases , transferases, hydrolases, lyases, isomerases and ligases (Wu et al., 2016). This classification of enzymes follows the kinds of reactions they catalyze namely redox reactions, transfer of functional groups, hydrolysis, elimination reactions, isomerization reactions and ligation reactions respectively.

For this project, the hydrolases are best suited as this process of waste management is through hydrolysis. Hydrolases are of many kinds and since enzymes are substrate specific, the hydrolases are classified according to the substrates whose reactions they catalyze (Wu et al., 2016). Fish being protein in nature requires an enzyme that specifically catalyzes the hydrolysis of proteins and that is the class of proteases. There are different commercial proteases and various isolates that have been established for different uses in the Kenyan market. Considering the severe environmental pollution caused by ever increasing fish waste and high costs incurred in waste disposal, there is an urgent need to isolate more efficient enzymes in order to minimize the wastes generated from the fish industry in an effort to combat environmental pollution problem cited earlier, reduce post-harvest losses and comply with set environmental norms. (Wanyonyi et al., 2014) reported a new eco-friendly and clean method of processing hides and fish skins into leather using alkaline protease enzyme isolated from Lake Bogoria. Extremophiles show the ability to grow in an exceedingly harsh environment characterized by high pH and temperature, high levels of salinity or salt, and pressure which invariably effect their growth (Dhakar & Pandey, 2016). The ability of crude protease enzyme that is an isolate of *Bacillus cereus strain wwcp1* was investigated in fish waste hydrolysis.

2.6. Mechanism of Enzyme Catalyzed Reactions.

The catalysis by proteases usually involves three consecutive reactions. The first is usually the formation of the Michaeli's complex between the peptide that is the substrate and the enzyme. This bond is then cleaved to release one of the peptides in the enzyme-substrate complex and this is usually the rate limiting step that requires catalysis (Kristinsson and Rasco, 2000).

The third step is usually a nucleophilic attack on what is left of the peptide to regenerate the enzyme. The breakage of the peptide bonds results to an increased presence of ionizable anionic and cationic groups like NH_3^+ and COO^- (Kristinsson and Rasco, 2000). An illustrated form of reaction showing the mechanism of enzymatic activity with regards to Michaeli's-Menten kinetics is given below.

$$E + S \leftrightarrow ES \rightarrow EP + H - P \rightarrow E + OH - P + H - P$$

This is where;

E – Enzyme

P – Product

H – Hydrogen

S – Substrate

ES – Enzyme substrate complex

OH^- – hydroxyl ion

The degradation of fish waste through enzyme hydrolysis has been studied to take several forms usually one to three step hydrolysis forms. The only variations in these forms is the number of

enzymes used as the first step applies the use of one enzyme while the two-step uses two and the three step uses two and an imitation of the gastrointestinal tract digestion (Ishak & Sarbon 2017). This form of hydrolysis offers selectivity and specificity as compared to its chemical counterparts. Amino acid damage is also limited as shown in studies on pony fish, yellow strip trevally and mackerel (Villamil et al., 2017).

Homogenous catalysis is where both the enzyme and the substrate exist in the same phase while in heterogeneous catalysis, the enzyme and substrate exist in different phases.(Ejikeme et al., 2010) The enzymatic fish hydrolysis is a heterogeneous kind of catalysis in that the enzyme is in the liquid phase while the fish substrate is in the solid phase (Ejikeme et al., 2010). The nature of the specificity of an enzyme to a substrate leads to the fact that enzymes have active sites in which chemisorption takes place and it is also on these sites that strong atomic or molecular bonds are formed between the substrate and the enzyme catalyst (Bartholomew and Farrauto, 2011).

The enzymatic catalyzed reactions occur in three steps namely adsorption, reaction and desorption which all take place on the surface of the catalyst (Wolf, 2004). In determining the rate law, the overall rate of the reaction is correspondent to the reaction rate of the rate-limiting step, usually the slowest of the reactions (Bartholomew and Farrauto, 2011). The important point in determining the rate limiting step is through establishing the concentration of the reactants. The rate is reliant on the concentration of the reactants and the reactants in this case are the enzymes, substrate and the enzyme-substrate complex formed.

An enzyme catalyzed reaction takes the general form as outlined in (Cornish-Bowden, 2014) as shown below;

$E + S \xrightarrow{K_1 / K_{-1}} ES \xrightarrow{K_2} E + P$ Equation 1

The constant is derived from the reaction constants by writing the individual rate laws for all the reactions.

$r_{1S} = k_1[E][S]$ Equation 2

$r_{2S} = k_{-1}[ES]$ Equation 3

$r_P = k_2[ES]$ Equation 4

When writing the overall rate law for the disappearance of the substrate then the first and the second rates are combined.

$r_{-3} = k_1[E][S] - k_{-1}[ES]$ Equation 5

The concentration of the enzyme substrate complex cannot be accurately determined and thus established as a function of the concentration of the substrate

$r_{1ES} = k_1[E][S]$ Equation 6

$r_{2ES} = k_{-1}[ES]$ Equation 7

$r_{3ES} = k_2[ES]$ Equation 8

The overall rate for the production of the complex is written as follows

$r_{ES} = k_1[E][S] - k_{-1}[ES] - k_2[ES]$ Equation 9

Because it is unstable and used very fast; thus is synonymous with

$r_{ES} = 0.$

$$0 = k_1[E][S] - k_{-1}[ES] - k_2[ES] \quad \text{Equation 10}$$

$$k_1[E][S] = k_{-1}[ES] + k_2[ES] \quad \text{Equation 11}$$

$$\frac{[ES]}{[E][S]} = \frac{k_1}{k_{-1}+k_2} = \frac{1}{K_M} \quad \text{Equation 12}$$

$$K_M = \frac{[E][S]}{[ES]} \quad \text{Equation 13}$$

$$[ES] = \frac{[E]_T[S]}{K_M+[S]} \quad \text{Equation 14}$$

The velocity with which a reaction catalyzed by an enzyme occurs is vital in that it points to the rate of product formation with. This implies that at the initial stages of reaction V_o point to an increase in product production when the product concentration is low (Berg et al., 2002).

$$V_0 = r_{0P} = k_2[ES] \quad \text{Equation 15}$$

Thus;$[ES] = {}^{V_0}/_{k_3}$

At maximal velocity the concentration of the complex is equal to the total enzyme concentration. This is because all the active sites are saturated with substrate.

Thus;

$$V_{max} = r_P = k_2[ES] = k_2[E]_T \quad \text{Equation 16}$$

The two scenarios are then substituted into the equation with [ES] as the subject to yield:

$$V_0 = \frac{k_2[E]_T[S]}{K_M+[S]}$$ Equation 17

$$V_0 = \frac{V_{max}[S]}{K_M+[S]}$$ Equation 18

2.7 Analytical Techniques

The resultant product after enzymatic hydrolysis of fish is abbreviated as FPH. Thus, to establish its true value, analyses such as proximate analysis, amino acid analysis and FTIR are necessary to establish the rate of hydrolysis, characterize further the metabolites and the functional groups present. Proximate analysis is described as the process of determining a collection of narrowly correlated elements (Hart & Fisher, 1971). Therefore, proximate denotes the chemical composition of a fed material. Its significance is important since it provides information regarding the delivery of essential nutrients for the maintenance of good health through feed formulation (Chalamaiah et al., 2012).

Amino acid analysis gives a pointer to both the quantity and sometimes the sequence of the amino acid present in a sample. FPH is usually produced from the dark muscle that is left of the fish after processing. It is usually rendered not useful because of the off flavor and its easy accessibility to oxidation (Chalamaiah et al., 2012). The AA analysis by the use of the HPLC is thus important to give the composition of FPH. It shows whether the FPH is abundant in free AA or short chain peptides thus this helps to determine the uses or applications of the FPH. The AA have different applications like as nutraceuticals and as functional foods depending on their profile as revealed through analysis by the HPLC (Chalamaiah et al., 2012). The establishment of the degree of hydrolysis is equally as important as the determination of the effectiveness of the process of

hydrolysis. The end result of the hydrolysis is to breakdown the longer peptide chains to individual amino acids.

The study of and analysis of the functional groups present in proteins has been aided by the Fourier Transform Infrared (FTIR) by providing the information on the secondary structure of the said protein (Gallagher, 1997). Proteins are distinguished by the number of amino acid units they contain and their sequence of the amino acid. FTIR therefore has the working principle of shining infrared beams of infrared light on a sample which are absorbed and each sample has characteristic set of absorption bands in its spectrum and in this case the focus will be on the wavelength absorbed by the protein sample (Gallagher, 1997). The peptides and polypeptide bands are the Amide I and Amide II that are as a result from bonds that link AA. The absorption that is allied to the former bond is that that comes from the stretching vibration of the carbonyl carbon while the Amide II is predominantly from the bending of the N−H bond. Therefore the locations of these bands are reliant on the secondary configuration of the peptide as a result of the carbonyl carbon and the NH bond being involved in the hydrogen bonding. The analysis of amino acids with the FTIR seeks to show the functional groups indicating that only the hydrogen bonds are severed during the process of hydrolysis and the functional groups remain unchanged.

Analyzing fatty acids proceeds using gas chromatography furnished with the mass spectrometry (GC-MS) or the gas chromatography fitted out with a flame ionization detector (GC FID). Fatty acids of both plant and animal source are known to contain even totaled chains ranging from 16 to 22 carbon chain with a maximum of6 double bonds in each chain while some chains may lack the double bonds (Christie, 1998). The GC-MS focuses on the qualitative investigation of the fatty acid methyl ester (FAME) that are derivatives of the fatty acids by taking advantage of their difference in polarity and thus their affinity to the stationary phase of the GC column whereas the

MS identifies the compound at their molecular level (Christie, 1998). The GC-FID on the other hand focuses on the quantitative analysis of the FAMEs where the GC column separates the fatty acids components and are quantified by the FID (Dodds et al., 2005). The FID element that has the GC equipped with it has had challenges with the misidentification of samples due to its lack of selectivity and thus has had improvements made with regards to retention time prediction, locking and the FAMEs chain lengths having the retention time dependent on them. (Dodds et al., 2005).

CHAPTER THREE

3.1 Materials and Methods

3.1.1 Raw Materials

The fresh water fish species tilapia (*Oreochromis niloticus*) commonly traded in the market were purchased from the fish market in Nairobi's City and Gikomba markets. The species was positively identified as the correct species of interest at The National Museums of Kenya, filleted and the head with the viscera and fins carried to the laboratory. These waste products were stored at -80ºC. When ready for use, the frozen viscera was thawed with the aid of running water for forty minutes. It was then left off to drain off excess water after which it was cut into sizeable pieces and weighed. This weight was recorded as the total weight to be hydrolyzed.

3.1.2 Chemicals

The chemical reagents preferred for use were purchased from Pyrex East Africa and were of analytical grade. They were; Trichloroacetic acid (TCA), sodium hydroxide, sodium carbonate and hydrochloric acid for the process of hydrolysis. Enzyme preparation chemicals included potassium hydrogen carbonate, potassium dihydrogen phosphate, magnesium sulfate, calcium chloride, urea, yeast, casein and glucose**.** Sulfuric acid, hexane and petroleum ether for proximate analysis. Other chemicals used were butyl hydroxytoluene (BHT), potassium hydroxide, triacylglycerol (TAG) and heptadecanoic (margaric) acid.

3.2 Methods

3.2.1 Enzyme Preparation

The preparation of the enzyme, crude alkaline protease from *Bacillus cereus* obtained from Lake Bogoria; was done according to the modified procedure as outlined by (Wanyonyi et al., 2014).The medium suitable for the growth of the bacteria species and this enzyme production contained casein and glucose in the ratios of 1:2 respectively in addition to other nutrients and chemicals. The reagents used were 5g of K_2HPO_4, 3.75g of KH_2PO_4, 0.75g of $MgSO_4.7H_2O$, $CaCl_2$ and urea. Lastly, 1.25g, 12.5g and 6.25g of yeast, casein and glucose respectively.

After measurement of the nutrients, 2500ml of distilled water was added to a 5l conical flask. This mixture thus acts as the medium that grows the *Bacillus cereus* and where the alkaline protease is harvested after successive growth. The mixture in the conical flask was stirred to facilitate dissolution of the water soluble components and pH adjusted to pH10.5 before transferring to the bioreactor. It was plugged using cotton wool and sterilized in an autoclave for 25 minutes at 121°C. The media was permitted to cool to room temperature and thereafter inoculated with 5% *Bacillus cereus stain wwcp1* seed bacteria culture that had been grown overnight. The medium was incubated at 45°C and stirred constantly at 150rpm (revolutions per minute) for three days. The crude enzyme was thus obtained for further studies.

3.2.2. Tyrosine Standard Calibration Curve Preparation

The reagents used were sodium carbonate, tyrosine and Folin and Ciocalteu′s phenol reagent (FC). 0.4M of Na_2CO_3, 1.1mM 33.3%v/v of FC reagent were prepared ((Wanyonyi et al., 2014). Varied amounts of tyrosine were measured using a micropipette and put into eleven falcon tubes. The amounts ranged from 0µl to 150µl and water also using a micropipette was measured and added to the falcon tubes to make the solution to 750µl as in the proportions shown in table 3. To this

solution, 1875µl of sodium carbonate and 375µl of water were added. The mixtures were agitated to completely mix them up to end up with a solution. The FC reagent was added and colour was developed in an incubator at 30°C. The absorbance values were taken at 660nm on a UV-VIS spectrophotometer to establish the amount of tyrosine corresponding to the various absorbance value. It should be noted that Folin & Ciocalteu′s phenol reagent contains no phenol. However, the reagent reacts with both phenols as well as non-phenolic reducing substances to obtain chromogens that can subsequently be detected using UV-VIS spectrophotometer. This was later used to define the degree of hydrolysis with regards to the optimization of the fish waste hydrolysis**.**

Table 3: Tyrosine Standard Curve Preparation Table

Reagent	1	2	3	4	5	6	7	8	9	10	11
1.1µM Tyrosine std	0	15	30	45	60	75	90	105	120	135	150
Reducing tyrosine volumes	0	5	10	15	20	25	30	35	40	45	50
Water (µl)	750	735	720	705	690	675	660	645	630	615	600
Na_2CO3 (µl)	1875	1875	1875	1875	1875	1875	1875	1875	1875	1875	1875
Diluted FC (µl)	375	375	375	375	375	375	375	375	375	375	375
Total Volume(ml)	3	3	3	3	3	3	3	3	3	3	3
Δ volume(ml)	1	1	1	1	1	1	1	1	1	1	1
[Tyrosine] (µM)	0	0.0055	0.011	0.0165	0.022	0.0275	0.033	0.0385	0.044	0.0495	0.055

3.2.3 pH Optimization

The fish samples were defrosted under running water for forty minutes until the samples were fully thawed after which they were washed to remove any particles that were not part of the fish waste. The samples were then weighed into portions of 2g each, wrapped in aluminum foil and left to acquire room temperature. The pH was investigated in triplicate and in comparison to acid and base hydrolysis. 100ml freshly prepared enzyme was measured into 150ml conical flasks, the pH adjusted from 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 13 using hydrochloric acid and sodium hydroxide of 1M concentrations; labeled appropriately and proceeded to be incubated at 80°C for thirty minutes to acquire the temperature at which hydrolysis was to take place (Vázquez et al., 2017). The acid and base hydrolysis experiment set up was done using 100ml of distilled water placed in 150ml and the pH adjusted from pH 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 13 using hydrochloric acid and sodium hydroxide of 1M concentrations respectively and labeled accordingly. The enzyme, acid and base solutions were then inoculated with the fish substrate and put in an oven regulated at 80°C**.** The pH values of the hydrolysis experiment samples were maintained at the labeled values by periodical adjustments using sodium hydroxide and hydrochloric acid. The sample aliquots were taken at intervals of two hours and put in falcon tubes of 10ml. 4ml of 10% Trichloroacetic

acid (TCA) w/v was added to the samples to halt the reaction, hydrolysis of proteins, and coagulate the un-reacted proteins. This mixture was then put in an ice bath for twenty minutes to aid in the coagulation process. What followed after was the centrifugation to separate the coagulated un-reacted proteins from the supernatant fluid. The supernatant was the separated from the solids and sodium carbonate added to neutralize the acid environment created by TCA. The degree of hydrolysis was then determined with the aid of the generated standard curve. The absorbance values correspond to an amount of tyrosine released thus showing the degree of hydrolysis.

3.2.4 Temperature Optimization

The frozen samples were thawed in running water, washed and then measured into pieces of two grams each after which they were wrapped in aluminum foil in order to acquire room temperature. This experiment was done in triplicate to reduce levels of error arising from inconsistencies. The temperatures that were evaluated were room temperature of 22°C, 30°C, 60°C and the highest being 80°C. 100ml of freshly prepared enzyme was measured and put in conical flasks of 150ml with the labels of their respective temperatures and their pH values adjusted to pH12. They were incubated in the ovens controlled at their respective temperatures for thirty minutes with the exception of the enzyme maintained at room temperature. Aliquots of sample were taken to determine tyrosine release before the beginning of hydrolysis after which the enzyme was inoculated with the fish substrate. The sample aliquots were taken at intervals of two hours and put in falcon tubes of 10ml and 4ml of 10% TCA w/v was added to the samples to end the reaction coagulate the un-reacted proteins. This mixture was put in an ice bath for twenty minutes to aid in the coagulation process followed by centrifugation to separate the coagulated un-reacted from the supernatant fluid. The supernatant was separated from the solids and sodium carbonate. The rate of hydrolysis was then established by comparing it to the values obtained from the standard

calibration curve. The absorbance values on the standard tyrosine calibration curve relate to the amount of tyrosine released and thus point to the degree of hydrolysis achieved.

3.2.5 Enzyme Concentration Optimization

500g of fish sample was defrosted using running water until it thawed completely after which it was weighed into portions of 2g each. This experiment was done at different concentrations of enzyme and a constant amount of substrate at a temperature of 80°C and pH 12. The enzyme was prepared by diluting the crude enzyme to the various concentrations from 100%, 50%, 25% and 12.5% with the resultant volume being 100ml for all the concentrations. The enzymes of different concentrations were adjusted to pH12 and incubated for thirty minutes in an oven maintained at 80°C. The enzyme samples had the fish substrate added to them and maintained at temperatures of 80°C and pH12. Aliquots of sample were taken to determine tyrosine release after which the enzyme was inoculated with the fish substrate. The sample aliquots were taken at intervals of two hours and put in falcon tubes of 10ml. 4ml of 10% TCA w/v was added to the samples to coagulate the un-reacted proteins and stop the reaction. The supernatant were obtained after centrifugation and the degree of hydrolysis determined with regard to the amount of tyrosine released.

3.3 Determination of Amino Acid Composition of the Fish Hydrolysate

2 kg of fish waste was place in a 5l conical flask and 2l of freshly prepared alkaline protease added to it. The fish waste were hydrolyzed for three days, while maintaining the pH at 12 and temperature at 80°C with periodic stirring after every two hours for the first 10 hours then after 24 hours.. After complete hydrolysis, oil was extracted and recovered. The FPH with the solid brittle bone particles were autoclaved for forty minutes to sterilize them. The contents were then left to cool and sieved to separate the solid particles from the liquid FPH which was then freeze

dried. This first involved reducing the volume of the FPH in an air drying oven at 40°C for 24.hours in which the volume of 2000ml reduced to 400ml. This was then left to cool after which it was frozen in the freeze dryer container and then loaded into the freeze dryer. The FPH sample was dried for 48 hours to obtain the dried FPH

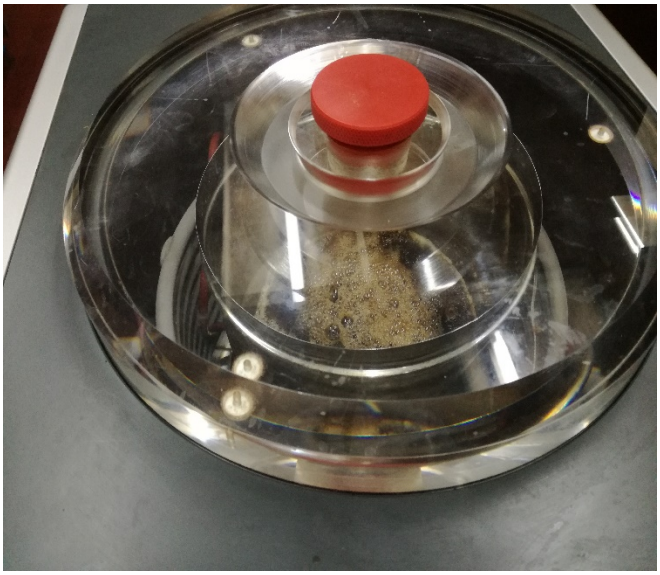


Figure 2: Freeze dryer with FPH

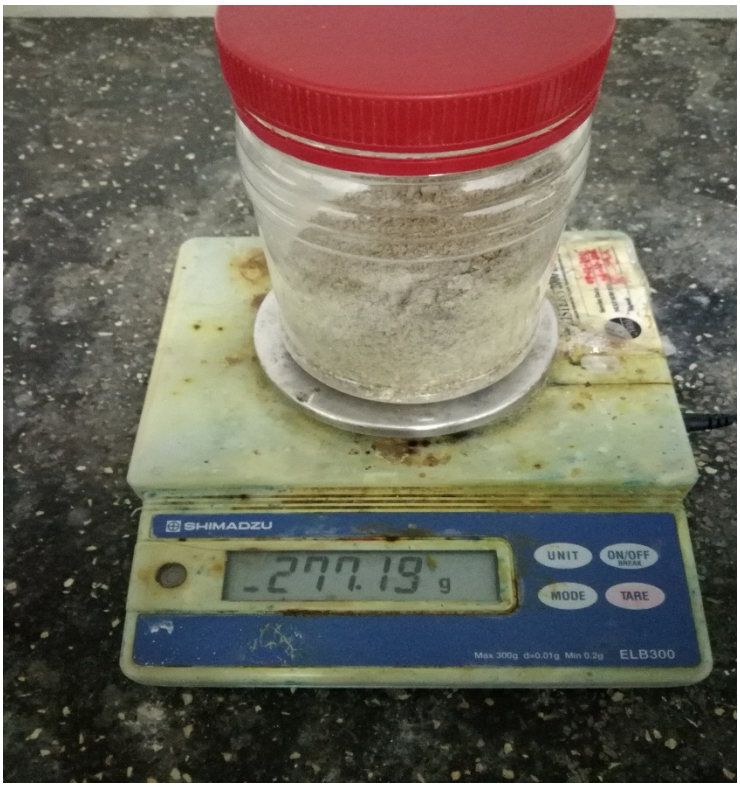


Figure 3: Freeze dried FPH
The FPH yielded 100g of dried FPH sample

For amino acid analysis by HPLC, the sample was weighed and digested in 1ml of 6N hydrochloric acid along with the internal standard. It then proceeded to be hydrolyzed at 100°C for 22 hours in line with the modified procedure by (Saidi et al., 2014). An aliquot of the hydrolysate of 10µl was dried and topped up in 200µl of 0.4N borate buffer. 1µl of the diluent was injected to the High Performance Liquid Chromatography (HPLC) machine and since the internal standard was added at the beginning of the assay, the volumes of the injections and diluents are of little or no significance. The pre-column derivation of the FPH using o-phthaldialdehyde (OPA) was done using the auto sampler. The OPA derived FPH samples was then measured to $2\mu l$ and injected into the column. The two mobile phases consisted of sodium phosphate whose pH was adjusted with sodium hydroxide while the other mobile phase consisted of acetonitrile/methanol/water in volumetric ratios of 45/45/10 (Bartolomeo & Maisano, 2006). These two phases constitute the

AccQ Tag Eluent A and B respectively. For this process, the HPLC was furnished with a Waters 410 Scanning Fluorescence and an AccQ Tag Column (3.9 x 150mm). The individual separation was then obtained by the setting the RP-HPLC at a flowrate of 2ml/min that at 0%B at 1.9min followed by the raising of B to 53% by a 16.3min step (Bartolomeo & Maisano, 2006).

3.4 Characterization of Oil Obtained From the Fish Hydrolysate

1kg of fish waste (head, skin bones and the entire viscera) was placed in a 2l conical flask and 1l of freshly prepared alkaline protease added. The contents of the flask were maintained at 80°C and at pH 12 for 3 days with periodic stirring for hydrolysis. After the completion of hydrolysis, the oil was recovered using a 3l separating funnel and water proteins soluble in water and other solid particles removed by washing thoroughly using hot distilled water. For preparation of the samples for analysis by the GC-FID, fatty acid methyl esters (FAME) were derivatized. The FPH oil samples were weighed and placed in safe 2ml safe lock tube. Thereafter, 300µl of 2.5% of sulfuric acid, 4µl of butylated hydroxytoluene (BHT) and $5\mu l$ of margaric acid ($C_{17:0}$) triacylglycerol (TAG) ($10mg/ml$ was added to each of the fatty acid samples. The resultant fatty acid methyl ester derivatives were extracted using $300\mu l$ of hexane and an equal amount of 0.9% sodium chloride. The analysis was then done with the GC-FID where 1µl was injected into the Hewlett Packard (HP) Innowax column of dimensions $0.25\mu m \times 30m$. The inlet and FID temperatures were 250°C whereas the temperature regime was 100°C for one minute followed with 40°C per minute ramp to 185°C and finally 7.5°C per minute to 235°C with a ten minute hold. The fatty acids were eluted at different retention times and identified against a standard chromatogram.

3.5 Determination of Fish Protein Hydrolysate Proximate Composition

5kg of fish waste was placed in a 10l conical flask and 5l of freshly prepared alkaline protease added. The flask contents were maintained at pH12 and 80°C with periodic stirring for three days.

After completion of hydrolysis, the oil was extracted and cleaned using a separating funnel and hot distilled water respectively. The FPH was the sterilized through autoclaving for forty minutes. The mixture of the FPH and the solid particles was then separated by decanting and sieving. The FPH was then evaporated at 40°C for a further 4days to facilitate a reduction in volume before freeze drying. After the volume of the FPH had reduced by three quarters to 1.25l, it was then loaded in volumes of 200ml into the freeze dryer. This was done for a week to obtain the dried FPH samples for proximate analysis.

3.5.1 Determination of Total Protein of FPH

The FPH sample to be used was obtained after having the FPH freeze dried after hydrolysis and sterilization using an autoclave. 0.5 grams of the freeze dried FPH was weighed on a nitrogen free filter paper in triplicate, carefully folded and put into a kjeldahl flask. For the blank, a nitrogen free filter paper was used. These were placed together with anti-bumping chips, a catalyst tablet and 20ml of concentrated sulfuric acid. The mixtures were heated slowly in a fume hood and then further boiled until a clear solution was attained. Once the clear solution had been obtained, heating continued for another hour and cooled. They were topped to the three quarter mark with distilled water after which phenolphthalein indicator was added. 10M NaOH was added to the kjeldahl flasks to change the colour of the indicator previously added to the mixture. The flask was connected to a distillation unit and distilled until the distillate no longer reacted with the 50ml mixture of 0.1N HCl in a conical flask. The distillate was back titrated with 0.1N NaOH and the content of the crude protein obtained. The calculation of the crude protein content followed the formula;

$$Crude\ Protein\ content = \frac{(\text{Blank} - \text{FPH titre})}{\text{FPH Weight}}\ X\ Kjeldahl\ constant$$

3.5.2. Determination of the FPH Moisture Content

To establish the moisture content of the FPH, the hydrolysate was obtained by separating the hydrolysate from the bones. The filtrate was freeze dried to remove all the excess water introduced during the process of hydrolysis. The determination of moisture content was also done in triplicate. Three dishes of same sizes were cleaned and placed in an oven to dry at 100°C. The freeze dried sample was weighed in portions of 5g each, dried and the dishes and contents placed in the air oven at 105°C for four hours. The dishes were cooled in a desiccator, weighed and the sample moisture content was then calculated as the formula;

$$\%\ Moisture = \frac{\text{Dry FPH weight}}{\text{Initial sample weight}}\ \text{X}\ 100\%$$

3.5.3 Crude Fat Determination

The crude fat content determination was carried out to establish the amount of fat present in the FPH. This proceeded through the extraction of fat by a soxhlet extractor and condenser. Three sets of samples were weighed of 5g and placed in an extraction thimble. The samples in the thimbles were covered with pieces of cotton wool and the whole thimble setup placed into the soxhlet extractor. To complete the setup of the soxhlet extractor and condenser setup, 200ml of petroleum ether in a flat bottomed flask was placed in a heating mantle and connected to the soxhlet extractor. The extraction fats was done for eight hours continuously after which the residue was dried in air oven for one hour at 105°C and the crude fat content calculated.

$$\%\ Crude\ fat\ content = \frac{\text{Weight of dried dried FPH after fat extraction}}{Initial\ FPH\ weight}$$

3.5.4 FPH Crude Fibre Determination

Three samples of 4g each were weighed and put into 600ml graduated beakers and 50ml of boiling distilled water. To each of the mixtures, 25ml of 2.04N of H_2SO_4 solution was added then filled to the 200ml mark with boiling distilled water. The mixtures were boiled on a hot plate for thirty minutes and the contents of the beakers were filtered with Buchner funnels that were packed slightly with glass wool and washed thrice with boiling distilled water. The glass wools with the residue were transferred back into the graduated beakers, 50ml of boiling distilled water added followed by the 25ml of 1.73N KOH solution. The volume was made up to the 200ml mark and left to boil for another half hour whilst maintaining the volume at 200ml after which the mixtures were again filtered with glass wool and washed with boiling distilled water and rinsed with small amounts of ethanol. The glass wools with residues were transferred into porcelain dishes and dried in an air oven at 105°C for two hours, cooled in a desiccator and weighed. The dishes were ignited at 550°C until they got to a constant weight. This was after four hours; they were cooled in a desiccator, weighed and the crude fibre was calculated as the formula;

$$\%\ Crude\ fibre = \frac{\text{Weight of wool + fish sample + crucible}}{\text{Weight of crucible + wool}}\ X\ 100\%$$

3.5.5 FPH Ash Determination

To determine the amount of ash present in the FPH sample, 4g of the freeze dried samples were weighed in three porcelain crucibles. The samples were slowly heated on a low Bunsen burner flame to begin ashing and transferred to continue ashing in a muffle furnace at 500^0C for four hours until a light grey white ash content of constant weight was obtained. The heating in the muffle furnace was left to continue for another six hours to establish if any change would be observed. The quantity of ash was then calculated using the equation below.

$$\%\ Ash\ Content = \frac{(\text{Weight of crucible} + \text{Ash}) - (\text{Weight of empty Crucible})}{\text{Weigt of Sample}}\ X\ 100\%$$

For the acid insoluble ash, the remnants of the crucible after heating in the muffle furnace were covered in concentrated HCl and evaporated to dryness and this was done twice. Three times, 25ml of 10%HCl was added to the ash and covered with a watch glass and boiled over a low flame gently for ten minutes after which the liquid portion was filtered using a filter paper with no ash. The residue was washed twice using hot distilled water and re-ashed in the muffle furnace for four hours and then up to until a constant weight was achieved. The % acid insoluble ash content was then calculated.

$$\%Acid\ insoluble\ Ash\ Content$$
$$= \frac{(\text{Weight of crucible} + \text{FPH Ash}) - (\text{Weight of empty Crucible})}{\text{Weigt of Sample}}\ X\ 100\%$$

3.6 FTIR Analysis of the FPH

5kg of fish refuse was placed in a 10l conical flask and 5l of freshly prepared alkaline protease added. The flask contents were maintained at pH12 and 80°C with periodic stirring for three days. After completion of hydrolysis, the oil was extracted and cleaned using a separating funnel and hot distilled water respectively.

The FPH was the sterilized through autoclaving for forty minutes. The mixture of the FPH and the solid particles was then separated by decanting and sieving. The FPH was then evaporated at 40°C for a further 4 days to facilitate a reduction in volume before freeze drying.

After the volume of the FPH had reduced by three quarters to 1.25l, it was then loaded in volumes of 200ml into the freeze drier for drying. The hydrolyzed fish waste was freeze dried and crushed into tiny particles and some dissolved in water to yield both a solid and a liquid FPH sample.

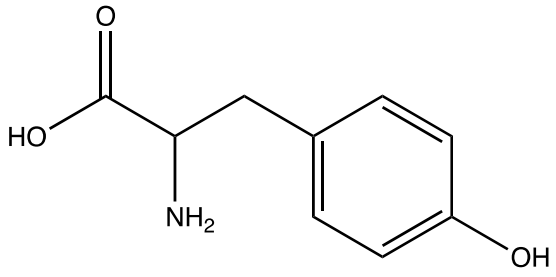
The samples were subjected to FT-IR analysis to identify the functional groups that are synonymous with the amino acid peaks. The IR beam was recorded using a single beam spectrometer between wave numbers 4000- 600cm^{-1}.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Tyrosine Release Standard Curve

Tyrosine was the known amino acid with which the optimization experiments were done with reference to the degree of hydrolysis. The Folin Ciocalteu′s (FC) method was used for this experiment making tyrosine the suitable amino acid due to its phenolic derivative. The presence of the hydroxyl phenol group present in the tyrosine enables it to reduce the green phosphor molybdate to the blue colour. This was then read calorimetrically using the UV/VIS spectrophotometer at 660nm. The absorbance values obtained were used to generate a standard curve. Known concentration values were measured and put in falcon tubes with water and sodium carbonate. The FC reagent was added and incubated for colour to develop, the absorbance values read and the standard curve in Figure 4 generated. This curve shows the amount of tyrosine liberated throughout the optimization process in relation to absorbance.



Tyrosine has a phenolic derivative and the OH group makes it possible to reduce green phosphor molybdate to blue.

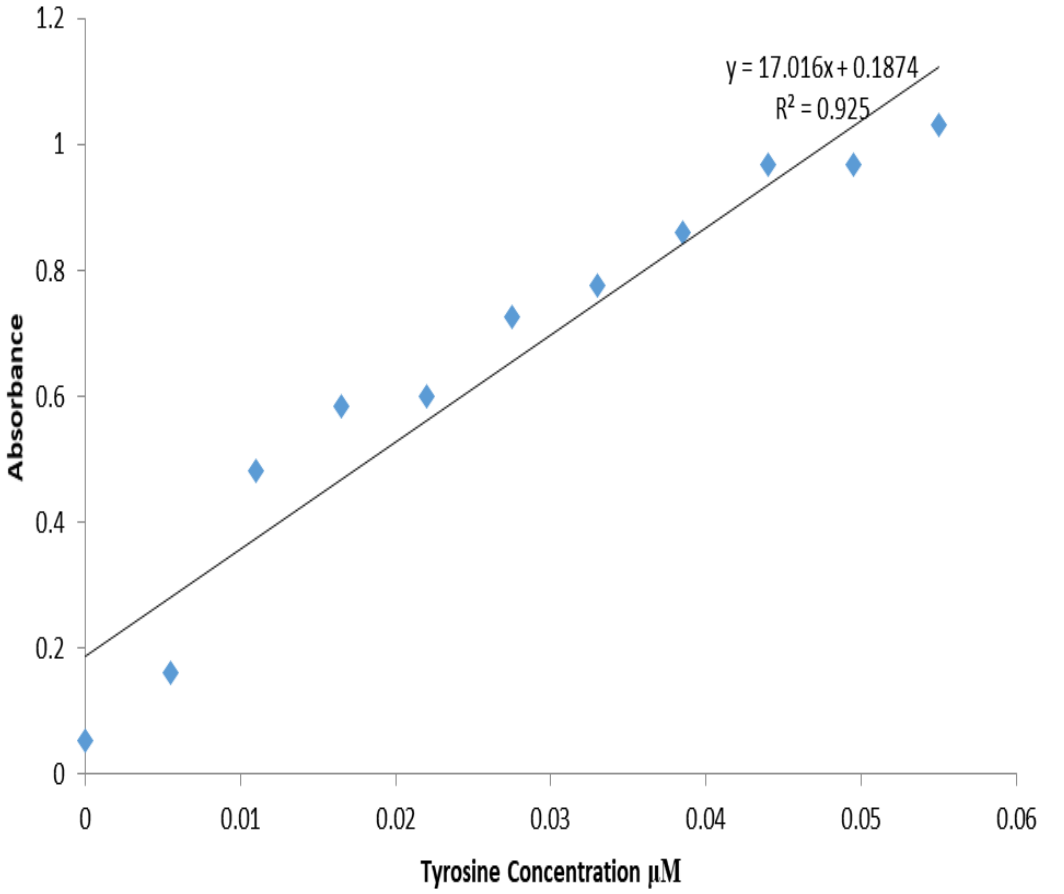


Figure 4: Tyrosine Standard Calibration curve.

It is through this standard curve that the degree of hydrolysis at different times was determined as it provided the tyrosine release for the subsequent results for the optimization of the parameters affecting the enzymatic hydrolysis of fish waste. The standard curve was also used to determine whether hydrolysis had taken place. The tyrosine standard curve has also been reported to be have been used in the establishment of the rate and extent of hydrolysis in various enzymatic hydrolysis reactions with the aim of producing protein hydrolysates (Fonseca et al., 2016) The concentrations

for the determination of the order of reaction and the K_M were obtained with the aid of the standard curve.

4.2 Effect of pH On Fish Waste Hydrolysis after 6 Hours

The pH parameter is important with regard to enzymatic activity as it makes up one of the parameters influencing the hydrolysis of the fish waste. It affects both the fish waste and the enzyme. In this case how hydrolysis of the fish waste is affected by the various changes in pH. The fish sample was thawed and weighed in 2g sizes and incubated at 30°C and thereafter inoculated with 100ml of the crude protease. The influence of pH on the hydrolysis of the fish waste is thus present in figure 5 below.

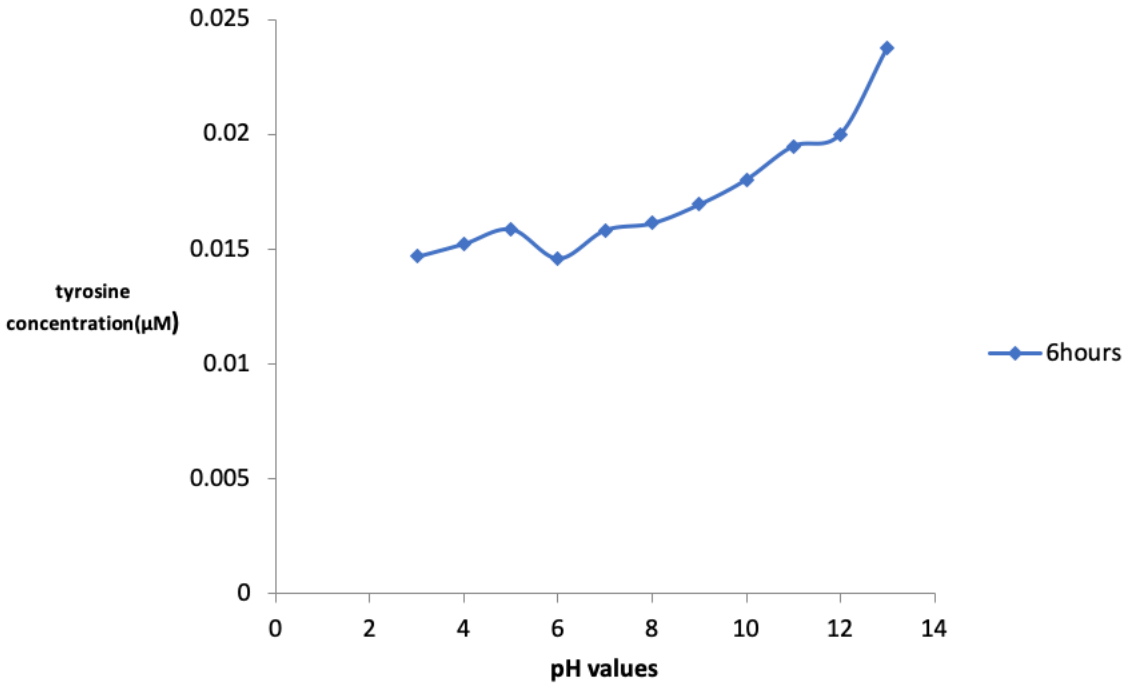


Figure 5: Effect of pH on fish waste Hydrolysis after 6 Hours

After six hours, the enzymatic activity of the protease enzyme steadily increases with the exception of pH6. The protease enzyme showed better activity over the alkaline region as the general trend of the enzymatic activity increases with an increase in pH. A considerable change in pH in the acidic region resulted in a decline in enzymatic activity. Other studies have also reported higher amount of amino acid production under alkaline conditions with pH of 8 and 9 (Bermúdez-Penabad et al., 2017). More recent studies as highlighted by (Ghaly, 2013) show pH 10 yields a higher amount of volatile fatty acids thus corresponding with the use of crude alkaline protease at pH 12 in hydrolysis of tilapia to yield both amino acids and fatty acids. Similarly, studies from (Halim et al., 2016) show the trends from 2011 to 2016 indicate that the effect of pH on the degree of hydrolysis is significant and that at high pH the yield increased. The pH has a considerable effect on both the structure of the substrate and the enzyme. An acidic environment affects the fish waste, for instance , the waste is protein nature and it renders the proton to remain on the carbonyl carbon and not on the amino group of the protein (Xu & Grassian, 2017). Denaturation is also likely to happen (Teo et al., 2016). This is the distortion of the primary structure of the protein which involves the sequence of the amino acids in the said peptide. This happens because the denaturation reactions are usually not strong enough to break the bonds of both the secondary structure from hydrogen bonding and tertiary structure. The protein in this environment will exist in a neutralization state; a salt bridge will be formed and this is where its final interaction with its environment is ionic between the H_3N^+ and the COO^-. Acids and bases thus disrupt this neutralization by replacing the positive and negative ions in the salt change partners with the new ions in acid or base introduced (Jenks, 2005). Enzymes also being protein in nature have extreme pH environments affecting their structure and thus their activity in terms of the reactions they catalyze (Balcão & Vila, 2015). Like for the amino acid, it results in the protonation of the amino

group plus deprotonation of the carboxyl group of the peptide while for the enzyme it results in the unfolding of the alpha helix and beta sheets of the enzyme thus uncoiling it into a random shape thereby destroying the active sites present on the enzyme structure (Stegeman et al., 1992).

4.3 Effect of Temperature on Hydrolysis of Fish Waste

Temperature like pH has effects on both the structure of the enzyme and that of the substrate which is protein in nature. Heat is known to disrupt the hydrogen bonds and the non-polar hydrophobic interactions present in protein structures (Teo et al., 2016). These protein structures also include enzymes. Thus an increase in temperature, causes an increase in the kinetic energy possessed by the molecules resulting into violent vibrations. This violent vibrations result to bond disruptions of both the fish waste substrate and the altering of the enzyme structure as well (Teo et al., 2016). Temperature as a factor affecting the hydrolysis of the fish waste was investigated by preparing the samples as the pH parameter investigation. They were then inoculated with $100ml$ of the enzyme. The effect of temperature on the hydrolysis on fish waste is as shown in Figure 6. The protease enzyme showed higher activity in the high temperature region, from 60°C to 80°C thus an increase rate of hydrolysis of the fish waste. This could be attributed to the fact that the enzyme is thermophilic with regards to where it was isolated from (Wanyonyi et al., 2014). Thus the optimum temperature for operation of the enzyme was found to be at 80°C. It is an enzyme that thrives in an extreme environment. At room temperature, the enzymatic activity was considerably lower as compared to the optimum temperature. This could be attributed to the high energy demand of the activation energy of the enzyme.

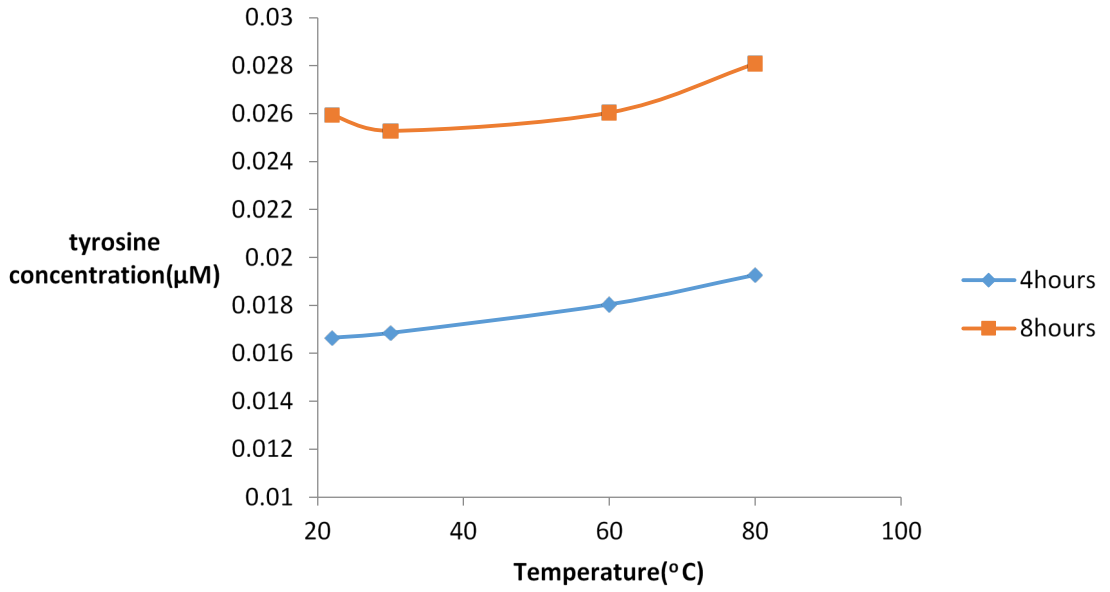


Figure 6: Effect of temperature on hydrolysis of fish waste

The effect of temperature has been reported to result in a similar fashion in that a rise in temperature results in a subsequent increase in rate of hydrolysis expressed in an increased yield (Srikanya et al., 2017). The degree of hydrolysis and yield subsequently increased over the range of temperature from 27°C to 70°C with 70°C producing the maximum yield. This is in line with other results like the (Gajanan et al., 2016) and (Tanuja et al., 2012) whose hydrolysate yield increased with an increase in temperature. Studies by (Linder et al., 2005) show that even with different enzymes at varied temperatures, the highest temperature is bound to have the highest yield. The enzymes used were Nutrease, Flavorzyme and alcalase all at the concentration of 0.5% had the yields of 17.2, 17.4 and 17.0% at temperatures of 45°C, 50°C and 55°C respectively. Research by (Halim et al., 2016) also shows that the degree of hydrolysis increases significantly by an increase in temperature.

4.4 Effect of Enzyme Concentration on the Hydrolysis of Fish Waste

Reaction rates, effects of various variables and formation of intermediates have a significant part in chemical kinetics of a reaction. The effect of concentration on reaction rates is the determinant of the rate laws because the rates are expressed as the concentrations of the reactants. The optimization of this factor affecting the hydrolysis of fish waste was done by varying the concentration of the enzyme and keeping the amount of the substrate constant. The graph in Figure 7 shows the effects of concentration of the enzyme on the rate of hydrolysis of fish waste.

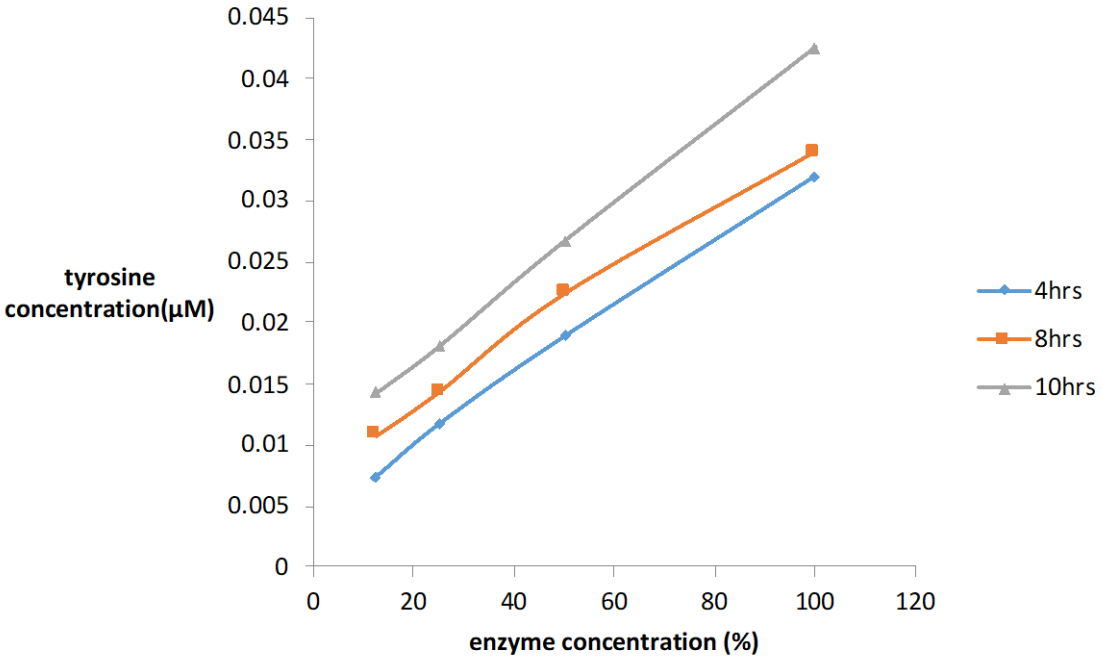


Figure7: Effect of concentration of enzyme on the hydrolysis of fish waste

The temperatures and the pH values were fixed at 80°C and 12 respectively. The tyrosine concentration rose with an increase in concentration of the enzyme. The tyrosine concentration also increased with the progression of time regardless of the concentration. This shows that

hydrolysis took place even in the samples with the least enzyme concentration. At four hours, the concentration of tyrosine obtained from 12.5% was 0.007431µM. The enzyme concentration experiment was performed with respect to crude enzyme with dilutions using distilled water to achieve the varied levels of concentrations. When the concentration was increased to 25%, the concentration of tyrosine liberated increased to 0.01172µM while a further increase in enzyme concentration to 50% also resulted in an increase in tyrosine concentration yield to 0.01889µM. 100% concentration of enzyme had the highest tyrosine concentration value of 0.03199µM at four hours. Generally, an increase in the concentration of crude enzyme resulted in an increase in the degree of hydrolysis. The results reported are comparable to (Ghaly, 2013) that disclosed that the oil yield increased with an increase in concentration in different parts of the fish and the whole fish. The degree of hydrolysis, measured in relation to the release of tyrosine, increased through all the concentrations present. It stabilized and formed a constant trend throughout the ten hours that this experiment was carried out. The amount of tyrosine released at the end of the eight hours was dependent on the concentration of the enzyme. Thus, the crude enzyme concentration with the highest value indicated the highest degree of hydrolysis. There exists a relatively linear relationship between concentrations of the enzyme and the degree of hydrolysis.

Time also has an effect on the degree of hydrolysis and yield in terms of concentration of tyrosine in that increasing the time taken for hydrolysis increased the concentration of tyrosine yielded also shown in Figure 7. The results showed that hydrolysis increased as time was increased. At four hours, the enzyme concentration at 12.5% had 0.007431µM while at eight hours, it increased to 0.1077µM and at ten hours the tyrosine concentration yield was at 0.01427µM. There were increases in the 25, 50 and 100% enzyme concentrations over time as shown from four to ten

hours. This is in line with (Ghaly, 2013) as it exhibited the yield of oil and protein from the enzymatic hydrolysis of different parts of mackerel fish increased with an increase in time.

4.5 Order of Reaction and Determination of K_M

To establish the reaction order, the product concentration which is the amount of tyrosine released was plotted against time at the optimum concentration of 100% as in Figure 8 below.

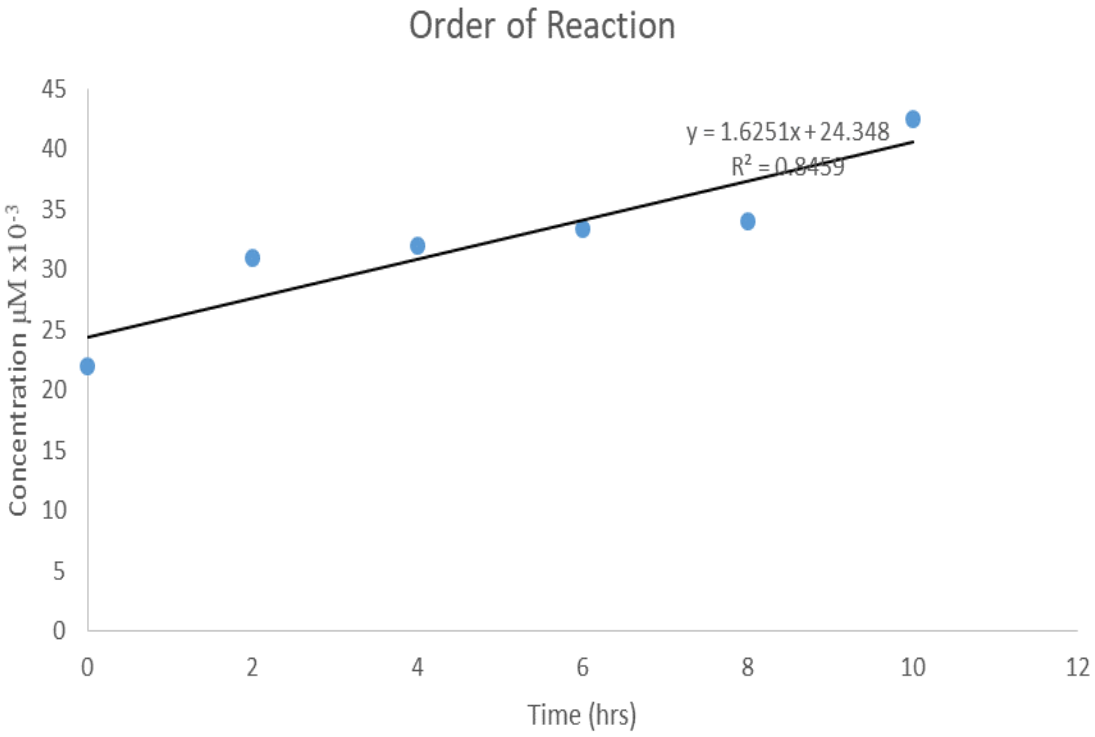


Figure 8: Graph showing the determination of order of the reaction and k

The graph shows an increase in the amount of tyrosine released, the product, increased as time progressed. It shows that the rate of the reaction is independent of the reactant concentrations and thus the plot gives a straight line indicating a zero-order reaction and from the gradient, the k is

determined from the slope as 0.004495μM/s. A further plot of the ln values of the concentration was plotted against time to further prove that it is indeed a zero order reaction. The graph in figure 9 shows the plot and the linear relationship between the concentrations of the product against time.

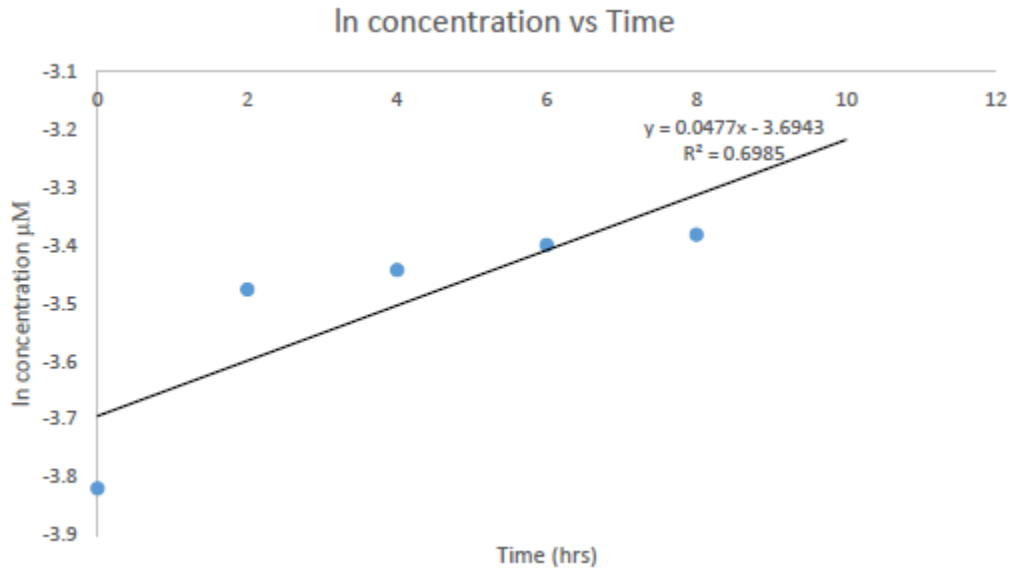


Figure 9: ln concentration of Tyrosine released at 10 hours vs Time

For the determination of the K_M value, the values chosen were after hydrolysis had fully taken place after ten hours. The substrate concentrations were obtained from the standard curve and the curve generated in the figure10 below of rate vs substrate concentration.

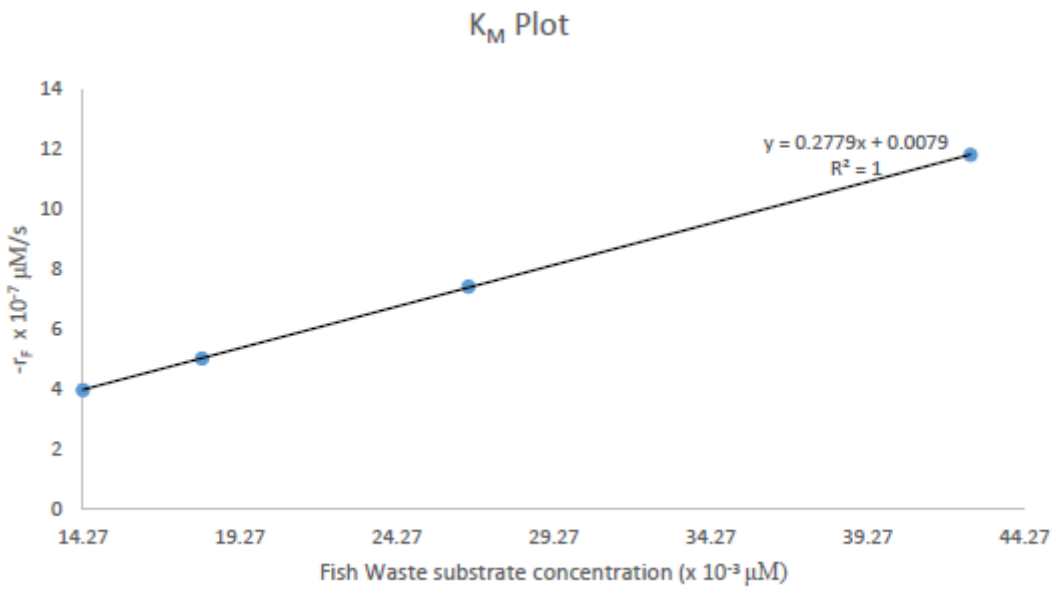


Figure 10: Michaeli's Menten curve determining V_{max} and K_M

The K_M value obtained from the plot is 2.1255 x 10^{-7} µM

The Kinetics of the reaction was established by monitoring the experimental courses of reaction and having the model equations fitted into respective times and concentrations (Yoshida et al., 2003). Studies on the kinetics of various hydrolysis reactions show that the kinds of reactions vary with regards to enzyme-substrate concentration as shown by (Yoshida et al., 2003). The K_M curve was obtained by plotting the concentration of tyrosine liberated at ten hours against the rate of tyrosine production in µM/s.

4.5 Amino Acid Composition

The vitality of amino acids cannot be overly stressed as they have different functionalities because different molecules have different amino acid sequences which influence both their characteristics and functionalities (European Pharmacopeia, 2005). The freeze dried samples were subjected to o-phthaldialdehyde (OPA) pre-column derivatization and analyzed using RP-HPLC. The chromatograms shown below indicate the amino acid distribution in the internal standard and the sample respectively.

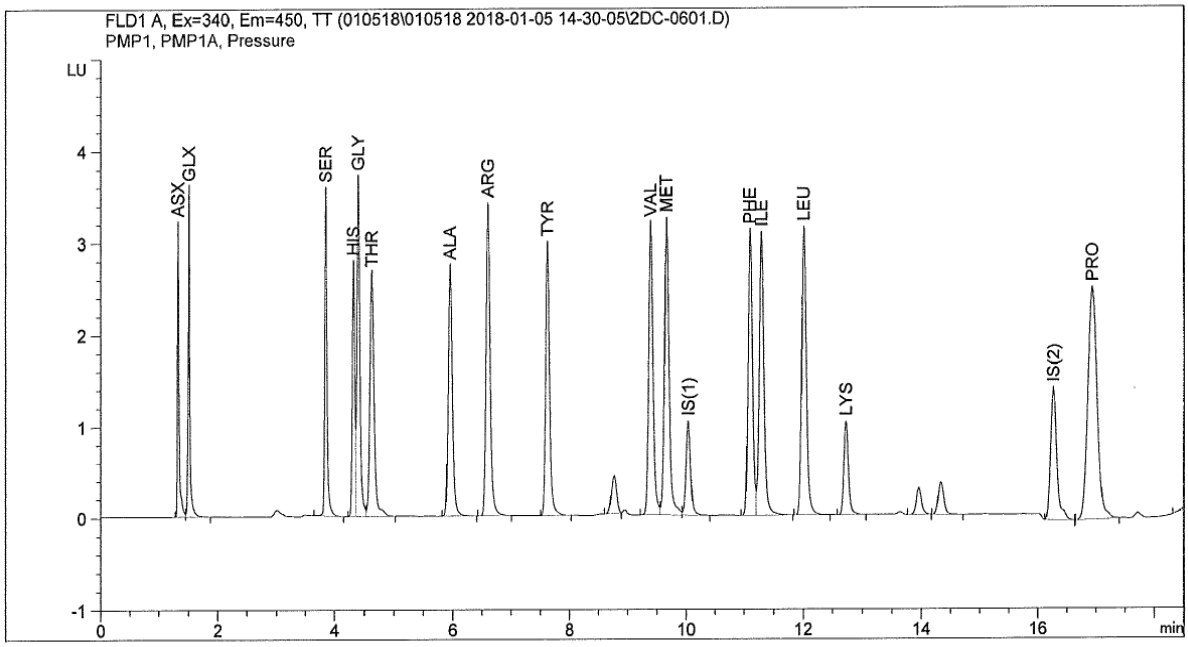


Figure 11: HPLC Chromatogram showing the Internal Standards.

The chromatogram in figure 11 shows the distribution of the amino in the standard while the chromatogram in 12 shows the amino acid distribution in the sample. The various peaks as outlined in the chromatogram and their corresponding areas indicate to the presence of the various amino acids present. The peaks appear in correspondence to their various retention time which points to the amount of time taken to elute.

The peaks that appear earlier in the chromatogram show that they are the least polar and thus elute faster than the most polar amino acids in a RP-HPLC. It is this standard chromatogram that shows the corresponding peaks to the various amino acids with which the sample was run against.

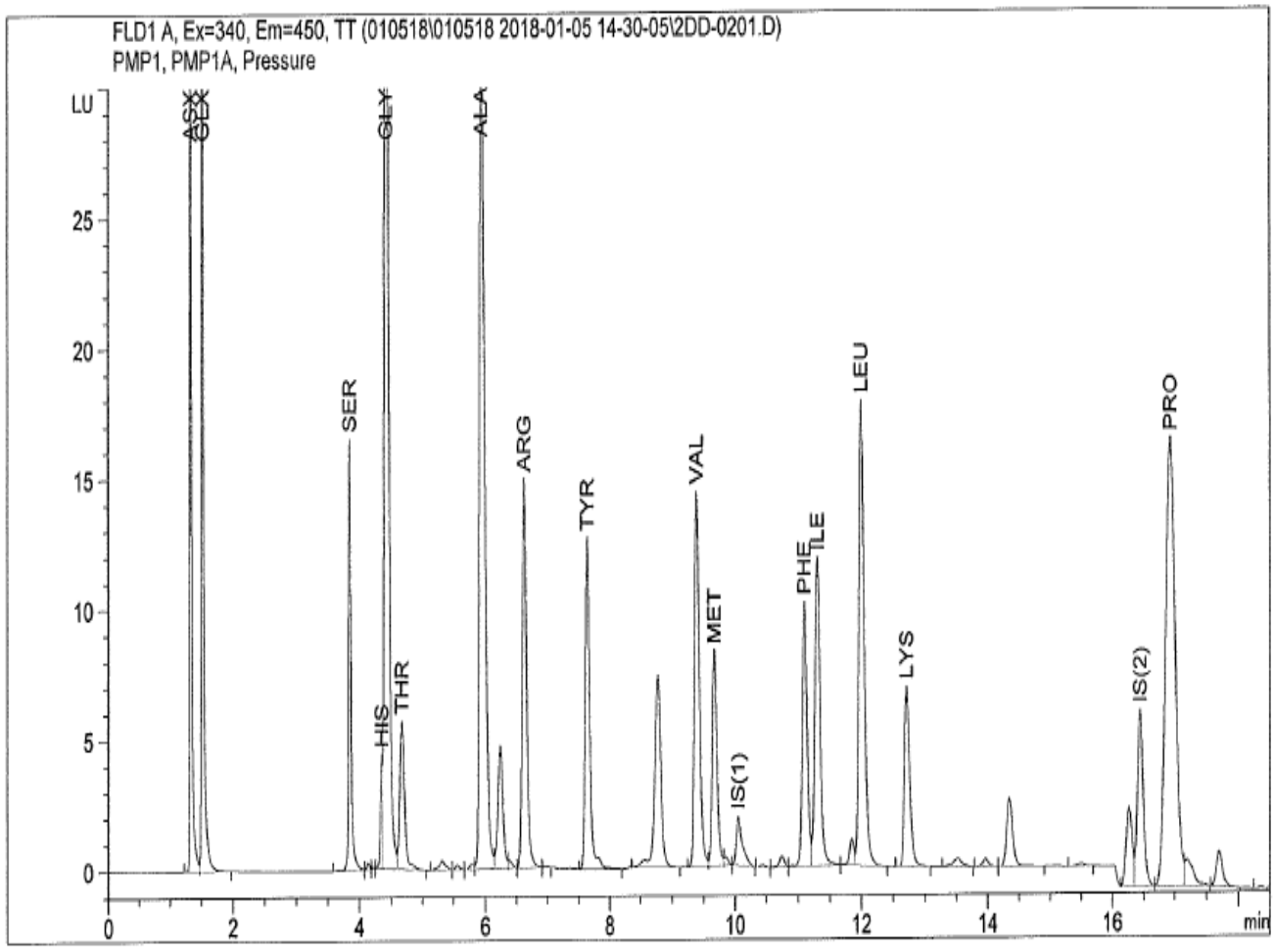


Figure 12: Chromatogram Showing the Amino Acid in the FPH.

The figure 12 shows the chromatogram of amino acids present in the FPH sample with their corresponding peak heights and areas in line with their retention time. The peak height and areas in the figure corresponds to the amount of amino present in the samples; thus, figure 11 and 12 show that the analytical method used in modification achieved the correct separation and

identification the amino acids as figure 12 peaks correspond to those of the standards. As per the chromatogram on figure 12, the highest amounts of amino acids were asparagine, glutamine, glycine and alanine while the lowest amounts were histidine and threonine.

The tables 4 and 5 show a breakdown of the essential amino acids present in the FPH and concentrations are dependent on the hydrolysis parameters (Asyiraf & Abdul, 2011). The tilapia fish hydrolysate was most rich in glycine and alanine with quantities of about 61 and 49µmol respectively. This is similar reports by other authors that the most concentrated amino acids were glycine and alanine (Asyiraf & Abdul, 2011). The least concentrated amino acid was histidine. This is also similar to research conducted on pseudo cereals in which histidine was also the least amino acid present (Mota et al., 2014). The importance of amino acids in both humans and animals in that they form the building clocks of proteins and thus various cells and cell components like glycine forms a major part of the human skin collagen component while the phenylalanine, proline, arginine, serine, alanine and isoleucine form a polypeptide that aids in healing and tissue repair in humans (Tenyang et al., 2014). The amino acids present in a sample also point towards the food item's ability to meet an organism's nutritional requirements. Amino acid composition and amount vary from different species as indicated by (Tenyang et al., 2014) in the analysis of various fish species from Cameroon

Table 4: Amino Acid composition in µ moles of *Oreochromis niloticus* of Lake Victoria, Kenya.

Amino Acid	Amount(µmoles) Trial 1	Amount(µmoles) Trial 2	Amount(µmoles) Trial 3
Asparagine	32.7725 ±2.4105	29.3963 ±0.02319	25.9218 ±2.4336
Glutamine	38.7930 ±2.3885	36.0416 ±0.4429	31.4109 ±2.8314
Serine	12.7628 ± 1.4542	11.6685 ±0.6804	7.6876 ±2.1345
Histidine	3.3652 ± 0.1043	3.0924 ±0.08864	3.1955 ±0.01567
Glycine	64.0413 ±1.7206	63.1158 ±1.0662	57.6669 ±2.7867
Threonine	6.9728 ±0.4794	6.4116 ±0.08252	5.5001 ±0.5619
Alanine	49.9475 ±2.2709	46.6965 ±0.02787	43.5637 ±2.2430
Arginine	12.9704 ±1.0077	11.603 ±0.04075	10.0626 ±1.04842
Tyrosine	12.6764 ±0.9839	11.3543 ±0.04908	9.8240 ±1.03299
Valine	15.1819 ±0.8373	14.2222 ±0.1587	12.5893 ±0.9959
Methionine	7.8501 ± 0.6032	7.4153 ±0.2958	5.7255 ±0.8990
Phenylalanine	10.416 ±0.7636	9.3517 ±0.01092	8.2409 ±0.7744
Isoleucine	12.6302 ±0.7708	11.6619 ±0.08609	10.328 ±0.8568
Leucine	19.357 ±1.2838	17.6603 ±0.08418	15.6066 ±1.3679
Lysine	22.878 ±1.6459	20.1866 ±0.2571	18.5861 ±1.3887
Proline	11.0117 ±4.0745	22.1655 ±3.8124	17.1445 ±0.2620

Amino acid analysis also as compared to the hydrolysate of other fish species by (Chalamaiah et al., 2012) contrasted in that the most abundant amino acid across board was glutamic acid while the least being methionine. This may be credited to the point that the species worked on were marine fish species and mostly pelagic and that the enzymes used to hydrolyze the fish waste (Villamil et al., 2017).

Table 5: Mean Amino Acid composition in µ moles of *Oreochromis niloticus* of Lake Victoria, Kenya

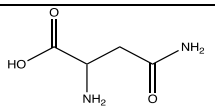
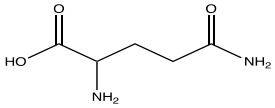
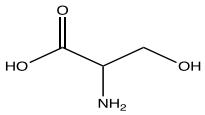
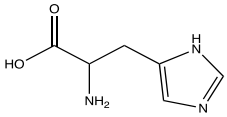
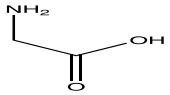
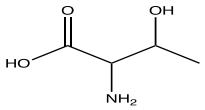
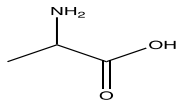
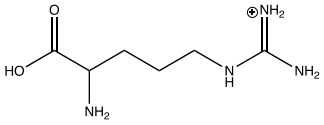
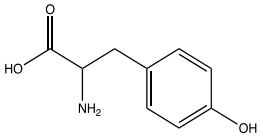
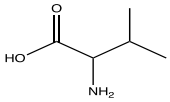
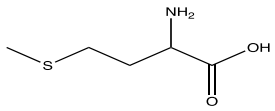
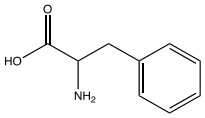
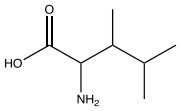
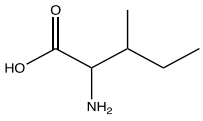
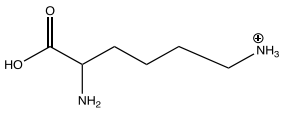
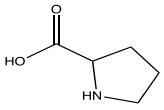
Amino Acid	**Average Amount(µmoles)**	**Structure**
Asparagine	29.36 ±1.15	
Glutamine	35.42 ±1.33	
Serine	10.71 ±1.00	
Histidine	3.22 ±0.27	
Glycine	61.61 ±1.31	
Threonine	6.30 ±0.26	
Alanine	46.74 ±1.07	
Arginine	11.55 ±0.49	

Table 5 continued: Mean Amino Acid composition in µ moles of *Oreochromis niloticus* of Lake Victoria, Kenya

Amino Acid	Average Amount(µmoles)	Structure
Tyrosine	11.28 ±049	
Valine	14.00 ±0.47	
Methionine	7.0 ±0.42	
Phenylalanine	9.34 ±0.37	
Isoleucine	11.54 ±0.40	
Leucine	17.54 ±0.79	
Lysine	20.55 ±0.78	
Proline	16.77 ±1.92	

Different enzymes used to hydrolyze fish waste also produce different amounts of amino acids like in (Ojha et al., 2016) shows the difference in using alcalase and protease as the degree of hydrolysis achieved is different. It also emphasizes the difference in the amounts of amino acids due to the difference in species.

The boar fish had histidine as the most abundant while the Nile tilapia had glycine as the most abundant amino acid present from both alcalase and protease (Ojha et al., 2016).

4.6 Fatty Acid Analysis

For the analysis of the fatty acids, their respective FAME derivatives were prepared as (Carvalho & Malcata, 2005). The fatty acid analysis was done by the GC equipped with the FID and the chromatogram obtained is as figure 13.

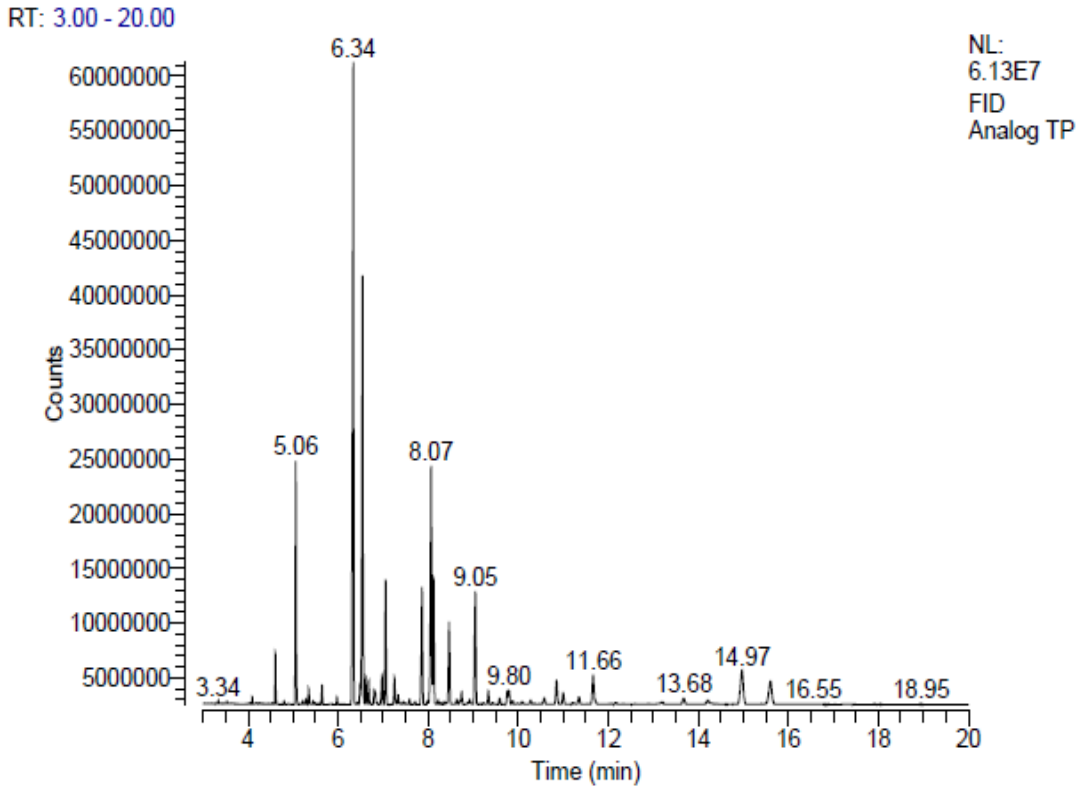


Figure 13: Chromatogram of fatty acid methyl esters from enzymatic hydrolysis of *Oreochromis niloticus* waste: 5.06, myristic; 6.34, palmitic; 8.07, oleic; 9.05, linoleic; 9.80, eruric; 11.66, eicosapentanoic & behenic; 14.97, osbond.

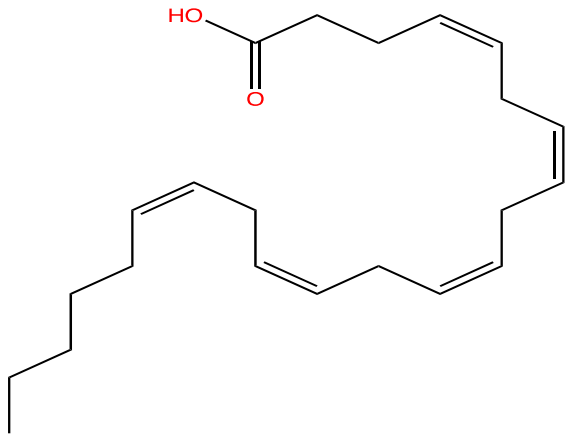
The peaks shown in the chromatogram in figure 13 are of fatty acids and the peaks assigned to their respective fatty acids with regards to their retention time which is the time taken to elute or time taken to reach the detector.

The peak height and area also symbolize the abundance of the fatty acid thus the most abundant fatty was that with retention time 6.34 minutes followed by the 5.06 minutes. The least abundant fatty acid was the one at retention time of 4.09 minutes and 2.57 minutes. The table 6 also gives a breakdown of the amino acid with regards to quantity, the ω (omega) shows the type of omega group it belongs to and identity: with the most abundant fatty acid was palmitic acid which is hexadecenoic acid. The fatty acids were identified with reference to a standard chromatogram of fatty acids.

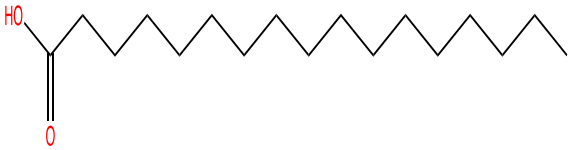
The Table 6 delivers a summary of the content of the saturated, monounsaturated, polyunsaturated fatty acids (PUFA) and ω-3/ω-6 PUFA ratios. The structures of the most predominant fatty acids are also listed. The *Oreochromis niloticus* waste comprised 17 fatty acids. The highest fraction of the fatty acid from the data shown in the table and from the chromatogram in figure 13 is the mono-unsaturated fats followed by the poly-unsaturated fats then the saturated fats. It is also prudent to say that the quality and the composition of the oil is dependent on the quality of the raw material and the conditions in which the hydrolysis and methods of extraction. The fatty acid that was in abundance relative to the rest in this case was the palmitic acid, followed by hexadecenoic acid. Stearic acid, oleic and oleic followed closely in terms of % abundance. Palmitic acid had an area of 29.76% while hexadecenoic acid had an (Routray et al., 2018) area of 17.99%. These results are however not similar to (Routray et al., 2018) in that the most abundnat fatty acids are the saturated fats followed by the mono-usaturated fats and the poly-unsaturated fats are the least abundant. This can be attributed to the difference in species analysed and the kind of enzyme which is also similar to the analysis of marine lipids investigated by (Carvalho & Malcata, 2005) on the fattyacid derivatization of their methyl esters for GC analysis as they had almost similar results.

Table 6: Identity, Area and %Area of Fatty acids.

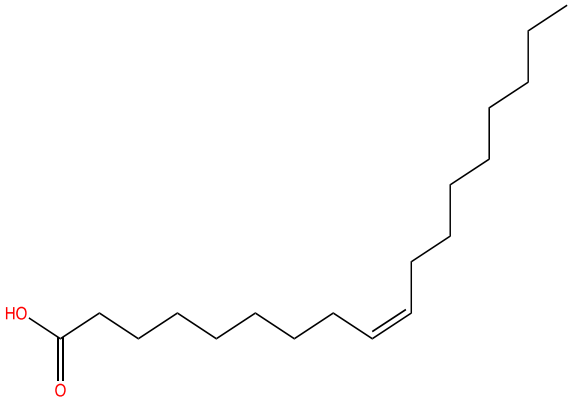
Fatty Acid	ω-n	Retention Time	Area	%Area
8:0 (caprylic acid)		2.57	590179	0.17
12:0 (lauric acid)		4.09	577652	0.16
14:0 (myristic acid)		5.06	26602588	7.49
16:0 (palmitic acid)		6.34	105764377	29.76
16:1 (palmitoleic acid)	ω-7	6.54	63940459	17.99
17:0 (Margaric acid)		7.05	18139199	5.10
18:0 (stearic acid)		7.86	19580362	5.51
18:1 (oleic/elaidic/vaccenic acid)	ω-9/ ω-9/ ω-7	8.07	42345824	11.92
18:2 (linoleic/ linolelaidic acid)	ω-6	8.46	13767140	3.87
18:3 (γ/α linolenic acid)	ω-6/ω-3	9.05	19391875	5.46
20:0 (arachidic acid)		9.59	1044518	0.29
20:1 (paullinic acid)	ω-9	9.80	2377379	0.67
20:5 (eicosapentanoic acid)	ω-3	11.66	8033860	2.26
22:0 (behenic acid)		11.66	8033860	2.26
22:1 (erucic acid)	ω-9	12.17	481191	0.14
22:5 (osbond acid)	ω-6	14.97	14042530	3.95
22:6 (docosahexaenoic acid)	ω-3	15.60	10681008	3.01



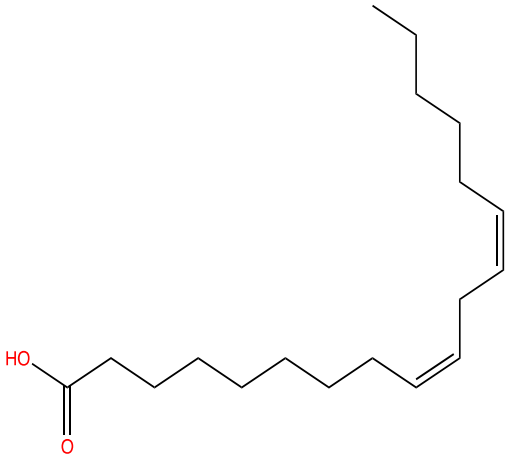
Osbond acid (PUFA)



Palmitic acid (Unsaturated fatty acid)



Oleic acid (monosaturated fatty acid)



Linoleic acid (18:2)

4.7 Proximate Analysis.

Proximate analysis essentially determines the nutritive value of a substance that is aimed at being used as food. The viscera hydrolyzed using the protease enzyme and the hydrolysate freeze dried in readiness for determining the proximate conformation and the results given as in table 7. The kjeldahl method for total crude protein determination was used with the aid of a nitrogen conversion factor (Latimer 2012). The factor was vital as it was used in the calculation of the crude protein content that was present in the sample. It was established that the hydrolysate had a relatively high crude protein content of an average of 54.2% and a high ash content of 21.8%. The high amount of ash could be attributed to existence of acid soluble and insoluble minerals. In comparison to other protein hydrolysates by other enzymes like alcalase showed that the crude protein content was higher like in 80% (Bhaskar et al., 2008; Chalamaiah et al., 2012). The high ash content is also in line with other researchers who had values of 20-24% (Shahidi & Han, 1995; Thiansilakul et al., 2007). The relatively high protein content could also be attributed to the dissolution of protein during hydrolysis coupled with the removal of lipid and the other insoluble and non-protenious substances (Thiansilakul et al., 2007). FPH have been investigated to have crude fat. Nile Tilapia (*Oreochromis niloticus*) was established to have a fat content of 12.8%.

This was relatively lower than the other fresh water fish like the Nile Perch (*Lates niloticus*) as the latter is considered a fatty fish. Moisture content making up the 10% of the fish protein hydrolysate. The least predominant items were the crude fibre and carbohydrates accounting for 0.6 and 0.2 respectively. It is also worth noting that different parts of the fish waste have different compositions and thus the difference in the values obtained in proximate analysis whereas the fish sample used for this was made up of the head, the viscera, skin and fins.

Table 7: FPH Proximate composition.

Parameters	**Trial 1 %**	**Trial 2 %**	**Average %**
Protein content	53.9054 ± 0.2946	54.4946 ± 0.2946	54.2
Moisture content	10.63 ± 0.23	10.17 ± 0.23	10.4
Total Ash content	20.3152 ± 1.4848	23.2848 ± 1.4848	21.8
Crude fat content	12.3735 ± 0.4265	13.2265 ± 0.4265	12.8
Crude fibre	0.7108 ± 0.1108	0.4892 ± 0.1108	0.6
Carbohydrate content	0.20651± 0.00651	0.19349 ± 0.00651	0.2

4.8 FT-IR Analysis of the FPH

The FT-IR analysis is vital in determining the functional groups present in the sample being analyzed. The usefulness in this is that it helps in determining the effectiveness of the hydrolysis. This is to ensure that hydrolysis only severed only the peptide bonds and the individual amino acids remain intact with unreacted functional groups. The freeze dried FPH sample was crushed and loaded on the FT-IR for analysis. The spectrum obtained is as figure 14. The FT-IR spectrum of the FPH showed peaks between 4000 – 600 cm^{-1}. The FPH showed the broad absorption band at 3259.70 cm^{-1} attributed to distending of the hydroxyl group. The peaks at 2924.09 and 2854.65

cm^{-1} are ascribed to the C-H stretching of an alkane. The peaks at 2360.87 and 2330.01 cm^{-1} peaks are attributed to O=C=O stretching. The amine bending resulted in peaks around the 1558.48 and 1647.21 cm^{-1} . The other peaks in the fingerprint region correspond to substituted C-H and amine bending and stretching respectively. Thus the FT-IR results indicate that the functional groups that make up an amino acid are present in the FPH and as such, the process of hydrolysis served to break the peptide linkages and change the structure of the amino acid. These results are correspondent to the amino acid analysis as by (Shankar & Rhim, 2015) on synthesis of nanoparticles of Hg mediated using amino acids and those of (Tesfaye et al., 2017) from protein hydrolysate obtained from the hydrolysis of chicken feathers. Therefore, the presence of proteinious functional group from the FPH renders it useful as an ingredient or blending material in producing animal feeds, pharmaceuticals, cosmetics and bioplastics.

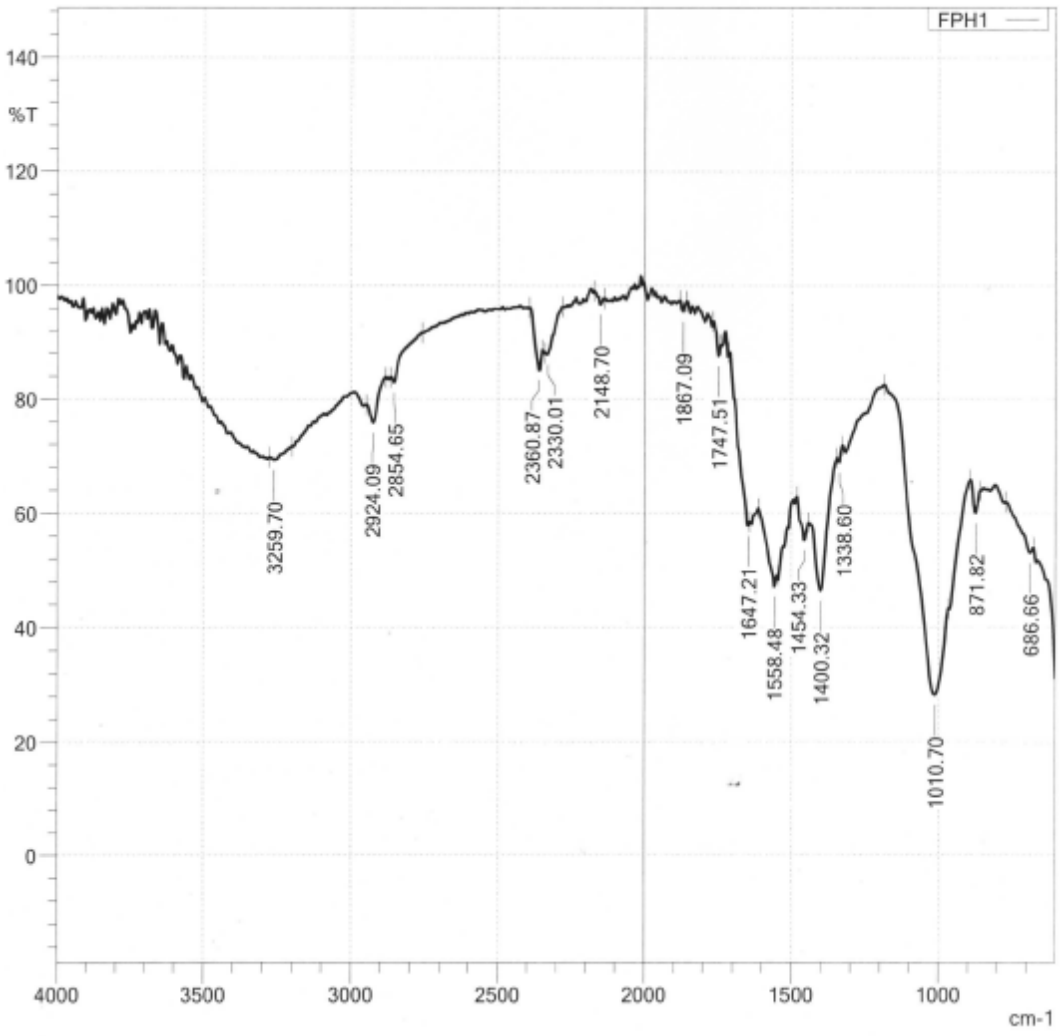


Figure 14: FT-IR spectrum of FPH

CHAPTER 5

5.1 CONCLUSION

The fish waste was hydrolyzed using the crude protease enzyme and the hydrolysate characterized using HPLC, GC-FID and IR to determine the amino acid, fatty acid and the efficiency of the enzyme in hydrolysis of the different kinds of fish waste (head, viscera, skins, bones and fins). The hydrolysate from the characterization was seen to contain the essential amino acids and poly unsaturated fats while the functional groups present on the IR spectra indicated that the hydrolysis did not alter the composition of the metabolites from hydrolysis.

The study revealed that the temperature, pH and enzyme concentration of 80°C, 12 and 100% of enzyme respectively was optimal for the process. Based on these outcomes from the characterization of the hydrolysate, enzymatic hydrolysis was considered suitable for fish waste hydrolysis over chemical hydrolysis as it achieved a higher degree of hydrolysis achieved over time. FPH would be considered suitable for application in the feed formulation industry to solve the need for protein foods.

5.2 Recommendations

1. Design ways of protease separation and purification so as to make the products fit for consumption by humans.
2. Methods of determining the fish waste substrate concentration and the enzyme concentration should be come up with for the proper assertion of the kinetics involved in these reactions.
3. Methods of determining time taken and quality of chemical hydrolysis product determination vis a vis products of enzymatic hydrolysis

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APPENDICES

Appendix 1

Table 8: Effect of pH on the Enzymatic Hydrolysis of Fish Waste

pH/Time	0 hours	1 hours	2 hours	4 hours	6 hours	8 hours	10 hours	12 hours
pH 3	0.237	0.393	0.806	0.525	0.396	0.426	1.049	0.944
pH 4	0.201	0.388	1.106	0.563	0.411	0.431	0.92	0.97
pH 5	0.245	0.41	0.647	0.656	0.428	0.496	1.088	1.024
pH 6	0.24	0.42	1.558	0.813	0.394	0.516	1.073	1.127
pH 7	0.235	0.429	2.206	0.66	0.427	0.49	0.193	1.096
pH 8	0.274	0.408	0.96	0.667	0.435	0.982	1.184	0.626
pH 9	0.486	0.369	0.748	0.687	0.457	0.736	1.112	0.989
pH 10	0.458	0.384	0.569	0.751	0.486	0.927	1.064	1.567
pH 11	0.362	0.369	0.564	0.587	0.525	0.463	1.039	0.964
pH 12	0.376	0.491	1.176	0.651	0.539	0.503	1.05	0.964

Appendix 2

Table 9: Effect of pH on Chemical Hydrolysis of Fish Waste

pH/Time	0 hours	1 hours	2 hours	4 hours	6 hours	8 hours	10 hours	12 hours
pH 3	0.015	0.012	0.03	0.203	0.134	0.521	0.203	0.133
pH 4	0.016	0.015	0.031	0.187	0.161	0.123	0.184	0.101
pH 5	0.013	0.012	0.017	0.194	0.152	0.098	0.163	0.091
pH 6	0.006	0.011	0.02	0.178	0.147	0.125	0.174	0.103
pH 7	0.006	0.007	0.227	0.181	0.149	0.094	1.043	0.095
pH 8	0.015	0.011	0.018	0.546	0.162	0.114	0.112	0.089
pH 9	0.012	0.01	0.019	0.184	0.153	0.1	0.212	0.112
pH 10	0.011	0.01	0.021	0.185	0.164	0.087	0.157	0.095
pH 11	0.013	0.01	0.02	0.2	0.168	0.084	0.13	0.107
pH 12	0.013	0.008	0.02	0.191	0.173	0.091	0.042	0.11
pH 13	0.013	0.011	0.022	0.219	0.182	0.127	0.241	0.191

Appendix 3

Table 10: Effect of Temperature on the Enzymatic Hydrolysis of Fish Waste

Temp/ Time	**0 hours**	**2 hours**	**4 hours**	**6 hours**	**8 hours**	**24 hours**
R.T						
Trial 1	0.41	0.399	0.472	0.607	0.776	0.428
Trial2	0.438	0.377	0.435	0.584	0.655	0.429
Trial 3	0.422	0.386	0.439	0.584	0.667	0.497
30 ^{0}C						
Trial 1	0.447	0.399	0.441	0.614	0.673	0.496
Trial 2	0.397	0.413	0.43	0.628	0.691	0.461
Trial 3	0.422	0.408	0.492	0.647	0.68	0.497
60 ^{0}C						
Trial 1	0.417	0.398	0.502	0.732	0.695	0.513
Trial 2	0.432	0.424	0.443	0.631	0.658	0.484
Trial 3	0.428	405	0.514	0.661	0.753	0.466
80 ^{0}C						
Trial 1	0.458	0.413	0.557	0.939	0.88	0.463
Trial 2	0.39	0.489	0.507	0.755	0.641	0.512
Trial 3	0.435	0.425	0.494	0.817	0.75	0.462

Appendix 4

Table 11: Tyrosine Standard Calibration Curve Absorbance Values

ABS/Tyrosine(µl)	0	10	30	45	60	75	90	105	120	135	150
Trial 1	0.032	0.105	0.187	0.293	0.234	0.262	0.244	0.360	0.349	0.468	0.512
Trial 2	0.058	0.138	0.480	0.596	0.486	0.698	0.704	0.850	0.980	0.942	1.009
Trial 3	0.061	0.226	0.531	0.622	0.664	0.762	0.834	0.930	0.969	1.003	1.049
Trial 4	0.059	0.124	0.434	0.533	0.650	0.718	079	0.802	0.954	0.958	1.031

Appendix 5

Table 12 Fish Protein Hydrolysate Amino Acid Distribution with Retention Time (IS)

Amino Acid	Retention Time	Area
Asparagine	1.322	6.12485
Glutamine	1.507	6.88673
Serine	3.846	9.96204
Histidine	4.315	8.42323
Glycine	4.397	13.55205
Threonine	4.625	13.61808
Alanine	5.954	13.47452
Arginine	6.602	16.79645
Tyrosine	7.625	14.41628
Valine	9.391	16.72500
Methionine	9.663	17.48376
Internal Standard (1)	10.036	5.55463
Phenyalanine	11.095	16.23794
Isoleucine	11.290	17.44531
Leucine	12.007	17.33976
Lysine	12.732	5.55954
Internal Standard (2)	16.264	10.91908
Proline	16.931	28.11209

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