UNIVERSITY OF EMBU

DENNIS CHERUIYOT

MSc

2025

EXTRACTION, CHARACTERIZATION, AND OPTIMIZATION OF PROTEIN FROM WASTE-ACTIVATED SLUDGE AND FOOD WASTE

DENNIS CHERUIYOT

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE IN CHEMISTRY OF THE UNIVERSITY OF EMBU

AUGUST, 2025

DECLARATION

This thesis is my original work and has not been presented elsewhere for a degree or any other award.

Signature…  ……Date.....27/08/2025

Dennis Cheruiyot

B523/1625/2022

Department of Physical sciences

University of Embu

This thesis has been submitted for examination with our approval as the University Supervisors.

Signature .....  ..... Date 28/08/2025

Dr. Seth Apollo

Department of Pure and Applied Chemistry,

Masinde Muliro University of Science and Technology

Signature .....  ..... Date 28/08/2025

Dr. George Simba Nyamato

Department of Physical Sciences

University of Embu

DEDICATION

This work is dedicated to my loving mother, Debora, and my dear siblings (Duncan, Sheila, and Vicky) for their unwavering support and prayers throughout my academic journey. Words alone cannot describe how much I am grateful for your constant belief in me. Your love, encouragement, and sacrifices have been my greatest strength. This achievement is not mine alone; it is yours as well.

ACKNOWLEDGEMENT

I express my deepest gratitude to the Almighty God for granting me the strength, good health, and perseverance throughout my academic journey. I sincerely thank the University of Embu for awarding me the scholarship opportunity to pursue my master's degree. I am especially grateful to my supervisor, Dr. Seth Apollo, for his excellent supervision, timely wake-up calls, and invaluable guidance and input throughout this work. I also wish to acknowledge Dr. George Nyamato for his exceptional supervision, consistent support, and invaluable input in this work. My heartfelt appreciation goes to the chemistry laboratory staff, led by Mr. Mukono, for their technical support and assistance during my lab work. I also extend my sincere thanks to my friends Hellen, David, Nancy, Mercy, Kelvin, Frankline, and Robin for their encouragement and moral support during this journey. Special thanks to Shadrack Kibet and Mercy for their support in the LC-MS analysis. My appreciation also goes to Dr. Sharon Kiprotich, Dr. Jane, and Mr. James of Muranga University of Technology for their unwavering support in FT-IR analyses.

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LIST OF ABBREVIATIONS/ACRONYMS

AS	Activated sludge
ANOVA	Analysis of variance
DESTW	Dandora Estate Sewage Treatment Works
EAAs	Essential amino acids
EU	European union
FAO	Food and agricultural organization
FSC	Food supply chain
FTIR	Fourier Transform Infrared Spectroscopy
FW	Food waste
HPLC-MS	High-performance liquid chromatography-Mass Spectrometry
ICP-OES	Inductively coupled plasma-optical emission spectroscopy
KSTW	Kariobangi Sewage Treatment Works
KW	Kitchen waste
MOWASSCO	Mombasa Water Supply and Sanitation Company
NEEAs	Non-essential amino acids
RSM	Response surface methodology
SCP	Single-cell protein
TS	Total solids
TSS	Total suspended solids
UN	United nations
VOCs	Volatile organic compounds
VSS	Volatile suspended solids
WAS	Waste-activated sludge
WHO	World Health Organization

ABSTRACT

There is a growing interest in the recovery of valuable biomaterials from waste in line with reaping the benefits of a circular economy. Organic wastes, such as waste-activated sludge (WAS) and food waste (FW) contain substantial amount of protein that can be recovered for various applications. The animal feed industry faces unprecedented challenges and might be interested in such a product, as the high demand coupled with the high costs of feeds has put so much pressure on the available resources. This study evaluated the potential of waste-activated sludge and food waste as alternative sources of proteins for animal nutrition. Thermal alkaline and acid hydrolysis methods were used in the extraction of protein from FW and WAS. The possibility of enhancing protein yield and quality through the co-extraction of protein from WAS and FW was also investigated. Response surface methodology (RSM) was used to optimize the extraction process. Before extraction, WAS was purified by removing heavy metals using acid pretreatment. Also, the energy efficiency of the extraction process was investigated. It was established that FW had 21.5 g/100 g protein, while WAS had 19.9 g/100 g protein. The two extraction methods had superior extraction of protein from WAS as compared to FW. In addition, co-extraction of protein from FW and WAS did not significantly increase the yield. Furthermore, optimization using RSM showed that the optimal yield of 15.8 g/100 g was obtained at a pH of 13 and a temperature of 120 °C, close to the experimental yield of 16.6 g/100 g for WAS. Besides, the extraction process was most energy efficient at a temperature of 120 °C. Moreover, LC-MS analyses of the extracted protein showed that WAS had a good essential amino acid profile with threonine, lysine, leucine, methionine, and valine in concentrations of 3.3, 2.7, 1.8, 1.1, and 2.7 g/100 g, respectively. The adequate concentration of threonine pointed to the potential beneficiation of WAS as an animal feed supplement. In addition, the ratio of the first limiting amino acids (lysine to methionine) of 2.5 met the standards for application as an animal feed supplement. Given this favorable amino acid composition, WAS presents a viable option for use as a protein source in animal nutrition. This study is significant in the management of the growing stream of waste-activated sludge and food waste. Repurposing these wastes into animal feeds not only contributes to sustainable waste management practices but also reduces the environmental burden associated with their disposal.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Animal feed is an important factor in the nutrition of animals, directly impacting their growth, health, and overall well-being. To achieve proper development levels, production animals need to be nourished with a balanced nutrition containing all the classes of nutrients (Carter & Lipman, 2018). However, the animal feed industry faces unprecedented challenges due to the high demand for feeds resulting from a broad mismatch between the supply of feeds and the rising livestock population. Subsequently, animal feed has become the largest single cost item in animal production, accounting for 60-85% of the total cost (Pinotti et al., 2021a). Of all the dietary components of animal feeds (protein, vitamins, minerals, and fiber), protein remains the most expensive and presents the greatest challenge for future feed formulation (El-Sayed, 2020; Van Krimpen & Hendriks, 2019). The main protein sources currently available in the market are fishmeal, meat and bone meal, soybean meal, wheat, corn, and barley (Karau & Grayson, 2014). However, these conventional sources are characterized by high prices and lack of sustainability because of their dependance on finite natural resources, and ecological impacts such as overfishing and deforestation (Malcorps et al., 2019). Additionally, using human-edible cereals, soybeans, and other oilseeds as animal feed is viewed as direct competition against human food security (Kim et al., 2019). Therefore, to meet the animal feed demands and enhance the self-sufficiency level, research on alternative sources of protein is needed.

Protein can be provided through the cultivation of various microbes, generally referred to as single-cell protein (SCP) (Ritala *et al*., 2017). Waste-activated sludge (WAS), a by-product of the activated sludge process used to treat wastewater, is rich in this type of protein, having a concentration of 20-60 g protein/100 g WAS (Gao et al., 2022a; Skripsts et al., 2024; H. Wang et al., 2021). Subsequently, protein can be recovered from WAS and potentially used to supplement animal feed (Chirwa & Lebitso, 2014; Singh *et al*., 2020). Valorization of WAS addresses the challenges associated with sludge treatment and recycling, such as high sludge generation,

pathogen removal, and disposal, which will make the overall process more environmentally friendly (Gupta et al., 2021).

Extraction of protein from waste-activated sludge involves the disruption of both the extracellular polymeric substances and intracellular materials to release protein into the bulk liquid (K. Xiao & Zhou, 2020a). This is because sludge is composed of a complex microbial structure with a hard cell wall. Therefore, a suitable method of protein extraction is required to break the cell walls and access the sludge protein. Consequently, WAS protein can be extracted from WAS via physical, chemical and biological methods or a combination of these techniques (Xiang et al., 2017a). Among these methods, chemical methods are famous due to their efficiency in breaking sludge flocs and enhancing protein solubilization into the aqueous phase (Taşeli, 2020). Studies have shown that hybrid methods such as acid-thermal and alkaline-thermal methods of hydrolysis have synergistic effect, leading to a greater WAS solubilization and protein yielding than single treatments (Kakar, Liss, et al., 2022; Siami et al., 2020).

The yield and quality of protein from WAS could be potentially improved by conducting co-extraction of protein from a mixture of WAS and other wastes such as food waste. As one of the most underutilized resources, proteins in food waste are lost at an alarming rate (Nath et al., 2023). Most researchers have focused on the reduction of food waste and food loss, but very little has been done on the extraction of valuable materials such as protein from food waste. This study, therefore, sought to improve the extraction of protein from WAS and food waste through optimization of extraction conditions, thus ensuring maximum protein recovery. Additionally, the study examined the amino acid profile of the extracted WAS protein.

1.2 Statement of the problem

The enormous amount of waste-activated sludge from municipal waste water treatment plants has become a critical issue globally, as sludge production is constantly growing due to rapid urbanization coupled with high population growth. The disposal of WAS is an environmental menace. Moreover, the treatment and management of WAS accounts for 25 – 60% of the total operating costs of municipal wastewater treatment plants (Otieno et al., 2019). Similarly, food waste impacts the environment by releasing harmful gases during degradation in open dumping sites

(Pandey, 2021). It has been reported that these organic wastes have valuable biomaterials such as proteins, which have the potential to be used to mitigate the challenges facing the animal feed industry (Rasool et al., 2023). In Kenya, up to 80% of the feed ingredients are imported, which has a significant effect on the price and quality of raw materials (Varol, 2024). Also, according to Kenya's ministry of agriculture and livestock reports, Kenya is grappling with a 60% feed deficit (Vidija, 2025). Moreover, farmers have raised concerns about the safety of animal feeds due to the entry of genetically modified commodities into the feed industry. To address these issues, an alternative is needed for a safe and cost-effective animal feed supplement. Protein extraction from WAS and FW, therefore, is a viable process to mitigate animal feed challenges. Thus, this research aimed at extracting protein from FW and WAS, optimizing extraction conditions, and assessing the nutritional properties of wastes for animal feed supplementation.

1.3 Justification of the study

This research was motivated by the urgent need to explore sustainable waste management practices. Thus, the recovery of protein from WAS will help in addressing the pressing need to manage the growing stream of WAS. As in many developing countries, in Kenya, sludge from on-site sanitation systems is emptied and discharged directly into natural channels or transported and disposed of untreated into the environment (Okoth et al., 2017). Consequently, the lack of appropriate sludge handling techniques highlights the need for valorization approaches. Similarly, the valorization of food waste will play a pivotal role in the management of food loss and food waste, as the FW contributes to greenhouse gas (GHG) emissions during final disposal in landfills (Girotto et al., 2015). Moreover, optimization of extraction conditions ensures maximum protein recovery. This will not only reduce the environmental footprint but also contribute to the production of raw materials required in the animal feed industry. Extracting protein from waste-activated sludge and FW as an animal feed supplement will therefore maximize resource utilization and provide a sustainable and cost-effective protein source for animal nutrition. Ultimately, it will lead to the realization of environmental benefits and economic viability through the utilization of the principles of blue economy.

1.4 Hypotheses

i. Waste-activated sludge (WAS) and food waste (FW) do not contain a significant amount of protein.
ii. There is no significant effect of interactive variables in protein extraction.
iii. There is no significant effect of co-extraction of protein from FW-WAS mixture on protein yield.
iv. Proteins extracted from WAS do not have essential amino acids.

1.5 Objectives

1.5.1 General objective

To extract, characterize, and optimize protein from waste-activated sludge (WAS) and food waste (FW).

1.5.2 Specific objectives

i. To extract proteins from waste-activated sludge and food waste using thermal alkaline hydrolysis and thermal acid hydrolysis methods.
ii. To optimize the process of protein extraction using response surface methodology.
iii. To determine the effect of co-extraction of protein from the combination of WAS and FW.
iv. To characterize proteins extracted from WAS and FW for selected physicochemical parameters.

CHAPTER TWO

LITERATURE REVIEW

2.1 Animal feeds

According to the latest United Nations projections, the global population is set to reach 9.7 billion in 2050, leading to a 60% rise in food demand and a 70% growth in the consumption of animal-derived products (Buccaro et al., 2023). This surge will ultimately lead to an increase in the demand for animal feeds necessary to increase livestock production. This is because animal feed and nutrition are the essential link in livestock production between crop cultivation and animal protein production and processing (Mengesha, 2022). However, the industry's ability to meet this rising demand is constrained by the persistent scarcity of raw materials and price volatility that undermines feed affordability and supply chain stability, particularly in low- and middle-income countries (Akintan et al., 2024). Additionally, Kim et al. (2019) reports that 685 million out of the 2.5 billion hectares of total agricultural land currently used for livestock feed production is under increasing pressure to be converted into cropland or other land uses. Furthermore, the conversations around the feed-food competition for resources have become particularly relevant in recent years, considering the planet's limited biophysical capacity and uncertainties associated with macroeconomic, geopolitical, and socioeconomic developments that threaten feed availability and food security, such as the ongoing Ukraine-Russia conflict, inflation, and energy shortages (Pexas et al., 2023). Subsequently, strategies and solutions, such as a "food recovery hierarchy," are needed to reduce the impact of feed production on the environment by reducing the use of natural resources and increasing their reuse (Pinotti et al., 2021b). Also, alternative sources of protein are needed to address such demands. These alternatives should focus on local, circular, and soilless protein feed sources that provide solutions for food security threats and key sustainability issues in the global livestock sector. One such approach is circular and cellular agriculture that involves the extraction of microbial protein, commonly referred to as single-cell protein due to its high protein content (Bajić et al., 2022a).

2.2 Single cell protein as alternative sources of protein

Single-cell protein (SCP) is a bulk of dried cells derived from the fast growth of biomass produced by microorganisms such as algae, yeast, fungi, and bacteria (Sharif *et al*., 2021). Single-cell proteins are gaining traction due to their high nutritional value, providing a balanced amino acid spectrum, a higher protein-to-carbohydrate ratio, and low fat content (Varol, 2025). Bacterial and fungal protein content as single-cell protein produced from specific substrates are shown in Table 2.1.

Table 2.1: Bacterial and fungal protein content from specific substrate.

Microorganism	Substrate	Protein content (%)
Marine yeast	Prawn shell wastes	61–70
Pleurotus florida	Wheat straw	63
Kluyveromyces marxianus	Orange pulp, molasses, brewer's, spent grain, whey, potato pulp	50
Candida tropicalis	Molasses	56
Kefir sp.	Cheese whey	54
Haloarcula sp. IRUI	Petrochemical wastewater	76
Methylococcus capsulatus, Ralstonia sp., Brevibacillus agri, Aneurinibacillus sp.	Methane (Natural gas)	67–73
Bacillus subtilis	Ram horn	71
Methylomonas sp.	Methane salt broth	69
Rhodopseudomonas palustris	Latex rubber sheet wastewater	55–65
Rhizosphericdiazotrophs (whole microbial community)	Brewery wastewater	>55

Source;(Raziq, 2020).

There is a growing interest in the utilization of SCP to meet the global demand for nutritious feed due to their advantages over conventional plant and animal proteins (Onyeaka *et al*., 2022). Compared to conventional protein sources, SCP requires a shorter production time, unlike the time intervals of weeks, months, or years required

for crop farming and animal husbandry (Bratosin et al., 2021; Gundupalli et al., 2024). Yeasts such as *Saccharomyces cerevisiae* are widely used in SCP production due to their high protein content. Parsons et al. (2020) reviewed the role of yeast-derived SCP protein, emphasizing their sustainability and potential to replace conventional feeds. Additionally, *Candida utilis* have been shown to be promising sources of protein in aquaculture feeds, capable of replacing 40% of the protein from high-quality fishmeal without adversely affecting growth performance, digestibility, and nutrient retention (Øverland et al., 2013). In another study by Saejung et al. (2018), *Rhodopeseudomonas faecalis*, a photosynthetic bacterium, led to the highest survival and growth rates of fairy shrimp while improving water quality by reducing ammonia, nitrate, and nitrite concentrations. These studies underscore the potential of single-cell protein in animal nutrition. SCP can be cultivated using a variety of substrates, including agricultural waste and industrial by-products, thus reducing pressure on traditional agricultural practices and mitigating issues related to land and water usage (Y. P. Li et al., 2024). Substrates play a key role in the cultivation of SCP, serving as the primary source of carbon, nitrogen, and energy required for microbial growth (Raziq, 2020). The choice of a substrate influences protein yield and overall cost-effectiveness of single-cell production (Chama, 2019).

2.3 Generation of waste-activated sludge as source of SCP

Waste-activated sludge (WAS) is the residual semi-solid material that is inevitably left over from municipal or industrial wastewater or sewage treatment processes using activated sludge technology (Zhang et al., 2018a). WAS is an intricate blend of numerous materials, such as organic and inorganic matter, microbes, and other substances in suspended states (Raheem et al., 2018). Waste-activated sludge is obtained through the activated Sludge (AS) process shown in Figure 2.1.

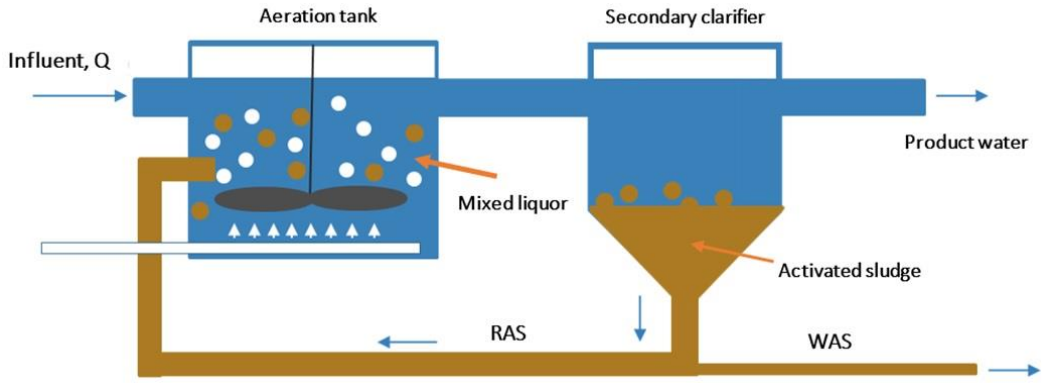

Figure 2.1: Activated sludge (AS) process

AS is a biological treatment process applied in wastewater treatment for the elimination of emerging organic compounds from wastewater (Mahlangu et al., 2023). It consists of two main units: the aeration basin and the secondary clarifier. The aeration tank is the heart of the activated sludge treatment process. In the aeration tank, a high concentration of microorganisms, bacteria, protozoa, and fungi, are present as a loose, clumped mass of fine particles that are kept in suspension by stirring to remove organic matter from wastewater (Rajasulochana & Preethy, 2016). The micro-organisms grow exponentially in the aeration tank as they aerobically consume the biodegradable organic contaminants such as proteins, carbohydrates, lipids, volatile fatty acids, and phenols in the wastewater leading to formation of flocs that develops into sludge. The sludge is then settled in the sedimentation tank (clarifier) while a clear treated wastewater is discharged (Yildiz, 2012). The main part of the sludge, called return-activated sludge (RAS), is recycled to the aeration basin to maintain a high population of microbes (Bajpai, 2017). The excess sludge that remains after treatment, removed from the system, is the waste-activated sludge (WAS) (Jafarinejad, 2017).

2.3.1 Characteristics and composition of waste-activated sludge

The physical and chemical characteristics of waste-activated sludge play an important role in the composition of WAS. The physical and chemical properties of WAS are given in Table 2.2.

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Table 2.2: Characteristics of the waste-activated sludge.

Parameter	Unit	Value
pH		6.5 –8.2
Total solids (TS)	% w/w	4.7–5.4
Volatile solids (VS)	% w/w	3.2–4
VS/TS		0.69–0.7
Organic matter	g/kg	418−592
Soluble sugar	Mg/L	80
Soluble protein	Mg/L	88
Fe	Mg/L	24,000–38,000
Mg	Mg/L	3,200–4,800
Zn	Mg/L	2,400–3,600
Cu	Mg/L	700–1,200
Cr	Mg/L	500–900
Ni	Mg/L	300–500
Mn	Mg/L	100–200
Pb	Mg/L	100–300

Source;**(Apollo, 2022; Eskicioglu et al., 2006).**

Generally, WAS is composed of inorganic components, microbial community, nutrients, trace elements, and organic components (Naorem et al., 2022). The inorganic fraction of WAS comprises silicates, carbonates, phosphates, and metal ions (Niinipuu et al., 2020). As shown in **Error! Reference source not found.**, Waste-a ctivated sludge contains total solids (TS) ranging from 4.7 to 5.4%, with the volatile solids (VS) fraction constituting 70-80% of TS. The VS comprises mainly organic matter such as microbial cells, proteins, extracellular polymeric substances (EPS), carbohydrates, lipids, and nucleic acids (Heindl, 2024; A. Kumar & Yadav, 2025; Tyagi & Lo, 2013). The extracellular polymeric substances in sludge, composed mainly of polysaccharides and proteins, offer enormous potential for biopolymer extraction and SCP production, particularly for feed application (A. Kumar & Yadav, 2025). Additionally, WAS contains significant amounts of nutrients such as nitrogen and phosphorus both in organic and inorganic forms that can be recovered and used in various applications including fertilizers (Boumalek et al., 2019). While WAS is

rich in recoverable resources, it contains heavy metals such as copper, lead, cadmium, nickel, chromium, and arsenic (Luo et al., 2024). The presence of these heavy metals in sludge is concerning due to their persistence, toxicity, and potential bioaccumulation in the environment underscoring the importance of pretreatment of WAS before its application.

2.3.2 Environmental challenges of waste-activated sludge

Huge amounts of waste-activated sludge have been produced each year, and the volume continues to increase due to rapid urbanization (K. Xiao & Zhou, 2020b). According to the European Commission (EC) Report, more than 10 million tons of dry solids of sludge were produced in the 26 Member States in 2008 (Gherghel et al., 2019a). The waste-activated sludge contains pathogens, heavy metal compounds, and persistent organic pollutants (Raheem *et al*., 2017), which are prone to secondary pollution. Heavy metals are extremely persistent in the environment, non-biodegradable, and can accumulate in the food chain, posing long-term ecological and health risks (Abdel-Rahman, 2022). The primary sources of heavy metals in WAS are industrial wastewater discharges and surface runoff (Fijalkowski et al., 2017). Industries such as mining, electroplating, metal plating, petroleum, wood processing, textile and tannery industry operations contribute significantly to the load of metals like cadmium, lead, chromium, copper, and zinc in municipal wastewater (Oladimeji et al., 2024). Ionescu et al. (2015) highlight that heavy metals such as copper (Cu), zinc (Zn), and chromium (Cr) can disrupt the metabolic functions of microorganisms and lower the effectiveness of the biological processes in wastewater treatment plants. Additionally, the traditional WAS disposal methods, such as incineration, landfilling, and soil application, have high processing costs and insufficiently utilize biological resources (Yan et al., 2020) such as the microbial biomass that can be processed to produce SCP for animal feed.

Landfilling of untreated WAS can lead to the release of significant amounts of methane (CH_4) and nitrous oxide (N_2O), both of which are potent greenhouse gases with high global warming potential (Lee et al., 2023). Al-Islmi et al. (2021) conducted a life cycle assessment (LCA) to evaluate the impact of activated sludge effluent on the environment. The study found that sludge contributed significantly to global warming, terrestrial toxicity, and eutrophication across multiple environmental impact

categories. Moreover, improper treatment and disposal of sludge can result in the leaching of harmful contaminants into the soil and water bodies, which can lead to long-term environmental degradation and public health risks (Bułkowska & Zielińska, 2024). To mitigate these challenges, sustainable practices such as valorization options are needed. Subsequently, by employing diverse techniques, various nutrients, enzymes, bio-plastics, bio-pesticides, and proteins can be recovered from WAS (Raheem et al., 2018).

2.3.3 Sustainability potential of proteins from Waste-Activated Sludge

Sustainability potential involves considering a balance between economic viability, environmental responsibility, and social acceptability (Vivien, 2023). In comparison with agricultural proteins, the production of single-cell protein from WAS biomass is more environmentally friendly, consumes less water, requires smaller land areas, and its effect on climate change is much less pronounced (Bajić et al., 2022b). Furthermore, the single-cell protein derived from waste-activated sludge can help reduce the protein gap in animal nutrition (Zha et al., 2021). This approach can reduce overreliance on imported feed commodities and lowers the environmental footprint associated with animal agriculture. Brown et al. (2010) used a greenhouse gas (GHG) calculator tool to determine GHG emissions for WAS and reported that WAS treatment and end use can constitute up to 40% of the total emissions associated with wastewater treatment. This percentage could be decreased if the circular economy concept involving valorization were applied in sludge management (Gherghel et al., 2019b), thus ensuring environmental sustainability.

2.3.4 Estimation of waste activated sludge production in Kenya

Kenya's urban population is growing at a faster rate than the country's general population, which is expected to intensify challenges related to waste generation and management (Haregu et al., 2017). In Kenya, sewerage coverage remains relatively low, with major cities like Nairobi, Kisumu, and Mombasa having 28%, 13%, and 4% sewerage coverage, respectively, contributing to a national average of 17% (K'oreje et al., 2016). According to data from Kenya's sewage treatment database, there are 49 sewage treatment systems in total (Bundi & Njeru, 2018), 6 of which use the activated sludge technology as shown in Table 2.3. The available waste management options

are inadequate compared to the country's growing stream of waste. On-site sanitation remains the predominant form of sanitation in Kenya, as sludge is often disposed into open drains, rivers, or informal dumping sites, posing serious environmental and public health risks (Mansour et al., 2017). Since waste generation is inevitable, the waste generated should be managed under the premise of valorization.

Table 2.3: Sewage treatment systems in Kenya

Technology	Number of public plants in the country
Constructed wetlands	10
Oxidation ditch	3
Conventional (activated sludge)	6
Aerated lagoons	3
Stabilization ponds	27
Total	49

Source: (Kilingo et al., 2021)

Nairobi, the capital of Kenya, is served by two main sewage treatment plants: Ruai Sewage Treatment Plant, also known as Dandora Estate Sewage Treatment Works (DESTW), with a capacity of 160,0000 m^3 /day, and Kariobangi Sewage Treatment Works (KSTW), with a capacity of 32,000 m^3/day (Wainoga, 2020). The Kariobangi treatment plant is a conventional plant based on biological aerated filters for wastewater treatment.

Table 2.4Table 2.4 shows the sludge production potential from Kenya's selected activated sludge treatment plants. Kisumu City is served by two types of sewer systems: conventional and lagoon, with an efficiency of less than 20% (IRO ONG'OR & LONG-CANG, 2007). According to Kisumu Water and Sanitation Company Limited (KIWASCO), Kisat Conventional Sewerage Plant, established in the year 1965, is the main sewerage treatment facility in Kisumu. The Kisat Conventional Sewerage Plant has a capacity of 8,000 m^3 per day. However, the sewerage capacity is low and serves less than 10% of the population as the two sewer systems do not accommodate most of the generated waste (Maoulidi, 2010), signifying inadequate waste disposal methods in the city.

Kipevu Sewer and Sewage Treatment Works is the main wastewater treatment facility in Mombasa, consisting of inlet works, aeration tanks, sludge thickening tanks, and sludge drying beds (Everlyne & Nairobi, 2014). According to Mombasa Water Supply and Sanitation Company Limited (MOWASSCO), the inflow is currently at less than 40% of the design average dry weather flow of 17,100 m^3/day. This report on the waste management situation in Kenya underscores the urgent need to adopt valorization as an alternative waste management option.

Table 2.4: An estimate of the sludge production potential of selected activated sludge treatment plants in Kenya.

Wastewater treatment plant	Capacity (ML/day)	Estimated COD load (ton/day)	Estimated sludge (ton/day)
Kariobangi	32	7.8	3.1
Ruai	160	46.6	18.6
Old town	6.6	0.4	0.2
Kisat	8	0.5	0.2
Kipevu	17.1	5.1	2.1

2.4 Challenges and opportunities associated with food waste

Food waste refers to wholesome edible material intended for human consumption, arising at any point in the food supply chain (FSC) that is instead discarded, lost, degraded, or consumed by pests (Parfitt et al., 2010). Food wastage represents a loss of valuable micronutrients (Ottomano Palmisano et al., 2021), including vitamins, minerals, proteins, and carbohydrates. Food wastage occurs at all levels, namely, farm, postharvest, processing, distribution, retail, and consumption levels (Chauhan et al., 2021). A report from the United Nations (UN) indicates that 1.3 billion tons of food are lost each year, which results in 795 million people going without food and nearly 1 billion people being undernourished (Manzoor et al., 2024). Figure 2.2 represents country-wise trends in food wastage around the globe. It is estimated that postharvest food loss and waste in Kenya is at 30% (Axmann et al., 2024). The paradox of food loss and food waste in Kenya against high levels of food and nutrition insecurity therefore calls for exploration of strategies such as valorization of FW.

According to Kamal et al. (2021a), the proportion of losses post-production includes up to 14% in the food service sector, 39% in food manufacturing, 42% at the household level, and 5% during distribution. Food waste has a direct impact on the environment, the economy, and society because it results in a one-third loss of the edible foods prepared for human consumption, and as a result, the developed world's higher rates of food waste and increasing food insecurity represent a contradiction of "scarcity within abundance" (Manzoor et al., 2024).

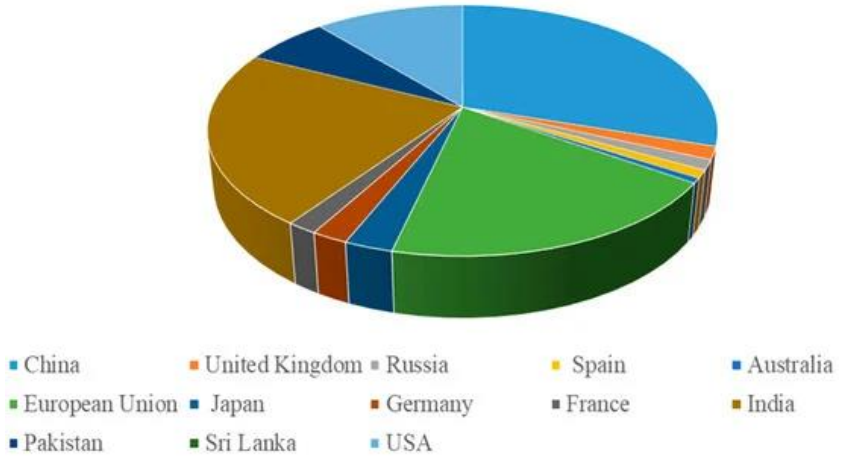

Figure 2.2: Global trends in the production of food waste by nation (Pal et al., 2024).

Food waste, a component of municipal solid waste, is generated from kitchens of residential households, restaurants, canteens, hostels, and food processing industries (Barik & Paul, 2017). Kitchen waste constitutes a significant portion of municipal solid waste contributing up to 50-60% of the total waste generated (Y. Wang et al., 2018). Food wastage is detrimental to the planet due to greenhouse gas emissions and the wasting of water and land used as inputs (von Braun et al., 2023). Although composting is proposed to reduce greenhouse gas emissions and is adapted to decentralized kitchen waste (KW) treatments, the composting process produces malodorous gases such as ammonia (NH_3) and hydrogen sulfide (H_2S) (Meng et al., 2022). KW is recognized as a pollutant because, in natural environments, it quickly decomposes, produces odors that leads to potential health risks such as headache, nausea, and respiratory irritation (He et al., 2023; Yaser et al., 2022). Despite the importance of decreasing FW, the circular economy, which sees waste as a valuable resource, calls for the reintroduction of food scraps into the food supply chain (Nath

et al., 2023). Subsequently, food and kitchen waste is rich in nutrients and organic materials that can be recovered for various uses (A. Sharma et al., 2025; Sindhu et al., 2019). The above-mentioned problems, therefore, could be mitigated by the reuse of protein extracted from FW in animal feed (Kamal et al., 2021b).

To work towards the goal of a circular economy, the inherent value of food waste, in the form of nutrients and energy, should be recovered through the conversion of food waste biomass into value-added products. Several techniques and processes, including mechanical processing, fermentation, enzymatic hydrolysis, extraction, and innovative biotechnological methods are employed in the valorization of food wastes into food products (Pal et al., 2024). Extraction and purification techniques isolate specific compounds, such as protein, from food waste for use in various food and feed products (Pal et al., 2024).

2.4.1 Characteristics of kitchen waste

Kitchen waste (KW) is characterized by its carbohydrate, total and volatile solids, pH, moisture, protein, fat, ash, and fiber content, as shown in Table 2.5. Primarily, KW consists of highly organic and biodegradable materials and consists of approximately 60% carbohydrates, 20% proteins, and 10 % lipids (Lisá et al., 2025). This biochemical composition highlights its potential for resource recovery applications, including protein extraction for use in animal feed (Rebello, 2019). Additionally, the moisture content of kitchen waste ranges from 48% to 95%, depending on the source and nature of the waste, with food waste from households and restaurants having a moisture content of 70%-85% (Selvam et al., 2021a). The high moisture content in kitchen waste is crucial for microbes to thrive and carry out their metabolic processes efficiently. Consequently, it facilitates dissolution of fats, proteins, and carbohydrates by providing an aqueous medium necessary for hydrolytic enzymes to break down the complex macromolecules into simpler soluble forms (Su et al., 2023). Moreover, the volatile solids content, often exceeding 80% of the total solids content in kitchen waste represents the biodegradable organic matter including proteins, carbohydrates, and lipids, which are valuable biomaterials for resource recovery (Y. Li et al., 2011).

Table 2.5: Characteristics of kitchen w aste (Alghashm et al., 2023; Hafid et al., 2015).

Parameter	Unit	Value
pH	-	5.2±0.32
Moisture content	%	70.06±2.33
Total solids (TS)	% w/w	15.73
Volatile solids (VS)	% w/w	12.78
VS/TS	-	0.81
Carbohydrate	%	60.78±2.02
Fat	%	13.65±1.74
Crude Protein	%	20.53±1.77
Ash	%	1.97±0.1
Fiber	%	1.67±0.05

2.4.2 Environmental impacts of kitchen waste

Kitchen waste poses significant environmental challenges when improperly managed (Batool et al., 2024). When kitchen waste is disposed of in landfills, it decomposes anaerobically generating methane, which accounts for more than 58% of fugitive methane emissions, exacerbating climate change (Kaplow, 2024). The decomposition of kitchen waste not only contributes to atmospheric pollution, but also interferes with the quality of soil and water through contamination (Meegoda et al., 2025). Additionally, as KW decomposes, it produces leachate, a liquid that can infiltrate surrounding soils and transport pathogens, nutrients, and toxic substances into groundwater, posing serious environmental and public health risks (Aderemi et al., 2011). Furthermore, the decomposition of kitchen waste in landfills contributes to air quality degradation due to the release of volatile organic compounds (VOCs) such as ammonia and sulfur compounds (Meegoda et al., 2025). Moreover, due to its high protein and carbohydrate content, kitchen waste is rich in organic nutrients, particularly nitrogen and phosphorus. While these nutrients are potentially useful for composting and bioenergy production, they become an environmental liability when mismanaged. According to Göncü et al. (2025), excess phosphorus and nitrogen from household waste, including kitchen waste, are major contributors to eutrophication in

aquatic ecosystems. Additionally, the organic matter, proteins, and carbohydrates in KW have the potential for resource recovery through composting, anaerobic digestion, and its potential in animal feed application (Piercy et al., 2023; Pradeshwaran et al., 2024). However, failing to harness these resources exacerbates environmental degradation by increasing reliance on natural resources.

2.5 Extraction of protein from food waste and waste-activated sludge

Organic matter is the major constituent of waste-activated sludge and food waste (H. Li et al., 2016). In WAS, protein accounts for about 40-60% of the organic matter (Hui et al., 2022b). Similarly, in food waste, protein contributes up to 42% of the organic matter (Selvam et al., 2021b). To fully recover the protein in FW and WAS, various physical, chemical, biological, and combinations of these techniques are available.

2.5.1 Physical methods of protein extraction

The physical methods include thermal, ultrasonic, electrical, and microwave (Gao, Weng, et al., 2020a). These methods aim to disrupt the extracellular polymeric substances and microbial cell walls, therefore improving solubilization of WAS proteins (Pondala & Botsa, 2025). In the thermal method, organic waste is exposed to heat at different temperatures and pressures (Kakar, Tadesse, et al., 2022). The heat introduced during heat treatment affects the chemical bonds of the cell wall and membrane, thus solubilizing the cellular components (Pilli et al., 2015). Xu et al. (2020) investigated the effects of temperature and time on soluble substances in sludge and found that the concentration of soluble protein increased with temperature. At 140 °C and 155 °C, there was no significant change in the concentration of the soluble protein; however, at 170 °C,185 °C, and 200 °C for 60 min, the concentration of soluble protein increased 1.4, 1.4, and 1.9 times, respectively. These results are consistent with the report by Bougrier et al. (2008).

Electrochemical treatment is a robust, scalable, and environmentally friendly approach that has been widely studied for wastewater treatment processes (Jafari & Botte, 2022). Through electrolysis, sludge cells can be ruptured, and intracellular substances, especially proteins and polysaccharides are solubilized (Ye et al., 2016). The electric pulse power can generate shockwaves, intense ultraviolet radiation, radicals, and strong electric fields, thus destroying the cell wall of sludge and releasing

the intracellular protein (Xiao et al., 2020). The cavitation and thermal effects caused by ultrasonication can generate high temperatures, high pressures, and mechanical shear force, which result in bacterial cell structure disruption and superior wall-breaking performance (Gao, Wang, Yan, & Li, 2021). Studies on intracellular and extracellular protein extraction and hydrolysis showed that ultrasonic pretreatment effectively reduced the EPS barrier by approximately 50% (F. Liu et al., 2023). Xiang et al. (2015) optimized ultrasonic extraction conditions using response surface methodology and achieved a protein extraction yield of 78.61% under conditions of 36.72 min sonication, pH 8.98, and 34.14 °C.

Microwaves are forms of non-ionizing radiation within 0.3-300 GHz (Bozkurt & Apul, 2020). Microwave (MW) technology is capable of disrupting sludge flocs and cells, releasing organic matter into the soluble phase (J. Xiao et al., 2017). The mechanism of microwave irradiation includes a thermal effect and an athermal effect. The term “athermal effect” for microwave relates to an effect that is not associated with an increased temperature, while “thermal effect” relates to the process that generates heat as a result of the absorption of the microwave energy by water or organic complexes marked by either constant or induced polarization (Yu et al., 2010). Thermal effects are generated via ionic conduction (in shorter frequencies) and dipole rotation (in higher frequencies), while a thermal effect is generated through the rotation of dipole molecules (like water) due to the constant and repeated changes in the polarity of the field (Jákói et al., 2021). Thus, whether the orientation of molecules comprising the cell membranes takes place, the hydrogen bonds assisting their structure break (Mitraka et al., 2022). Inevitably, intracellular substances are released, while changes in the secondary structures of microorganism proteins occur by virtue of denaturation (Mitraka et al., 2022). The microwave energy is converted to heat by internal resistance to rotation, possibly triggering bond decomposition and reorienting (Bozkurt & Apul, 2020). Yu et al. (2010) studied the effect of microwave irradiation on extracellular polymeric substances. Proteins and polysaccharides were initially estimated to be present at concentrations of 585.21 mg/L and 97.70 mg/L, respectively, in the sludge samples examined. At 900 W and a contact time of 140 s, there was a 294% protein increase and a 654% polysaccharide level after sludge disintegration by microwave irradiation. The physical methods discussed so far play

a role in the extraction of sludge protein, but high energy consumption makes them unsuitable for practical production (Qin et al., 2018).

2.5.2 Biological methods of protein extraction

Biological methods of protein recovery involve the use of enzymes to hydrolyze microbial cells and EPS, releasing the soluble proteins into the aqueous phase (Elayaperumal et al., 2025). The application of an enzyme in protein extraction is dependent on the operational conditions, including substrate and enzyme ratio, enzyme-specific temperature and pH, and extraction time (Kamal et al., 2023). As the enzyme dosage is increased, the extracted protein increases up to a certain concentration, beyond which not much extraction is observed (Sesay et al., 2006). Enzymatic hydrolysis offers the advantages of mild reaction conditions and is environmentally friendly (F. Liu et al., 2023). According to Qin et al. (2018), the maximum protein extraction rate was 58% at an enzyme dosage of 2%, an enzymolysis time of 4 h, pH 8, a temperature of 55 °C, and sludge moisture content of 4%. The high cost of enzymes remains a major barrier to large-scale application, especially when compared to acid or alkaline extraction methods (Fasim et al., 2021). Furthermore, enzymatic hydrolysis is sensitive to temperature and pH fluctuations, which affects reproducibility and protein recovery (Suzuki, 2019).

2.5.3 Chemical methods of protein extraction

Chemical methods utilize chemical reagents such as acids, alkalis, and oxidants to hydrolyze sludge (Khanh Nguyen et al., 2021). Among the chemical methods, alkaline-thermal hydrolysis has realized industrial application with high extraction efficiency and mature technology (Yan, Zhang, Gao, et al., 2023). In alkaline-thermal hydrolysis method, alkaline compounds such as calcium hydroxide ($Ca(OH)_2$), potassium hydroxide (KOH), and sodium hydroxide (NaOH) are used (K. Xiao & Zhou, 2020a). NaOH is the most frequently used chemical method due to its high solubilization rate using relatively low dosages (de Sousa et al., 2021). NaOH ionizes charged groups in proteins and polysaccharides, increases repulsion among polymeric matrix components, and increases their solubility (García Becerra et al., 2010). At different pH or alkaline concentration, different amounts of thermally alkaline-treated sludge results in different disintegration degrees (Shi et al., 2015). According to Xiao

& Zhou (2020) and Gao, Wang, et al. (2020), alkaline hydrolysis at a pH of 12 and a temperature of 120 °C for 4 hours were the most effective conditions for solubilization of sludge protein.

The acid thermal hydrolysis method is also used to solubilize protein. Compared to other chemical methods used in protein extraction, the acid hydrolysis method is cheaper and more environmentally friendly (Gao, Wang, Yan, Li, et al., 2021). Previous studies focused on the extraction of protein from an individual substrate, and often, the benefit of the extraction of protein from the FW-WAS mixture has been overlooked. It is therefore necessary to explore the effect of co-extraction of protein from FW and WAS. Gao et al. (2020b) compared the methods of extraction of protein from waste-activated sludge and found that the chemical methods of extraction were more effective in the solubilization of sludge and extraction of protein.

2.6 Characterization analysis of the products of hydrolysis

To study the elemental composition, functional groups, and amino acids of hydrolysis products, inductively coupled plasma-optical emission spectroscopy, Fourier transform infrared spectroscopy, and high-performance liquid chromatography-mass spectrometry techniques were used, respectively.

2.6.1 Fourier Transform Infrared Spectroscopy (FTIR)

Fourier transform infrared spectroscopy (FTIR) is an instrumental technique used to identify the functional groups present in organic and inorganic compounds by measuring their absorption of infrared radiation over a range of wavelengths (Berna, 2017). FTIR is convenient, non-destructive, requires less sample preparation, and can be used under a wide variety of conditions (Kong & Yu, 2007). It is useful in identifying a wide range of compounds (liquids, solids, and gases), especially organic molecular groups and compounds such as proteins (S. A. Khan et al., 2018; Rakhee et al., 2018).

The fundamental principle of the FTIR technique is based on the vibration and rotation of atoms, and it has become a universal and widely used spectral methodology for the detection of internal molecular structures in a diverse range of fields (Pasieczna-Patkowska et al., 2025). When a beam of infrared light with a continuous wavelength

is passed through the sample, the light with a certain wavenumber, providing an energy difference between two energy levels, will be absorbed in an absorbance spectrum (Gong et al., 2024). An interferometer identifies the sample by producing an optical signal with IR frequencies encoded in it. The Fourier transformation (a system-based or generated process) technique is used to decode the signal and helps in the generation of the spectral information (Selvan et al., 2021).

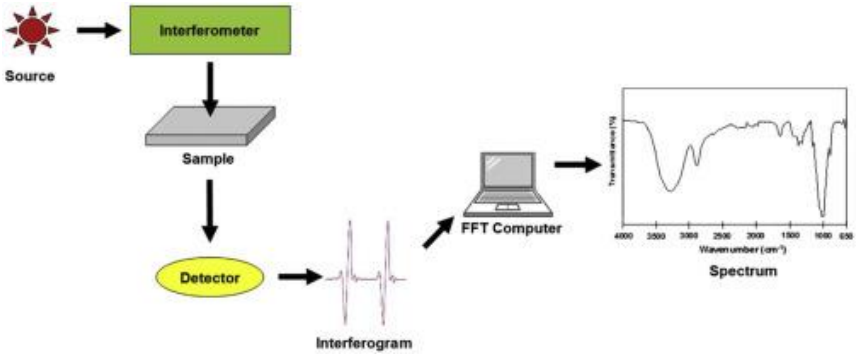

Figure 2.3: A schematic representation of FTIR (Undavalli et al., 2021).

FTIR spectroscopy has become a major tool in determining the secondary structure of protein (De Meutter & Goormaghtigh, 2021). Protein molecules exhibit nine characteristic IR absorption bands, and they have been identified as amide A (~3,300cm^{-1}), amide B (~3100cm^{-1}), amide I (~1650cm^{-1}), amide II (~1550cm^{-1}), amide III (~1300cm^{-1}), amide IV (~735cm^{-1}), amide V (~635cm^{-1}), amide VI (~600cm^{-1}), and amide VII (200cm^{-1}). Amide I and II bands are the most prominent and sensitive vibrational bands of the protein backbone, and they relate to protein secondary structural components (H. Yang et al., 2015).

2.6.2 Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES)

Inductively coupled plasma spectroscopy is a powerful tool for the determination of trace and ultratrace elemental concentrations in a wide variety of samples, specifically for multielement analysis (Douvris et al., 2023). It offers the least detection time, a broader linear dynamic range, lower detection limits, negligible chemical interferences, and a greater matrix tolerability (S. R. Khan et al., 2022). This technique utilizes inductively coupled plasma to generate excited atoms that emit characteristic electromagnetic radiation (S. R. Khan et al., 2022). The fundamental components of the ICP-OES instrument are the sample introduction system, pump, nebulizer, spray

chamber, ICP torch, monochromator/polychromator, and detector, arranged in a definite order (I. Sharma, 2020) shown in Figure 2.4. In ICP-OES, samples are introduced as acid solutions that are transported into the plasma, where they undergo desiccation, vaporization to molecular gases, and dissociation into atoms that can be ionized (de la Guardia & Armenta, 2011). The ions are excited in the plasma, reverting to their ground state with the emission of light, which is measured by the optical spectrometer. The element type is measured depending on the position of the photons, and the concentration of each element is determined based on the intensity of emitted photons (K. F. Khan, 2019). Therefore, by determining the types of wavelengths emitted by a sample and by determining their intensities, the analyst can quantify the elemental composition of the given sample relative to a reference standard (Wittig et al., 2008).

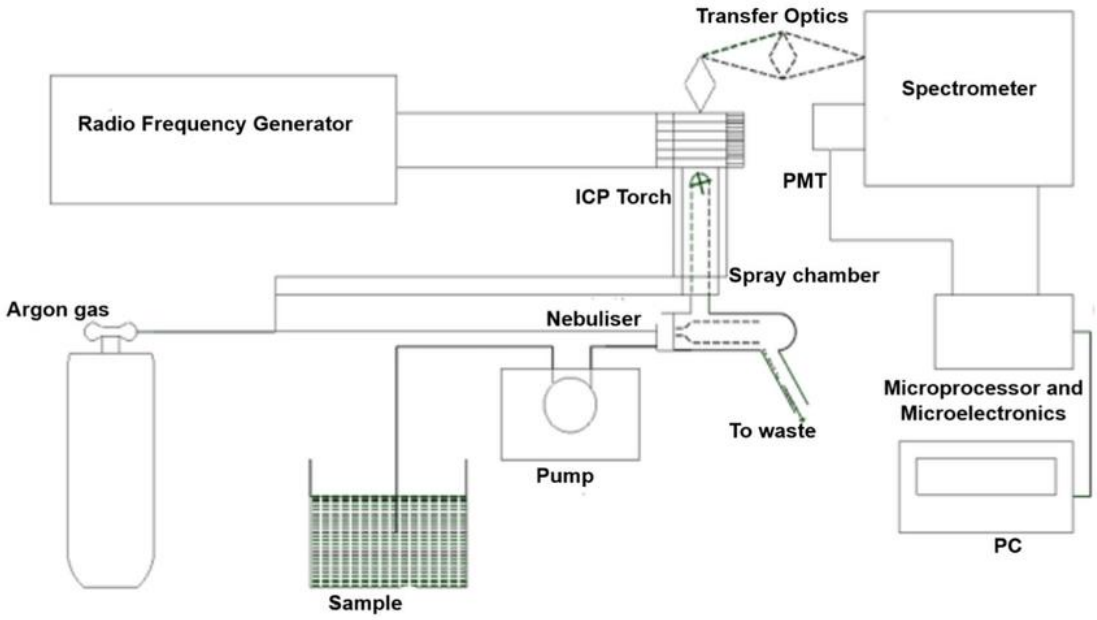

Figure 2.4: Schematic representation of ICP-OES system (Bini et al., 1997).

2.6.3 High-performance liquid chromatography-mass Spectrometry (HPLC-MS)

Chromatography is an analytical method often used for the separation of mixtures of chemical substances into their pure components (Gaffney & Marley, 2018). In chromatography, the components of a sample are separated into two phases: a

stationary phase and a mobile phase (Singh et al., 2022). As the solvent flows through the column, the solutes travel at speeds depending on their relative affinities to the stationary phase used (Doran, 2013). Figure 2.5 shows a schematic representation of HPLC-MS.

High-performance liquid chromatography (HPLC) is an advanced form of column chromatography that is frequently used to separate, identify, and quantify different compounds (Guijt, 2017). HPLC-MS has emerged as a powerful technique in analytical chemistry, combining the physical separation capabilities of liquid chromatography with the high selectivity and sensitivity of mass spectrometry (Vanitha Madhuri T et al., 2024). This hyphenated technique is the most reliable, versatile, and robust analytical technique used to analyze amino acids (Violi et al., 2020). The sample is injected into the liquid chromatography system, where it is separated based on the interaction of amino acids with the stationary phase in the packed LC column (Senyuva, 2014). The separated amino acid exits the column and enters the mass spectrometer. The mass spectrometer generates multiple ions from the sample, followed by identification by their mass-to-charge ratio (*m/z*) and relative abundances (Malik et al., 2016).

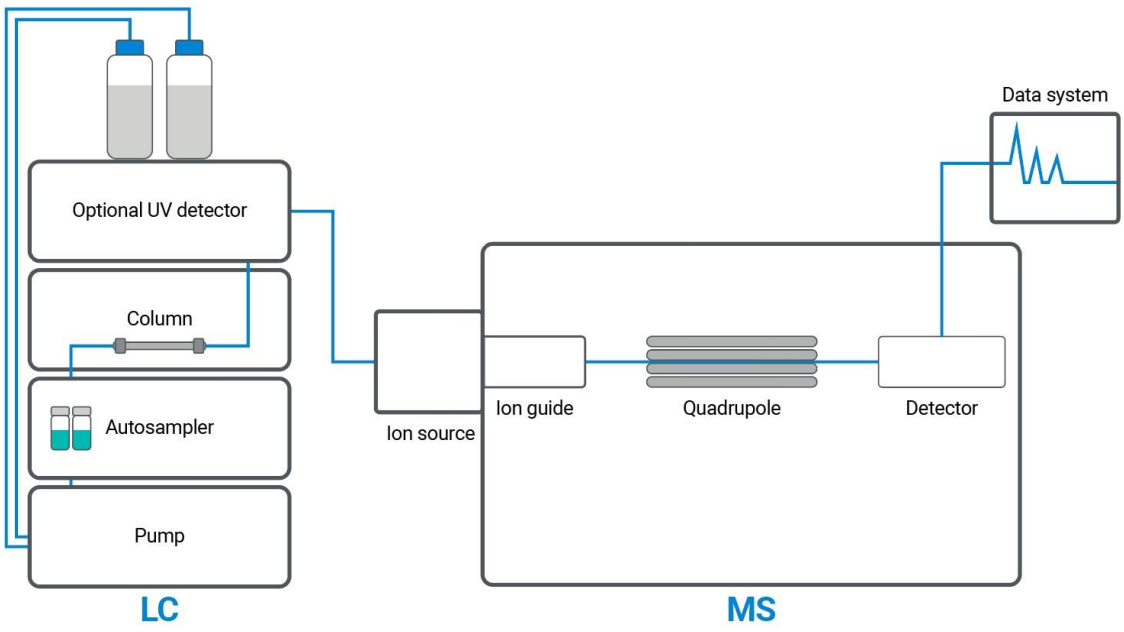

Figure 2.5: A schematic representation of HPLC-MS (Agilent, 2025).

2.7 Quantification of proteins

2.7.1 Kjeldahl method of protein quantification

The Kjeldahl method for estimation of nitrogen is used worldwide as a standard method to determine the amount of protein from a wide variety of materials, which include animal or human food, fertilizer, wastewater, and fossil fuels (H. Kumar, 2022). In this method, first, the nitrogen concentration of a sample is determined. The protein content is then estimated by multiplying the nitrogen content by a nitrogen-protein conversion factor, usually 6.25, since the nitrogen content of protein in feeds is 16% (Sáez-Plaza et.al., 2013). The Kjeldahl method has been an essential analytical tool for protein determination in research, academic, and industrial laboratories since its introduction in 1883 (Aguirre, 2023). Though the Kjeldahl procedure is hazardous, lengthy, and labor-intensive, it has become the industry standard as it remains an accurate and reliable method used to standardize other methods (Sáez-Plaza et.al., 2013).

The Kjeldahl method involves three main steps: digestion, distillation, and titration (Chromý et al., 2015). Digestion of organic material is achieved using concentrated H_2SO_4, heat, K_2SO_4 (to raise the boiling point), and a catalyst such as copper to speed up the reaction (Goulding et al., 2020). The digestion aims to break all the nitrogen bonds in the organic material into ammonium sulfate (Marwat, 2008). The resulting solution is distilled in the presence of excess sodium hydroxide to release ammonia; the liberated ammonia is trapped in an acid and quantified by titration using a standard mineral acid (H. Wang et al., 2016).

2.8 Optimization of the process of protein extraction using RSM

A proper experimental design plays a vital role in developing every product or process (R. Kumar & Reji, 2023). In every experiment, some inputs (x) transform into results that have one or more observable response variables (y) (A, 2023). To examine the impact of several factors and interactions among them on the response quantity, the experiments need to be performed systematically using factorial experiments (factorial experimental designs/arrays), where several factors are altered during each experimental run (Jankovic et al., 2021). Statistical approaches such as response

surface methodology can be employed to optimize products towards maximizing a relevant response variable, such as increasing yield and efficiency of a process (Giacalone, 2018).

The response surface methodology (RSM) is a collection of mathematical and statistical techniques useful for constructing models and analyzing problems in which several independent variables influence dependent variables or responses (Gaitonde et al., 2012). The primary goal of optimization is to enhance the overall sustainability including the environmental, social, economic, and energy resources sustainability, while effectively satisfying defined objective functions (Sadollah et al., 2020). Optimization by the RSM method involves three major steps: statistically designing the experiments, estimating the coefficients in a mathematical model, predicting the response, and checking the adequacy of the model (Pereira et al., 2021). RSM offers seamless statistical analysis, good visualization of the responses, associated empirical models, prediction, and interaction of variables is easily presented with clear curves and other visual aids (Lamidi et al., 2023).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials and reagents

The materials used include waste-activated sludge obtained from sludge drying beds of the Kariobangi Sewerage Treatment Plant, a local wastewater treatment facility in Nairobi, Kenya. The plant uses an activated sludge system to treat wastewater, and its treatment capacity is 32,000 m^3 per day. Food waste material included kitchen waste and peels obtained from various eateries. Reagent-grade alkali (sodium hydroxide) and acid (sulfuric acid) were used for protein extraction from the wastes. A heating mantle was used for the extraction process. H_2SO_4, Cu catalyst, K_2SO_4, NaOH, H_3BO_3, and HCl were used in protein quantification. All the reagents were Analytical Reagent (AR) grade.

3.2 Sample collection and pretreatment

3.2.1 Waste-activated sludge sample collection and pretreatment

The activated sludge samples were collected and stored at 4 °C in a refrigerator to prevent any changes in sample's composition until use. The sludge was pretreated with 1N HCl to remove heavy metals. The solution was filtered and rinsed with distilled water to recover the pretreated sludge as supernatant and heavy metals as filtrate. Heavy metal concentrations were determined in the filtrate.

3.2.2 Food waste sample collection and pretreatment

Similarly, food waste was collected from various eateries and stored in a refrigerator at 4 °C. To prepare the sample for analysis, food waste was dried in an oven at 50 °C (Hui et al., 2022a) to reduce the moisture content to 60% (Kamal et al., 2023). The dried sample was then ground into small particles. Ethanol was added to FW in a ratio of 1:1 (Saini et al., 2021) to remove fats and oils, and the mixture was centrifuged to separate the solvent from the food waste.

3.3 Sludge and food waste characterization

The characterization of WAS and FW was done following the American Public Health Association (APHA) prescribed standard methods (Bridgewater et al., 2017). To determine the total suspended solids (TSS), the samples were filtered through a pre-

weighed filter paper (initial weight denoted W_1). The filter paper was gently rinsed with distilled water and dried in an oven at 105 °C for 1 hour. After drying, the filter paper was allowed to cool in a desiccator, and the weight (W_2) was determined. The TSS was calculated using equation 1. To determine the volatile suspended solids (VSS), the dried filter paper was carefully transferred into a crucible and ignited in a muffle furnace at 550 °C for 1.5 hours. The filter was allowed to cool in a desiccator, and weight (W_3) was determined (Adjovu et al., 2023). The VSS content was calculated using equation 2. To determine the moisture content, the weight (W_1) of the dried filter paper was determined. The sample was weighed into the filter paper and recorded as W_2. The sample was dried in an oven at 105. After 24 hours, the sample was removed from the oven, allowed to cool in a desiccator, and the final weight (W_3) was recorded. The moisture content was calculated using equation (3).

$$\text{TSS (mg/L)} = \frac{(W_2 - W_1) \times 1000}{\text{Volume of the sample (mL)}} \quad (1)$$

$$\text{VSS (mg/L)} = \frac{(W_2 - W_3) \times 1000}{\text{Volume of the sample (mL)}} \quad (2)$$

$$\text{Moisture content (\%)} = \frac{(W_2 - W_3)}{(W_2 - W_1)} \times 100 \quad (3)$$

Protein content was determined by the Kjeldahl method through direct measurement of nitrogen and subsequent multiplication by a conversion factor, usually 6.25 (Mæhre et al., 2018). The method involves three steps: digestion, distillation, and titration. The sample was digested in 20 mL sulfuric acid to convert the protein nitrogen to ammonium sulfate at a boiling point elevated by the addition of 7.0 g potassium sulfate and 0.8 g of copper catalyst as the digestion mixture. After the digestion was complete, 50 mL of 40% NaOH was added to release ammonia (NH_3). The liberated ammonia was trapped in a conical flask containing 4% H_3BO_3, which was then quantified titrimetrically using 0.1N HCl. Elemental analysis was also done to establish the presence of heavy metals. Finally, the amino acid analysis of the protein supernatant was done to determine the nutritional properties of the sludge.

3.4 Alkaline thermal hydrolysis experiments

According to Hui et al. (2022a), the study of protein yield involved a series of tests were conducted under different reaction conditions to establish the best operation point. In the first place, the dried, pretreated sludge was ground into fine powder and then sieved through a 100-mesh sieve. Protein was then extracted using NaOH at different temperatures and pH ranges. The extraction temperature was varied from 90 °C to 130 °C, while pH was varied from 10 to 13. Under these conditions, each reaction was allowed to proceed for 4 hours with periodic hourly sampling. After each reaction period, the mixtures were allowed to cool to room temperature before being centrifuged at 4000 rpm for 30 min. After centrifugation, the total nitrogen measurements on the supernatant were carried out by the Kjeldahl method using equation (4). To convert the measured N to protein, a conversion factor of 6.25 was used (Latimer, 2023). Similar experiments were carried out for acid thermal hydrolysis using H_2SO_4. In acid thermal hydrolysis, the pH of the sample was adjusted using 98% H_2SO_4 to a pH range of 2-5 under a similar temperature range as that for the alkaline thermal hydrolysis. The reaction was allowed to proceed for 4 hours. Both the alkaline thermal hydrolysis and acid thermal hydrolysis experiments were repeated for food waste.

$$\text{Kjeldahl nitrogen, g/100g} = \frac{(V_S\text{-}V_B)\times M\times 14.01}{W\times 10} \tag{4}$$

$$\text{Crude protein, g/100g} = \frac{g}{100}\ \text{Kjeldahl N}\times F \tag{5}$$

Where V_S is the volume (mL) of standard HCl used to titrate a test, V_B is the volume (mL) of standard HCl used to titrate a reagent blank, M is the molarity of standard acid (0.1N HCl), 14.01 is the atomic molecular weight of nitrogen, W is the weight (g) of the sample, 10 is a factor used to convert mgN/100 g to gN/100 g sample, and F is a factor to convert the measured N to protein. Similarly, the protein content in the raw sludge was determined using the Kjeldahl method. Protein extraction efficiency was determined by comparing the extracted protein yield to the protein yield in raw sludge using Equation (6).

$$Y=\frac{c_1}{c_o}\times 100 \quad (6)$$

Where C_1 and C_o are the protein content in the supernatant and raw feed sample, respectively.

3.5 Analysis of energy efficiency of the process of protein extraction

The power usage of the hydrolysis process was calculated by fixing the desired yield to a value common across all temperature ranges. The power rating of the heating mantle used was 250W and the common yield across different temperature ranges was 3.72 g/100g. The energy consumption was calculated based on equation (7), based on the duration required to reach the common yield at each temperature setting.

$$E = p \times t/x \quad (7)$$

Where E is the energy requirement in kWh/g, p is the power rating of the mantle (kW) used to supply heat, t is the operation time in hours, and x is the amount of protein extracted within time t.

3.6 Co-extraction of protein

To study the extraction of protein from waste-activated sludge and food waste (FW), food waste and sludge were combined in the ratios of 1:0, 3:1, 1:1, 1:3, and 0:1 (S, 2009). In this study, alkaline and acid methods of hydrolysis were used under similar conditions as explained above. The performance of the two hydrolysis methods on protein recovery was compared.

3.7 Method Validation of the Kjeldahl Method for Protein Determination

To ensure the accuracy, precision, and reliability of the results of protein quantification, verification of nitrogen recovery tests was done (Latimer et al., 2023). To check the digestion efficiency, 0.18 g of tryptophan with 0.67 g of sucrose was added to a digestion flask. All the digestion reagents described above were added and distilled under the same conditions. Similarly, to check the distillation and titration efficiency, 0.12 g of $(NH_4)_2SO_4$ was distilled, omitting the digestion step. For all the tests, nitrogen recoveries must be ≥ 98% (Latimer et al., 2023). The nitrogen recovery was calculated based on equation (8).

$$\text{N recovery (\%)} = \frac{\text{Measured N}}{\text{Theoretical N}} \times 100\% \quad (8)$$

3.8 Optimization of protein extraction using RSM

Response surface methodology (RSM) was used to optimize the process of protein extraction. To analyze the process parameters, Design Expert software version 13.0 was used to study the interactive effect of pH, temperature, and extraction time on protein yield and extraction efficiency.

3.9 Sample analysis

3.9.1 Characterization analysis using FTIR

Fourier transform infrared spectroscopy (FTIR) analysis to identify the functional groups in the raw waste and extracted protein was conducted using the Shimadzu IR spectrometer (IRSpirit-X model). Attenuated total reflection (ATR) mode was used. Prior to sample analysis, a background scan was done to account for any atmospheric interference. Subsequently, each sample was placed onto the ATR crystal surface. The sample was scanned over the wavenumber range of 4000-400 cm^{-1} with a spectral resolution of 4 cm^{-1}, yielding characteristic absorption spectra (Fuji, 2021).

3.9.2 Determination of heavy metals using ICP-OES

Heavy metals were analyzed using an Inductively Coupled Plasma (ICP) spectrometer, model ICPE-9000. Analysis was done according to the method described by Turek et al. (2019). Sludge was dried to a constant weight at 105 °C, then a 1 g aliquot of sludge was digested using a 4 cm^3 HNO_3 and 12 cm^3 HCl mixture. The samples were boiled for 2 h in beakers inside a fume hood. All solutions with undissolved residual phases were transferred into the 100 cm^3 volumetric flasks and filled to the mark with deionized water, followed by filtration. Heavy metals in the solutions were analyzed using ICP.

3.9.3 Amino acids analysis using HPLC-MS

Amino acid analysis was conducted using Agilent 1290 High-Performance Liquid Chromatography (HPLC) paired with a 6120 series single quad Mass Spectrometer (MS) (Agilent Technologies Inc., Santa Clara, CA, USA). The recovered protein

supernatant was analyzed for its amino acid composition using the HPLC-MS technique. The crude protein supernatant was hydrolyzed into amino acids with 1.5 mL of 6N HCl at 110°C for 24 h under nitrogen (Mustea et al., 2019). The hydrolysates were then evaporated at 40 °C to dryness in a stream of nitrogen, and the residues were reconstituted in 1 mL of 0.01% formic acid/acetonitrile (95:5). The mixture was vortexed for 30 s, sonicated for 30 min, and then centrifuged at 14,000 rpm. The supernatant was filtered through a 0.45 μm syringe filter and analyzed on an Agilent 1290 HPLC coupled to a 6120 series single quad MS. The chromatographic separation was achieved using a Zorbax RX-C18, 4.6 × 250 mm, 5 μm column, operated at 40 °C. The mobile phase consisted of water (A) and acetonitrile (B), all supplemented with +0.01% formic acid.

The gradient elution adopted was as follows: 0-6 min, 10% B; 6-7.5 min, 10-80% B; 7.5-10.5 min, 80% B; 10.5-13 min, 80-100% B; 13-18 min, 100% B; 18-20 min, 100–10% B; 20–25 min, 10% B. The flow rate was programmed as follows: 0-13 min; 0.25 mL/min, 13-25; 0.5 mL/min, and the injection volume was 5 μL. The mass spectrometer was operated in API-ES positive mode at a mass range of m/z 50-600 at a 70 eV cone voltage. An authentic standard of amino acids (Sigma-Aldrich, St. Louis, MO, USA) was analyzed by LC-MS and used to externally quantify the amino acids. All the analyses were performed in triplicates (Kibet et al., 2024).

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Characteristics of WAS and food waste

The characterization of FW and WAS included the determination of the following parameters: pH, moisture content, total suspended solids (TSS), volatile suspended solids (VSS), heavy metals, and protein content, as shown in Table 4.1.

Table 4.1: Characteristics of WAS and FW

Parameter	Units	WAS	FW
Moisture content	%	69.22	75
Protein	%	19.92	21.45
pH		6.5	5.5
TSS	Mg/L	4673	10350
VSS	Mg/L	3080	7580
VSS/TSS	Mg/L	0.66	0.73
Cd	Mg/L	7.41	-
Fe	Mg/L	18705	569
Ni	Mg/L	13.9	-
Mn	Mg/L	6705	1200
Pb	Mg/L	42.35	-
Cr	Mg/L	53.50	-
Zn	Mg/L	867.65	291
Cu	Mg/L	155	6.95

A moisture content of 69% in WAS implied that the sludge used in this study had been partially dewatered but still retained a substantial amount of water. A VSS range between 50-70% indicates that a sample contains high content of organic matter (Ahnert et al., 2021). Thus, the VSS of approximately 66% of the TSS in this study indicated that a significant portion of the sludge is organic. This suggests a healthy microbial population required to break down the biodegradable organic contaminants in an activated sludge system (Ahnert et al., 2021). The VSS/TSS ratio of 0.73 suggests that a large portion of the total suspended solids in food waste is organic and often contains nutrients such as proteins, carbohydrates, cellulose, and lipids (Cicekalan et al., 2024). In comparison with WAS protein, FW had 1.6 g/100 g protein more than WAS. This confirms that the high organic matter in food waste, as shown by a VSS/TSS of 0.73, contributed to high protein content. Additionally, the results of

elemental analysis in food waste demonstrate high levels of contamination against the safety standards set by the World Health Organization (WHO) and the Food Agriculture Organization (FAO). The concentrations of Cu, Zn, Mn, and Fe were above the WHO and FAO limits (Mundi et al., 2019). According to Scutarașu & Trincă (2023), the high levels of these elements are primarily due to their natural abundance in food, and their levels may have been elevated by industrial growth and the excessive use of chemicals in agriculture. However, Cr, Ni, Cd, and Pb were present in low concentrations below detectable levels.

4.2 Pre-treatment for heavy metals removal

The metals detected in WAS included Ni, Cr, Cd, Mn, Pb, Zn, Fe, and Cu, which were above the permissible levels for animal feeds. However, after a two-stage removal process involving pretreatment using dilute acid followed by removal during protein extraction, substantial amounts of heavy metals were reduced to allowable limits, as shown in Table 4.2.

Table 4.2: Removal of heavy metals during pre-treatment

Element	Raw sludge (Mg/L)	Pretreated sludge (Mg/L)	Extracted Protein (Mg/L)	Removal Efficiency (%)	WHO/FAO /EU limits (Mg/L)
Cr	53.50	24.80	0.37	53.64	1
Ni	13.9	7.80	0.16	43.80	2
Cd	7.41	ND	ND	100	0.5
Mn	6705	37.70	ND	99.44	150
Pb	42.35	2.50	ND	94.10	10
Zn	867.65	38.60	1.59	95.55	150
Cu	155	121	0.68	21.94	170
Fe	18705	5890	26.5	68.51	100

ND: Not detectable

A removal efficiency of more than 90% was recorded for lead, manganese, cadmium, and zinc. Conversely, the pre-treatment step had the least removal efficiency for copper, 22%. These results coincides with the findings by (Yoshizaki & Tomida, 2000). The low removal efficiency of Cu during pre-treatment may be attributed to its binding affinity to organic matter in the sludge to form stable complexes, which can make it resistant to leaching by acid (Ashworth & Alloway, 2007). Despite this observation, the extraction step after pretreatment led to further purification due to the

selectivity of the alkali used towards protein extraction (Cruz-Solis et al., 2023). This led to a further reduction in the amount of the heavy metals in the extracted protein, with concentrations of Cd, Mn, and Pb below the limits of detection. Unlike copper, zinc, lead, manganese, and cadmium do not strongly bind to the extracellular polymeric surface matrix and are therefore available for leaching by acid (Deng et al., 2024; Hullebusch, 2006). This explains the high extraction efficiency of these elements. Additionally, Fe recorded the highest concentration among the elements analyzed. The elevated levels of iron can be attributed to industrial effluents containing iron-rich materials discharged from the facilities surrounding the sampling location. Kariobangi Sewerage Treatment Works is located near the Kariobangi light industries that deal with steel manufacturing and processing.

4.3 Results of Kjeldahl method validation

To check the accuracy of the procedure and equipment used in protein extraction, nitrogen recovery tests were conducted to assess potential nitrogen loss during digestion, distillation, or titration. Acceptable nitrogen recovery rates fall within 95-105%, depending on the complexity of the matrix and the analytical conditions (Latimer & Latimer, 2023). To verify the accuracy of the digestion step, tryptophan was used as a standard organic nitrogen compound. Tryptophan has 13.98% nitrogen content (Corgar, 2010). In this step, 13.95% nitrogen was recovered, corresponding to a 99.8% recovery rate. This indicates that the digestion step was highly effective and all the nitrogen in the samples was converted to ammonium sulfate $(NH_4)_2SO_4$. The digestion step is critical in protein quantification, and incomplete digestion can lead to underestimation of protein, which compromises the reliability of the protein quantification process (Sáez-Plaza, Michałowski, et al., 2013). In addition to that, a nitrogen recovery rate of 102.6% was recorded during analysis of the distillation and titration efficiency. This shows that the distillation and titration were precisely controlled, ensuring efficient trapping of ammonia into boric acid solution. This emphasizes the reliability of the Kjeldahl method of quantification.

4.4 Effects of temperature and pH on protein extraction

4.4.1 Effect of temperature on protein extraction

It was observed that the amount of protein extracted increased from 11 g/100 g to 16 g/100 g when the temperature was increased from 90 °C to 120 °C, after which there

was a remarkable decline to 7 g/100 g when the temperature was increased stepwise to 130 °C as shown in Figure . A similar trend has previously been reported (Gao et al., 2022a). As the temperature increases from 90 to 120, the structure of the sludge is destroyed, and microbial cells are lysed, releasing organic material into the liquid phase and thus increasing the protein content (Gao, Weng, et al., 2020c). However, a decline in protein concentration after 120 °C could be due to favorable conditions that promote the Maillard reaction (MR) between amino acids and carbohydrates, thus lowering the protein yield (N. Yang et al., 2023).

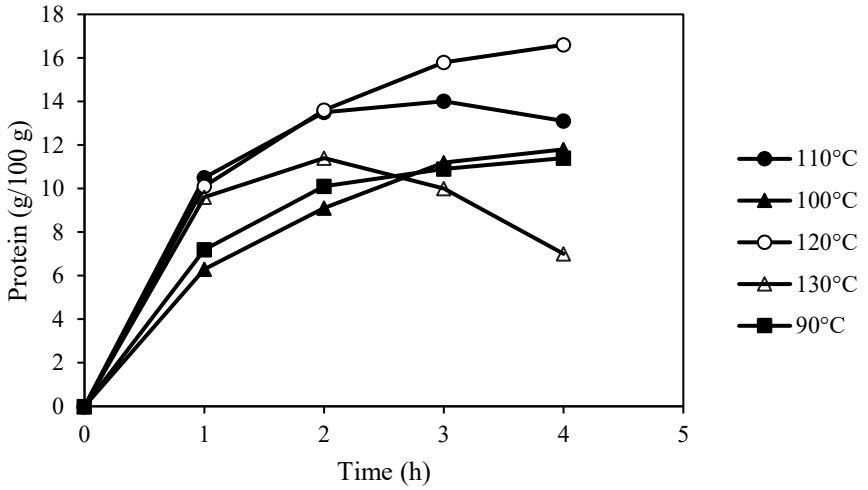

Figure 4.1: Effect of temperature on protein extraction at a constant pH of 13

4.4.2 Effect of pH on protein extraction

The effect of pH on protein concentration was investigated at a constant temperature of 120 °C. Figure shows that as the pH was increased from 10 to 13, the amount of extracted protein increased from 4 g/100 g to 16 g/100 g. Compared to pH 12 and 13, the rates of extraction at pH 10 and 11 were lower. This can be attributed to the low alkali concentration that was insufficient to break or lyse the cell membrane of the cells in the sludge to release protein (K. Xiao & Zhou, 2020a). Conversely, at pH 13, the alkali concentration was adequate, resulting in an effective release of protein into the aqueous phase since a high alkaline concentration facilitates the solubility of sludge proteins (Gao, Weng, et al., 2020c). Varying pH can lead to increased protein concentration, as protein solubility is highly pH-dependent due to the amphoteric nature of amino acids that make up the protein (Kramer et al., 2012). High alkaline concentrations, particularly within the pH range 10-13, have been reported as optimal

for extraction of protein from waste-activated sludge. Under such conditions, bacterial surfaces of sludge become increasingly negatively charged, creating a high electrostatic repulsion that causes cell wall disruption for protein release (N. Yang & Yang, 2025). X. Liu et al. (2015) reported a 42.2% decrease in soluble protein when pH was decreased from 12.0 to 3.0, showing the effect of high alkaline conditions on WAS. In another study by Xiao et al. (2017), soluble protein concentration increased by 23 times under an alkaline treatment of pH 12, while Yan, Zhang, Wan, et al. (2023) achieved a maximum of 68% extraction efficiency at a pH of 13. As shown in Figure , compared with the effect of temperature, pH had a greater influence on the extraction of protein from sludge. This is due to the direct influence of pH on the solubility of WAS proteins (Gao, Weng, et al., 2020c).

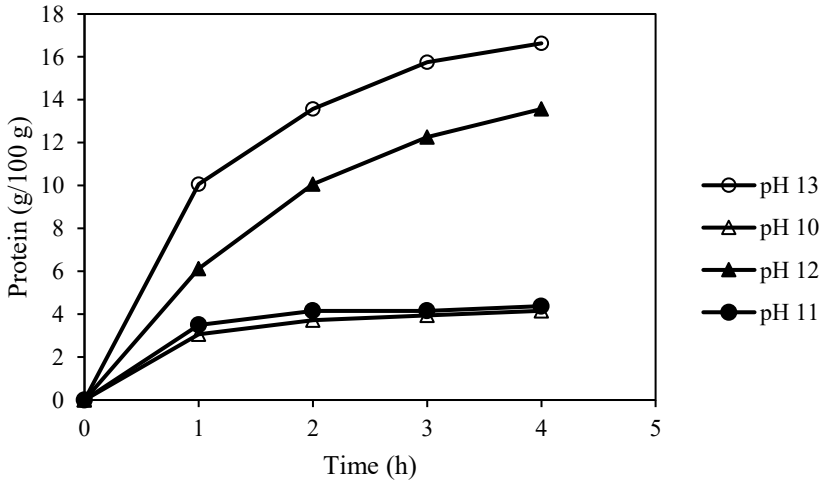

Figure 4.2: Effect of pH on protein extraction at 120 °C.

4.4.3 Combined effect of temperature and pH on protein extraction

In Figure the protein extraction increased gradually with an increase in both temperature and pH. When the pH was increased from 10 to 13, with a subsequent increase in temperature from 90 °C to 120 °C, the protein yield increased fourfold, peaking at 16.6 g/100 g. The increase in protein concentration as a result of an increase in both alkali concentration and temperature can be attributed to the destruction of sludge structure as a result of high temperatures, resulting in ease of permeation of alkali into the sludge flocs (Gao, Wang, et al., 2020b). As a result, it leads to solubilization of the membrane proteins, saponification of the membrane lipids, and destruction of microbial cells, resulting in a higher release of protein into the liquid phase (Song et al., 2019). Additionally, proteins unfold at high temperatures (Lapidus,

2017), exposing charged groups within the sludge structure, which enhances the solubility of WAS protein through electrostatic repulsion (García Becerra et al., 2010).

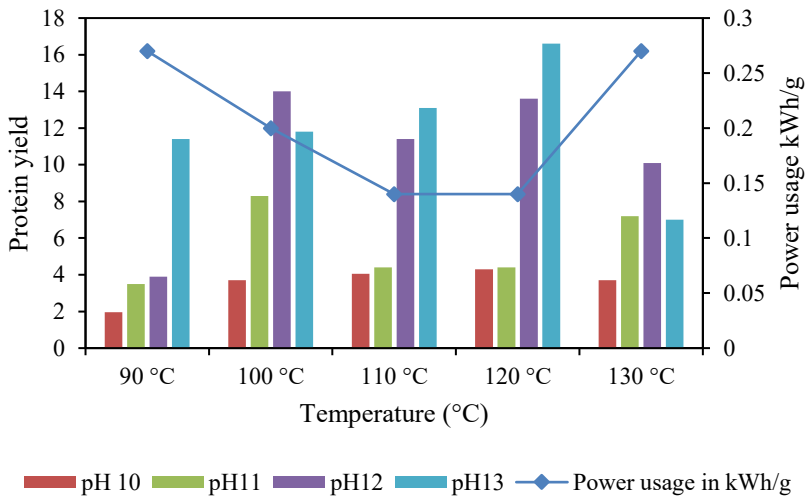

Figure 4.3: Power usage and the combined effect of temperature and pH on protein extraction.

There was a notable decline in protein yield at 130 °C and a high pH of 13. At this temperature and pH, it was observed that the extracted solution was dark brown, which could be attributed to the Maillard reaction (MR), where protein degrades into amino acids and reacts with simple sugars to form dark brown-colored melanoidins (Song et al., 2019; N. Yang et al., 2023). Therefore, the availability of amino acids and protein is significantly reduced by the MR compounds formed under high temperatures and pH (Al-abbasy et al., 2024). Under these conditions, the MR can affect the quality of the recovered protein (N. Yang et al., 2022). Subsequently, optimization of pH and temperature is crucial in maintaining maximum protein yield and quality. Several studies recommend a pH above 10 and an extraction temperature greater than 80 °C for maximum protein extraction efficiency (Hadinoto et al., 2024; Xiang et al., 2017b).

At lower temperatures of 90 °C and 100 °C, there was a higher protein yield at high pH values of 12 and 13 than at pH values of 10 and 11. This could be because at high pH, proteins are negatively charged, which increases their solubility (Ferreira Machado et al., 2007). Moreover, at low temperatures, the stability of proteins is high, and therefore, this contributes to high protein concentrations at low temperatures and high alkaline concentrations.

4.5 Energy efficiency of Thermal alkaline hydrolysis

Power consumption in kWh/g was calculated to assess the energy efficiency of thermal alkaline hydrolysis across different temperatures. Although thermal alkaline hydrolysis gave maximum protein yield, the overall energy consumption could offset the benefits if not carefully managed. While the previous research focused on improving protein recovery, little attention has been given to the energy consumption of the extraction process. This gap is critical, as the thermal alkaline hydrolysis is energy-intensive, requiring heat input.

At a temperature of 90 °C, the power requirement was 0.27 kWh/g. However, as the temperature was increased to 100 °C, the power consumption dropped significantly to 0.2 kWh/g because of the increase in protein yield. With a further increase in temperature to 120 °C, the power consumption decreased to 0.14 kWh/g. However, increasing the temperature beyond 120 °C led to higher power consumption due to a decrease in yield, as shown in Figure 4.3. Based on these observations, the process was most energy efficient at 120 °C. Thus, the best conditions for protein extraction were a temperature of 120 °C, a pH of 13, and a hydrolysis time of 4 hours. Under these conditions, the extraction yield and protein concentration of protein were 83% and 16.6 g/100 g, respectively.

4.6 Co-extraction of protein from sludge and food waste by alkaline hydrolysis

Co-extraction of protein from food waste (FW) and WAS was studied to establish if there could be any synergy that would improve the protein yield. The ratios of FW to WAS studied were 1:0, 3:1, 1:1, 1:3, and 0:1 (S, 2009) at a pH of 13. The protein concentration in raw sludge was 19.9 g/100 g, while food waste had a protein concentration of 21.5 g/100 g, as shown in Table 4.3. Even though FW had a higher protein content, thermal alkaline hydrolysis had lower protein extraction efficiency from food waste than WAS. Subsequently, when the ratio of FW to WAS was lower, the protein yield was higher. This shows that the thermal alkaline hydrolysis has higher extraction for WAS protein. This may be due to the difference in composition between food waste and sludge, as the FW protein often co-exists with compounds such as starch, cellulose, pectin, and lipids in the cells, which may lower the protein extraction efficiency (Kamal et al., 2021b). Conversely, alkaline thermal hydrolysis

has a good sludge lysis ability, thus releasing protein into the aqueous phase (Gao et al., 2022b).

Table 4.3: Co-extraction of protein from sludge and food waste by alkaline hydrolysis

Mixing ratio (FW: WAS)	Protein (g/100 g)	Extracted protein (g/100 g)	Efficiency (%)
1:0	21.45	11.38	53
3:1	19.26	5.69	30
1:1	17.07	9.63	56
1:3	17.95	14.01	78
0:1	19.92	16.63	83

4.7 Co-extraction of protein from sludge and food waste by Acid hydrolysis

The acid method of hydrolysis was used to investigate the effect of acid on protein concentration in FW and WAS. The extracted protein in food waste and sludge was 10.5 g/100 g and 13.1 g/100 g, respectively. A FW:WAS ratio of 3:1 had a protein extraction of 8.8 g/100 g, while at a FW:WAS ratio of 1:1, 11.4 g/100 g protein was recovered, as shown in Table 4.4. Subsequently, extraction of 11.8 g/100 g was achieved when the FW:WAS ratio was 1:3. Generally, the amount of protein recovered increased as the ratio of FW to WAS decreased. This shows that acid thermal hydrolysis is more efficient in extracting WAS protein than the food waste protein. This efficiency may be due to the composition of waste-activated sludge, which contains a higher proportion of microbial cells and extracellular polymeric substances that are more readily broken down by acid thermal hydrolysis (Gao, Wang, et al., 2020c). In contrast, food waste often contains a variety of complex organic materials, including fats, oils, and carbohydrates, which can make protein extraction more challenging (Tasaki, 2021).

Table 4.4: Co-extraction of protein from sludge and food waste by Acid thermal hydrolysis.

Mixing ratio	Protein (g/100g)	Extracted protein (g/100 g)	Efficiency (%)
1:0	21.45	10.51	49
3:1	19.26	8.75	45
1:1	17.07	11.38	67
1:3	17.95	11.82	66
0:1	19.92	13.13	66

4.8 Comparative analysis of the use of alkaline and acid methods of protein extraction

A comparative study on the performance of alkali and acid hydrolysis of mixed FW and WAS was carried out to determine the best extraction method. As shown in Figure , there was no significant increase in protein yield in co-extraction. Although food waste had higher protein content than waste-activated sludge, the analysis of variance (ANOVA, $p = 0.09$) revealed no significant increase in the protein yield during co-extraction from the food waste-sludge mixture, as shown in Table 4.5. This suggests that substrate mixing did not enhance protein extraction under the tested hydrolysis conditions.

Table 4.5: ANOVA table for the analysis of the use of alkaline and acid methods of hydrolysis.

SUMMARY	Count	Sum	Average	Variance
1:0	2	21.89	10.94	0.38
3:1	2	14.44	7.22	4.69
1:1	2	21.01	10.51	1.53
1:3	2	25.81	12.91	2.39
0:1	2	29.76	14.88	6.13
Acid yield	5	55.59	11.12	2.64
Alkaline yield	5	57.34	11.47	17.47

ANOVA Source of Variation	SS	df	MS	F	P-value	F crit
Rows	65.64	4	16.41	4.43	0.09	6.39
Columns	0.31	1	0.31	0.08	0.79	7.71
Error	14.82	4	3.71			
Total	80.78	9				

Generally, it was observed that thermal alkaline hydrolysis was superior to the thermal acid hydrolysis method at all ratios tested except at FW:WAS ratios of 3:1 and 1:1. The highest protein extraction of 16.3 g/100 g was attained at a FW:WAS ratio of 0:1. This observation can be linked to the acid solubilization, which has limited floc disruption compared to alkaline hydrolysis (De Sousa et al., 2021). Apart from breaking sludge floc, thermal alkaline hydrolysis damages the cell membrane, hence robust protein release (De Sousa et al., 2021). Moreover, acid hydrolysis is less effective in protein extraction and often causes protein precipitation, thus lowering the protein yield (Novák & Havlíček, 2016).

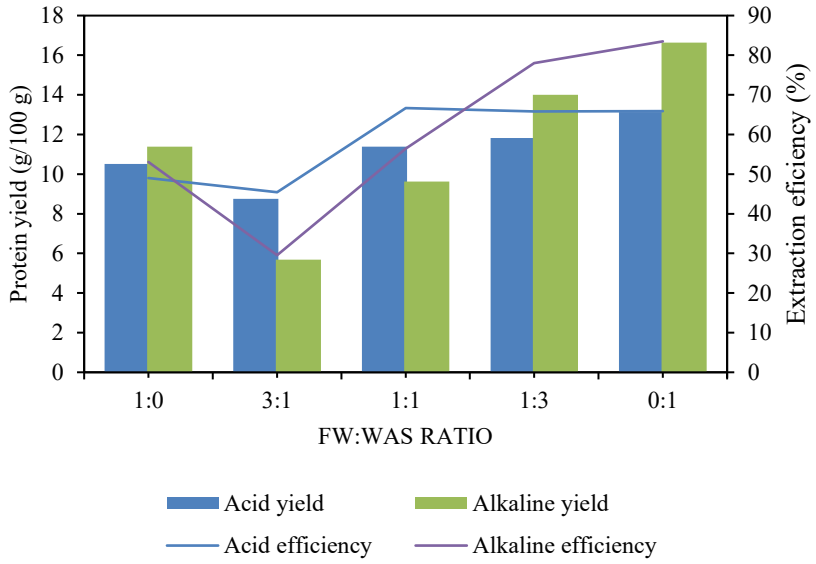

Figure 4.4: Comparative analysis of the use of alkaline and acid methods of protein extraction

4.9 Characterization analysis of hydrolysis products

FTIR spectra of sludge and the extracted protein are displayed in Figure 4.6. FTIR was essential for analyzing the structural changes that occur during the process of extraction of protein from WAS. The broad, mid-intense band between 3400 and 3200 cm^{-1} represents O–H stretching vibrations of hydroxyl groups. An intense peak at around 1640 cm^{-1} was assigned to the stretching vibrations of the –C=O group of amide I, while the peaks at 1560-1520 cm^{-1} are attributed to the N–H bending vibrations of the amide II band (Berthomieu & Hienerwadel, 2009). The absorption bands in the region from 3000 to 2800 cm^{-1} can be linked to the presence of hydrocarbon chains on the organic matter of the sludge (De Oliveira Silva et al., 2012). These bands are associated with the asymmetric stretching of the C–H methyl bonds

(2955 cm^{-1}) and methylene (2920 cm^{-1}) groups and are also related to the symmetric C–H stretching of methylene (2850 cm-1) groups. Double peaks at 1030 and 1009 cm^{-1} suggest specific functional groups, which can be linked to C–O–C and C–O stretching vibrations of carbohydrates and polysaccharides in the biomass (Grube et al., 2006; Hui et al., 2023). The appearance of peaks in the treated sludge at 1400 cm^{-1} represents symmetric stretching vibrations of carboxylate groups (COO–) commonly found in proteins and other organic compounds broken down during the hydrolysis process (C. Liu et al., 2024). Similarly, the appearance of a small peak at 1340 cm^{-1} can be assigned to the bending vibrations of CH_3 groups or the C–N stretching vibrations in amides, which are also components of proteins.

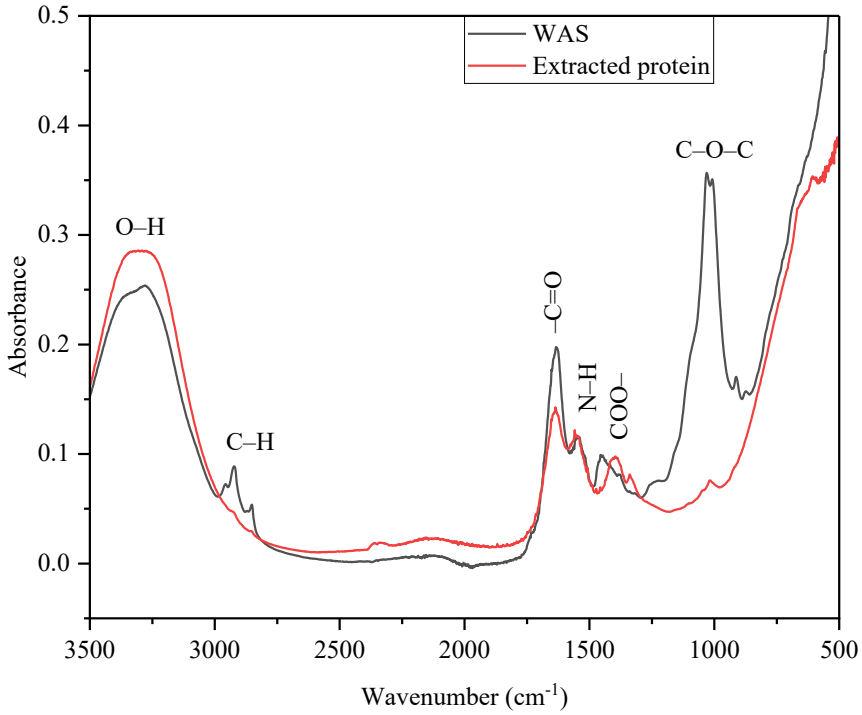

Figure 4.6: FTIR spectra of raw sludge and protein supernatant.

It is also noteworthy that the shift in peaks, as well as the appearance and disappearance of certain peaks, are indications of organic matter transformation during the protein extraction process. Subsequently, all the peaks except the amide I and amide II bands, which are characteristic bands that indicate the presence of protein, disappeared. This observation highlights the selectivity of the thermal

alkaline hydrolysis method employed in this study for targeting and isolating proteins from the sludge matrix.

4.10 Amino acid analysis

The high levels of protein in WAS make it a rich source of amino acids (Zhang et al., 2018; Zhou et al., 2023). The amino acid distribution in protein obtained from WAS compared to conventional protein sources is presented in **Table 4.6**. 15 amino acids, consisting of 9 essential amino acids (EAAs) and 6 non-essential amino acids (NEEAs), were detected. Among the EAAs, threonine was the most abundant. Threonine plays a critical role in macromolecular biosynthesis, gut homeostasis, and the modulation of nutritional metabolism (Tang et al., 2021). It also promotes body weight gain, feed intake, and carcass weight when used as a feed supplement (Qaisrani et al., 2018). Furthermore, threonine works synergistically with lysine and methionine by playing a critical role in antioxidant and immune functions (Ji et al., 2019), thus complementing the growth and health benefits provided by lysine and methionine, which are considered co-limiting amino acids (Erickson & Kalscheur, 2020).

The ratio of lysine to methionine is critical for optimal growth and development of body tissues, particularly in young animals (Lisnahan et al., 2017). The current study found a ratio of 2.5, which is above the 1.9 threshold recommended by FAO. This value correlates with the fishmeal's ratio of 2.6, indicating the possibility of adding WAS protein to the fishmeal. Moreover, the presence of other essential amino acids like valine, arginine, histidine, phenylalanine, and leucine in WAS protein emphasize its potential as a feed supplement. Supplementation of animal feeds using protein derived from WAS will not only reduce the environmental burden associated with the disposal of waste-activated sludge but also contribute to global food security by reducing the reliance on human-edible protein sources for animal feeds.

Table 4.6: The amino acid distribution (g/100g) in WAS compared to conventional protein sources.

Amino acid	Extracted WAS protein	Soybean meal	Fishmeal	FAO Ref. protein
Valine	2.7	5.2	4.7	4.2
Threonine	3.3	4.4	3.8	2.8
Phenylalanine	2.2	5.3	3.5	2.8
Methionine	1.1	1.3	2.9	2.2
Lysine	2.7	6.6	7.6	4.2
Leucine	1.8	7.6	6.5	4.8
Isoleucine	0.2	5.8	3.9	4.2
Histidine	0.5	2.7	2	-
Alanine	4.3	-	-	-
Arginine	1.6	7.3	6.8	-
Glutamic acid	5.7	-	-	-
Serine	2.1	-	-	-
Glycine	3.2	-	-	-
Proline	1.8	-	-	-
Tyrosine	1.5	4.1	3	2.8

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Vriens et al. (1989) carried out feeding experiments on pigs, rats, poultry, and steers using waste-activated sludge. In their report, satisfactory results were obtained with tests on poultry, as no adverse effects on the growth and health of chicks and on layers were noted. Similarly, Nkhalambayausi-Chirwa & Lebitso. (2012) assessed the nutritional value of single-cell protein from WAS after removal of heavy metals and noted that chicks fed with WAS gained more weight than chicks fed with conventional feed. The current study, therefore, sought to determine the potential of WAS protein as a nutritional supplement in chicken feed. However, to date, there is no existing literature about the protein content of WAS-derived protein, and therefore, the extracted protein should undergo further testing to determine if there are any antinutritional factors as well as to determine its digestibility.

4.11 Statistical optimization of protein extraction using RSM

Response surface methodology (RSM) was used in the optimization of the thermal alkaline hydrolysis process. There was no significant increase in protein yield in the co-extraction of protein from FW and WAS, as shown in Figure hence, only optimization of protein extraction from WAS using RSM was considered. The protein extraction analysis using custom design in RSM could be best described by second-degree polynomial models. Equations 8 and 9 represent quadratic models that guide yield (Y) and extraction efficiency (E), respectively, where A, B, and C are temperature, pH, and time, respectively.

$$Y= 7.96+1.31A+4.48B+1.64C+0.60AB+0.31AC+0.99BC-2.05A^2+0.82B^2-0.67C^2 \quad (8)$$

$$E=39.93+6.35A+22.45B+8.04C+3.15AB+1.97AC+5.06BC-10.00A^2+3.86-3.11C^2 \quad (9)$$

The ANOVA for protein yield is presented in Table 4.6. The significance of the model was checked by examining the p-factor and the F-factor values. The model was significant as suggested by a P value of 0.0001 for both protein yield and efficiency and F values of 46.94 and 45.67 for protein yield and extraction efficiency, respectively. Also, the model terms A, B, C, A^2, and the interactive effect of BC are significant in the model equation

Table 4.6: ANOVA table for the protein yield.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1192.11	9	132.46	46.97	< 0.0001	significant
A-Temperature	68.63	1	68.63	24.34	< 0.0001	
B-pH	893.38	1	893.38	316.79	< 0.0001	
C-Time	119.50	1	119.50	42.37	< 0.0001	
AB	7.93	1	7.93	2.81	0.0981	
AC	2.18	1	2.18	0.7733	0.3822	
BC	23.93	1	23.93	8.49	0.0048	
A^2	58.89	1	58.89	20.88	< 0.0001	
B^2	10.58	1	10.58	3.75	0.0568	
C^2	7.10	1	7.10	2.52	0.1171	
Residual	197.41	70	2.82			
Cor Total	1389.52	79				

In addition to the p-value, other statistical parameters, including the coefficient of determination (R^2), the coefficient of variation (CV%), predicted R^2 (R^2_{pred}) of, and adjusted R^2 (R^2_{adj}) were used to analyze the effectiveness of the generated model. R^2 of 0.8579, predicted R^2 of 0.8227, and adjusted R^2 of 0.8397 were calculated as shown in Table 4.7. R^2 and adjusted R^2 values close to one and smaller standard deviation values indicate the level at which the empirical model fits the experimental data (Nahemiah et al., 2015). R^2 value of 0.86 in this study shows that 86% of the variations can be explained by the model. Also, the predicted R^2 and the adjusted R^2 calculated are consistent since their difference is less than 0.2, indicating a better predicting response of the quadratic model in both responses. Adeq precision evaluates the signal-to-noise ratio, and a value greater than 4 is desirable (Roudi et al., 2021). Thus, 25.76 in this study represents an adequate signal to navigate the design space.

Table 4.7: Fit Statistics for protein yield

Std. Dev.	1.68	**R^2**	0.8579
Mean	7.02	**Adjusted R^2**	0.8397
C.V. %	23.92	**Predicted R^2**	0.8227
		Adeq Precision	25.7594

Additionally, at a 95% confidence level, there exists a correlation between the predicted models and observed response values, as shown in Figure 4.7. This suggests that the quadratic model can accurately predict both responses

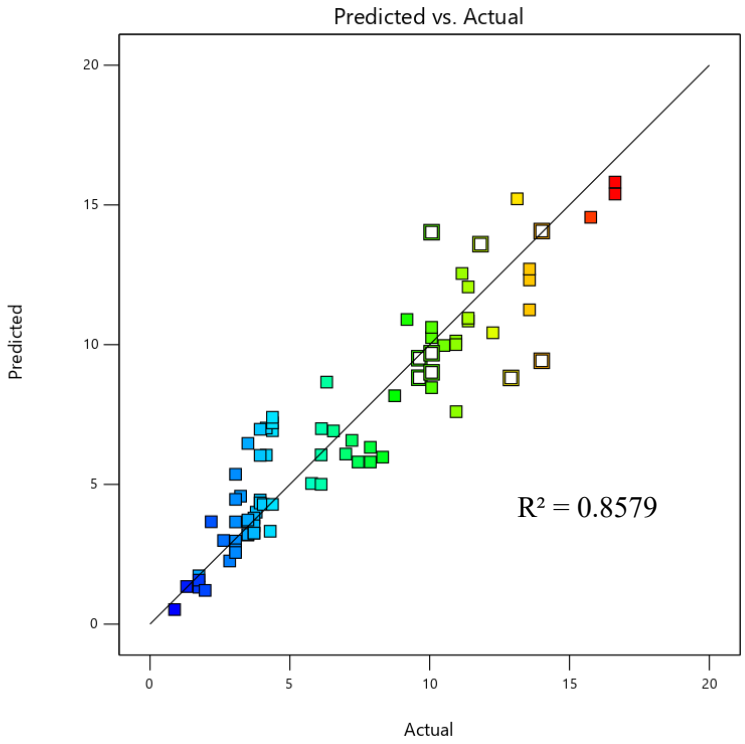

Figure 4.7: Plot for predicted values against experimental values of Protein yield.

4.12 Response surface plots

3D surface plots were used to investigate the combined effect of independent variables on the responses. Figure 4.8 shows the effect of increasing temperature from 90 °C to 130 °C and pH from 10 to 13 on protein yield. At a temperature of 120 °C and pH < 12, the contour lines were widely spaced, which implied that the protein yield and extraction efficiency were relatively low. However, at pH above 12 and temperatures greater than 120 °C, the contour lines became dense, and the shading on the response curves became intense (Montgomery, 2017). This shows that an increase in both temperature and pH leads to an increase in protein yield up to around 120 °C and pH 13, where maximum protein yield and extraction efficiency are recorded. Beyond this temperature, the conditions become unfavorable in the extraction of protein from WAS. This is depicted by a decline in yielded protein at 130 °C. Also, the curves close

to pH were denser, suggesting that pH had more influence than temperature during thermal alkaline hydrolysis (Gao, Weng, et al., 2020c).

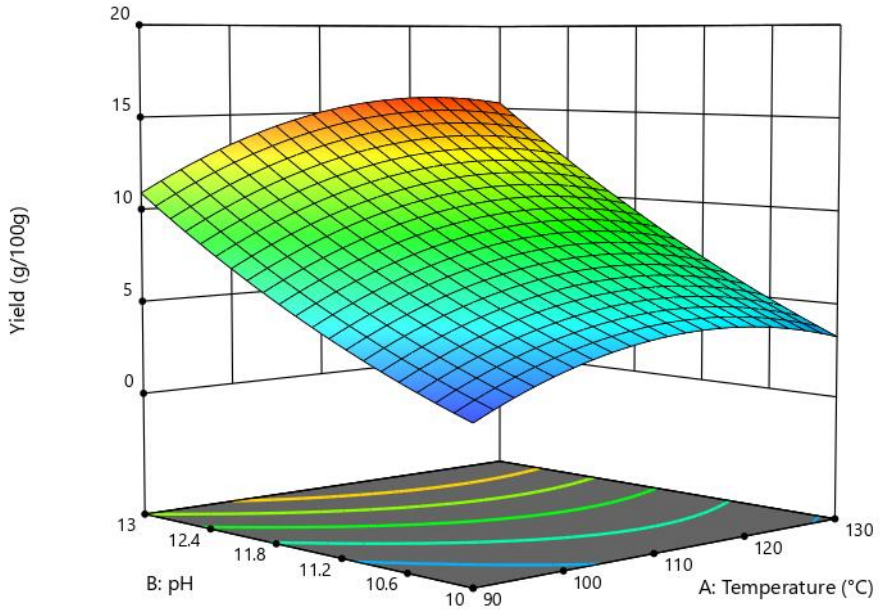

Figure 4.8: 3D surface plots showing the interactive effect of pH and temperature on protein yield.

4.13 Optimization of thermal alkaline hydrolysis

The desirability of the model was observed at optimum conditions of pH at 13 and a temperature of 120 °C, as shown in Figure 4.9. At the optimized conditions, 15.8 g/100 g and 79.5% protein yield and extraction efficiency were obtained by the model, respectively. To validate the reliability of the model, experimental runs conducted at the identified optimal conditions, g a protein yield and extraction efficiency of 16.6 g/100 g and 83.4%, respectively. This is consistent with the predicted values shown by the ramps, confirming the accuracy and precision of response surface models in predicting the optimal conditions.

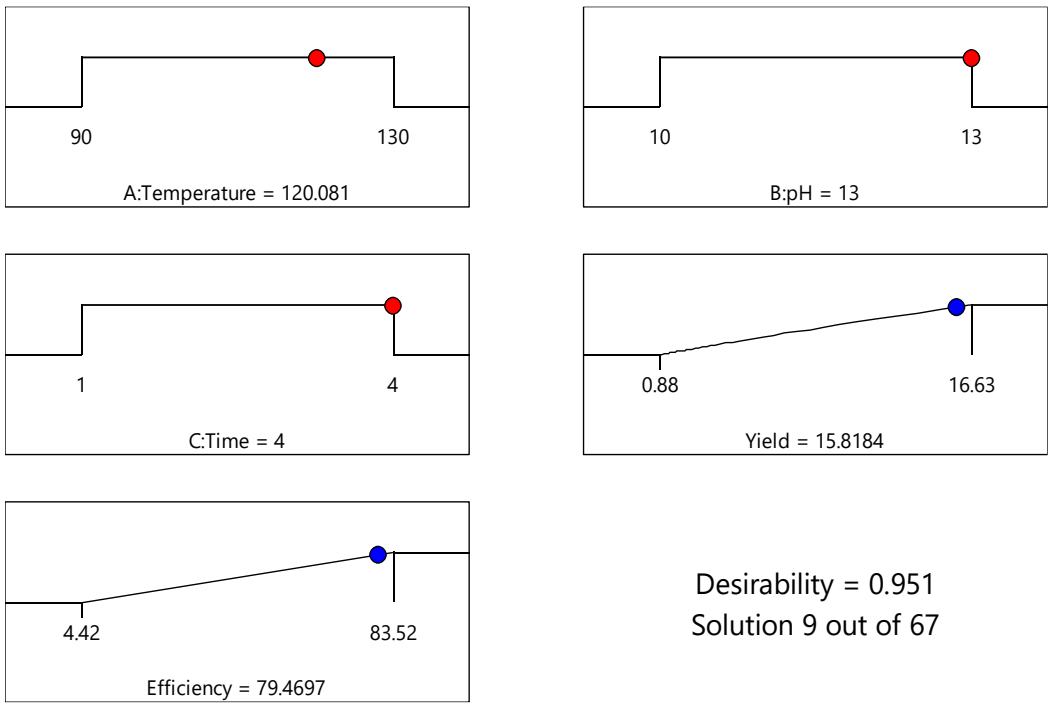

Figure 4.9: Optimization of thermal alkaline hydrolysis

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusion

Protein from food waste and waste-activated sludge was extracted and characterized, and the process of extraction was successfully optimized using the response surface methodology. It was found that FW had 21.45 g/100 g protein, while WAS had 19.92 g/100 g protein. Although the food waste had 1.6 g/100 g more protein than WAS, the two methods of extraction had a superior extraction for WAS protein than for FW. This was attributed to the differences in composition of the two substrates. The optimal conditions for maximum protein recovery were identified at a temperature of 120 °C and a pH of 13. Under these conditions, extracted protein yield and efficiency were 16.6 g/100 g and 83.4%, respectively. Furthermore, there was no significant increase in protein yield during the co-extraction of protein from the FW-WAS mixture. In addition to this, amino acid profiling of the extracted protein revealed the availability of nearly all essential amino acids. The ratio of the first limiting amino acids (lysine and methionine) met the standards set by the Food and Agricultural Organization (FAO), affirming the nutritional quality of WAS protein and its potential application in feed supplementation. The results of this study, therefore, emphasize the potential of WAS protein in feed supplementation.

5.2 Recommendations

The thermal alkaline hydrolysis led to the highest extraction efficiency and is therefore recommended as the most effective method for protein recovery from the waste-activated sludge. Also, the optimized conditions of pH 13 and a temperature of 120 °C demonstrated high protein extraction efficiency. These optimized conditions should be adopted for implementation in pilot or industrial applications to enhance the valorization of food waste and waste-activated sludge. Additionally, owing to its high nutritional quality, WAS protein could be used to supplement animal feeds.

5.3 Suggestions for future studies

i. The nutritional composition of protein extracted from waste-activated sludge underscores its potential as a supplement in animal nutrition. However, prior to full application of WAS protein, digestibility and anti-nutritional factor tests should be conducted in the forthcoming investigations.

ii. Given the lower efficiency of chemical methods of extraction in assessing food waste protein, further research is recommended on alternative methods of extraction. This will improve the overall protein recovery and facilitate the reintroduction of food waste into the circular economy, contributing to both economic and environmental sustainability

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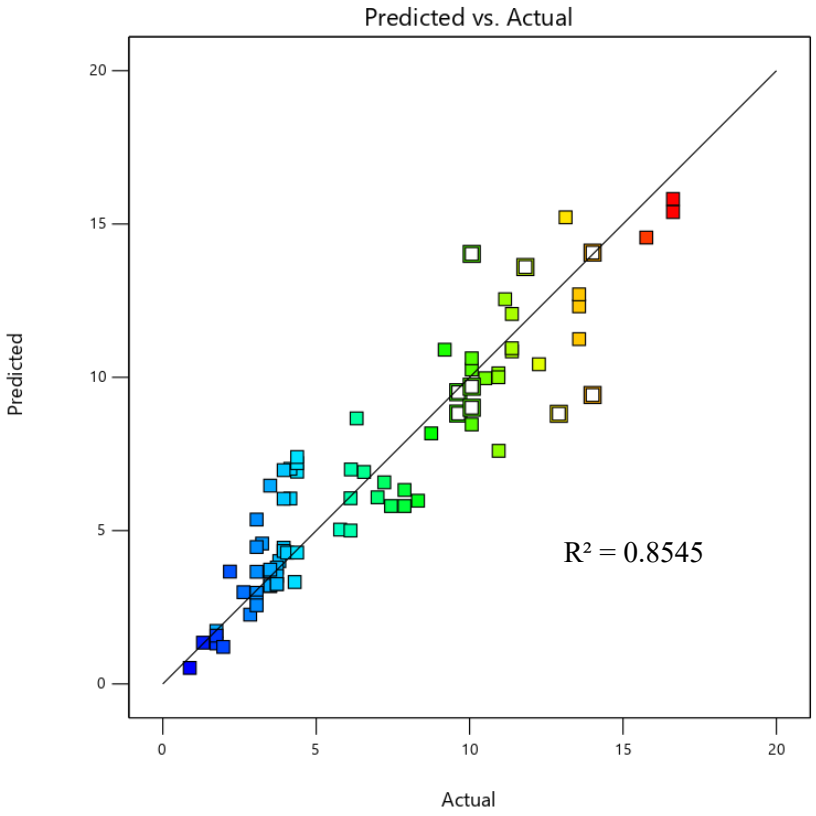
APPENDICES

Appendix 1. ANOVA table for extraction efficiency.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	29600.75	9	3288.97	45.67	< 0.0001	significant
A-Temperature	1612.71	1	1612.71	22.39	< 0.0001	
B-pH	22392.43	1	22392.43	310.95	< 0.0001	
C-Time	2869.85	1	2869.85	39.85	< 0.0001	
AB	220.15	1	220.15	3.06	0.0848	
AC	86.31	1	86.31	1.20	0.2774	
BC	632.99	1	632.99	8.79	0.0041	
A^2	1398.95	1	1398.95	19.43	< 0.0001	
B^2	234.89	1	234.89	3.26	0.0752	
C^2	152.46	1	152.46	2.12	0.1501	
Residual	5040.93	70	72.01			
Cor Total	34641.68	79				

Appendix 2*:* Plot for predicted values against experimental values of extraction efficiency.



Appendix 3: 3D surface plots showing the interactive effect of pH and temperature on extraction efficiency.