UNIVERSITY OF KWAZULU-NATAL ™

INYUVESI YAKWAZULU-NATALI

CHARACTERIZATION AND ANALYSIS OF KERATINOUS MATERIAL FROM WASTE CHICKEN FEATHERS AS PROTEIN INGREDIENT FOR ANIMAL FEED

By

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For

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in the

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PREFACE

The research work contained in this thesis was carried out by the candidate for a PhD degree while based in the Discipline of Chemical Engineering, School of Engineering of the College of Agriculture, Engineering and Sciences, University of KwaZulu-Natal, Howard Campus, Durban, South Africa. The research was financially supported by Biorefinery Industry Development Facility (BIDF) Council for Scientific and Industrial Research (CSIR), and Technology Innovation Agency (TIA).

This work represents the original work of the author and has not been submitted in any form to another university. Where the work of others was used, it was accordingly acknowledged in the text.

Signed: _____ Date: 21/07/2022

Prof. Bruce Sithole (Supervisor)

COLLEGE OF AGRICULTURE, ENGINEERING AND SCIENCE DECLARATION

1: PLAGIARISM

I, Lizzy Mpho Kekana, declare that:

i. the research reported in this thesis, except where stated otherwise, is my original work.

ii. this dissertation has not been submitted for any degree or examination to any other university.

iii. this thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons;

iv. this thesis does not contain other person's writing, unless specifically acknowledged and referenced as being sourced from other researchers. Where other written sources have been quoted, then:

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2: PUBLICATIONS

Journal Publications:

- Chapter 2.
  L.M Kekana, B.B Sithole, R. Govendin, Characterization and analysis of keratinous material for animal feed production: A review (Submitted and under review in Animal Nutrition and Feed Technology).

- Chapter 3.
  Kekana L.M, Sithole B.B, Govinden R, Khumalo M, Fagbemi O.D , Mnguni O and Dlume T, Keratinous hydrolysate profiling: Comparison of the differences obtained from different extraction methods**.** Published online in Biomass Conversion and Biorefinery, 2022.

- Chapter 4
  Kekana L. Mpho, Mnguni Olwethu, Sithole B. Bruce and Govinden Roshni, Characterization and analysis of enzymatic chicken feather keratin for animal feed production. Submitted in Journal of Basic Microbiology.

Conference Presentations:

Mpho L. Kekana, B.B Sithole, Characterization and analysis of keratinous biomass with the objective of beneficiating biomass into animal feed, Poster presentation at The 7th International Conference on Biorefinery, at Johannesburg; 18-21 August 2019 (Won most Popular Poster).

Mpho Kekana, Bruce Sithole and Roshni Govendin, Characterization and analysis of keratinous materials for animal feed production, Poster presentation at Sustainable Bioenergy and Processes 2021, 13-15th December 2021, Cape Town International Convention Centre, Hybrid (Won 3rd Poster Price).

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Yours Sincerely,

Lizzy Mpho Kekana

ABSTRACT

Keratin is one of the most abundant proteins, which is derived from wool, feathers, nails, hair, and other sources. Chicken feathers are a well-known keratin waste by-product, produced in large quantities by poultry slaughterhouses. Their disposal is expensive, and includes incineration of the waste thus contributing to greenhouse gases; or disposal in landfills, also leading to environmental pollution or they can be recycled into low-quality feeds for animals. Research is done worldwide for the beneficiation of waste chicken feathers into commercial products; these include cosmetics, pharmaceutical products, and biomedical products, and it is also useful in the production of animal feed. The focus of this research was to characterize and analyze keratinous hydrolysates formed from waste chicken feathers using enzymatic and chemical hydrolysis for their suitable applications in different industries. The novelty of this project is based on looking at analytical techniques of the keratinous hydrolysate produced from newly formed keratinolytic microorganisms and newly optimized chemical methods from the waste chicken feathers.

Different fungal and bacterial strains were tested for the degradation of waste chicken feathers. The quality and quantity of the hydrolysate formed were determined by using a combination of analytical techniques, where the characterization is done via proximate and ultimate analysis. We used Fourier Transform Infrared Spectroscopy (FTIR), which showed the presence of the keratinous structure, which is known to have high protein content. Thermogravimetric Analysis (TGA), showed that a thermally stable hydrolysates were obtained, which is known to be formed by the hydrophobic hydrolysate, which is best for animal feed. CHNS analysis showed evidence that we have high protein content in the hydrolysate. Bradford assay revealed different quantities of the hydrolysate while Sodium Dodecyl Sulphate–Poly-Acrylamide Gel Electrophoresis (SDS-PAGE), showed mostly medium to low molecular weight, due to the presence of amino acids and small peptide chain. A low Ash Content was obtained which means a cleaner fraction of keratin. The hydrolysate formed from the enzymatic hydrolysis contains a mixture of amino acids and peptides. These peptides and essential amino acids formed are known to play a special role in various biological activities.

The hydrolysates formed from different degradation methods were also compared, focusing on the qualities and quantities formed from enzymatic and chemical hydrolysis. While looking at all the characterization techniques, enzymatic was the best and suitable for animal feed due to

the obtained keratin structure, which is more soluble, contains high protein content, has low molecular weights, and has a cleaner fraction of keratin. Future work will be based on obtaining a peptide chain using Liquid Chromatography with tandem Mass Spectrometry (LC-MS/MS), then testing the hydrolysates for bioactivities.

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CHAPTER 1. INTRODUCTION

1.1 Background of the study

Keratin is one of the most abundant proteins, which has the characteristics of greater mechanical stability, chemical resistance, and low solubility. These properties are due to that keratin has the presence of hydrogen bonds, compact microfibrils, and high disulphide crosslinks between two cysteine residues (Cardamone 2010). Chicken feathers are known to contain 91% of pure keratin, which can be considered a suitable protein source (Ramya, Thangam, and Madhan 2020). The high protein source can be used in cosmetics, biomedicals, pharmaceuticals, leather tanning, detergents, fertilizers, and animal feeds. Feathers are a major waste in the poultry industry and their disposal causes environmental problems.

There are various methods that are applied for the extraction of keratin from keratinous waste. Chemical hydrolysis is one of the methods used to extract keratin using strong acids, which is known to damage the keratin and destroys some of the important amino acids (Zhang et al. 2013).

Thermochemical is used mostly to improve the yield of keratin which also ensure the structure is not destroyed, while supercritical water and high steam flash explosion treatments disintegrate the keratin (Ramya et al. 2020). Oxidation and reduction methods are used to break the disulphide bonds and they don't damage the peptide chain but they use a large quantity of oxidizing and reducing agents.

While enzymatic hydrolysis is known to have the potential for high productivity, less effluent generation, and low energy consumption (Srivastava et al. 2020). The microorganisms like bacteria, fungi, and actinomycetes are used to produce keratinases which are important in the degradation of keratin. The hydrolysis of keratin with enzymatic hydrolysis process has two main step, which includes, sulphitolysis, which is the reduction of the disulphide bond followed by the breakdown of the protein into amino acids, which is known as proteolysis (Kurnert, 1976).

The keratin produced is characterized by various techniques like FTIR, TGA, CHNS, SDS PAGE, Ash content and Bradford Assays. All these techniques combined gives an overview of the structure, quality and quantity of the hydrolysate produced.

1.2 Thesis Rational

The poultry industry generates 5 billion tonnes of waste chicken feathers globally, while South Africa generates 230 million kg per annum (Khumalo M 2019). This causes environmental problems, where costly strategies are used to get rid of the waste feathers which in turn causes all types of pollution. Waste chicken feathers can be valorized to valuable products, like keratin which is rich in protein and have high quantities of peptides and amino acids which are suitable for numerous applications. There are industrial applications for keratin hydrolysate produced from different methods. Cosmetics, bio-medicals, pharmaceuticals, fertilizers, detergents, leather industries, bio-adhesives and animal feed are some of the industries which are applicable to keratin. There are methods used to degrade the waste feathers to valuable products and have been extensively studied, with the focus on the optimization of the processes.

Using enzymatic hydrolysis is known to be one of the biotechnological processes, where it uses enzymes for the degradation of keratinous biomass, where the applications are focused in fertilizers, detergents and animal feeds. There are a variety of keratinases that degrade keratin to high quality products. Most of the researchers focuses on the production of the keratinase and the optimization of the processes. There is a wide gap in characterizing the hydrolysates produced using modern techniques and comparing the hydrolysate produced from various methods for different applications.

This research is aimed to answer the following questions,

- Can the enzymatic hydrolysis produce a keratin hydrolysate that has the animal feed quality?
- Which analytical techniques can be used to determine the quality and the quantity of the keratin hydrolysate?
- What is the difference between the chemical and enzymatic hydrolysate?
- What quality determines the applications of the hydrolysates produced?
- Do fungal and bacterial strains produce the same hydrolysate quality?

1.3 Research Objectives

The objective of this research is to fully characterize the hydrolysate from the enzymatic and chemical hydrolysis and to determine the quality of the hydrolysate produced.

The objectives of the thesis are:

- To use newly produced and characterised keratinase from a Masters thesis for the degradation of waste chicken feathers, the keratinase were produced from waste chicken feathers.
- To use hydrolysate from published optimised chemical hydrolysis method for analysis.
- To fully analyze the enzymatic and chemical hydrolysates using analytical techniques.
- To characterize the hydrolysates using the following techniques; FTIR, TGA, CHNS analysis, SDS PAGE, ash content and Branford assays.
- To compare the analyzed enzymatic hydrolysate with the chemical hydrolysate produced.
- To determine the suitability of the enzymatic hydrolysate as ingredient for the protein animal feed.

1.4 Thesis Overview

The structure of the thesis comprises of manuscripts. Each chapter is a manuscript submitted to a specific journal which is under review process or published online. All the manuscripts are structured according to the specification format of the journal submitted to.

Chapter 1. Introduces the background, thesis rational, the objectives and the overview of the thesis.

Chapter 2. Paper 1: Characterization and analysis of keratinous material for animal feed production: A Review.

Chapter 3. Paper 2: Keratinous hydrolysate profiling: Comparison of the differences obtained from different extraction methods.

Chapter 4. Paper 3. Characterization and analysis of enzymatic chicken feather keratin for animal feed production**.**

Chapter 5. Conclusions and future work

CHAPTER 2

LITERATURE REVIEW

Chapter Overview

Chicken feathers are known to consist of keratin protein. The keratin protein can be extracted from the waste chicken feathers to more valuable products which can be used in industrial applications. The applications of the end product obtained from the extraction methods depend on the quality and quantity of the protein hydrolysate formed.

In this review we critique the enzymatic hydrolysis, how most researcher focuses on the optimisation processes and neglect the full analysis of the protein hydrolysate formed. And how most of the techniques and their suitability to determine the quality and the quantity of the protein hydrolysate are not mentioned in their analysis.

This chapter reviews the importance of the quality of the enzymatic hydrolysate as a protein ingredient for animal feed, where we focus on characterization techniques. The use of a combination of analytical techniques is crucial to determine the quality for a suitable industrial application.

PAPER 1:

ANALYTICAL TECHNIQUES AND ANALYSIS OF KERATINOUS HYDROLYSATE AS SUPPLEMENT FOR ANIMAL FEEDS: A REVIEW

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Abstract

Keratin is one of the most abundant proteins that can be derived from wool, feathers, nails, hair, and other sources. A large number of keratinous by-products are mostly disposed in landfills. These disposal methods cause environmental pollution, which is air, water, and soil pollution. Various hydrolysis or extraction methods can be applied to keratinous by-products during industrial processing applications. The focus of this study is the enzymatic hydrolysis of keratinous material as protein ingredients for animal feeds which is of biotechnological interest due to the quality and quantity of the hydrolysate formed. These hydrolysate parameters are determined using a combination of analytical techniques, where the characterization is done via proximate and ultimate analysis. The enzymatic hydrolysate contains a mixture of amino acids and peptides which are key in several biological activities. This review focuses on the analytical techniques for the characterization of the enzymatic hydrolysates produced by diverse microorganisms for their quality and the quantity of the animal feed.

2.1 Introduction

Animal feeds are foods with high nutritious components and are used to feed a variety of animals. Some factors are known to determine the composition of the animal feeds, which are the prices of raw material, nutritional value of the components, nutritional requirement of the specific animal as well as rules and regulation of the government (DAFF 2015).

The production of animal feed requires the use of various agricultural raw materials, whose provenance is from industrial mills or simple farm mixes. The global animal feed market is experiencing a huge demand owing to the growth of animal-based products. Between 2015 and 2016 a growth rate of 3.7% was reported with around 1032 million tons animal feed per annum produced globally in 2016. China was the highest producer in 2016, with 187.20 million tons per annum and South Africa was ranked 22nd with 11.74 million tons per annum (DAFF 2015).

The South African feed industry is known to be about 87 years old, and came into existence after the severe droughts and depression in the1930's (DAFF 2015). The industry produces feeds for five different significant categories, including poultry, dairy, beef, sheep, and pigs (Figure 2.1). The broiler feed volumes were the highest (29%) followed closely by beef and sheep feed (28%). Globally pig feed represents the 2nd largest share of the animal feed produced, while in SA, it's only represents 8%. The remaining 5% includes feed for dogs, horses, ostriches, and aquaculture.

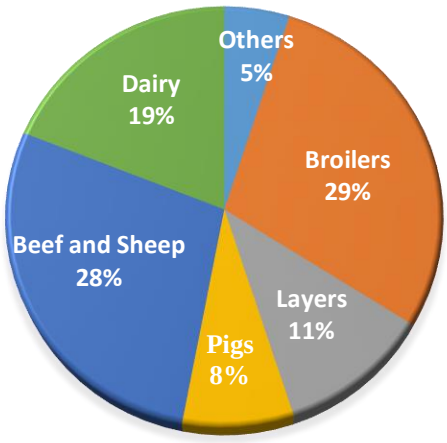

Figure 2.1 Major categories of South African animal feed industries for the year 2015/2016 (DAFF 2015)

South Africa does not import compound animal feed, which is a mixture of products of vegetable or animal origin in their natural state derived from industrial processing for oral feeding, it is mostly the feed ingredients or additives that are imported from other countries. The export and import markets of these ingredients play a significant role in animal feed production. The major primary ingredients used in animal feeds include oilcake, maize, and fishmeal. Oilcake is the protein used in most animal feeds and its ingredients includes oilseeds, soybean, groundnut cotton, and sunflower. Most of these ingredients are expensive and either imported or exported.

Due to the impact of drought, disease outbreak and an increase in population the demand for meat, milk and eggs has increased. This has led to farmers being keen on enhancing the performance and the health of their livestock to meet this increased demand and also the potential for an increase in demand for additives, minerals, vitamins, proteins, and antioxidants.

Keratin is one of the most abundant proteins present in higher vertebrates (mammals, birds, and reptiles. Food industries (meat markets and slaughterhouses) and wool industries produce millions of tons of keratin-containing biomass globally. Keratin biomass is derived from living organisms or their body parts after death. The major source of keratin includes skin, hides, wool, nails, hooves, claws, scales, and feathers. Large amounts of keratin by-products are disposed off as waste, which is a potential threat to the environment. The environmental problems lead to landscape degradation and local disturbance, which in turn leads to soil and water pollution. Chicken feathers are a well-known keratin waste by-product, produced large quantities by poultry slaughterhouses. Their disposal is expensive, and includes incineration of the waste thus contributing to greenhouse gases; or disposal in landfills, also leading to environmental pollution or they can be recycled into low-quality feeds for animals. Research is done worldwide for the beneficiation of the waste chicken feathers produced. The keratin by-products from different industries can be converted into commercial products; these include cosmetics, creams, shampoos, hair conditioners, biomedical products, and it is also useful in the beneficiation of animal feed.

2.2 Keratinous material as a protein ingredient for animal feed production

Proteins are a class of macromolecules that perform a diverse range of functions for the cell, where amino acids are the building blocks of proteins. There are 20 commonly occurring amino

acids. A protein's size, shape, and function is determined by the sequence and number of amino acids. Two amino acids are bonded together by a peptide bond which is formed by the dehydration reaction (Figure 2.2). The more the amino acid sequence grows through the peptide bonds, the resulting chain is called a polypeptide. A combination of polypeptide chains forms a protein.

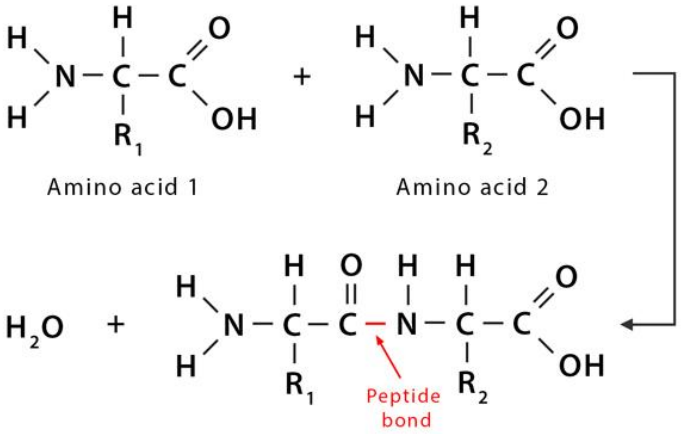

Figure 2.2. Peptide bond between two amino acids (biologydictionary.net').

Proteins are organized at four levels: primary, secondary, tertiary, and quaternary structure, depending on the complexity of the polypeptides and their conformations. The protein keratin is constituted of one polypeptide and is described as having a secondary structure. It is mainly found in two forms, α-helix and β- pleated sheet (Wang et al. 2016), (Figure 2.3). Where the α-helix its helical structure is stabilized by a hydrogen bond, red bonds in structure B, causing the chain to twist and exhibit a helical shape and β-pleated sheet consist of β-strands which are either parallel or antiparallel where chains are held together by intermolecular hydrogen bonds (red dotted bonds in structure A). Keratin is one of the proteins being researched. It is a fibrous protein and a major constituent of animal biomass in the form of hair, nail, feathers, wool, horns, and hooves. It is highly stable and insoluble in most organic solvents and is a cysteine-rich protein

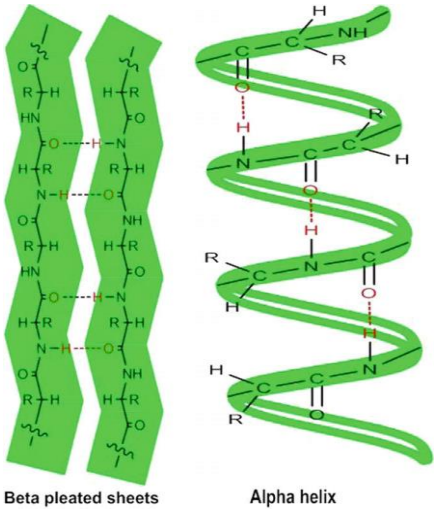

Figure 2.3. The structure of keratin A) β-pleated sheet B) α-helix (Wang et al. 2016).

Glycine and alanine, the smallest amino acids are found in high concentration in the α-helix where the keratin molecules are held together by hydrogen bonds and disulphide cross-linked bonds. These bonds form a more rigid structure and contributes to the insolubility of keratin. The α-form is mostly found in mammals in wool, hair, nails, hooves, and horns while the β-pleaded sheet is a major component of birds and reptile tissues such as feathers, claws, and beaks (Greenwold et al. 2014).

Keratinous materials contain protein consisting of amino acids and peptides, similar in composition to soybean and cotton seed extracts; hence, they can be used for nutritional purposes in animal feed. However, the materials are not easily digestible due to the highly disulphide cross-linked structure of the polypeptides, which must be cleaved before utilization. Degradation of keratin waste can, therefore, provide an inexpensive source of digestible protein and amino acids.

There are different methods of extraction for the keratinous biomass, including chemical (acid and alkaline hydrolysis), thermal, and enzymatic hydrolysis [(Fontoura et al. 2019a), (Fontoura et al. 2014a),(Lo, Too, and Wu 2012a)(Eremeev et al. 2009a),(Alahyaribeik and Ullah 2020),(Sharma, Gupta, Chik, et al. 2017)]**.** Ionic liquids are green and promising materials for the potential application in various fields because of their functionality. They are typically non-volatile, non-flammable, chemically and thermally stable and highly soluble [(Wang and Cao 2012a),(Idris et al. 2013b),(Sinkiewicz et al. 2017)]**.** Keratin from chicken feathers can be

extracted using hydrophobic ionic liquids. The extracted keratin is known to have good solubility in water while the ionic liquid itself is immiscible with water. This makes for the easy separation of the extracted keratin to be separated from the reaction system by water. But the ionic liquids are expensive compared to the inorganic reagents but since they can be reused this improves the efficiency of the whole process leading to lower overall cost. The disadvantage of this method is the low yield of the keratin extracted 75.1% vs 95% for extraction with inorganic chemicals [(Sinkiewicz et al. 2017)(Ji et al. 2014)].

The chemicals used to extract keratin from chicken feathers are reducing agents. These reducing agents facilitate the reduction in the stability of the solid keratin fibres in feathers. The reagent breaks down the keratin fibre disulphide bonds, hydrogen bonds, and salt linkages to dissolve it into a protein solution. This method utilising reducing agents causes the dissolution of chicken feathers in chemicals followed by the separation of the protein from the chemicals. The most common reducing agents are sodium sulphide, potassium cyanide, and thioglycolic acid, and others used by different authors (Khumalo et. al, 2019).The most widely used reducing agent is sodium sulphide reported to completely dissolve chicken feathers. However, the chemical and thermal hydrolysis methods are known to destroy and convert the essential amino acids required in animal feed.

The quality and the quantity of the keratin hydrolysate obtained depend on the extraction method used. Hence this review focuses on enzymatic hydrolysis due to the quality of the hydrolysate formed. The characterization techniques will be used to determine the quality and quantity of the keratin hydrolysate for future modification purposes and for the level of industrial upscaling.

2.3 Enzymatic hydrolysis

The hydrolysis of keratin waste by keratinolytic microorganisms is considered a beneficial biotechnological alternative for keratin recycling and valorization. Keratinases are enzymes that disrupt the disulphide bonds of the major amino acid in the keratin, cysteine, where they are more readily available to the extracellular microbial hydrolytic enzymes. These microorganisms are grown in a basal medium containing keratinous substrate, where they produce keratinolytic enzymes. Enzymatic hydrolysis is a reaction where the keratinolytic enzyme catalyzes the hydrolysis of the peptide bond resulting in the formation of a C-terminal (COO^-) and N-terminal (NH_3^+) and also revealing the hydrophobic groups of the amino acids residues (Patterson *et al.*, 1988). This reaction changes both the primary and secondary

structure of the protein peptide. The release of the peptide can be promoted by the activity of the microorganism and the enzyme. There are factors which play a major role in the production of active enzymes, and these include the kind of microbial strain used, the fermentation method, the basal medium composition, pH, and temperature.

The microorganisms producing the keratinases are bacteria, fungi, and actinomycetes. They are known to catalyze the release of peptides from keratin. *Bacillus* strains, like *Bacillus pumilus*, *Bacillus subtilis*, and *Bacillus licheniformis* known to degrade keratin effectively, produce feather degrading enzymes (Brandelli et al. 2015). Chickens fed by feather hydrolysate produced by Bacillus sp. and supplemented with amino acids grow as well as chicken fed by soybean meal.

Enzymatic hydrolysis of waste keratin material is thus, an attractive means of generating high quality, small or large peptides that have both nutritional and physiological or regulatory functions in livestock, poultry, and fish. Some peptides of plant or animal sources also have antimicrobial, antioxidant, antihypertensive, and immunomodulatory activities (Hou et al. 2017). It is where an enzyme is used for the hydrolysis of the feather to protein hydrolysate that can be used as a supplement in animal feed and the production of keratin peptides with biological activities (Brandelli et al. 2015). In 1995 Kida *et al.* developed an apparatus and set of conditions for effective enzymatic hydrolysis of horn and hoof proteins. The resulting enzymatic hydrolysate displayed anti-oxidative activity (Kida et al. 1995).

Protein hydrolysates are a mixture of peptides and amino acids resulting from the cleaving of the peptide bonds of keratinous biomass. The protein hydrolysate contains an enhanced level of free amino acids and with exposed hydrophobic groups, viz., their amino and carboxylic groups. The presence of hydrophobic and aromatic groups of the amino acids in the peptide chain is known to increase their antioxidant potential (Callegaro, Brandelli, and Daroit 2019)(Callegaro, Welter, and Daroit 2018). Bioactive peptide potential is sought after in animal feed formulations.

The peptides which confer biological functions beyond their nutritional value are called bioactive peptides, usually with 2 to 20 amino acid residues bonded by peptide bonds (Hou et al. 2017). Enzymatic hydrolysis can improve the solubility, viscosity and emulsification of these peptides. Most peptides generated from animal and plant proteins are incorporated in diets for feeding pigs, poultry, fish, and domesticated animals. They are feed peptides to

improve the nutrition status, gut function, and ability to resist infectious diseases (Bhat, Kumar, and Bhat 2015).

Protein food such as milk and soybean are known to produce hydrolysates which have biological activities due to the bioactive peptides which are significant for animal and human health and nutrition and relate to the feed and food industries (Kida et al. 1995) (figure 2.4). The bioactive peptides affecting human health are known to have the same effect as the bioactive peptides in the enzymatic hydrolysate, such as antimicrobial, antioxidant, antihypertensive, and immunomodulatory. This affects the quality of feed formulation and hence is widely researched.

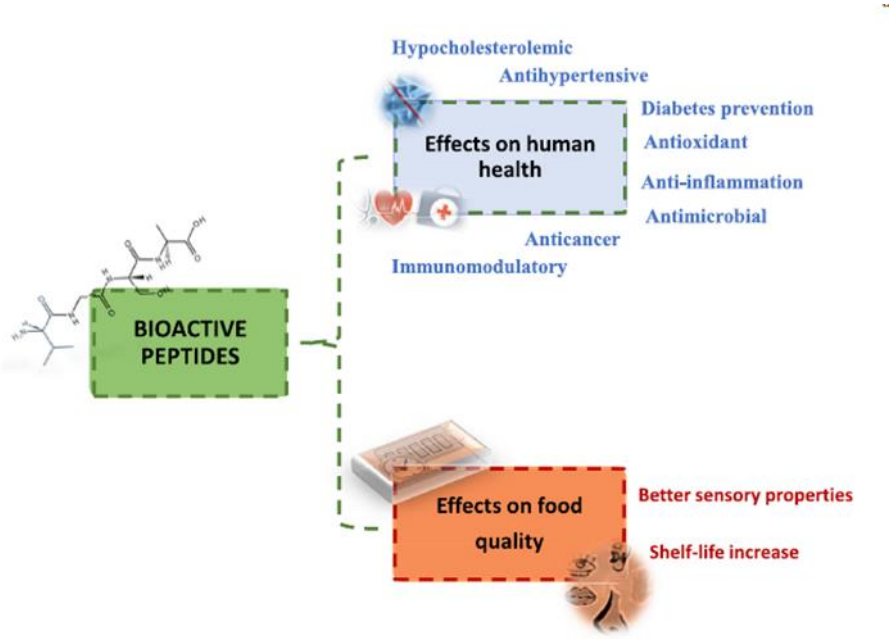

Figure 2.4. The effect of the bioactive peptide on human health and the quality of food (Kida et al. 1995).

Antioxidant peptides from food are known to be healthy compounds and are safe with low cost, easy absorption, low molecular weight, and higher activities. While the antioxidant peptides obtained from enzymatic hydrolysis are known to be more stable and have a simple structure. Besides antioxidant activities, they also present nutritional and other functional properties.

The mechanism behind the antioxidant activity of the peptide is not yet fully understood. Most studies showed that they are inhibitors of lipid peroxidation, chelators of transition metal ions, scavengers of free radicals and that they keep cells safe from damage by reactive oxygen species (Table 2.1).

Table 2.1. Amino Acid composition in relation with their peptide antioxidant activities (Sarmadi and Ismail 2010a).

Amino Acids (AAs)	Mechanism of action
Aromatic AAs	Radicals are converted into stable molecules by donating electron, due to their ability to serve as hydrogen donors. This improves the properties of amino acids.
Hydrophobic AAs	The solubility of peptides in lipids is enhanced, which in turn facilitates the accessibility of hydrophobic radical species.
Acidic and Basic AAs	Side chain carboxyl and amino groups act as hydrogen donors and metal ion chelators.
Cysteine	SH group acts as a radical scavenger, protecting tissue from oxidative stress and improves the glutathione activities.

After the enzymatic hydrolysis reaction, there is a need for the identification and characterization of the primary and secondary structures formed as well as the peptide sequence released during hydrolysis, which will provide information about the bioactive peptide and the amino acids formed. In order to understand the enzymatic degradation of keratin, there is a need to understand the molecular structure of keratin at a microscopic level. The understanding of the peptides and amino acids formed during enzymatic hydrolysis will provide information about the quality of the feed formulation required for animal feeds. However, most authors don't provide sufficient information about the identity and characteristics of the peptides and amino acids formed for feed formulation. With the use of intensive characterization techniques, more information can be obtained for modification and industrial production purposes to determine the best feed.

2.4 Characterization of keratinous biomass

2.4.1 Characterization of keratinous biomass via proximate and ultimate analysis

Biomass characterization is mostly done by proximate and ultimate analysis, to determine their compositional and structural properties. Proximate analysis is known to provide the physical characteristics of biomass, such as the moisture content, ash, volatile matter, total solids, decomposition temperature, and crystallinity of biomass. All these parameters affect the composition behaviour of the biomass, while the ultimate analysis determines the chemical composition of the biomass. It usually determines the major elemental components such as carbon, hydrogen, nitrogen, oxygen, and sulphur in the biomass (Singh et al. 2017).

2.4.2 Characterization of keratinous biomass using different techniques

Characterization is important in determining the characteristics and behavioural properties of the biomass. Following the hydrolysis from the keratinous biomass, different techniques are used to determine the quality and quantity of the hydrolysate produced. The most widely used techniques are Fourier Transform Infrared Spectroscopy (FTIR), CHNS analysis, Sodium Dodecyl Sulphate Polyacryamide Gel Electrophoresis (SDS-PAGE)**,** High Performance Thin Layer Chromatography (HPTLC), High performance Liquid Chromatography (HPLC), Matrix-Assisted Laser Desorption/ Ionization Time of Flight Mass Spectroscopy (MALDI-TOF-MS), Nuclear Magnetic Resonant Spectroscopy (NMR) and Liquid Chromatography Mass spectrometry (LC-MS). Subsequent to enzymatic hydrolysis, the characterization techniques will determine the quality of the peptides and amino acids required for animal feed production.

From literature, it is evident that the bioactivity of the keratinous hydrolysate is related to peptides of 2-20 amino acid residues and a molecular mass of less than 6 KDa (Sarmadi and Ismail 2010a) in addition the presence and content of the aromatic and hydrophobic amino acid residues appear to be involved in the antioxidant activities of the peptides and the hydrolysate. While keratin is known to contain 50-60% of hydrophobic and aromatic amino acid residues (Arai *et al.*, 1983), there are 20 well known amino acids with their different characteristic side chains, 14 of which are essential amino acids as they are not synthesized by the body and have to be supplied by the diet. Amino Acids are widely distributed in biological fluids and are involved in many biological processes where certain amino acids are known to participate in those biological activities (Song et al. 2018).

I. Fourier Transform Infrared Spectroscopy (FTIR)

This is an analytical technique used to identify polymers and organic materials and is an example of ultimate analysis. The absorption bands identify molecular components and structures. This review, highlights the important functional groups present in the keratinous materials of the peptide chains by focussing on the fingerprints of the molecular components of the keratin structure which are required to profile the animal feed and understand the chemical structure of the keratinous material.

FTIR analysis will aid in the detection of any changes in the chemical composition of the peptide. Keratin samples display spectral bands corresponding to peptide bonds (-CO-NH), which are identified as Amide A, which is Amide I–III (figure 2.5). The bands represent the secondary structure of the extracted keratin β-sheet.

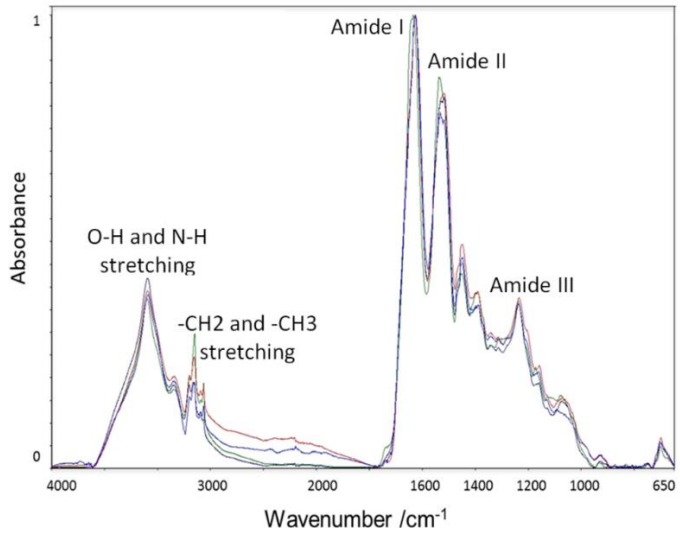

Figure 2.5. FTIR curve for keratin hydrolysate (Eslahi et al. 2013).

Table 2.2 below illustrates the different functional groups present in the keratin structure and their assigned transmission bands [(Alahyaribeik and Ullah 2020),(Tesfaye, Sithole, and Ramjugernath 2018),(Sharma, Gupta, Chik, et al. 2017)].

This technique will also help with the determination of the disulphide bonds in the hydrolysate, as the presence of these bonds is indicative of the poor digestibility of the hydrolysate in animal feed. From the enzymatic degradation, we don't expect to see this band as the disulphide bond will be degraded by the enzymes and also the S=O which is formed due to a reaction of sulphides and cysteine in protein.

Calian *et al.* used FTIR and observed that only the S-S bonds were affected by different microorganisms. It was reported that the different strains disrupted the peptides bonds of the

keratin chain, where the breaking of the S-S bonds in the range of 600-620 cm^{-1} and the appearance of the bands around 1035-1075 cm^{-1} which are signed to sulphoxide bond (S=O) and seen as a sign of biodegradation (Lo, Too, and Wu 2012b). Alahyaribeik *et.al.*, and Tesfye *et al*., presented similar results of the chemical extraction where the disulphide bonds were observed, as chemical extraction partially breaks the disulphide bond linkage, or not shown on the spectrum and similar results were seen with other authors [(Sharma, Gupta, Chik, et al. 2017),(Wang and Cao 2012b),(Sharma, Gupta, Bin Tuan Chik, et al. 2017)]. Most authors are only interested in the fingerprint of the structure and do not mention any disulphide bond breakages.

The only drawback of this technique is that it is only an ultimate analysis and does not represent the whole keratin structure but it is widely used as it is easily accessible.

Table 2.2. Important functional groups present in keratinous material shown in the FTIR

Functional groups	**Transmission bands (cm^{-1})**	**Type of peptide group**
stretching vibration of O-H And –N-H	3400 – 3250	Amide A
-C=O	1750-1610	Amide I
-C-H stretching and N-H bending	1590-1470	Amide II
C-N stretching and N-H bending	1310-1200	Amide III
S=O stretching	1021–1076	cysteine-S-sulfonated residues
-S-S- bridge	500–600	cross linking disulphide group

II. CHNS analysis

This is a technique used for organic elemental analysis, which is an ultimate analysis and determines the amount of Carbon, Hydrogen, Nitrogen, and Sulphur present in the sample ref. It can be both seen as qualitative and qualitative analysis as it can be used to determine the protein content in the keratinous materials. This is an important technique as it can predict the protein content of the animal feed from keratinous material.

Tiwary et *al.* 2012 , reported on a keratinase from *Bacillus* sp., which degraded chicken feathers to feather meal. The quality of the feather meal was determined by CHNS analysis, where they obtained 14% N, 44% C, 3.2% S, and 1.4% H. Their feather meal contained 87% protein, which constitutes a protein-rich meal. While Kakkar *et al.*, obtained a hydrolysate containing 13.3% N, 45.3%, and 6.84% H, which has 83% of protein after chemical hydrolysis. However, most authors neither consider nor mention the sulphur content of their hydrolysate. Enzymatic hydrolysis, produces a higher protein content compared to chemical hydrolysis. This technique only quantifies (the percentage) the protein present in the hydrolysate but does not identify the amino acids or peptides present nor the absolute protein content.

III. Sodium Dodecyl Sulphate Polyacryamide Gel Electrophoresis (SDS-PAGE)

This is an electrophoretic technique that uses polyacrylamide gel to separate proteins based on their molecular weight. It is used to determine the different range of molecular weights present in the hydrolysate after degradation of the keratinous material. This is the proximate analysis as the molecular weight of the hydrolysate is a physical property of the material. Protein and peptides of different molecular weights are visible as different bands on the gel as shown in figure 2.6.

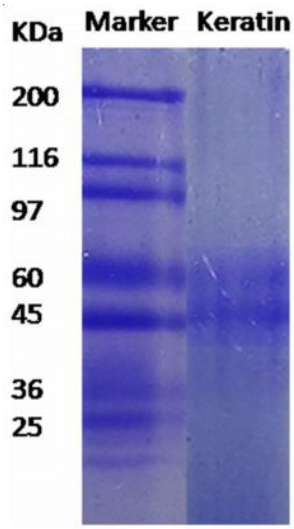

Figure 2.6. SDS-PAGE gel for keratin compared to a marker (Kakkar, Madhan, et al. 2014a).

Literature reports that the bioactivity of the keratinous hydrolysate is related to peptides of 2-20 amino acid residues with molecular mass of less than 6 kDa (Sarmadi and Ismail 2010b). The use of SDS-PAGE has been reported for the analysis of the protein hydrolysates generated by both chemical and enzymatic hydrolysis. The chemical hydrolysate usually shows a higher molecular weight of over 10 kDa due to the cross-linking of keratin units, and many other bands are seen at 10 kDa and but not lower than 5 kDa [(Alahyaribeik and Ullah 2020),(Idris et al. 2013a),(Zoccola, Aluigi, and Tonin 2009a)]. Fontoura *et al*. used a bacterial strain for the degradation of the feathers. The molecular mass of the hydrolysate showed that the bands were lower than 2 KDa, but this method could not show specific bands below 2KDa. Most of the bands reported by the authors which are below 10 KDa are not sufficiently distinct.

The technique is quantitative, and a disadvantage is that the preparation of the gel is a lengthy procedure and that lower molecular weights are not easily seen as they are estimated. The bands are presented in a range and not clear enough to determine the molecular weight of the specific hydrolysate.

IV. Size Exclusion Chromatography (SEC)

Size Exclusion Chromatography a technique that is used to separate molecules according to their different sizes. It helps to quantify the hydrolysate and also provides information about the quality through the size. It is a proximate analysis technique that presents the range of different sized peptides contained in the hydrolysate, which is important for animal feed composition. Figure 2.7, shows the keratin elution profile with a higher molecular weight

region around 10 min and a lower molecular weight region around 21 min. According to the protein standard calibrated curve, peptides of approximately 10 kDa elute around 21 min (Zhang et al. 2015).

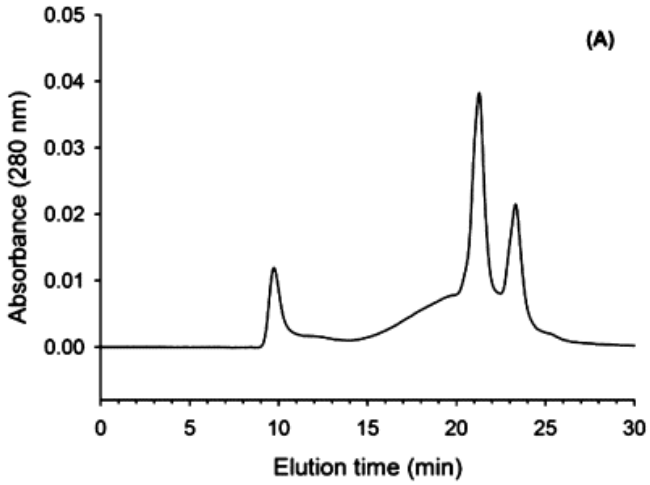

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Figure 2.7. Size exclusion chromatography of keratin (Zhang et al. 2015).

The limitation of this technique is that it usually has poor selectivity and the number of peaks are limited since the running time of the chromatogram is short.

V. High-Performance Liquid Chromatography (HPLC)

It is a chromatographic technique used to separate a mixture of compounds to quantify the individual components in a mixture. When applied to keratin hydrolysates for animal feed, it separates and identifies the different amino acids present. It is an ultimate analysis as it can identify the amino acids, which in turn can inform on the chemical composition. It is capable of determining the quality of the hydrolysate as the essential amino acids present in the hydrolysate required for animal feed can be identified. Figure 2.8, shows an example of the chromatogram of HPLC, where different peaks represent different amino acids, while the area under the peak can help us determine how much there is of each amino acid in the hydrolysate.

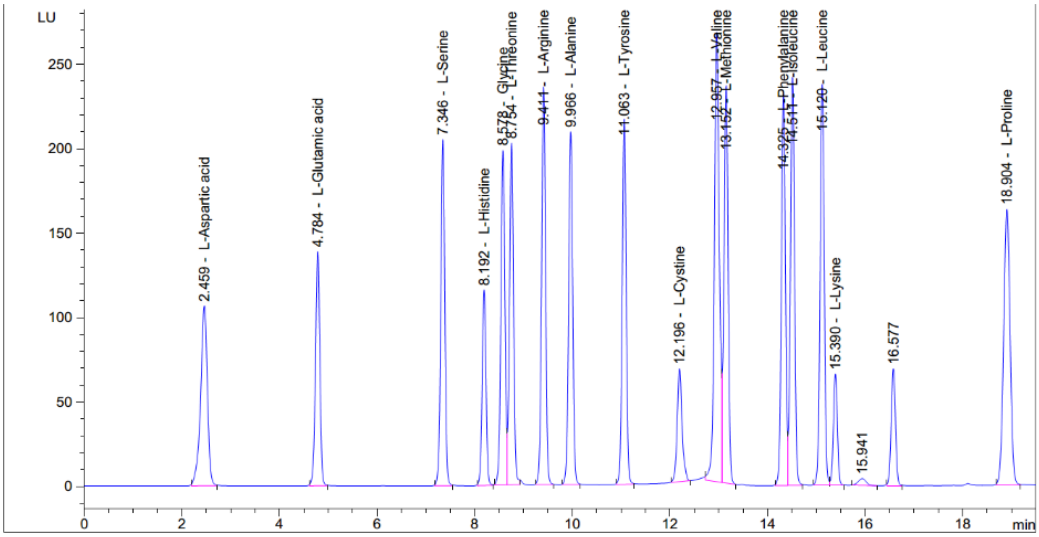

Figure 2.8. HPLC chromatogram for amino acid analysis (Slobodianiuk et al. 2021).

The limitations associated with technique is the issue of sensitivity and selectivity, as most HPLC use different detectors that are incapable of detecting all the amino acids and peptides. The solution towards sensitivity was resolved by using the derivatizing agent but their reaction products are often unstable and affect quantification [(Kubáň and Hauser 2006),(Sharma et al. 2014)].

VI. Liquid Chromatography with tandem Mass Spectrometry (LC-MS/MS)

This is a technique that combines physical separation with mass analysis owing to the application that allows sequencing of the peptides and proteins. This technique is both qualitative and quantitative, providing information on both the quality and quantity of the hydrolysate obtained. It also provides the proximate and the ultimate analysis of the hydrolysate

Fontoura et al 2019, used a feather degrading bacterium to produce a feather hydrolysate and through LC-MS/MS analysis, different peptide sequences comprised of 8-2 amino acids residues with molecular masses of around ~1 kDa were identified. LC-MS/MS shows precise molecular weights and can be compared to the calculated masses from the peptides obtained. The hydrophobic amino acid content of the peptides identified from the feather hydrolysates was between 20% to 66%, and the presence and content of aromatic and hydrophobic amino acid residues appear to be involved in the antioxidant activities of the peptides and protein hydrolysate. The hydrophobic amino acid residues contained valine or leucine at the N-terminus and proline, histidine, or tyrosine within the peptide sequence, which is consistent

with higher antioxidant activities. This is the best technique to profile the amino acid and peptide content of the hydrolysates, with the limitation of being not easily accessible and has high operational costs.

VII. Thermogravimetric Analysis (TGA)

Thermogravimetric analysis measures the weight changes in a material as a function of temperature under a controlled atmosphere (Sarfraz et al. 2022). It is used to determine the thermal stability of the sample, which is a proximate analysis. It only provides information about the degradation of the hydrolysate when exposed to higher temperatures.

The quantification of the thermal degradation of the keratinous hydrolysate can be obtained. It was reported that the S-S bonds have the highest energy of all the bonds, and their cleavage represents the rate-determining step of the keratin degradation (Călin et al. 2017a). There are 3 stages that appear in the TGA chromatogram of the keratinous substrate. Figure 2.9 illustrates the 1st stage which is due to water evaporation, the 2nd stage due to the denaturing of the polypeptide chain, where the keratin suffers organic degradation and the 3rd stage is where complete degradation occurs.

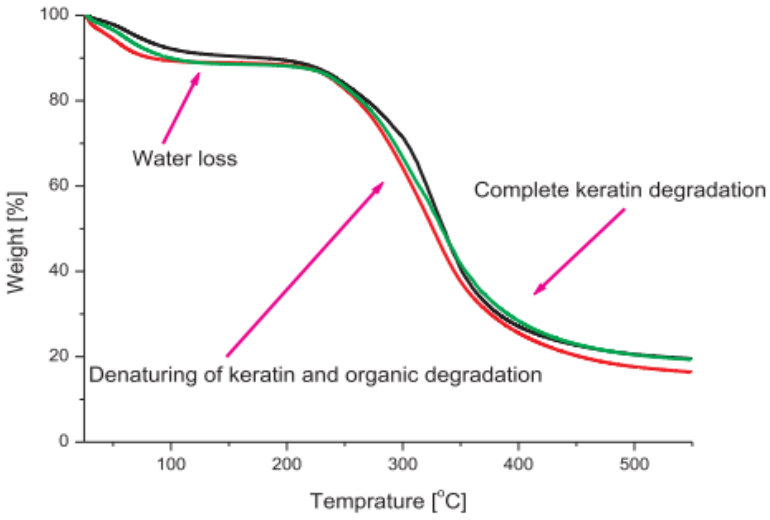

Figure 2.9. TGA curve for keratin (Tesfaye, Sithole, Ramjugernath, et al. 2018a).

Tesfaye *et al.*, reported the same trend where the 1st stage occurred between 25ºC and 230ºC, with a 12% -13% weight loss and the 2nd stage from 230ºC to 380ºC with a mass of about 46%.

And the last 3rd stage was from 380ºC to 550ºC with a loss of between 81 to 83%. Sharma *et al.*, also studied TGA of the raw feathers where only 2 stages were observed. The 1st stage was due to water loss as a result of water evaporation around 150ºC, followed by rapid decomposition between 220ºC and 345ºC representing the degradation of the protein molecule chain as a result of breakage of disulphide bond and release of sulphur dioxide and hydrogen sulphide.

The stability of the hydrolysate is known to be affected by molecular mass and the aromatic groups present (Sharma et al. 2017). When heated under nitrogen atmosphere, polymers with aromatic rings are known to form char residues which are stable up to 600°C (Durukan et al. 2019).

Calian *et al.*, isolated eight fungal strains for the degradation of keratin (Table 2.3). Of the eight isolated strains, the least active strain in terms of the degradation process was number 9, *Chrysosporium sp.* as the highest amount of keratin residue (25.28%) was observed for this culture. While strain number 3, *Fusarium sp. strain 1A* was the most active, producing the strongest denaturing of the polypeptide chains from the hydrolysate after producing the lowest amount of the residue, 17.51%, and the highest total weight loss after 700 ºC.

Table 2.3. TGA results for the weight loss of the hydrolysate using different fungal strains

Tested Fungal Strain	**Residue (%)**	**Total weight loss (%)**
Control (virgin keratin)	22.46	77.55
Trichophyton sp.	21.22	78.71
Fusarium sp. *strain 1A*	*17.51*	*82.45*
Trichoderma sp.	19.44	80.48
Cladosporium	19.92	80.04
Microsporum sp.	23.53	75.52
Fusarium sp.	20.85	79.08
Phytophthora sp.	19.35	80.82
Chrysosporium sp.	*25.28*	*74.73*

However, the authors did not elucidate the amino acids present or the molecular weight of the hydrolysate. Such information is required to clearly appreciate the higher activity produced by the strain. To understand the thermal stability of the hydrolysate other techniques are required to grasp the decomposition process. The disadvantage with this technique is that the data obtained is difficult to interpret and not straightforward.

VIII. X-Ray Powder Diffraction (XRD)

It is a technique that is used to provide detailed information about the crystallographic and chemical composition of the materials and it is an ultimate analysis technique. It is used to determine the structure, the phase, crystallinity of the material, and sizes of the crystallites. This technique only useful if the material has crystalline properties.

Several authors reported on the XRD of raw feathers and keratin hydrolysates. Indris et al. 2013 reported that both the raw feathers and keratin hydrolysates occur in an amorphous form. Their diffraction patterns were observed at 11 º and 22 º, which are due to the presence of α-helix at 11 º. After reduction or extraction, they both underwent a shift to 9 º, which is assigned to both the α-helix and β-sheet structures and 20 º which is due to the β-sheet structure. The strong diffraction at 9 º and 20 º (figure 2.10), represents the β-sheet keratin structure, where is indicative that we have a β-sheet structure of keratin.

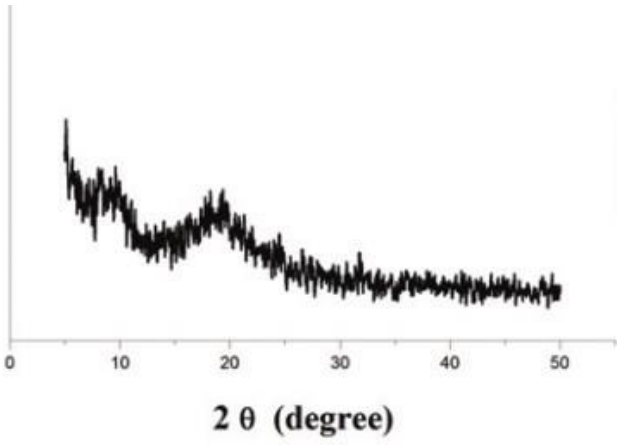

Figure 2.10. X-ray diffraction pattern of keratin (Idris et al. 2013a).

The disadvantage of using this technique is that it does not give any information about keratinous material as they are not crystalline and that it cannot differentiate between the two types of secondary protein structures available in the hydrolysate.

IX. Nuclear Magnetic Resonance spectroscopy (NMR)

This is a spectroscopic technique that applies a magnetic field to an atomic nucleus, most commonly, ^{1}H, ^{13}C, and uses a radiofrequency pulse to characterize the resonant frequency of the atomic nucleus according to the chemical and environmental surroundings. This technique is mostly used in quality control to determine the purity and the content of a sample and most importantly the molecular structure of the sample. It studies the physical properties of the sample at the molecular level, conformational exchange, phase change, and solubility. It is a proximate analysis and an ultimate analysis. For the analysis of the hydrolysate, the ^{13}C atomic nucleus will be useful for the determination of the carboxylic carbon group in the peptide and the determination of the hydrophobicity of the peptide obtained.

The ^{13}C NMR of the feather hydrolysate shows the different functional groups present in the structure of the keratin. An example is shown in figure 2.11 below where the carbonyl groups present from amino acids and peptide chains in the hydrolysate can be seen near 175 ppm. While 130 ppm represents the aromatic group carbons, which is more important in the enzymatic hydrolysis to determine its hydrophobicity. Between 60 to 65 ppm represents the α-Carbon. The peak at 40 ppm represents the β-carbon, which suggests the presence of leucine and cysteine, the disulphide bridge cleavage of cysteine reduces the β-carbon signal from 40 ppm to between 20 – 29 ppm to produce a thiol signal. The δ-carbon is presented at around 20 ppm, which is mixed up with the aliphatic carbon side chain between 10 – 35 ppm [(Idris et al. 2013a),(Nuutinen 2017),(Ghosh et al. 2019)].

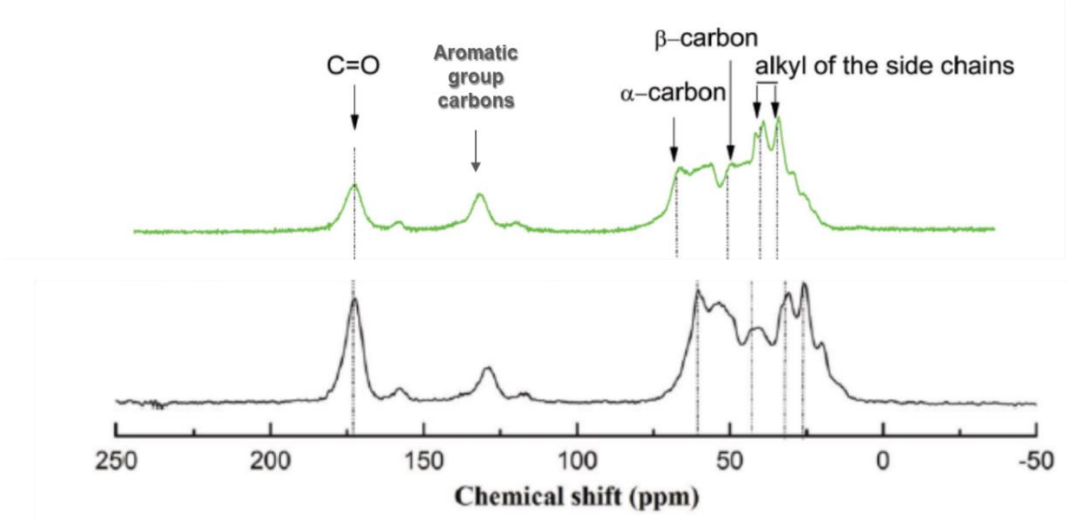

Figure 2.11. ^{13}C NMR of raw feathers, bottom, and extracted keratin top (Idris et al. 2013a).

The limitation with this technique is that the whole molecular structure cannot be determined using NMR as the structure of keratin is very complex and it cannot differentiate which secondary protein structure is present.

X. Pyrolysis- Gas Chromatography-Mass Spectrometry (Py-GC/MS)

It is an analytical technique used to determine structural information by analyzing the thermally degraded products. It usually involves the heating of the sample at high temperatures, where they degrade to smaller molecules which are then separated by the gas chromatography and identified by mass spectrometry. It can be used to analyze most of the materials and even complex materials at trace levels.

Tesfaye et al. reported the Py-GC/MS of chicken feathers to identify the degraded products and other potentially toxic compounds which may be found in the feathers after decomposition (Tesfaye et al. 2019).

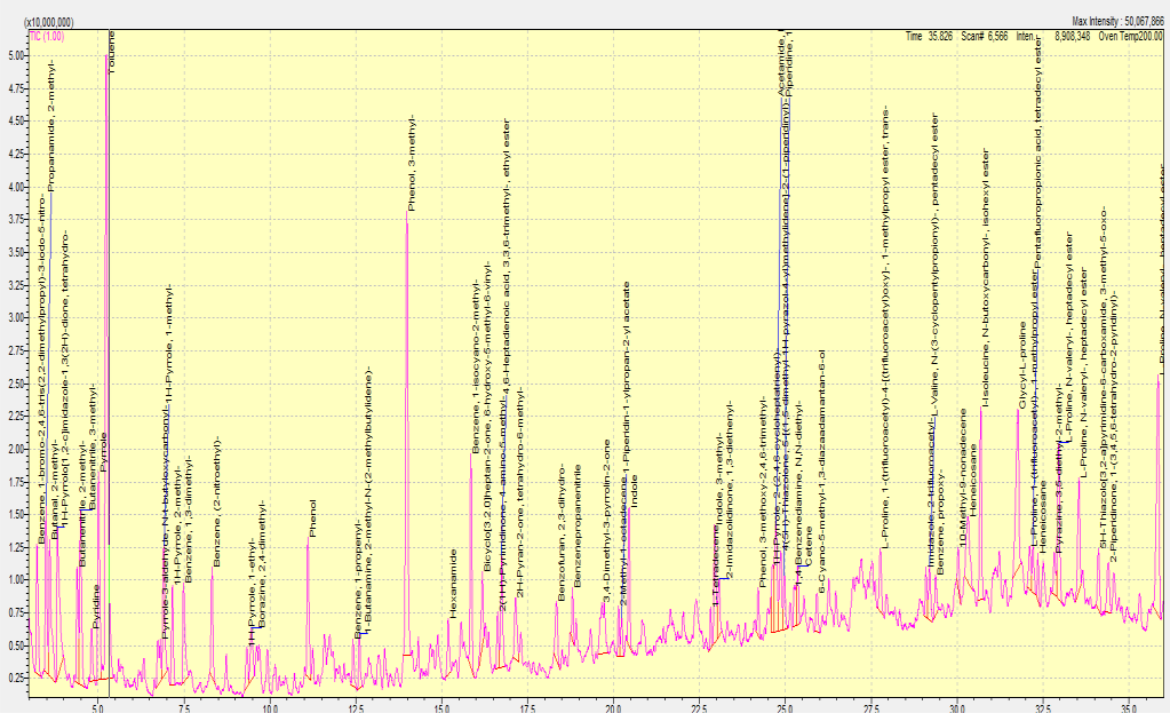

Figure 2.12. Py-GC-MS chromatogram of chicken feathers (Tesfaye et al. 2019).

He identified a variety of degraded products, including amino acids in the degraded feather, figure 2.12. The existence of toxic compounds in feathers was also noted.

The limitation of Py-GC/MS is that it is a high cost analytical instrument and that is not easily available and there is a limited number keratin hydrolysate analyses.

A summary of the different analytical techniques available for various analyses is shown in figure 2.13. Chemical analysis which is an ultimate analysis uses techniques like, FTIR and HPLC to study the chemical structure. While the physical analysis is a proximate analysis which uses techniques like, TGA and XRD, to elucidate the physical properties of the materials. LC-MS/MS, can be for the analysis of both physical and chemical properties.

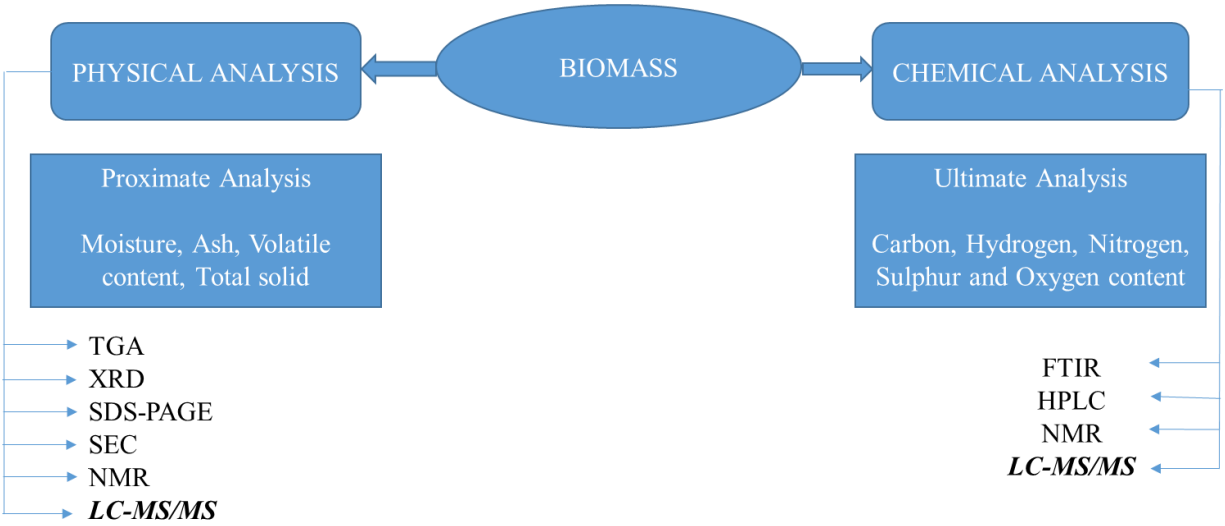

Figure 2.13. An example of the characterization techniques used in the biomass analysis [Edited from ref. (Singh et al. 2017)]

Lo *et al.*, reported on a feather degrading bacterium, which was isolated from the soil of a poultry farm. The keratinase produced degraded feathers under optimum conditions of 40ºC, pH of 5.3 over 96 h. The hydrolysate obtained was characterized by reverse phase-HPLC for amino acid analysis and FTIR for functional groups present in the hydrolysate. The hydrolysate was rich in glutamic acid, aspartic acid, proline, glycine, and serine. Lysine, methionine, and threonine which are essential nutritional amino acids were also obtained. The author only employed two characterization techniques to conclude on the composition of the keratin hydrolysate.

Essential amino acids differ from different microorganisms. For the feed formulation to have nutritional and functional properties, the amino acids present should be hydrophobic and aromatic. In Table 2.5, most of the microorganisms produce aromatic amino acids like phenylalanine, tyrosine, histidine, and tryptophan also the hydrophobic amino acids like glycine, proline, isoleucine, leucine, valine, and alanine. Peng *et al.*, reported that a combination of the two microorganisms, *B. licheniformis BBE11-1* and *S. malt- ophilia BBE11-1*, increased the degree of hydrolysis of the chicken feathers. And also there was an increase in the concentration of the essential amino acids and soluble peptides compared to the individual microorganisms (table 2.5) (Peng et al. 2019). These amino acids have a biological function, the hydrolysate produced using these microorganisms is suitable for the animal feed additives/formulation.

Table 2.4. Different microorganisms producing different essential amino acids for feed formulation.

Micro-organisms	Essential Amino Acids	References
Bacillus licheniformis ER-15	histidine, phenylalanine, methionine, threonine	(Tiwary 2012)
β-Keratinase	cysteine, valine, threonine, leucine, isoleucine, phenylalamine, methione	(Mukherjee, Rai, and Bordoloi 2011)
Streptomyces coelicoflavus	threonine, methionine, histidine, leucine	(Fakhfakh et al. 2011)
Bacillus subtilis S1-4	cystine, glycine, proline, arginine	(Nahed et al. 2012)
Acremonium chrysogenium	methionine, isoleucine, glycine, glutamic acid, aspartic acid, lysine	(Eremeev et al. 2009a)

Chryseobacterium sp. Kr6	valine, threonine, leucine, glycine, phenylalanine, isoleucine	(Fontoura et al. 2019b)
Bacillus pumilus GRK	tryptophan, isoleucine, lysine, valine, methionine, arginine, histidine	(Ramakrishna Reddy et al. 2017)
Bacillus licheniformis BBE11-1 and *Stenotrophomonas maltophilia* BBE11-1	tyrosine, valine, phenylalanine, leucine	(Peng et al. 2019)

Fontoura et al 2019., used a bacterial strain for the degradation of the feathers. The hydrolysate obtained was characterized using SDS-PAGE but did not produce clear enough profiles while RP-HPLC showed the higher intensity peaks related to peptides with increased hydrophobicity and the eluted solute was in the order of increasing hydrophobicity. The HPLC profile of the with less than 10 KDa presented the presence and accumulation of the peptides with higher hydrophobic properties, which resulted in the observed bioactivities of the hydrolysate obtained, but could not identify which peptide sequence were found to be bioactive.

Villa *et al.*, also reported the same trend for the enzymatic hydrolyate analyzed with MALDI-TOF and HPTLC. They reported lower molecular weights between 800 to 1079 Da (0.8- 1 kDa), where the lower molecular weight bands were clear compared to SDS-PAGE. HPTLC showed that all the peptides and amino acids had lower molecular masses, but they we not identified.

A combination of more than two techniques is required for the analysis and characterization of enzymatic hydrolysates, whereas most authors only investigated at-most two techniques to determine the animal feed quality (Table 2.5). Most of these techniques can be used in combination as they all have limitations. Like FTIR, which will help in determining the breakage of the disulphide bonds which is necessary for animal feeds and using Size Exclusion Chromatography (SEC) and LC-MS/MS to determine, the molecular weight of the hydrolysate as SDS-PAGE is limited for lower molecular weights. The lower the molecular weight of the protein hydrolysate, the more biologically active they are. Also, LC-MS/MS can also be used

to profile the peptide chain. HPLC is appropriate for amino acid profiling as hydrophobic and aromatic amino acids in the hydrolysate, make for good quality animal feed, and also the specific amino acids in the hydrolysate determines the quality of the feeds. Characterization techniques like SEM, for the degree of degradation on the surface of the keratinous biomass, and TGA, for the thermal degradation of the keratin, can be used in combination with other characterization techniques to obtain information on the quality of the hydrolysate. Py-GC/MS helps in identifying the by-products after degradation and also the presence of the amino acids. All these different techniques are necessary for the further investigation of the quality, quantity, and safety of the hydrolysate for animal feed formulation.

There are few studies on the determination of the quality and quantity of the hydrolysate produced by enzymatic hydrolysis. Most research is focused on the production, characterization, and activities of the keratinases obtained. There is a huge gap in using different analytical techniques to obtain a full analysis of the hydrolysate to determine its quality for animal feed additives or formulations.

Table 2.5. Research done on different strains and the techniques used for the analysis for the hydrolysate

Microbial strain	Techniques used	References
Bacterial strain	HPLC, FTIR	(Kida et al. 1995)
Fungal strain	SEM, FTIR	(Călin et al. 2017a)
Bacterial strain	SDS-PAGE, HPLC	(Fontoura et al. 2014b)
Bacterial strain	MALDI-TOF-MS, HPTLC	(Villa et al. 2013)
Bacterial strain	LC-MS/MS	(Fontoura et al. 2019b)
Bacterial strain	CHNS, HPLC	(Tiwary 2012)
Bacterial strain	HPLC	(Ramakrishna Reddy et al. 2017)
Bacterial strain	GC-MS, MALDI-TOF-MS, SEM	(Mukherjee et al. 2011)

2.5 Conclusions

In conclusion, the chemical and enzymatic hydrolysates shows a trend of produced peptides with different molecular mass, which are confirmed by several researchers through different characterization techniques. The enzymatic hydrolysate is known to contain a complex mixture of amino acids and peptides, which are derived from the cleavage of the peptide bonds (Hou et al. 2017). Enzymatic hydrolysis by different microbial enzymes enhances the production of free amino acids and carboxyl groups and also exposes the hydrophobic groups of the amino acid residues (Callegaro et al. 2018). The peptides and amino acids formed during hydrolysis can be employed as a supplement in animal feed and also have nutritional and physiological functions in animals. These potential applications demand technical analysis and characterization of the enzymatic hydrolysate and also the purification and identification of the peptides formed during hydrolysis to achieve the quality and quantity required for animal feed production [(Fakhfakh et al. 2011),(Nahed et al. 2012)].

The review showed how most of the authors focuses on the optimization of the enzymatic hydrolysate and only a few techniques are used to determine the quality then draw their

conclusion. And also shows the importance of the analytical techniques in combination can be very useful in determining the type of the protein hydrolysate required for the animal feed.

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CHAPTER 3

PAPER 2: HAS BEEN PUBLISHED IN BIOMASS CONVERSION AND BIOREFINERY JOURNAL

KERATINOUS HYDROLYSATE PROFILING: COMPARISON OF THE DIFFERENCES OBTAINED FROM DIFFERENT EXTRACTION METHODS

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Abstract

The extraction of keratin from chicken feathers is of research interest due to the beneficiation of biomass. This study investigates the impact of the keratin hydrolysate obtained using four different methods. There are two different chemical extraction methods, CH1 and CH2, and two enzymatic hydrolyses, EH1 and EH2. The investigation includes the characterization and analysis using different types of equipment for different applications. The keratin hydrolysates formed were all characterized using FTIR, TGA, SDS PAGE, CHNS analysis, Bradford assay, and ash content. All the keratin hydrolysate from different methods showed all the amides bonds present in the keratinous structure from the FTIR, while TGA followed the three-phase trend loss of the keratinous structure. Protein concentrations obtained from CH1, CH2, EH1, and EH2 were 1.40, 1.02, 1.08, and 0.45 mg/ml respectively and their protein content was 86.56, 67.63, 78.06, and 90.00%. Their molecular weights were all in different ranges while

the ash content for CH1 was 20.7, CH2 was 5.27 and EH1 was 9.19. All the results obtained were compared to the pure keratin azure and EH2 showed high protein content but low protein concentration. CH1 showed the second-highest protein content but with high impurities from the extraction method shown from the ash content.

Keywords

Chicken Feathers, Extraction Methods, Keratin Hydrolysate, Analytical Techniques

3.1 Introduction

Keratinous biomass is of interest to most researchers due to its wide applications in different industries. Poultry industries generate five billion tonnes of waste chicken feathers annually, which leads to a potential threat to the environment, while in South Africa nineteen million broiler chickens are killed every month (Khumalo et al. 2019). The waste feathers produced are ground into feather meals or end up in landfills while others are incinerated which causes air pollution leading to greenhouse gas effects. The chemical composition of chicken feathers is 91% β-Keratin protein and the other components, like lipid, fibre, ash, and moisture content (Tesfaye et al. 2017). This is a very valuable rich protein and the reason why the extraction of keratin is one of the most researched topics. The extracted keratin has different technological applications, including cosmetics, pharmaceuticals, fertilizers, biofuels, biomedical, animal feed, and others.

Keratin is a fibrous protein derived from hair, nails, feathers, wool, horns, and hooves and is insoluble in most organic solvents. It is found in two different secondary structures, the α-helix, and the β-pleated sheet (Saha et al. 2019).

The extraction of keratin has attracted much interest, where there are different extraction methods, including chemical hydrolysis, ionic liquids, enzymatic hydrolysis, and thermo-chemical. The choice of extraction method depends on several factors, the chief being the application of the hydrolysate, the cost of the process as well as the yield of the desired product. All the different methods have their advantages and disadvantages. The most widely used methods are the chemical methods using reducing agents (Alahyaribeik and Ullah 2020; Idris et al. 2013a; Sharma, Gupta, Chik, et al. 2017; Wang and Cao 2012a), and enzymatic hydrolysis using different keratinases (Abdel-Fattah et al. 2018; Bach, Lopes, and Brandelli 2015; Bhari et al. 2018; Eremeev et al. 2009b; Fontoura et al. 2019b). The main process of chemical hydrolysis involves, dissolving the chicken feathers in different reducing agents such as

thioglycolic acid or thioglycolate salts, 2-mercaptobisulphite, sodium sulphite, sodium bisulphite, and sodium followed by separation of the protein from the chemicals, which cannot be recycled. (Gupta et al. 2012). The chemical reagents break down the disulphide bonds, hydrogen bonds, and salt linkage of the keratin fibers. While in enzymatic hydrolysis, the keratinase is known to disrupt the disulphide bonds, where an enzyme hydrolyzes the peptide bond resulting in the C-terminal (COO^-) and N-terminal (NH_3^+) and also the formation of the hydrophobic amino acids residues (Patterson *et al.*, 1988).

Alahyaribeik *et al.,* (2020) also used reducing agents to produce keratin hydrolysates. They found that the different reducing agents influenced the molecular mass, surface morphology, and crystallinity of the keratin hydrolysate, which in turn affects the bioactivity. Keratin hydrolysate with antioxidant bioactivity can be used in a variety of industries including cosmetics, pharmaceuticals, food processing, and agriculture. Sharma *et al.,* (2017) extracted keratin from chicken feathers using sodium sulphide in an alkaline hydrolysis. The keratin obtained had a higher glass transition temperature and most of the disulphide bonds were broken. The keratin in this hydrolysate has application in coating, packaging, and biodegradable composites. Sinkiewicz *et al*., (2017) used various reducing agents for the extraction of keratin and obtained a high yield of soluble keratin was for the application as the formation of biodegradable film for food applications. Wand and Cia (2012) employed a different chemical agent, viz., hydrophobic ionic liquids for extraction, to produce keratin that was highly soluble in water and had uniform molecular weight with lower molecular weights amino acids. While most of the enzymatic hydrolysates are used for animal feed and fertilizers.

The keratin hydrolysate formed from different methods all show different molecular weights, different quality and quantity of the protein formed, different thermal activities, and different morphologies. The quality and quantity of the keratin hydrolysates formed can be determined using different analytical techniques, like FTIR, CHNS Analysis, TGA, SDS PAGE, Bradford Assays, and the determination of the ash content. This article focuses on the different extraction methods used and their effect on the keratin hydrolysate formed. The keratin hydrolysate formed will be determined by using different techniques to characterize and analyze the chemical composition and physical properties from each method used. The effect each method has on the keratin hydrolysate obtained has not been compared to other keratin hydrolysates from other methods. Most authors focus most on the extraction methods and optimization of

the methods but not the detailed comparison of the characteristics of the keratin hydrolysate obtained for a specific application. To focus on a specific application of the keratin hydrolysate, we need to have an understanding of what quality and quantity we get with different methods, this is what this article is focusing on.

3.2 Experimental

3.2.1 Methods

Materials:

Waste chicken feathers were collected from Chicken meat processing plant at Hammarsdale in KwaZulu-Natal, where they were washed, disinfected, milled and stored at -6°C. The cleaned feathers were used for all the different methods. Keratin Azure, 5g in a glass bottle, was purchased from Sigma Aldrich.

Chemical Treatment:

CH1 represents the keratin hydrolysate extracted using an alkaline method (Fagbemi et. al.., 2020), where feathers were weighed and added to an alkaline solution containing sodium hydroxide and sodium, the pH of the solution obtained was between 12 and 13. The resulting solution was filtered and then neutralized using HCl where the filtrate was dialyzed for 72 h then freeze-dried to collect the keratin hydrolysate. The quantities of all the chemicals used and detailed method is from an article (Fagbemi *et al.,* 2020)

CH2 represents the reduction extraction method (Khumalo *et al.*,, 2020) where chicken feathers were immersed in sodium bisulphite, sodium dodecyl sulphate, and urea. The resulting mixture was shaken and heated in an oil bath. After the reaction, the mixture was centrifuged and then filtered. The filtrate obtained was dialyzed for 5 days, and the keratin solution obtained was then freeze-dried to obtain the keratin hydrolysate. The quantities of all the chemical and detailed method is from the article (Khumalo *et al.,* 2020).

Enzymatic Hydrolysis:

EH1 represents an enzymatic hydrolysis method (Dlume, 2021). A basal salt medium containing K_2HPO_4, KH_2PO_4, $MgCl_2$, $CaCl_2$; and chicken feathers were inoculated with the

bacteria isolates, Exiguobacterium species and incubated. The mixture of bacterium and keratin hydrolysate obtained were then isolated.

EH2 (Mnguni, 2021).

A subset of waste feathers, which were not washed, were used to isolate the microbial flora indigenous to chicken feathers. The feathers were suspended in minimum media which was composed of (g/l): NaCl, 0.5; KH_2PO_4, 0.7; K_2HPO_4, 1.4; $MgSO_4.7H_2O$, 1 and incubated at 30°C with shaking. The suspended samples were plated out on feather meal agar (FMA) plates composed of agar (%) and 0.1% feather meal made up in minimum growth medium. The plates were incubated at 30°C, pH8, for 7 days (Mnguni, 2021). Fungal and bacterial strains were used to produce hydrolysate by enzymatic hydrolysis.

3.2.2 Characterizations

Fourier Transform Infrared spectroscopy from PerkinElmer (Frontier Universal model) in an attenuated total reflection mode (ATR) was used for the analyses of the functional groups, where spectra were collected over a frequency range of 35000-400 cm^{-1}. TGA profiles were determined using Simultaneous Thermal Analyser (STA) STA 6000. The temperature range of the profiles was 28-750 °C with a heating rate of 10°C/min under nitrogen with a purge flow of 20ml/min. Elemental compositions were analyzed using PerkinElmer, series II CHNS elemental analyzer, where the protein content was determined using the nitrogen content obtain by multiplying it with the conversion factor of 6.25 (Mariotti, Tomé, and Mirand 2019). Bradford Assays were done on the UV/Visible spectrophotometer operation Cary 50 CONC. All the absorbance for the calculated concentrations were taken at a wavelength of 595 nm. Ash content was determined using the convection drying oven, with temperature control of 105 ±3°C, ignited in a muffle furnace at 525 °C. Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis (SDS-PAGE). Keratin samples were dissolved in distilled water. Followed by adding a 15 µL keratin sample into a solution of 5 µL of NuPAGE LDS sample buffer (4X) containing 5% β-mercaptoethanol, and the mixture was boiled for 7 min. The polyacrylamide gels used were 16% and 12% for low and mid-high molecular weight determination where denatured samples were then loaded. The gels were exposed to 80 V for 30 min, followed by 120 V for 2 h. The gels were then stained, for 30 min, with Coomassie Brilliant Blue (CBB) G-250 followed by an overnight de-staining with a mixture of ethanol-acetic acid solution. Imaging software was used on both gels for analysis.

3.3 Results and discussion

3.3.1 FTIR (Fourier Transform Infrared Spectroscopy)

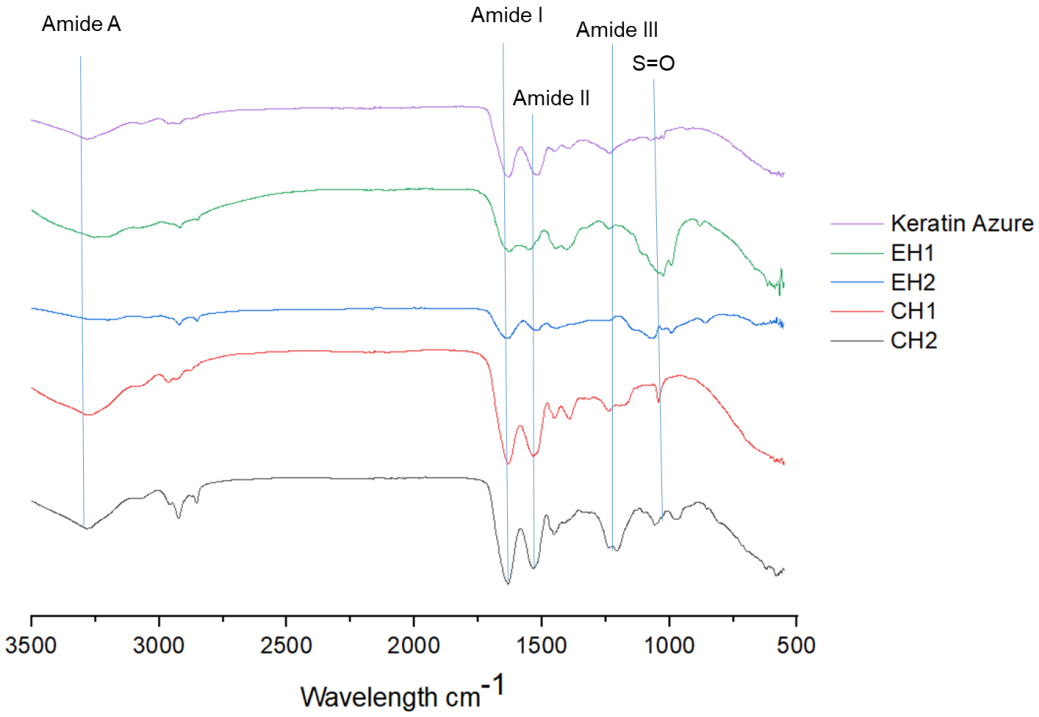


Figure 3.1. FTIR of different keratin hydrolysate when compared to keratin azure

The keratin hydrolysates formed from the different methods are shown in figure 3.1. They all show the presence of the keratinous structure with the functional group's Amide A at 3250 cm^{-1} representing the stretching vibration of O-H and –N-H. Amide I at 1 632 cm^{-1} shows the presence of C=O, while Amide II at 1510 cm^{-1} represents -C-H stretching and N-H bending and Amide III at 1240 cm^{-1} shows C-N stretching and N-H bending. This is in agreement with most reported keratinous materials (Călin et al. 2017b; Nuutinen 2017; Sharma, Gupta, Chik, et al. 2017; Zoccola, Aluigi, and Tonin 2009b).

There is also the presence of the band around 1069 cm^{-1}which is assigned to the sulphoxide bond, S=O, which represents the breakage of the disulphide bond. This technique helps with the determination of the disulphide bonds in the hydrolysate. The presence of the S=O is

formed due to a reaction of sulphides and cysteine in protein, showing the breakage of the disulphide bonds.

EH1 has the largest peak of S=O, while EH2, CH2, and CH1 have a similar peak. And keratin azure the S=O peak is almost non-existence.

3.3.2 TGA (Thermogravimetric Analysis)

There are 3 different stages of weight losses, where the 1st stage, 28 ºC - 150 ºC, is due to water evaporation and the 2nd stage,150 ºC - 500 ºC, represents the denaturing of the polypeptide chain, where it is known that keratin suffers organic degradation. The last stage, the 3rd stage, between 500 ºC - 700 ºC is where complete degradation occurs as shown in figure 3.2. Most authors (Gupta et al. 2016; Sharma, Gupta, Chik, et al. 2017; Tesfaye, Sithole, Ramjugernath, et al. 2018b, 2018a) present the same trends from their keratin hydrolysates.

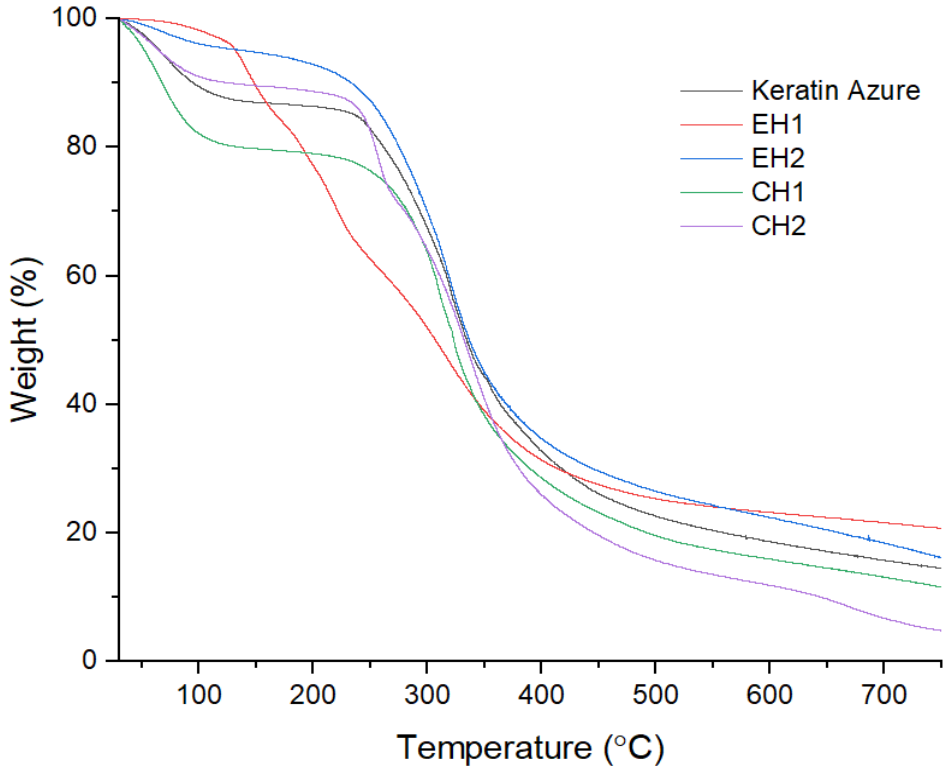
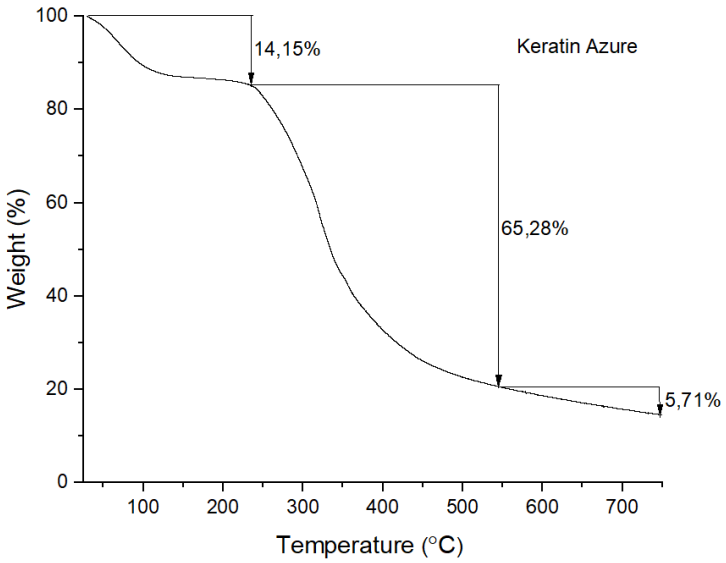
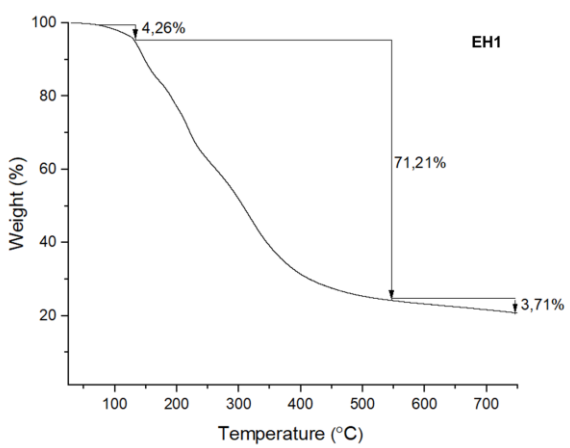
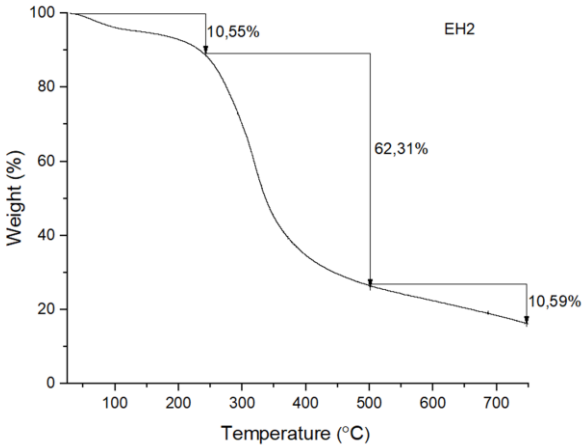


Figure 3.2. The TGA curves of different keratin hydrolysate when compared to keratin azure

All four different keratin hydrolysates show the trend of keratin degradation, Figure 3.3. Where CH1 shows the lowest weight loss of the organic degradation from the 2nd stage, with 59.90%. The keratin hydrolysate from CH2 shows the highest weight loss with 73.62%, followed by EH1 at 71.21% and EH2 at 62.31%.







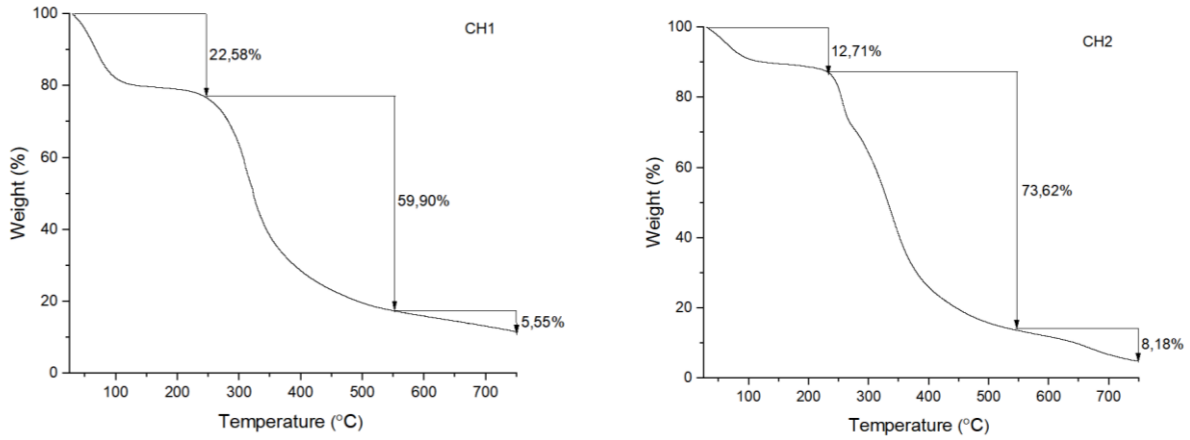


Figure 3.3. TGA curves show the mass percentage loss of different keratin hydrolysates with an increase in temperature**.**

At 600°C, CH1 and CH2 are more thermally stable than EH1 and EH2. Such high thermal stability is known to be cause by the closely packed polypeptide chain in the β-sheet and that large particle sizes also play a huge role. It is also known that polymer with aromatic rings are known to yield char residue which is stable at 600°C under nitrogen (Alahyaribeik and Ullah 2020). The difference in molecular weights also plays a role in the thermal stability of the hydrolysates as smaller solid residues tend to have longer residence time which is seen with EH1 and EH2.

3.3.3 Elemental analysis (CHNS analysis)

The protein content in the keratin hydrolysate was determined using this technique. The protein content in the hydrolysate was found to be 86.85% for CH1, 67.63 for CH2, 78.06% for EH1, 90.00% for EH2, and 93.68% for keratin azure, Table 3.1. The keratin hydrolysate from keratin azure was found to have the highest protein content, which was followed by EH2 as it had the high nitrogen content shown in Figure 3.4 and Table 3.1.

Table 3.1. Elemental analysis of Keratin hydrolysate

Keratin Hydrolysate	**Carbon %**	**Hydrogen %**	**Nitrogen %**	**Sulphur %**	**Protein %**

Keratin Azure	43.21	6.50	14.99	4.13	**93.68**
EH1	35.08	3.14	12.49	4.15	**78.06**
EH2	43.55	5.99	14.40	2.51	**90.00**
CH1	47.25	6.90	13.85	2.80	**86.56**
CH2	46.64	7.72	10.82	5.02	**67.63**

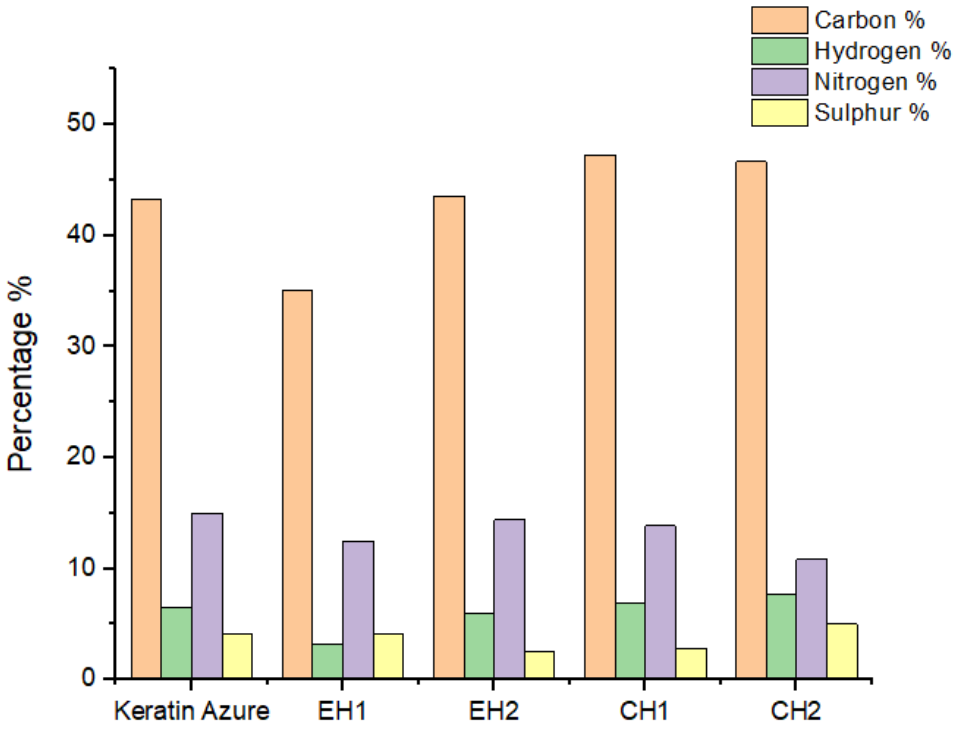


Figure 3.4. The bar graph shows different elemental analyses of different keratin hydrolysates.

From Table 3.1 and Figure 3.4, the four keratin hydrolysates from the different methods were compared. Where keratin azure is the highest, EH2 is the second-highest protein content. This is due to the factors used for the process involved in the production of the keratin hydrolysate, where enzymatic hydrolysates are known to have high protein content. While keratin azure has the highest protein content as it is keratin extracted from wool compared to keratin extracted from chicken feathers, which correlates with the literature.

3.3.4 Bradford Assay

This technique was used to determine the concentration of the protein in the hydrolysate. Figure 3.5., represents the standard curve for the Bradford assays for the analysis of protein content. The curve was used to determine the unknown concentration of the keratin hydrolysate. The curve represents the absorbance taken at the wavelength of 595 nm. Bovine Serum Albumin (BSA) was used as a protein standard with increasing concentration. The protein was used with the coomassie blue staining to determine the binding of the protein.

The hydrolysates from the different methods were tested and the unknown concentrations were determined using a standard curve shown in Figure 3.5. From the standard curve, the concentrations from table 3.2 were obtained

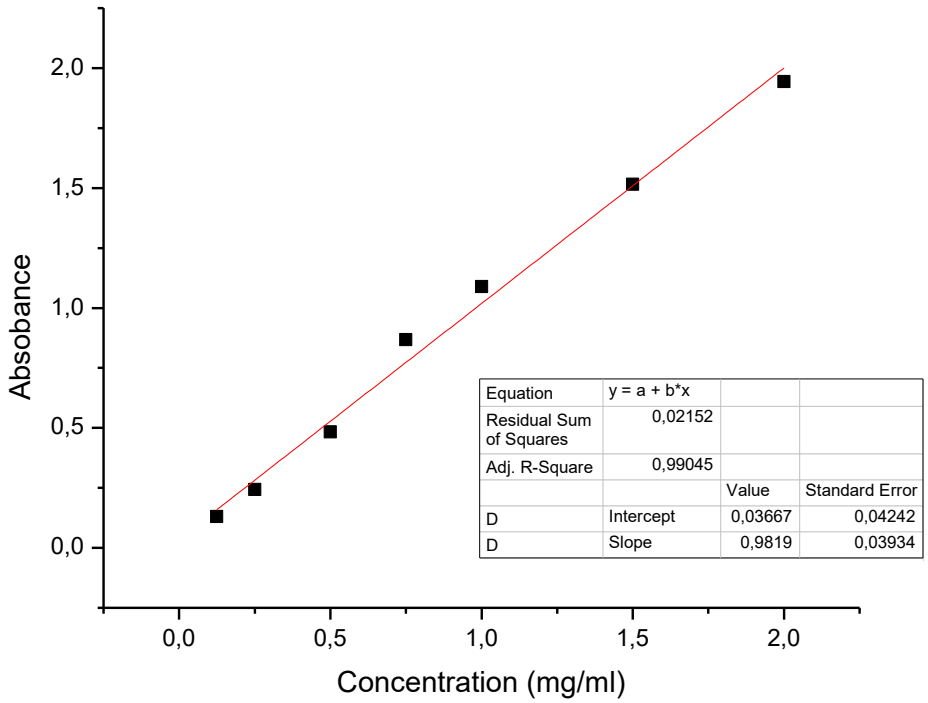


Figure 3.5. Standard Curve for Bradford Assays to determine the unknown protein concentration

All four keratin hydrolysates were tested to determine the concentration of the protein, Table 3.2. CH1 shows the highest protein concentration of 1.40 mg/ml and EH2 shows the lowest protein concentration of 0.45 mg/ml.

Table 3.2. The protein concentration obtained from different methods

Keratin Hydrolysate	**Concentration mg/ml**
EH1	1.08
EH2	0.45
CH1	1.40
CH2	1.02

3.3.5 Ash Content

Ash content measures the inorganic matter and minerals content of the biomass that remains after the complete oxidation of organic matter. The ash content of all three keratin hydrolysates from different methods was determined and shown in Table 3.3.

Table 3.3. The ash content of the hydrolysate obtained from different keratin hydrolysates

Keratin Hydrolysate	**Ash content (%)**
Keratin Azure	0
EH1	9.19
EH2	NA
CH1	20.7
CH2	5.27

The ash content of keratin azure was found to be zero, where a low ash content is known to be from a clean fraction of the keratin, where this was followed by CH2 at 5.27 and EH1 at 9.19. Keratin hydrolysate from CH1 has a high ash content which is known to be caused by the alkaline environment and also the salts formed from the chemical used. The ash content of EH2 could not be determined due to the low yields obtained.

3.5.6 SDS PAGE (Sodium Dodecyl Sulphate–Poly-Acrylamide Gel Electrophoresis)

The molecular weights of the hydrolysate from CH1, CH2 and EH1 were determined. Where CH1 there is a big band around 5–10 kDa and other higher molecular weight

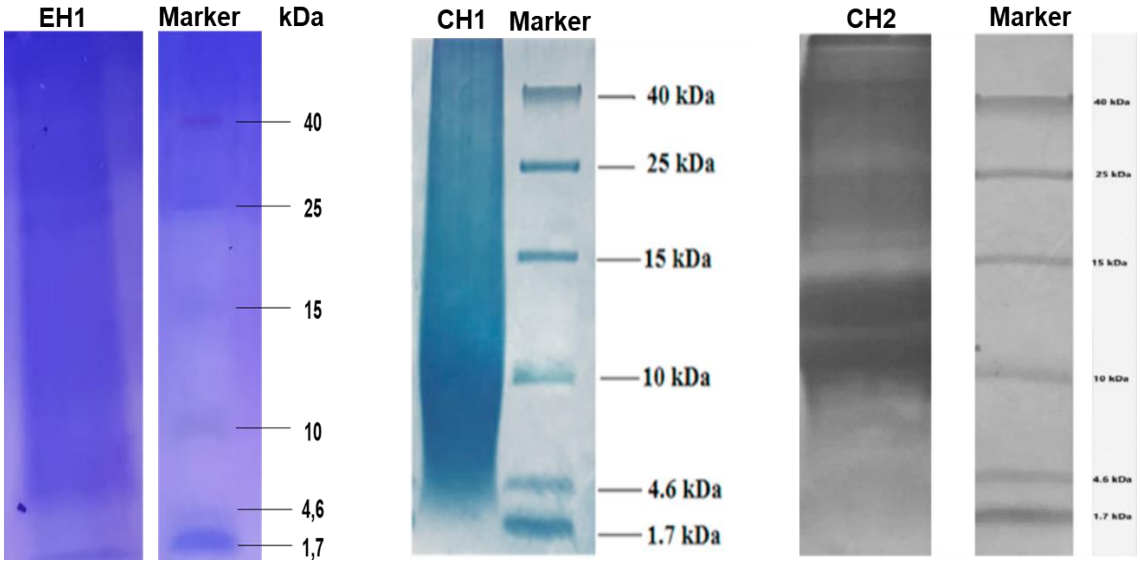


Figure 3.6. SDS PAGE of the keratin hydrolysate from three different methods.

While CH2 10–15 kDa, 15–25 kDa, and higher molecular weight. And EH1 is a band around 5 kDa and 10 kDa and also contains higher molecular, which are not clearly separated. Enzymatic hydrolysis is known to produce medium to low molecular weight due to the production of amino acids and small peptide chains while chemical hydrolysis contains mostly higher molecular weight

3.4 Discussion and conclusion

From the FTIR it is seen that the keratin hydrolysate from all the three different methods shows the presence of the keratin structure as shown in Figure 3.1. There is a large peak around 1069 cm^{-1}, S=O, which is due to the breakage of the disulphide bonds from all four keratin hydrolysate, while in EH1 the peak is the largest and non-existing in keratin azure

The breakage of the disulphide bonds helps with the solubility of the keratin hydrolysate and it was found that EH1 was highly soluble in water while the keratin hydrolysates from the EH2, CH1, and CH2 are partially soluble and keratin azure is insoluble in water, which explains the non-existence of the S=O peak.

The protein concentration and protein content of the hydrolysates were also determined. From the results obtained it was observed that the keratin hydrolysate from EH2 had the highest protein content of 90.00%, this is because the hydrolysis conditions are mild, when compared to chemical, and does not damage any amino acids. The low protein concentration from EH2, 0.45 mg/ml, is due to low extraction yields. While CH1 has the highest concentration, high yields, and second-highest protein content,1.40 mg/ml, and 86.56%. CH2 showed the lowest protein content of 67.63 %. The concentration of the keratin azure couldn't be determined as it is insoluble in water.

The ash content was also determined with keratin azure 0% which shows a clean organic fraction of the keratin hydrolysate. CH2 and EH1 had the second-lowest, 5.27%, and 9.19%. CH1 having the highest ash content, 20.7%, which is due to the alkaline environment of the keratin hydrolysate meaning there are more inorganic matter and minerals. The high ash content might be due to the keratin hydrolysate from CH2 being dialyzed for 5 days which removed most of the inorganic and mineral contents while CH1 was only dialyzed for 72 hours.

From the TGA curves, it is observed that the hydrolysate from CH1 showed the lowest weight loss of organic degradation,59.90%, with CH2 the highest, 73.62%, and EH1 the second highest. This correlates with the ash content where complete oxidation of most of the organic matter is observed with CH2 and EH1. The TGA of keratin azure is used as a standard. The molecular weight, polypeptide chain and disulphide bonds present also play a major role in the thermal stability of the hydrolysates, where it is observed with CH1 and CH2 being thermally stable when compared EH1 and EH2.

In the SDS-PAGE it is observed that EH1 hydrolysate showed the presence of lower molecular weights when compared to the other two and also high due to the enzymes which were not separated from the hydrolysate. While CH1 and CH2 had both high and low molecular weights. Enzymatic hydrolysis controls the degree of hydrolysis to certain amino acids and peptide chains, where the low molecular weights are due to amino acids obtained. While chemical hydrolysis is known to destroy some of the amino acids, leaving behind the peptide chains which results in higher molecular weights.

The qualities of the keratin hydrolysates obtained from the four different methods showed that they apply to fertilizer, animal feed, bio-adhesives, and nanofibres. CH1 and CH2 can be applicable to bio-adhesives and nanofibres due to their high molecular weights, which will have a high number of functional groups expected to interact with the polymer for bio-adhesives applications (Medronho and Fonseca 2019). For nanofibers, high chemical and thermal stability are the qualities required, which the two chemical hydrolysates show. EH1 and EH2, apply to fertilizers and animal feeds, this is shown by their quality of high nitrogen content which is important in both application, high solubility, and low molecular weights. All four keratin hydrolysates can have other applications based on the quality of the hydrolysates produced. In conclusion, the four different methods produced four different hydrolysates, this comparison helps with determining which hydrolysate is suitable for which application.

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CHAPTER 4

PAPER 3: UNDER REVIEW

CHARACTERIZATION AND ANALYSIS OF ENZYMATIC CHICKEN FEATHER KERATIN AS A SOURCE OF ANIMAL FEED PROTEIN INGREDIENT

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Abstract

Valorization of waste chicken feathers to keratin is of biotechnological interest. Microbial keratinase in feather processing is of interest due to its environmentally friendly technology. Bacterial and fungal strains were isolated from chicken feathers and screened for keratinase activity on feather meal agar. They were then tested for the degradation of chicken feathers where they were tested for their ability to degrade chicken feathers and produce keratin

hydrolysates. The enzymatic hydrolysates produced from the strains were characterized and analyzed using analytical techniques to profile their quality and quantity for their applicability as ingredient in animal feed. The keratin structure was confirmed using Fourier Transform Infrared Spectroscopy (FTIR). The protein concentration and protein content were determined using the Bradford assay and CHNS analysis, Thermogravimetric Analysis (TGA) was used for the determination of weight change with temperature. All the hydrolysates produced by enzymatic hydrolysis from the fungal and bacterial strains showed qualities suitable for protein ingredient in animal feed, with CFF1 showing the best qualities of the keratinous hydrolysate as a protein ingredient, with the highest protein content and maximum amino acid concentration.

Keywords

Chicken Feathers, Keratinase, Keratinous Hydrolysate, Analytical Techniques

4.1 Introduction

Keratin is one of the insoluble structural proteins which is highly stable and resistant because of the extensive cross-linkages of disulphide, hydrogen, and hydrophobic interactions, and it is resistant to microbial degradation (Abdel-Fattah et al. 2018). It is found mainly in two forms, α-helix and β-sheets. The structure of chicken feathers is comprised of 91% β-keratin (Wang et al. 2016). The most abundant keratinous materials are known to be chicken feather waste with billions of tonnes produced in slaughterhouses annually (Herzog et al. 2016). Feathers are protein-rich products, however most waste feathers are disposed off in landfills, dumps and some are incinerated resulting in environmental pollution. Waste feather processing and valorisation is required to avoid this. Their biotechnological application via microbial fermentation and enzymes would serve as an environmentally friendly technology to valorise the waste (Srivastava et al. 2020) .There are other hydrolytic methods such as chemical hydrolysis, however, this method is known to destroy some important amino acids (Mustățea, Ungureanu, and Iorga 2019). Microbial keratinases are intensively applied in feed, fertilizer, leather, pharmaceuticals, and biomedical applications (Călin et al. 2017b; Fang et al. 2013; Jani et al. 2014; Mazotto et al. 2013; Sharaf and Khalil 2011). Waste from keratin represents a source of valuable protein and amino acids and are mostly applicable in fodder and additives for animals and also as a source of nitrogen for plants. Most microorganisms or strains have

their special conditions for maximum enzyme production (Bach et al. 2015; Bhari et al. 2018; Fontoura et al. 2014b, 2019b; Ramakrishna Reddy et al. 2017). In this article, we look at the quality of the enzymatic hydrolysates for animal feed, produced by bacterial and fungal strains.

Keratinases belong to a class of proteases and are keratinolytic enzymes produced by micro-organisms like bacteria, fungi, yeast, and actinomycetes and have widespread application in feed, fertilizers, leather, pharmaceuticals, and biomedical industries (Călin et al. 2017b; Fang et al. 2013; Jani et al. 2014; Mazotto et al. 2013; Sharaf and Khalil 2011) due to their low cost, availability and free from contaminants. The unique characteristic that distinguishes keratinases from other proteases is the ability to bind to the complex and insoluble substrates (feathers, wool, silk, collagen, elastin, horns, stratum corneum, hair, azokeratin and nails). Their production is carried out by fermentation, utilizing chicken feathers as the only source of carbon and nitrogen (Srivastava et al. 2020). Of key importance in the production process is microbial strain capable of production of high activity keratinases thus screening of microbes is required to identify a strain that can produce a non-toxic, highly active and less expensive enzyme. The important factors that play a role in the production of the highly active keratinase enzymes are the microbial strain, aeration, , medium composition, temperature, pH, and fermentation method (Srivastava et al. 2020). They are a class of proteolytic enzymes, which are more advanced than the normal proteases due to their stability over a range of alkaline pH and temperature conditions (Herzog et al. 2016). They are mostly active in the presence of the keratin substrate, where they are known to attack the peptide bond in the structure of the keratin and convert it into small peptide chains and amino acids. Keratin decomposition requires proteolytic reaction and disulphide bonds reduction (Srivastava et al. 2020).

Waste from keratin represents a source of valuable protein and amino acids and are mostly applicable in fodder and additives for animals and also as a source of nitrogen for plants. Most microorganisms or microbial strains have their special conditions for maximum enzyme production (Bach et al. 2015; Bhari et al. 2018; Fontoura et al. 2014b, 2019b; Ramakrishna Reddy et al. 2017).

Laba *et. al*. (2018) isolated a feather degrading bacterium where a single strand was identified as *Kocuria rhizophila* p3-3, which exhibited significant keratolytic properties. The culture conditions were optimised in order to maximise the production of soluble proteins and free amino acids. The bacterium degraded chicken feathers within four days, and the resultant hydrolysate was tested for the amino acids present, ferric reduction and radical scavenging

activities (Łaba et al. 2018). (Riffel and Brandelli 2006) isolated four keratinolytic bacteria, where three belonged to the genera *Burkholderia*, *Chryseobacterium* and *Pseudomonas* and one was *Microbacterium* species. Keratinase activities were detected in all four strains. Complete degradation of chicken feathers was observed with *Microbacterium* sp. and *Chryseobacterium* sp., while the *Pseudomonas* sp. only disintegrated feather barbules but not all rachises and minor degradation was observed with *Chryseobacterium* species. Only proteolytic activities of the keratolytic strains were determined, and the hydrolysate was not characterized. Eights strains of *Bacillus* were isolated by (Nagal and Jain 2010) from decomposing feathers and were tested for hydrolysis of feather waste. Among them *Bacillus cereus* KB043 was the best feather degrading microorganism. It also showed the highest level of keratinase activity, but again the hydrolysate formed was not analysed.

Keratinolytic fungal strains were isolated from the soil by (Călin et al. 2017b), followed by evaluating their ability to degrade keratin substrate using SEM, FTIR and TGA. *Fusarium* sp. 1A was found to be the most active in the degradation process, while once more the hydrolysate was not characterized.

Most of these researchers focus on the optimisation processes and activities of the keratinases, but detailed analysis on the hydrolysate formed is lacking. The qualities and quantities of the hydrolysate are very important in determining their applicability formed from the keratinases produced.

In this article, we look into detailed analysis of the hydrolysate formed from the keratinolytic bacterial and fungal strains. This is done by using analytical characterization techniques like FTIR, TGA, CHNS analysis and Bradford assay.

4.2 Experimental

4.2.1 Methods

Screening for the keratinase activity

Waste chicken feathers used were collected from Rainbow Chicken, in KwaZulu-Natal, Durban. A subset of waste feathers, which were not washed, were used to isolate the microbial flora indigenous to chicken feathers. The feathers were suspended in minimum media which was composed of (g/l): NaCl, 0.5; KH_2PO_4, 0.7; K_2HPO_4, 1.4; $MgSO_4.7H_2O$, 1 and incubated at 30°C with shaking. The suspended samples were plated out on feather meal agar (FMA)

plates composed of agar (%) and 0.1% feather meal made up in minimum growth medium. The plates were incubated at 30°C, pH8, for 7 days (Mnguni, 2021). Following incubation, the plates were stained with 10% Trichloroacetic acid (TCA) for 15 min. Keratinolytic activity was confirmed by clear zones appearing around the isolates. Five fungal and four bacterial isolates exhibiting activity were chosen for time course

Chicken feather meal was prepared from washed, autoclaved (15 min at 121 °C) and dried (overnight in a hot air oven at 50°C) waste chicken feathers dry. The dried feathers were powdered and sieved was used as feather meal. The isolates were then identified by isolation of the genomic DNA and analysed using blast analysis. All the bacterial (CFB1 and CFB3) and fungal (CFF1 and CFF4) isolates were then tested for feather degradation in a basal medium with chicken feathers as the only source of carbon and nitrogen. The concentration of the free amino groups was determined using the ninhydrin method, with glycine as a standard. The assay mixture comprised of 2 mL of crude supernatant and 1 ml 8% ninhydrin reagent. The mixture was boiled for 10 min then cooled for 15 min. Five millilitres of 95% ethanol were then added to the mixture and the absorbance measured at 570 nm (Spedding et al. 2013). The hydrolysates formed were characterized and analysed using different analytical techniques which are FTIR, TGA, Bradford assay and CHNS analysis.

4.2.2 Characterization Techniques

FTIR spectroscopy in an attenuated total reflection mode (ATR), was used for the analyses of the functional groups, where spectra were collected over a frequency range of 35000-400 cm^{-1}. TGA profiles were determined using Simultaneous Thermal Analyser (STA) STA 6000. The temperature range of the profiles was 28-750 °C with a heating rate of 10°C/min under nitrogen with a purge flow of 20ml/min. Elemental compositions were analyzed using PerkinElmer, series II CHNS elemental analyzer, where the protein content was determined using the nitrogen content obtain multiplying it with the conversion factor of 6.25. Bradford Assays were done on the UV/Visible spectrophotometer operation Cary 50 CONC. All the absorbances for the calculated concentrations were taken at a wavelength of 595 nm.

4.3 Results and Discussions

The four isolates were screened for keratinase activity and they all showed activity by forming a clear zone around the feather meal agar plate. Figure 4.1, shows an example of one of the

screened keratinolytic strains on a feather meal agar plate and this showed that the keratinase was active.

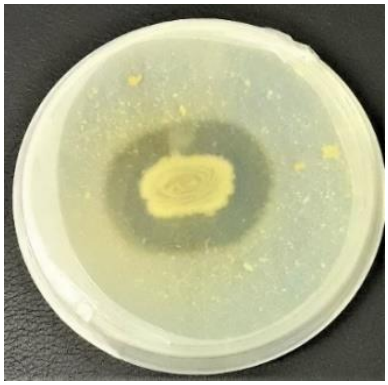

Figure 4.1. Screening for the keratinase activity on feather meal agar plate

The four bacterial and fungal isolates were identified by isolation and were analyzed using BLAST analysis of 16S and 18S rRNA gene sequencing, respectively. Their identities are CFB1: *Bacillus cereus,* CFB3: *Chryseobacterium* sp., CFF1: *Penicillium marquandii*, and CFF4: *Fusarium solani.*

All the strains were then tested for chicken feather degradation in shake flask fermentations Varying degrees of degradation were displayed by all. The deterioration of feathers in the medium is shown in Figure 4.2, where complete degradation was seen after three days. After 3 days, the flask showed a milky solution with no chicken feathers in the flask, meaning complete degradation was observed.

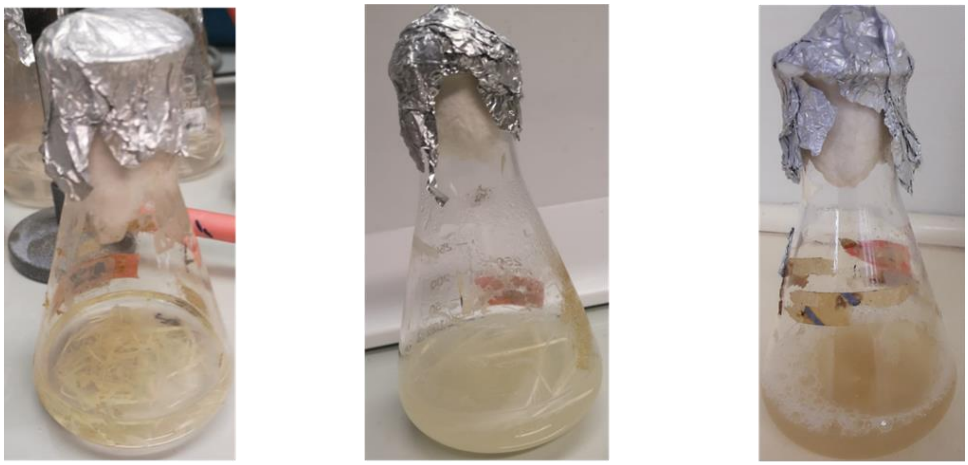

Figure 4.2. Feather degradation by the keratinases from day 0: A, day 1: B, day 3: C

The free amino acid concentration was observed to increase in a culture medium with time. The breakdown of peptide into amino acids continued with time of degradation. After 3 days, the maximum concentration of the free amino acids in the culture meduim was found to be 2.3 mg/ml. Amino acids formation is a good quality for ingredient in the protein animal feed as they are known for their antioxidant activities. The hydrolysates formed were further characterized and analysed using different analytical techniques which are FTIR, TGA, Bradford assay and CHNS analysis.

4.3.1 Fourier Transform Infrared Spectroscopy (FTIR)

It represents important functional groups present in the keratinous materials of the peptide chains. It shows the fingerprint of the molecular component in the keratin structure. The FTIR profiles of the hydrolysates produced by the different bacterial and fungal strains are shown in figure 4.3. They all show the presence of the keratinous structure, where the feather meal and keratin azure serve as standards for the keratinous structure (Brebu and Spiridon 2011; Kakkar, Madhan, and Shanmugam 2014b; Sharma, Gupta, Chik, et al. 2017; Wang and Cao 2012a; Zoccola et al. 2009a). Figure 4.4, shows the functional groups of the keratinous structure in the hydrolysates produced by the fungal and bacterial strains with Amide A at 3277 cm^{-1} representing the stretching vibration of O-H and –N-H. Amide I at 1 632 cm^{-1} shows the presence of C=O, while Amide II at 1535 cm^{-1} represents -C-H stretching and N-H bending and Amide III at 1240 cm^{-1} shows C-N stretching and N-H bending.

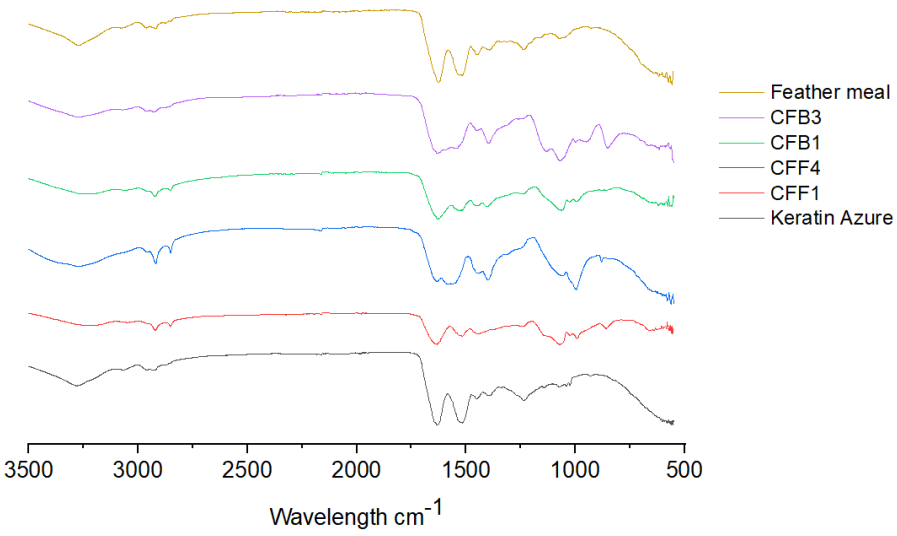

Figure 4.3. FTIR profiles of the enzymatic hydrolysates produced by the bacterial (CFB1 and CFB3) and fungal (CFF1 and CFF4) strains compared to feather meal and keratin azure.

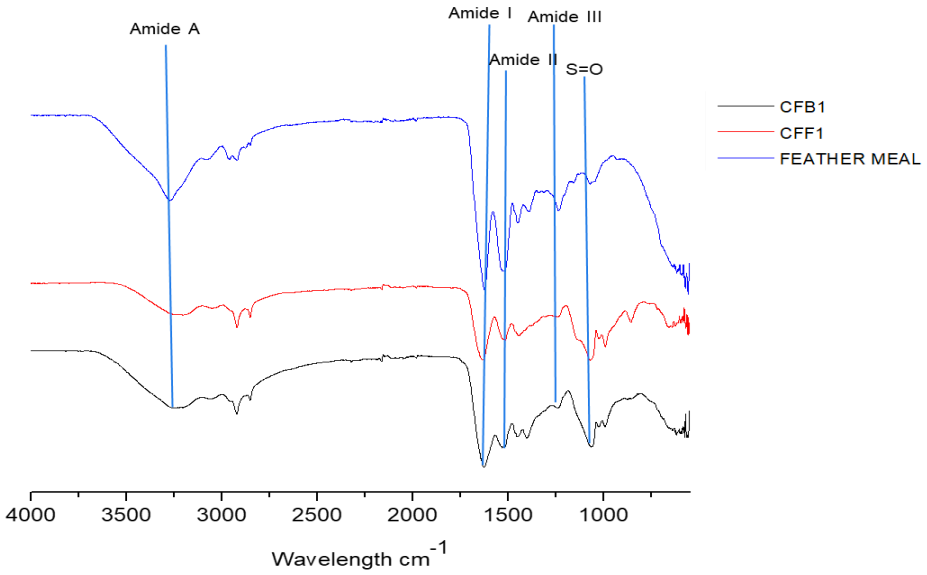

Figure 4.4. Comparison of the FTIR profiles of the enzymatic hydrolysates produced by fungal (CFF1) and bacterial (CFB1) strains and feather meal

There is also the presence of the band around 1069 cm^{-1}which is assigned to the sulphoxide bond, S=O, which represents the breakage of the disulphide bond.

This technique is important for the determination of the disulphide bonds in the hydrolysate, as the presence of these bonds is indicative of poor digestibility of the hydrolysate in animal feed. Following the keratinase degradation, the presence of the S=O which formed as a result of the reaction of sulphides and cysteine in protein, thus demonstrating the breakage of the disulphide bonds.

4.3.2 Thermogravimetric Analysis (TGA)

TGA measures weight changes in a material as a function of temperature under a controlled atmosphere. It only provides us with information about the degradation of the hydrolysate when exposed to higher temperatures. Figures 4.5 and 4.6 show, the TGA profiles of the hydrolysates produced by the fungal and bacterial strains with that of the feather meal and keratin azure.

There are three different stages of weight losses, where the 1st stage, 28 ºC - 150 ºC, is due to water evaporation and the 2nd stage, 150 ºC - 500 ºC, represents the denaturing of the polypeptide chains, where it is known that keratin suffers organic degradation. The 3rd and last stage, between 500 ºC - 700 ºC is where complete degradation occurs (Brebu and Spiridon 2011; Idris et al. 2013b; Kakkar, Verma, et al. 2014; Tesfaye, Sithole, Ramjugernath, et al. 2018b).

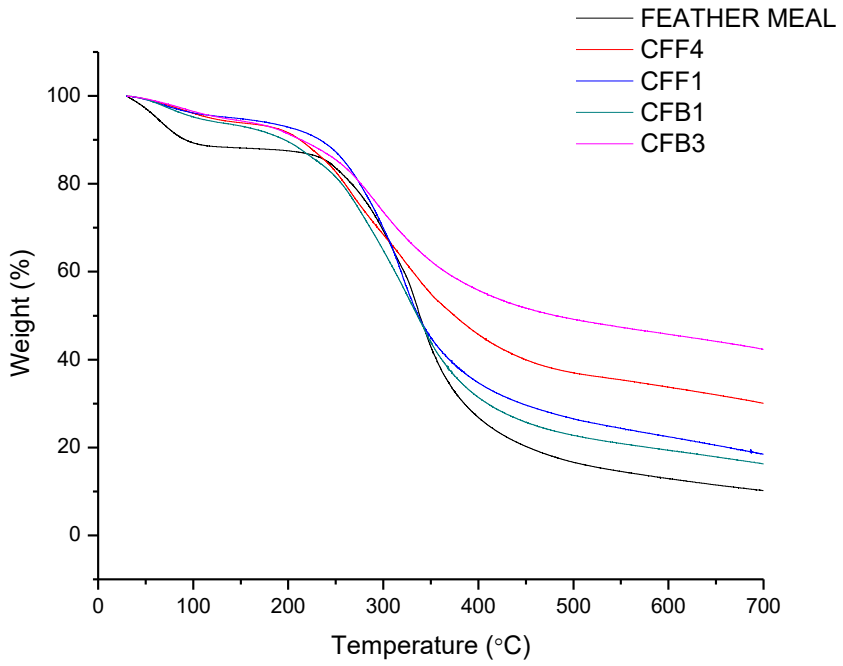

Figure 4.5. The TGA curves of the enzymatic hydrolysates produced by fungal (CFF1 and CFF4) and bacterial strains (CFB1 and CFB3), compared to feather meal.

In figure 4.6, keratin azure shows a rapid water loss in the 1st stage which is due to loosely bonded water in the structure, while the hydrolysate shows a different trend. The second stage and third stage only show the difference in stability of the hydrolysate from different strains, when compared to keratin azure. Keratin azure (in black) and CFB1 (in red) shows the hydrolysate which is easily degradable, meaning the hydrolysate will be easily digestible, as it is proven in Table 4.1 with the highest weight loss

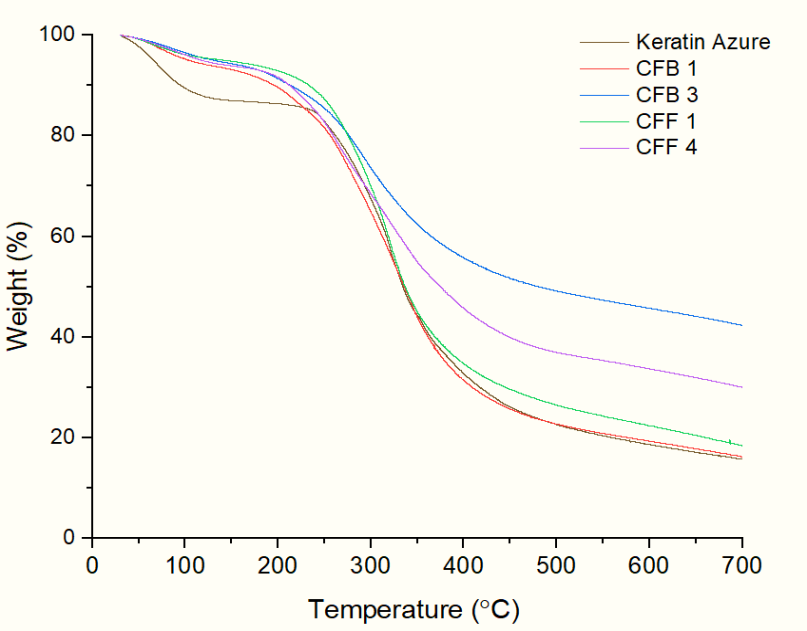

Figure 4.6. TGA profiles of the enzymatic hydrolysates produced by fungal (CFF1 and CFF4) and bacterial strains (CFB1 and CFB3), and keratin azure.

The TGA profiles show different weight losses at the different temperatures. Figure 4.7 and Table 4.2, show the strains with different weight loss shown in percentages. It also shows that the highest weight loss was achieved at the 2nd stage from all different strains, where keratin is known to suffer organic degradation.

Table 4.1. TGA results for the weight loss of the hydrolysates produced by the fungal and bacterial strains

Tested strains	*1st stage weight loss %*	*2nd stage weight loss %*	*3rd stage weight loss %*	*Total weight loss (%)*
CFB1	6.84	70.39	6.51	*83.74*
CFB3	5.66	45.16	6.79	*57.61*
CFF1	5.20	68.28	8.04	*81.52*
CFF4	6.10	56.91	6.93	*69.94*

The total weight loss was obtained after heating the samples at 700 ºC. The most active organism in the degradation process was the CFB1 strain, figure 4.7a, which is due to the strongest denaturing of the keratin chain and showed the highest weight loss of 83.74%. While CFB3, figure 4.7b, showed the lowest total weight loss at 57.61%.

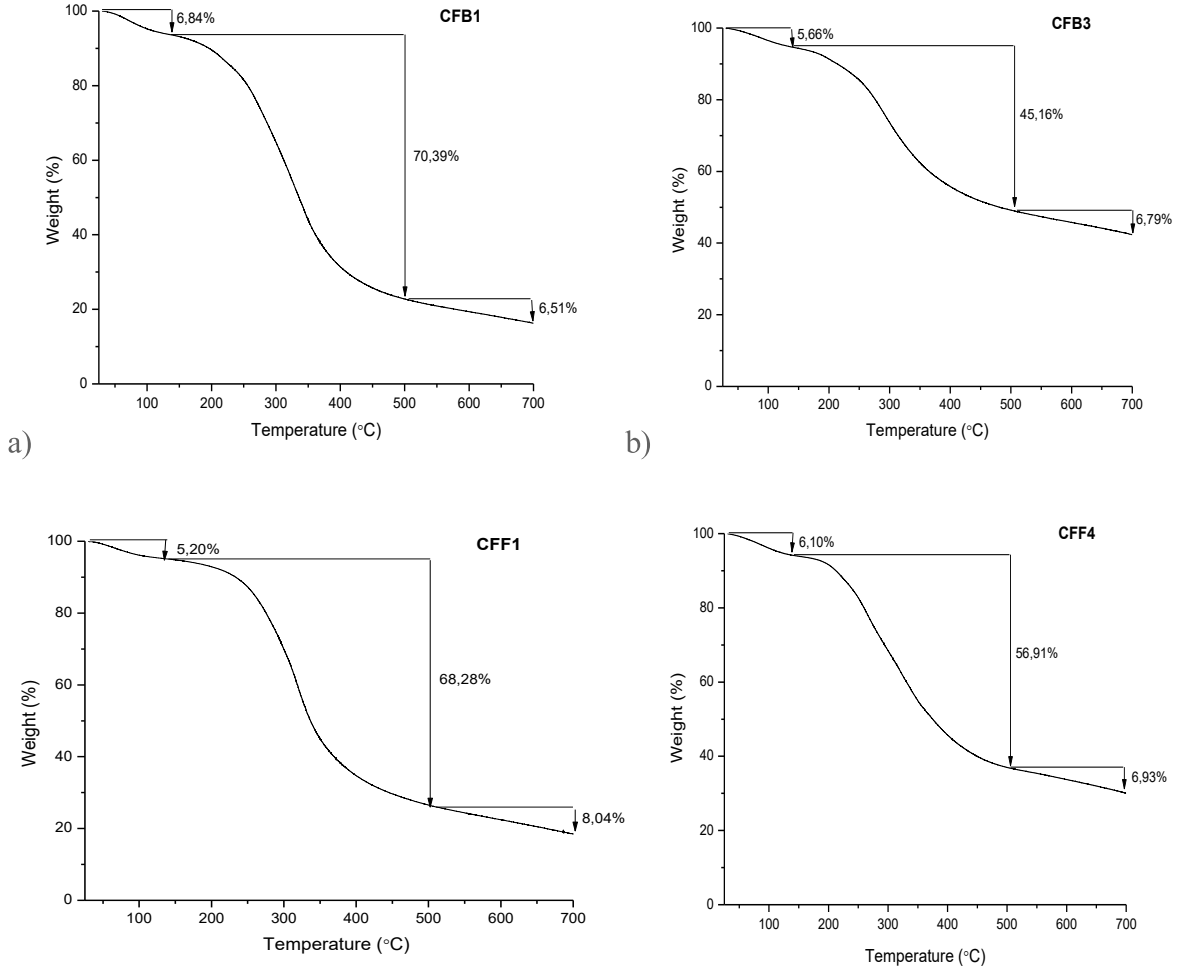

Figure 4.7 TGA profiles of hydrolysates produced by the bacterial strains (a) CFB1 strain and **(***b)* CFB3 strain and fungal strains (c) CFF1 strain and (d). CFF4 strain.

The stability of the hydrolysate is known to be affected by molecular mass and the aromatic groups present in the hydrolysate. The aromatic group stability is due to the fact that polymers with aromatic rings when heated under nitrogen atmosphere, are known to form char residues which are stable up to 700 °C. The more aromatic groups there are the more hydrophobic the hydrolysate is, which is the best hydrolysate for the animal feed.

4.3.3 Bradford Assay

This technique was used to determine the concentration of the total proteins in the hydrolysates. The hydrolysates of the four strains were tested and the unknown concentrations were determined using a standard curve shown in Figure 4.8. The curve represents the standard protein with the known concentration and absorbance taken at the wavelength of 595 nm. From the standard curve, the concentrations from table 4.2 were obtained.

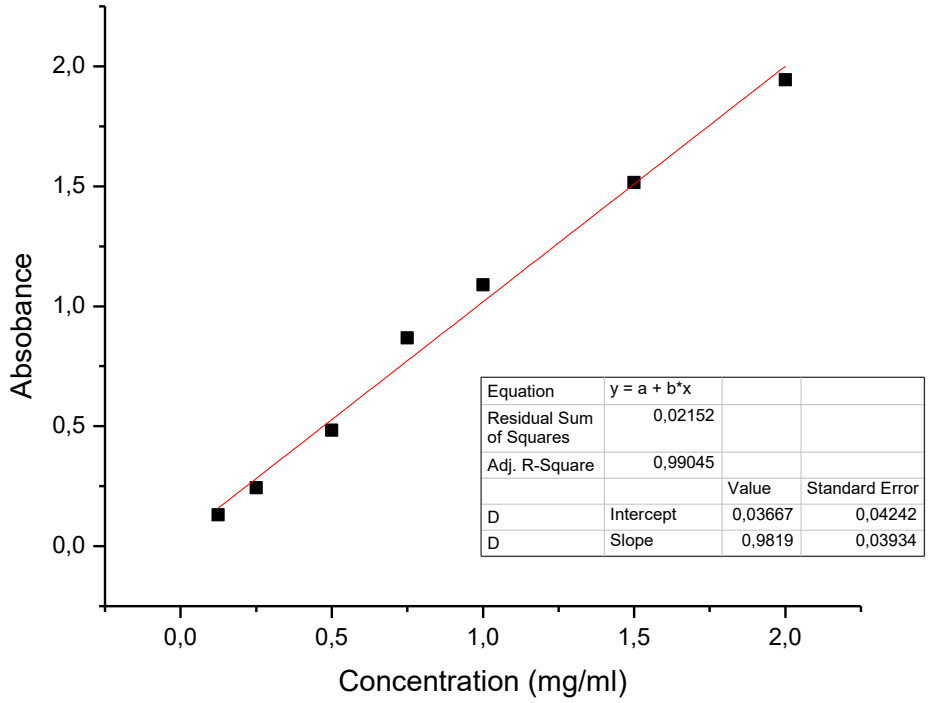

Figure 4.8. Standard Curve for Bradford Assays to determine the unknown protein concentration.

the concentration of the protein in the hydrolysate produced by all four strains were determined (Table 4.2). This characterization technique is important to determine the quantity of the hydrolysate produced, which will be open to optimization for industrial applications.

The CFB3 strain showed the highest protein concentration of 0.85 mg/ml and the lowest protein concentration of 0.45 mg/ml was obtained with the CFF1 strain.

Table 4.2. Total protein concentration in the hydrolysates produced by the different strains

Strains	*Concentration (mg/ml)*
CFF1	0.45
CFF4	0.78
CFB1	0.51
CFB3	0.85

4.3.4 CHNS Analysis

The elemental analysis profiles the proportion of the key elements C, H, N and S and using a formula (using the percentage of nitrogen multiplying with 6.25 factor) estimates percent protein in the enzymatic hydrolysates The results which are shown in table 4.3 and figure 4.9 with keratin azure included as a control for comparison

Table 4.3. Elemental analysis of enzymatic hydrolysates.

Enzymatic Hydrolysate	*Carbon %*	*Hydrogen %*	*Nitrogen %*	*Sulphur %*	*Protein %*
CFF1	43.55	5.99	14.40	2.51	*90.00*
CFF4	35.63	5.75	10.29	2.02	*64.31*
CFB1	36.95	5.87	12.78	1.85	*79.88*
CFB3	23.14	3.57	7.05	1.0	*44.06*
Keratin Azure	43.21	6.50	14.99	4.13	*93.68*

. The highest from the different strains was found to be CFF1 with 90%, where Tiwary *et al.* (2012) reported 87% with *Bacillus* strain, with the lowest being CFB3 with 44.06%. The

protein content produced in the CFF1 hydrolysate corresponds to what is expected to be found in the enzymatic hydrolysate, for the highest protein quality for animal feed.

The keratin azure has the highest protein content as it is keratin extracted from wool compared to keratin extracted from chicken feathers, which correlates with the literature (Călin et al. 2017a). But the keratin azure has the highest sulphur content, as keratin extracted from wool has a β-pleated sheet structure which is different from the feathers with α-helix structure. The use of keratin azure as a comparison is to determine the overall structure of keratin and to confirm the presence of keratin in chicken feathers.

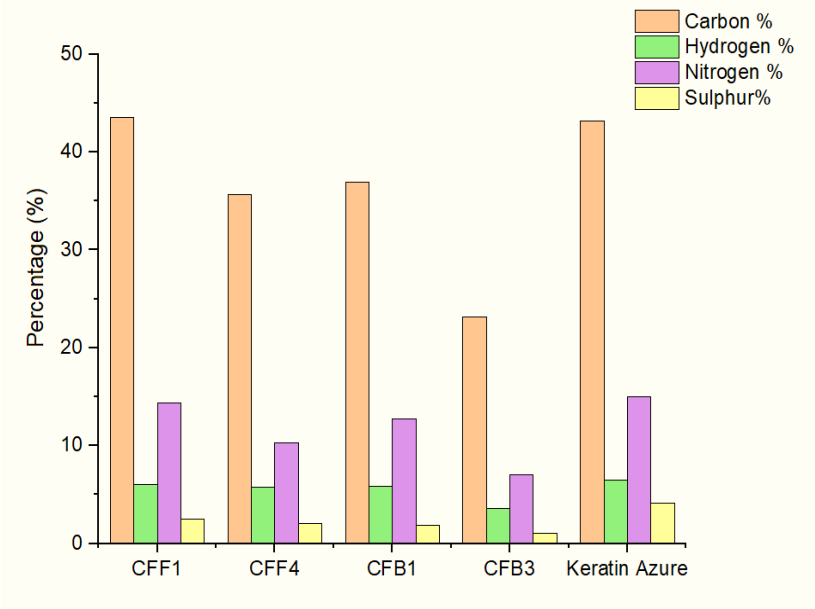

Figure 4.9. Bar graph of elemental analyses of different enzymatic hydrolysates.

The analysis also shows that the protein concentration in the different hydrolysates does not correlate with the protein content. From our results, CFF1 has the highest protein content, where the concentration of the total protein was the lowest. While CFB3 has the lowest protein content but the highest protein concentration.

And also the CFB3 strain showed the lowest activity when compare to all strains and also the lowest protein content. This proves that the strain is not active as there is low protein content.

CFF1 has the highest protein content when compared to the reported strain (Tiwary 2012), meaning optimization has to be done in order to improve the content, the amino acid and the concentration of the protein in the hydrolysate.

4.4 Conclusion

All the four bacterial and fungal strains showed keratinolytic activities. The degradation of feathers was also observed for all four strains.

The FTIR from the four strains showed that the keratin hydrolysates with the highest peak of the S=O was from CFB1, which means more disulphide bonds were broken. This was also shown by the solubility test where CFB1 was more soluble compared to all keratin hydrolysates produced by the four strains, meaning most of the disulphide bonds were broken. And from the TGA, CFB1 suffered the most organic degradation implying that the strain was the most active. CHNS analysis showed that CFB1 and CFF1 had the most protein content, which correlates with the TGA about the organic content. All produced strains showed the production of the keratinous hydrolysate with CFF1 being the best quality obtained for animal feed products due to high protein content and showing the maximum amino acid concentration.

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CHAPTER 5

CONCLUSIONS AND FUTURE WORK

Keratin hydrolysates from enzymatic, reduction and alkaline hydrolysis were characterized and analysed to determine the quality and quantity of the hydrolysates. This is important to determine their industrial applications. The characterization techniques used included FTIR for the determination of the chemical structure, Bradford Assay for the concentration of the hydrolysate, CHNS Analysis for the protein content in the hydrolysate, SDS PAGE for the molecular weight of the keratin, TGA to determine their thermal stability and ash content.

From the analysis the FTIR showed the presence of the keratin structure, which also showed the breakage of the disulphide bonds. This is important for the solubility of the hydrolysate, as enzymatic hydrolysis showed the most soluble hydrolysate as most of the disulphide bond were broken when compare to chemical hydrolysis. Protein content was high with enzymatic hydrolysis and ash content showed complete oxidation of organic matter. The chemical hydrolysates were thermally stable when compare to enzymatic hydrolysate, which was confirmed by TGA. The molecular weights of the hydrolysate were mostly low for enzymatic while chemical were medium to high. All the qualities obtained from the enzymatic hydrolysate are suitable for animal feed production, due to high protein content, high solubility, low ash content and lower molecular weights.

Then we looked closer at the enzymatic hydrolysis using bacterial and fungal strains to produce the best quality hydrolysate for animal feed production. The fungal strain showed higher activities and the best hydrolysate qualities for animal feed production.

To answer the questioned aimed at this research,

- The enzymatic hydrolysis produced the protein hydrolysate, which was high with the protein content and the presence of the amino acids were detected.
- The quality of the hydrolysate was determined using CHNS analysis, FTIR, ash content and TGA, and the quantity was determined by the Bradford assay, ninhydrin method and SDS PAGE.
- We have observed that enzymatic hydrolysis obtained the highest protein content from the CHNS analysis when compared to the reported literature and was highly soluble in

water when compared to the chemical hydrolysates. The FTIR confirmed the solubility with of the enzymatic hydrolysate with the disulphide bond breakage.

- The industrial applications can be determined by looking at the molecular weight, the protein content, the ash content and the solubility of the hydrolysate. And also the TGA shows the stability of the hydrolysate under controlled atmosphere and increased temperatures.
- The fungal hydrolysate showed more of the protein ingredient for animal feed due to high protein content and maximum amino acid concentration. And combined with all the other techniques it confirms the quality and quantity of the enzymatic hydrolysate.

Recommendations

For further characterization techniques we require the use of:

- 13C NMR for the presence of the functional groups in the hydrolysate, which shows the carbonyl groups from the amino acids and the peptide chain in the hydrolysate. The aromatic carbons can also be detected using this technique, which is important for the hydrophobicity of the hydrolysate.
- LC-MS/MS, which determines the peptide sequence of the hydrolysate which comprises of amino acids and their molecular masses.

Then testing the enzymatic hydrolysate for bioactivity of the peptides which is important in animal feed production, and then extending the work into pilot scale for industrial production into animal feed.

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APPENDICES

Appendix A: Submitted article

Biomass Conversion and Biorefinery
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ORIGINAL ARTICLE

Keratinous hydrolysate profiling: comparison of the differences obtained from different extraction methods

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Abstract
The extraction of keratin from chicken feathers is of research interest due to the beneficiation of biomass. This study investigates the impact of the keratin hydrolysate obtained using four different methods. There are two different chemical extraction methods, CH1 and CH2, and two enzymatic hydrolyses, EH1 and EH2. The investigation includes the characterization and analysis using different types of equipment for different applications. The keratin hydrolysates formed were all characterized using FTIR, TGA, SDS PAGE, and CHNS analysis, Bradford assay, and ash content. All the keratin hydrolysate from different methods showed all the amide bonds present in the keratinous structure from the FTIR, while TGA followed the three-phase trend loss of the keratinous structure. Protein concentrations obtained from CH1, CH2, EH1, and EH2 were 1.40, 1.02, 1.08, and 0.45 mg/ml respectively and their protein content was 86.56, 67.63, 78.06, and 90.00%. Their molecular weights were all in different ranges while the ash content for CH1 was 20.7, CH2 was 5.27, and EH1 was 9.19. All the results obtained were compared to the pure keratin azure and EH2 showed high protein content but low protein concentration. CH1 showed the second-highest protein content but with high impurities from the extraction method shown from the ash content.

Keywords Chicken feathers · Extraction methods · Keratin hydrolysate · Analytical techniques

Novelty Statement The main objective of this article is to compare the different keratin hydrolysates produced from different extraction methods. Most authors focus on the extraction methods and optimization but not the detailed characteristics of the keratin hydrolysate obtained for a specific application.

The quality and quantity of the keratin hydrolysate are dependent on the method used. Comparing the different hydrolysates helps with determining which method to use to obtain a specific hydrolysate with the quality and quantity required for the application in the study.

This research has not been done before where the same characterizations techniques are used on different keratin hydrolysate from different methods, then comparing their qualities and quantities. The applications of the keratin hydrolysates are all dependent on the quality of the hydrolysate obtained which is dependent on the method used, this is the reason why this research is important.

1 Introduction

Keratinous biomass is of interest to most researchers due to its wide applications in different industries. Poultry industries generate five billion tonnes of waste chicken feathers annually, which leads to a potential threat to the environment, while in South Africa nineteen million broiler chickens are killed every month [13]. The waste feathers produced are ground into feather meals or end up in landfills while others are incinerated which causes air pollution leading to greenhouse gas effects. The chemical composition of chicken feathers is 91% β-keratin protein and the other components, like lipid, fibre, ash, and moisture content [21]. This is a very valuable rich protein and the reason why the extraction of keratin is one of the

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most researched topics. The extracted keratin has different technological applications, including cosmetics, pharmaceuticals, fertilizers, biofuels, biomedical, animal feed, and others.

Keratin is a fibrous protein derived from hair, nails, feathers, wool, horns, and hooves and is insoluble in most organic solvents. It is found in two different secondary structures, the α-helix and the β-pleated sheet [19].

The extraction of keratin has attracted much interest, where there are different extraction methods, including chemical hydrolysis, ionic liquids, enzymatic hydrolysis, and thermo-chemical. The choice of extraction method depends on several factors, the chief being the application of the hydrolysate, the cost of the process as well as the yield of the desired product. All the different methods have their advantages and disadvantages. The most widely used methods are the chemical methods using reducing agents [2, 12, 20, 24], and enzymatic hydrolysis using different keratinases [1, 3, 4, 7, 9]. The main process of chemical hydrolysis involves dissolving the chicken feathers in different reducing agents such as thioglycolic acid or thioglycolate salts, 2-mercaptobisulphite, sodium sulphite, sodium bisulphite, and sodium followed by separation of the protein from the chemicals, which cannot be recycled [10]. The chemical reagents break down the disulphide bonds, hydrogen bonds, and salt linkage of the keratin fibres. While in enzymatic hydrolysis, the keratinase is known to disrupt the disulphide bonds, where an enzyme hydrolyzes the peptide bond resulting in the C-terminal (COO^-) and N-terminal (NH_3^+) and also the formation of the hydrophobic amino acid residues (Patterson et al., 1988).

Alahyaribeik et al. (2020) also used reducing agents to produce keratin hydrolysates. They found that the different reducing agents influenced the molecular mass, surface morphology, and crystallinity of the keratin hydrolysate, which in turn affects the bioactivity. Keratin hydrolysate with antioxidant bioactivity can be used in a variety of industries including cosmetics, pharmaceuticals, food processing, and agriculture. Sharma et al. [20] extracted keratin from chicken feathers using sodium sulphide in an alkaline hydrolysis. The keratin obtained had a higher glass transition temperature and most of the disulphide bonds were broken. The keratin in this hydrolysate has application in coating, packaging, and biodegradable composites. Sinkiewicz et al. (2017) used various reducing agents for the extraction of keratin and obtained a high yield of soluble keratin for the application in the formation of biodegradable film used for food applications. Wand and Cia (2012) employed a different chemical agent, viz., hydrophobic ionic liquids for extraction, to produce keratin that was highly soluble in water and had uniform molecular weight, with lower molecular weight being amino acids, while most of the enzymatic hydrolysates are used for animal feed and fertilizers.

The keratin hydrolysate formed from different methods shows different molecular weights, different quality and quantity of the protein formed, different thermal activities, and different morphologies. The quality and quantity of the keratin hydrolysates formed can be determined using different analytical techniques, like Fourier transform infrared spectroscopy (FTIR), CHNS analysis, thermogravimetric analysis (TGA), SDS PAGE, Bradford assays, and the determination of the ash content. This article focuses on the different extraction methods used and their effect on the keratin hydrolysate formed. The keratin hydrolysate formed will be determined by using different techniques to characterize and analyse the chemical composition and physical properties from each method used. The effect each method has on the keratin hydrolysate obtained has not been compared to other keratin hydrolysates from other methods. Most authors focus most on the extraction methods and optimization of the methods but not the detailed comparison of the characteristics of the keratin hydrolysate obtained for a specific application. To focus on a specific application of the keratin hydrolysate, we need to have an understanding of what quality and quantity we get with different methods; this is what this article is focusing on.

2 Experimental

2.1 Methods

2.1.1 Materials

Waste chicken feathers were collected from Rainbow Chicken in KwaZulu-Natal, where they were washed, disinfected, milled, and stored at – 6 °C [8, 14]. The cleaned feathers were used for all the different methods.

2.1.2 Chemical treatment

CH1 represents the keratin hydrolysate extracted using an alkaline method (Fagbemi et. al.., 2020), where feathers were weighed and added to an alkaline solution containing sodium hydroxide and sodium; the pH of the solution obtained was between 12 and 13. The resulting solution was filtered and then neutralized using HCl where the filtrate was dialyzed for 72 h then freeze-dried to collect the keratin hydrolysate. The quantities of all the chemicals used and the detailed method are from an article [8].

CH2 represents the reduction extraction method (Khumalo et al.., 2020) where chicken feathers were immersed in sodium bisulphite, sodium dodecyl sulphate, and urea. The resulting mixture was shaken and heated in an oil bath. After the reaction, the mixture was centrifuged and then filtered. The filtrate obtained was dialyzed for 5 days, and the keratin

solution obtained was then freeze-dried to obtain the keratin hydrolysate. The quantities of all the chemical and detailed method are from the article (Khumalo et. al., 2020).

2.1.3 Enzymatic hydrolysis

EH1 represents an enzymatic hydrolysis method [6]. A basal salt medium containing K_2HPO_4, KH_2PO_4, $MgCl_2$, and $CaCl_2$ and chicken feathers were inoculated with the bacteria isolates, Exiguobacterium species, and incubated. The mixture of bacterium and keratin hydrolysate obtained was then isolated.

EH2 (Mnguni, 2021).

Fungal and bacterial strains isolated from chicken feathers were used to produce hydrolysate by enzymatic hydrolysis.

2.2 Characterization

Fourier transform infrared spectroscopy from PerkinElmer (Frontier Universal model) in an attenuated total reflection mode was used for the analyses of the functional groups, where spectra were collected over a frequency range of 35,000–550 cm^{-1}.

TGA profiles were determined using Simultaneous Thermal Analyser 6000. The temperature range of the profiles was 28–750 °C with a heating rate of 10 °C/min under nitrogen with a purge flow of 20 ml/min.

Elemental compositions were analysed using PerkinElmer, series II CHNS elemental analyser, where the protein content was determined using the nitrogen content obtain by multiplying it with the conversion factor of 6.25 [16].

Bradford assays were done on the UV/Visible spectrophotometer operation Cary 50 CONC. All the absorbance for the calculated concentrations was taken at a wavelength of 595 nm.

Ash content was determined using the convection drying oven, with temperature control of 105 ± 3 °C, ignited in a muffle furnace at 525 °C.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis. Keratin samples were dissolved in distilled water, followed by adding a 15-µL keratin sample into a solution of 5 µL of NuPAGE LDS sample buffer (4×) containing 5% β-mercaptoethanol, and the mixture was boiled for 7 min. The polyacrylamide gels used were 16% and 12% for low and mid-high molecular weight determination where denatured samples were then loaded. The gels were exposed to 80 V for 30 min, followed by 120 V for 2 h. The gels were then stained, for 30 min, with Coomassie Brilliant Blue G-250 followed by an overnight de-staining with a mixture of ethanol-acetic acid solution. Imaging software was used on both gels for analysis.

3 Results and discussion

3.1 FTIR

This is an analytical technique used to identify polymers and organic materials and is an example of ultimate analysis. The absorption bands identify molecular components and structures. It also shows the importance of functional groups present in the keratinous materials of the peptide chains by focussing on the fingerprints of the molecular components of the keratin structure which are required to profile the animal feed and understand the chemical structure of the keratinous material.

The keratin hydrolysates formed from the different methods are shown in Fig. 1. They all show the presence of the keratinous structure with the functional group's amide A at 3250 cm^{-1} representing the stretching vibration of O–H and –N–H. Amide I at 1 632 cm^{-1} shows the presence of C = O, while amide II at 1510 cm^{-1} represents –C–H stretching and N–H bending and amide III at 1240 cm^{-1} shows C–N stretching and N–H bending. This is in agreement with most reported keratinous materials [5, 18, 20, 25].

There is also the presence of the band around 1069 cm^{-1} which is assigned to the sulphoxide bond, S = O, which represents the breakage of the disulphide bond. This technique helps with the determination of the disulphide bonds in the hydrolysate. The presence of the S = O is formed due to a reaction of sulphides and cysteine in protein, showing the breakage of the disulphide bonds.

EH1 has the largest peak of S = O, while EH2, CH2, and CH1 have a similar peak. And in keratin azure the S = O peak is almost non-existence.

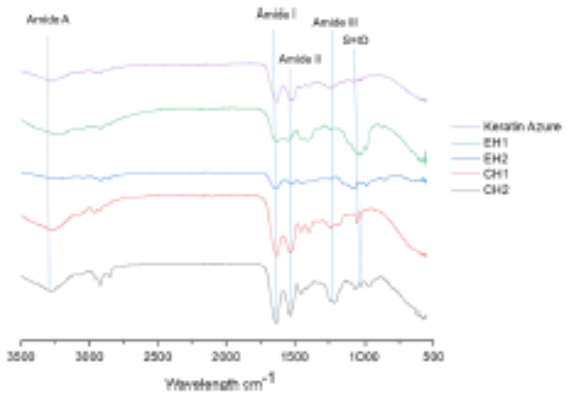

Fig. 1 FTIR of different keratin hydrolysate when compared to keratin azure

3.2 TGA

It is an analytical technique used to determine thermal stability and volatile components of the material by monitoring weight change as a function of temperature under inert. There are 3 different stages of weight losses, where the 1st stage, 28–150 °C, is due to water evaporation and the 2nd stage, 150–500 °C, represents the denaturing of the polypeptide chain, where it is known that keratin suffers organic degradation. The last stage, the 3rd stage, between 500 and 700 °C is where complete degradation occurs as shown in Fig. 2. Most authors (Gupta et al., 2016; [20, 22, 23] present the same trends from their keratin hydrolysates.

All four different keratin hydrolysates show the trend of keratin degradation (Fig. 3), where CH1 shows the lowest weight loss of the organic degradation from the 2nd stage, with 59.90%. The keratin hydrolysate from CH2 shows the highest weight loss with 73.62%, followed by EH1 at 71.21% and EH2 at 62.31%.

At 600 °C, CH1 and CH2 are more thermally stable than EH1 and EH2. Such high thermal stability is known to be caused by the closely packed polypeptide chain in the β-sheet and that large particle sizes also play a huge role. It is also known that polymer with aromatic rings are known to yield char residue which is stable at 600 °C under nitrogen [2]. The difference in molecular weights also plays a role in the thermal stability of the hydrolysates as smaller solid residues tend to have longer residence time which is seen with EH1 and EH2.

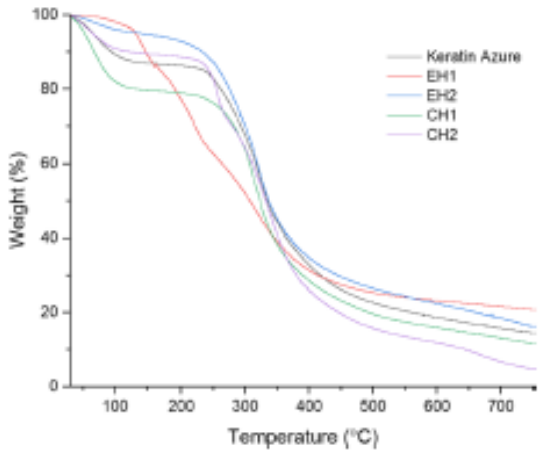

Fig. 2 The TGA curves of different keratin hydrolysate when compared to keratin azure

3.3 Elemental analysis (CHNS analysis)

The protein content in the keratin hydrolysate was determined using this technique. The protein content in the hydrolysate was found to be 86.85% for CH1, 67.63 for CH2, 78.06% for EH1, 90.00% for EH2, and 93.68% for keratin azure (Table 1). The keratin hydrolysate from keratin azure was found to have the highest protein content, which was followed by EH2 as it had the high nitrogen content shown in Fig. 4 and Table 1.

From Table 1 and Fig. 4, the four keratin hydrolysates from the different methods were compared. Where keratin azure is the highest, EH2 is the second-highest protein content. This is due to the factors used for the process involved in the production of the keratin hydrolysate, where enzymatic hydrolysates are known to have high protein content, while keratin azure has the highest protein content as it is keratin extracted from wool compared to keratin extracted from chicken feathers, which correlates with the literature.

3.4 Bradford assay

This technique was used to determine the concentration of the protein in the hydrolysate. Figure 5 represents the standard curve for the Bradford assays for the analysis of protein content. The curve was used to determine the unknown concentration of the keratin hydrolysate. The curve represents the absorbance taken at the wavelength of 595 nm. Bovine serum albumin was used as a protein standard with increasing concentration. The protein was used with the Coomassie blue staining to determine the binding of the protein.

The hydrolysates from the different methods were tested and the unknown concentrations were determined using a standard curve shown in Fig. 5. From the standard curve, the concentrations from Table 2 were obtained.

All four keratin hydrolysates were tested to determine the concentration of the protein (Table 2). CH1 shows the highest protein concentration of 1.40 mg/ml and EH2 shows the lowest protein concentration of 0.45 mg/ml.

3.5 Ash content

Ash content measures the inorganic matter and mineral content of the biomass that remains after the complete oxidation of organic matter. The ash content of all three keratin hydrolysates from different methods was determined and is shown in Table 3.

The ash content of keratin azure was found to be 0, where a low ash content is known to be from a clean fraction of the keratin, where this was followed by CH2 at 5.27 and

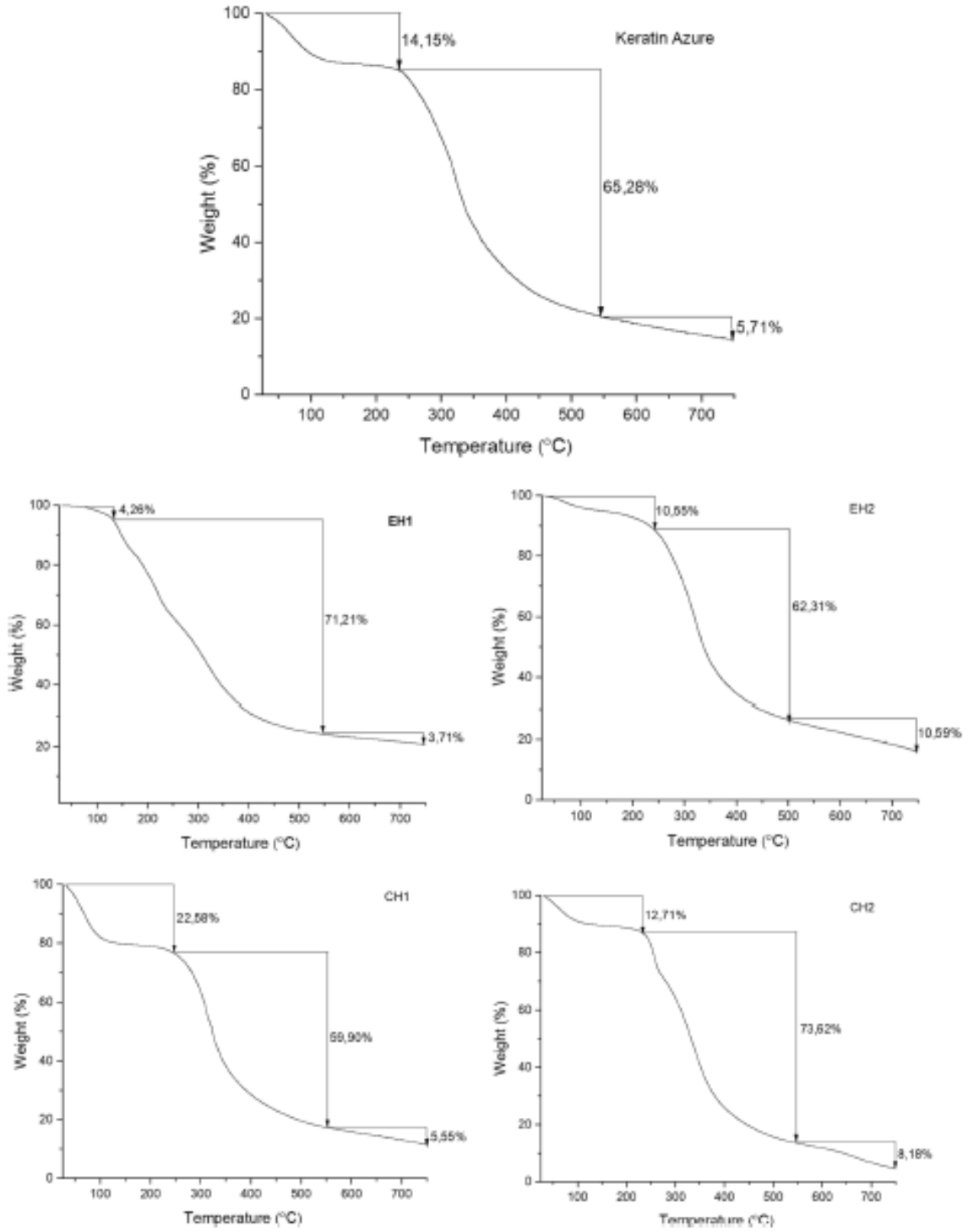

Fig. 3 TGA curves show the mass percentage loss of different keratin hydrolysates with an increase in temperature

Table 1 Elemental analysis of keratin hydrolysate

Keratin hydrolysate	Carbon %	Hydrogen %	Nitrogen %	Sulphur %	Protein %
Keratin azure	43.21	6.50	14.99	4.13	93.68
EH1	35.08	3.14	12.49	4.15	78.06
EH2	43.55	5.99	14.40	2.51	90.00
CH1	47.25	6.90	13.85	2.80	86.56
CH2	46.64	7.72	10.82	5.02	67.63

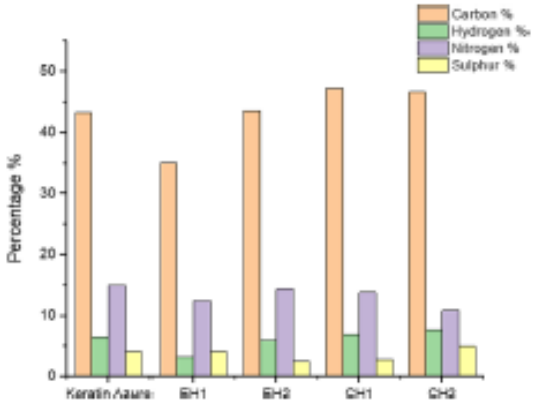

Fig. 4 The bar graph shows different elemental analyses of different keratin hydrolysates

EH1 at 9.19. Keratin hydrolysate from CH1 has a high ash content which is known to be caused by the alkaline environment and also the salts formed from the chemical used. The ash content of EH2 could not be determined due to the low yields obtained.

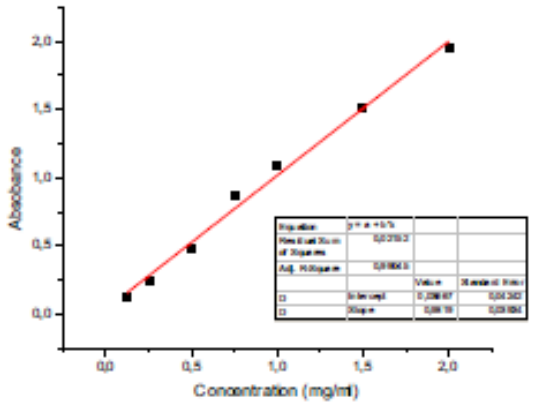

Fig. 5 Standard curve for Bradford assays to determine the unknown protein concentration

Table 2 The protein concentration obtained from different methods

Keratin hydrolysate	Concentration mg/ml
EH1	1.08
EH2	0.45
CH1	1.40
CH2	1.02

3.6 SDS PAGE

It is used to determine the different ranges of molecular weight present in the hydrolysate (Fig. 6).

With CH1, there is a big band around 5–10 kDa and other higher molecular weight, while CH2 10–15 kDa and 15–25 kDa, and higher molecular weight. And EH1 is a band around 5 kDa and 10 kDa and also contains higher molecular weight, which are not clearly separated. Enzymatic hydrolysis is known to produce medium to low molecular weight due to the production of amino acids and small peptide chains while chemical hydrolysis contains mostly higher molecular weight.

4 Discussion and conclusion

From the FTIR, it is seen that the keratin hydrolysate from all the three different methods shows the presence of the keratin structure as shown in Fig. 1. There is a large peak around 1069 cm^{-1}, S=O, which is due to the breakage of the disulphide bonds from all four keratin hydrolysates, while in EH1 the peak is the largest and non-existing in keratin azure.

Table 3 The ash content of the hydrolysate obtained from different keratin hydrolysates

Keratin hydrolysate	Ash content (%)
Keratin azure	0
EH1	9.19
EH2	NA
CH1	20.7
CH2	5.27

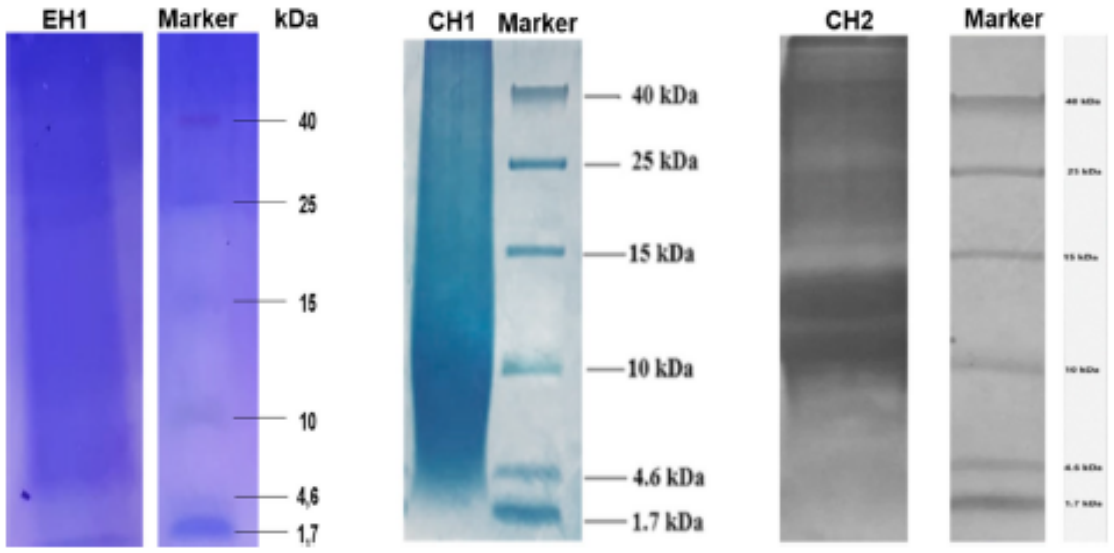

Fig. 6 SDS PAGE of the keratin hydrolysate from three different methods

The breakage of the disulphide bonds helps with the solubility of the keratin hydrolysate and it was found that EH1 was highly soluble in water while the keratin hydrolysates from the EH2, CH1, and CH2 are partially soluble and keratin azure is insoluble in water, which explains the non-existence of the S=O peak.

The protein concentration and protein content of the hydrolysates were also determined. From the results obtained, it was observed that the keratin hydrolysate from EH2 had the highest protein content of 90.00%; this is because the hydrolysis conditions are mild, when compared to chemical, and does not damage any amino acids. The low protein concentration from EH2, 0.45 mg/ml, is due to low extraction yields. While CH1 has the highest concentration, high yields, and second-highest protein content, 1.40 mg/ml, and 86.56%, CH2 showed the lowest protein content of 67.63%. The concentration of the keratin azure could not be determined as it is insoluble in water.

The ash content was also determined with keratin azure 0% which shows a clean organic fraction of the keratin hydrolysate. CH2 and EH1 had the second-lowest, 5.27%, and 9.19%. CH1 having the highest ash content, 20.7%, is due to the alkaline environment of the keratin hydrolysate meaning there are more inorganic matter and minerals. The high ash content might be due to the keratin hydrolysate from CH2 being dialyzed for 5 days which removed most of the inorganic and mineral contents while CH1 was only dialyzed for 72 h.

From the TGA curves, it is observed that the hydrolysate from CH1 showed the lowest weight loss of organic degradation, 59.90%, with CH2 the highest, 73.62%, and EH1 the second highest. This correlates with the ash content where complete oxidation of most of the organic matter is observed with CH2 and EH1. The TGA of keratin azure is used as a standard. The molecular weight, polypeptide chain, and disulphide bonds present also play a major role in the thermal stability of the hydrolysates, where it is observed with CH1 and CH2 being thermally stable when compared EH1 and EH2.

In the sodium dodecyl sulphate polyacrylamide gel electrophoresis, it is observed that EH1 hydrolysate showed the presence of lower molecular weights when compared to the other two and also high due to the enzymes which were not separated from the hydrolysate, while CH1 and CH2 had both high and low molecular weights. Enzymatic hydrolysis controls the degree of hydrolysis to certain amino acids and peptide chains, where the low molecular weights are due to amino acids obtained, while chemical hydrolysis is known to destroy some of the amino acids, leaving behind the peptide chains which results in higher molecular weights.

The qualities of the keratin hydrolysates obtained from the four different methods showed that they apply to fertilizer, animal feed, bio-adhesives, and nanofibres. CH1 and CH2 can be applicable to bio-adhesives and nanofibres due to their high molecular weights, which will have a high number of functional groups expected to interact with the polymer for bio-adhesive applications [17]. For nanofibres, high chemical and thermal stability are the qualities required, which the two chemical hydrolysates

show. EH1 and EH2 apply to fertilizers and animal feeds; this is shown by their quality of high nitrogen content which is important in both application, high solubility, and low molecular weights. All four keratin hydrolysates can have other applications based on the quality of the hydrolysates produced.

In conclusion, the four different methods produced four different hydrolysates; this comparison helps with determining which hydrolysate is suitable for which application.

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Authors contributions I am responsible for the study, conception and design. Material were supplied by M. Khumalo, O. Fagbemi, O. Mnguni and T. Dlume. All data collection and analysis were performed by L. Kekana. The first draft and the final version of the manuscript were written by L. Kekana and all authors read and approved the final manuscript.

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Data availability The data used to support the findings of the study are all included in this article.

Declarations

Competing financial interests The authors declare the following financial interests/personal relationships which may be considered as potential competing interests.

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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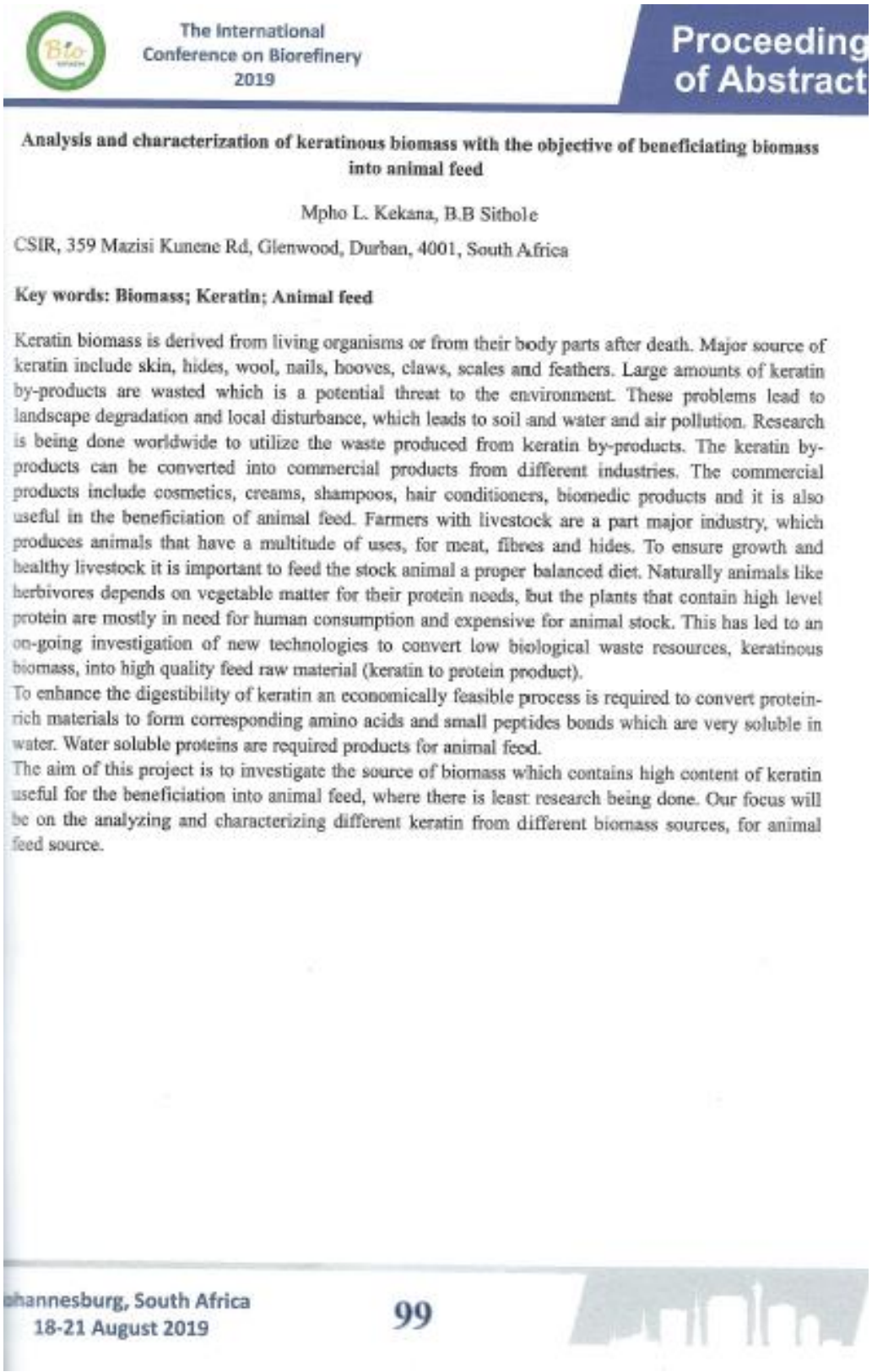
Appendix B: Conferences Abstract

Conference (18-21 August 2019): 7th International Conference of Biorefinery



The International Conference on Biorefinery 2019

Proceeding of Abstract

Analysis and characterization of keratinous biomass with the objective of beneficiating biomass into animal feed

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Key words: Biomass; Keratin; Animal feed

Keratin biomass is derived from living organisms or from their body parts after death. Major source of keratin include skin, hides, wool, nails, hooves, claws, scales and feathers. Large amounts of keratin by-products are wasted which is a potential threat to the environment. These problems lead to landscape degradation and local disturbance, which leads to soil and water and air pollution. Research is being done worldwide to utilize the waste produced from keratin by-products. The keratin by-products can be converted into commercial products from different industries. The commercial products include cosmetics, creams, shampoos, hair conditioners, biomedic products and it is also useful in the beneficiation of animal feed. Farmers with livestock are a part major industry, which produces animals that have a multitude of uses, for meat, fibres and hides. To ensure growth and healthy livestock it is important to feed the stock animal a proper balanced diet. Naturally animals like herbivores depends on vegetable matter for their protein needs, but the plants that contain high level protein are mostly in need for human consumption and expensive for animal stock. This has led to an on-going investigation of new technologies to convert low biological waste resources, keratinous biomass, into high quality feed raw material (keratin to protein product).

To enhance the digestibility of keratin an economically feasible process is required to convert protein-rich materials to form corresponding amino acids and small peptides bonds which are very soluble in water. Water soluble proteins are required products for animal feed.

The aim of this project is to investigate the source of biomass which contains high content of keratin useful for the beneficiation into animal feed, where there is least research being done. Our focus will be on the analyzing and characterizing different keratin from different biomass sources, for animal feed source.

Sustainable Bioenergy and Processes 2021
13-15th December 2021
Cape Town International Convention Centre
Hybrid

Characterization and Analysis of Keratinous Material for Animal Feed Production

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Abstract

Keratin is known to be one of the most abundant proteins which can be derived from wool, feathers, nails, hair, and other sources. A large number of keratinous by-products are mostly disposed and others are found in landfills. The disposal methods cause environmental pollution like air, water, and soil pollution. There are different degradation methods of the keratinous by-product to industrial processing for different applications.

Our focus is on the research done for the degradation of the keratinous material into animal feeds using enzymatic hydrolysis, which is known to be of biotechnological interest due to the quality and quantity of the hydrolysate formed. The quality and quantity of the hydrolysate are determined by using a combination of analytical techniques, where the characterization is done via proximate and ultimate analysis. And to focus on the importance of using different characterization techniques and the analysis of the enzymatic hydrolysate from the different microorganisms to determine the quality and the quantity of the animal feed.

The hydrolysate formed from the enzymatic hydrolysis is known to contain a mixture of amino acids and peptides. These peptides and essential amino acids formed are known to play a special role in some of the biological activities.

Different fungal and bacterial strains were tested for the degradation of chicken feathers for the beneficiation of animal feed. We used Fourier Transform Infrared Spectroscopy (FTIR), Thermogravimetric Analysis (TGA), CHNS Analysis, and Bradford assay for the characterization of the enzymatic hydrolysate formed.