Hydrolysis of Chicken Feathers by Microbial Keratinase for Production of Value-added Compounds

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Hydrolysis of chicken feathers by microbial keratinase for production of value-added compounds

A thesis submitted in partial fulfillment of the requirements for the Degree of

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In

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In the name of Allah, the Most Gracious, Most Merciful

Dedicated To

My Late Father

My Beloved Mother

My Brothers

&

My Loving Husband

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Marium Saba

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List of Acronym/abbreviations

^{o}C	Degree Celsius
μ	Micro
α	Alpha
β	Beta
FRAP	Ferric reducing ability of plasma
SEM	Scanning electron microscope
TEM	Transmission electron microscope
FTIR	Fourier transform infrared spectroscopy
DLS	Dynamic light scattering
XRD	X-ray powder diffraction
U.V	Ultra violet
OD	Optical density
nm	Nanometer
rpm	Rotation per minute
rRNA	ribosomal Ribose Nucleic Acid
RT	Retention Time
U	Unit
SDS	Sodium Dodecyl Sulphate
%	Percentage

Abstract

This study was aimed to isolate and screen efficient keratinolytic bacterial strains for bioconversion of chicken feathers waste into value-added products. Based on screening results C1M isolate was the best to hydrolyze feathers keratin and was identified as *Pseudomonas aeruginosa.*-C1M. By conventional optimization, chicken feathers (carbon source), urea (nitrogen source), sodium sulfite (sulfur source), and zinc chloride (salts) were significant media components, while 37°C temperature and pH 8 were significant physiological factors for the enhanced keratinase production. Further optimization was carried out through Plackett-Burman Design, chicken feathers were found to be the most substantial nutritional factor and specific activity of keratinase was found as 14.64 U/mg. Feathers keratin was converted into hydrolysate through keratinase of *Pseudomonas aeruginosa* C1M, and bioactive peptides having antioxidant properties were obtained.

Keratinase was purified from the fermentation media through ammonium sulfate precipitation followed by column chromatography. After partial purification of keratinase from *Pseudomonas aeruginosa* C1M, specific activity was found 25.06 U/mg on 60% ammonium sulfate saturation. However, after purification from gel filtration chromatography 74U/mg specific activity was obtained. Keratinase characterization revealed its stability at broad range of temperature (30-50°C) and pH (6-9). Besides feather keratin, the enzyme was active upon the soluble proteins keratin azure, casein, and bovine serum albumin (BSA); however, no conspicuous activity was observed in the presence of insoluble hair. Keratinase displayed high compatibility and stability with detergents by efficiently removing the proteinaceous stains from fabric pieces and exhibited activity improvements 40-73% compared with commercial detergents.

Crude keratinase from *Pseudomonas aeruginosa*-C1M was used for the biogenic synthesis of zero-valent silver nanoparticles (AgNPs). These particles appeared transparent to dark brown with single, distinctive peak of UV-Visible spectroscopy was observed at ~450nm. FTIR inference proved that silver nanoparticles were capped with proteins (crude keratinase). X-ray diffraction indicated that biogenic synthesized AgNPs were crystalline in nature and lattice planes were face-centered and cubic. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) indicated that

silver nanoparticles (AgNPs) were found as mono-dispersed spheres of variable sizes. Likewise, Dynamic light scattering (DLS) analysis showed that the diameter of AgNPs was ~ 119 nm. A high negative zeta potential value supports good colloidal properties, long-term stability, and high dispersity of the particles. Green synthesized silver nanoparticles exhibited antibacterial activity against pathogenic strains of *Staphylococcus aureus* and *Escherichia coli* with zone of inhibition of 22 and 25mm, respectively. The synthesized zero-valent silver nanoparticles (AgNPs) aided in the decontamination of azo-dyes in the presence of the reductant sodium borohydride ($NaBH_4$) and reduced azo-dyes (methyl orange, methyl red) to a less hazardous form. Similarly, bioremediation of other dyes (Safranin O, and methyl violet) by silver nanoparticles was carried out through light catalyzed processes.

Keratin hydrolysate produced as a result of keratinase from *Pseudomonas aeruginosa*-C1M was used in different biotechnological applications such as biogas and biofertilizers. Hydrolysate consists of polypeptides and amino acids and therefore has the ability to be used as precursor for biogas and biofertilizers production. For the biogas production, chicken feathers, rice husk and green grocery waste served as substrates in batch and continuous reactors. Pretreatment of chicken feathers through microbial keratinolytic *Pseudomonas aeruginosa*-C1M increased biogas yield. The pretreatment of rice husk RH significantly increased the biogas yield by 23.8%. The co-digestion process also addressed the enhancement and stability of anaerobic digestion as biogas production. The highest biogas yield was obtained in a batch reactor with co-digestion of pretreated rice husk and microbial pretreated chicken feathers (333.6 N ml/g VS) and it significantly increased 34% from calculated value (249Nml/g VS). Co-digestion of chicken feathers hydrolysate with green-grocery waste in continuous fermentation mode has also enhanced the biogas yield compared to the mono-digestion (Chicken feather hydrolysate and green-grocery waste) and therefore improves the efficiency of the overall process.

Similarly, keratin hydrolysate potential was also evaluated on the growth improvement of spinach plant as a bio-fertilizer. Beside hydrolysate reactor digestate, a byproduct obtained after biogas production from hydrolysate and grocery waste, was also used as biofertilizers. A greenhouse study was carried out to examine the comparative efficacy of

reactor digestate and feather hydrolysate on the growth of spinach plants. The application of keratin hydrolysate-based fertilizers observed a remarkable increase in the agronomic parameters. Plants treated with dry hydrolysate indicated a 50% increase in leaf breadth as compared to control plant provided with normal fertilizer. Plant height, number of leaves per plant, root length, chlorophyll content, protein content, biomass, and nutrient content in the spinach plant were also considerably enhanced with keratin hydrolysate-based fertilizers. However, results were more promising with applying *Pseudomonas aeruginosa*-C1M derived hydrolysate, indicating its application as a potential bio-stimulant in the agroindustry.

This study concluded that chicken feathers waste was efficiently converted into keratin hydrolysate with the help of the keratinase of *Pseudomonas aeruginosa*-C1M. The potential of keratinase was exploited for bioactive peptide extraction and as a bio-additive in detergent formulations. Moreover, keratinase was used for the biogenic synthesis of silver nanoparticles which served as antibacterial agents and in dye degradation studies. The prospect of keratin hydrolysate was used for biogas generation and bio-stimulant for plant growth.

Chapter 1: Introduction

1.1.Introduction and Background of the study

In the modern era, it is essential to mitigate the anthropological stress on the ecosystem and reduce the risk associated with continuous environmental crisis. Many governmental and non-governmental organizations suggested plans and strategies to encourage constructive changes and execute novel concepts, technologies, and models for future economies. The most aspiring and comprehensive model articulated to date is Sustainable Development (SD) (–Six Transformations to achieve sustainable Development). The United Nations (UN) preliminary plan was also based on sustainable development goals to design the strategies and actions that can serve as a linker between environmental protection problems and socio-economic growth. Ecological stability can be achieved by massive transformations that include reducing greenhouse gas emissions, protecting biodiversity and natural habitats, access to fresh food and clean water for all living organisms, de-carbonizing the energy production system, and combating land, air, and water pollution.

The food industry significantly enhances environmental pollution. The food industry is not only related to producing meat, dairy, and other food items but also produces considerable waste. Similarly, the harmful impact of the food industry on the environment is also through releasing toxic gases, eutrophication of water reservoirs, and damaging land through waste generation. Poultry industry is directly linked with the major food industry constituents. In the same way, poultry industry is growing in Pakistan. According to Pakistan production of poultry meat statistics, poultry meat production reaches to 1.66 million tons in 2020 and increasing annually at the rate of 10.72%. Thus, alternatively increasing an abundant volume of waste in the form of poultry excreta, feed that is spilled and majorly feathers. The poultry industry is an essential and diversified constituent of food. Chicken is the primary food source; its daily consumption is increasing annually as one of the inexpensive and healthiest protein sources. However, along with its valuable aspects, this sector generates considerable

waste (Sypka, Jodłowska, & Białkowska, 2021). The most critical waste in a poultry processing plant is feathers.

Previously, it has reported that every year approximately 24 billion chickens are slaughtered worldwide (Fellahi, 2014) and thus producing feathers which are roughly 125 grams of a chicken. The feather waste consists of 8% of the live weight of chicken (Jayathilakan, Sultana, Radhakrishna, & Bawa, 2012). Incineration, landfilling, and composting are a few strategies adopted by several countries to dispose of feathers. These approaches reduce waste, but no valuable products are obtained, and potential revenues are also lost (Kornilłowicz-Kowalska & Bohacz, 2011b). As plants' byproducts and agro-industrial waste are used in several industries, less consideration is given to animal waste. Therefore, a significant aesthetic and catastrophic health problem arises due to the non-utilization of animal byproducts, and a colossal asset is being economically lost.

Feathers consist of keratin —hard to degrade. Keratin is an unsolvable recalcitrant protein, most abundant in poultry feathers. The arrangement and composition of amino acids guarantee their structural rigidity(Kornilłowicz-Kowalska & Bohacz, 2011b). Primarily keratin exists in two forms α-keratin and β-Keratin (Mokrejs, Svoboda, Hrncirik, Janacova, & Vasek, 2011). However, feathers keratin consists of 41–67 % α-keratins, 33–38 % β-keratin, and amorphous keratin (McKittrick et al., 2012). The composition of amino acids in the keratin of chicken feathers varies depending on age and breed. Major amino acids present are glycine, cysteine, proline and threonine (I. Sharma & Kango, 2021). As mentioned earlier, keratin is being degraded chemically, which is not an eco-friendly and cost-effective method. Meanwhile, chemical and physical treatments have worst effect on nutritional properties of amino acids.

Traditional methods of feathers waste management include landfilling, incineration, and disposal in sewage lines. Landfilling or dumping of feathers is responsible for the growth of pathogenic microorganisms, which are agents for the transmission of diseases. Moreover, after dumping, the soil would not be available for the cultivation of crops and farming. Thus a productive land may become useless. During the dry season, the landfilled area may become the habitat of several birds, which is ultimately a reason for bird waste excreta production, which can cause many infections. In highly populated

areas burying feathers may cause congestion leading to other health-related issues. In a case study, it has been noticed that many humans were infected with mycoplasma and the underlying cause was poor disposal of feathers (Fasina, Rivas, Bisschop, Stegeman, & Hernandez, 2011).

Discarding feathers in sewer lines also has disadvantages. During the rainy season, flooding may cause blockage of sewer lines which can lead to the spread of diseases like cholera and dysentery. Meanwhile, the burning of feathers is another reason for air pollution. Feathers contain a disulfide bond; upon incineration, it releases sulfur dioxide and carbon mono-oxide into the air. Therefore, the global poultry industry is looking for ways to feathers disposal in an eco-friendly manner.

Chemical degradation of feather methods includes acid, alkali, and hydrothermal hydrolysis. While treating with acid and alkali strong disulfide bond of keratin is broken, and protein becomes soluble. The result of this treatment is not only toxic gases emission into the environment, but also nutritional properties of keratin may also have deteriorated. Chemical hydrolysis of keratin under high pressure and temperature also reduces nutritive properties. During redox hydrolysis, chemicals like cyanide, bromine, and sodium sulfide are toxic to the environment and cause severe problems.

Therefore, proper feather management procedures are essential to minimize potential environmental issues and the depletion of a potential protein supply (peptide source). Enzyme hydrolysis of feathers keratin is an environmentally friendly and less expensive method. Microbial enzyme potential for keratin hydrolysis is being explored, which is considered eco-friendly. Initially, Goodrich and lee used keratinase for the hydrolysis of feathers keratin(Mwakazi Eva Dama, 2020).

The diversity of keratinolytic bacteria is significantly high, *Bacillus and Streptomyces* are the most critical genera in this regard (Jeevana Lakshmi, Kumari Chitturi, & Lakshmi, 2013). Many fungal species also possess keratinolytic activity for the conversion of feathers biomass. Among them included are *Chrysosporium keratinophilum, Doratomyces microsporus*, *Engyodontium album* (*Tritirachium album*), *Onygena Corvina,* and *Paecilomyces marquandii* (Lange, Huang, & Busk, 2016a)). Microbial

keratinases efficiently cleave the disulfide bond between keratin monomers to produce small peptides and amino acids that can serve as value-added products (Verma et al., 2017).

Based on their mode of action, keratinases are classified into endo-keratinase and exo-keratinases. However, on the basis of their biochemical properties keratinases lie into metalo and serine peptidase class (Zaraî Jaouadi et al., 2015). Based on keratinase activity, keratinolytic microorganisms are also classified into potent keratinolytic and true keratinolytic microorganisms. Potent keratinolytic microorganisms have the ability to degrade non-kerataniceous constituents along with hard keratin. However, true keratinolytic microbes only hydrolyze the keratin substrate (Jagadeesan, Meenakshisundaram, Saravanan, & Balaiah, 2020).

For obtaining better keratin hydrolysate, keratinase was required. Therefore, the natural sites containing indigenous flora of keratinolytic microorganisms were explored. Chicken feathers dumping site and leather tannery sites were mainly considered as isolation sites due to the presence of keratin raw substrate. After isolation of keratinolytic microorganisms‘ potent strain was selected and identified.

In this study, keratinase from *Pseudomonas aeruginosa*-C1M was used for keratin hydrolysate production and effect of keratinase and keratin hydrolysate was explored in various applications. Keratinase production was enhanced by optimizing culture conditions. In this study, the optimization of cultural conditions for increased keratinase synthesis was conducted using both the one-factor-at-a-time and multifactor optimization approaches. Using the Plackett-Burman design, the influence and contribution of independent process factors were investigated. Keratinases and keratin hydrolysate obtained from feathers are employed in several biotechnological applications.

After enhanced production of keratinase, the aim was to assess the keratinolytic properties of purified enzyme. Several parameters of keratinase activity were characterized by using keratin waste as substrate source. Keratinase can be purified, and its potential in multiple industries like detergents, cosmetics and pharmaceuticals can also be assessed (Lateef, Adelere, Gueguim-Kana, Asafa, & Beukes, 2015).

A number of keratinases were purified from various microorganisms for their potential use in detergent formulations. However, in this study the potential of keratinase was considered for laundry application.

Presently, nanotechnology is the fast growing trend in the field of science and technology. To better apply antimicrobial and antioxidant agents, nanoparticles were synthesized using various plants and microbial by-products. In this study, the redox potential of crude keratinase of *Pseudomonas aeruginosa*-C1M was used for the biosynthesis of silver nanoparticles. After characterization, their prospect was employed for antimicrobial and antioxidant activities.

By involving chemical treatments, keratin hydrolysate was used to extract amino acids, peptides, and antioxidants. These bioactive peptides, such as amino acids and antioxidants, were incorporated in broiler and fish feed (Vijay Kumar, Srijana, Chaitanya, Harish Kumar Reddy, & Reddy, 2011) However, this study used enzymatically produced keratin hydrolysate to extract bioactive peptides named keratin microparticles. These microparticles were characterized, and their potential was exploited as antimicrobial and antioxidant agents.

Keratin hydrolysis yields a rich supply of polypeptides and amino acids as keratin hydrolysate. As feathers contain around 15% nitrogen, keratin hydrolysate is utilized to manufacture fertilizers. Therefore, feathers were used as slow-releasing nitrogen fertilizers in greenhouse and nursery businesses. However, the potential of keratin hydrolysate in combination with other wastes as fertilizers was explored in this study.

As energy is the biggest issue of this era, one attempt can be made to produce bio-gas by utilizing feathers as a substrate to meet all energy crises. Researchers have also found applications in the feed and fuel industry (Rai and Mukherjee, 2011). Previously, by utilizing keratin, hydrolysate biogas was produced. Nevertheless, the co-digestion and other raw substrates improved the quality and quantity of bio-methane. Rice husk, green grocery waste, and chicken feathers were incorporated into the anaerobic digester to increase biogas yield.

The aim of this project is to convert the chicken feathers keratin into keratin hydrolysate through eco-friendly and less expensive approach. In this study, keratinase producing microbes were isolated and screened. Best keratinolytic strain was identified through

biochemical and 16srRNA molecular technique. Several physical and nutritional parameters essential for the production of keratinase of *Pseudomonas aeruginosa*-C1M were optimized. After optimizing culture conditions, keratinase was purified and its physical and chemical properties were evaluated. Furthermore, keratinase application was evaluated as bio-additive in laundry detergent formulations. Meanwhile, keratinase was utilized for the environmentally friendly production of silver nanoparticles, due to its redox properties. Silver nanoparticles were characterized through several techniques. Potential of AgNPs were explored for antimicrobial and dye-degrading applications. By the help of *Pseudomonas aeruginosa*-C1M, keratin from chicken feather waste was converted into keratin hydrolysate. Hydrolysate contains bioactive molecules, amino acids, peptides having anti-oxidant potential.

Chicken feathers hydrolysate along with rice husk and green grocery waste was used for the generation of bioenergy (biogas). The digestate, effluent from biogas digester and keratin hydrolysate was used as biofertilizers. Potential of biofertilizers was evaluated on spinach plant. Effect of feathers based fertilizers on the growth and properties of spinach plant was assessed.

1.2. Significance of the study

The poultry business in Pakistan is expanding so rapidly that it creates millions of tons of trash. Waste management is the greatest challenge. Every day, chicken feathers are either burnt or dumped. Despite the fact that waste control is practiced, these processes cannot provide useful goods. Not only is improper waste management responsible for the emission of several pollutants and odorous gases into the environment, but it also raises energy consumption and operational costs (Sahoo et al., 2012). Therefore, an efficient system must be developed to remove recalcitrant chicken feathers waste and convert them into valuable products economically. Bioconversion of waste to valuable goods is a novel process, and it could not only manage waste but also be beneficial in several industries.

Instability at high temperatures, limited specificity, high cost, and less production is the most common reasons which limit microbial keratinase from being used on an industrial

scale. If these issues are managed by cultural conditions optimization large-scale production and industrial utilization could be possible, which would be a green alternative to conventional chemical methods. Moreover, the product of keratin hydrolysis has more nutritional value. Utilization of remarkable properties of enzyme provides the effective exploitation of keratin waste into food, feed and fertilizers.

1.3.Aims and Objectives of the study

Aim of the study was "To enhance the keratin hydrolysate bio-production from chicken feathers by the help of microbial keratinase for obtaining value-added compounds".

Objectives were as follow:

1. Isolation, screening of keratinolytic microorganisms and optimization of culture conditions for the enhanced production of microbial keratinase.
2. Purification and characterization of microbial keratinase.
3. Utilization of microbial keratinase for the production of value-added compounds.
4. Exploitation of feather hydrolysate potential in biogas production.
5. Keratin hydrolysate utilization for biofertilizers production.

1.4.References

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Chapter 2: Literature Review

2.1. Facts and figures about Poultry industry

The poultry processing industry has boomed in response to rising demand for chicken products, leading to widespread dumping of feathers as a keratin solid waste (Brandelli et al., 2010). As per projections from the United States Department of Agriculture (USDA), the global production is forecast 2 percent higher in 2023 to a record 102.7 million tons., resulting in the production of about 5 million tons of chicken feathers, and this rate is expected to increase by two percent every year (Qiu et al., 2020).

2.2. Poultry industry waste threats to the environment

The persistence of feathers poses a serious challenge to those responsible for getting rid of chicken feathers (Brandelli et al., 2015). In addition, if this waste is dumped at an unsafe rate, it might lead to an outbreak of waterborne diseases caused by bacteria or viruses (Tamreihao et al., 2017). Nitrates seep into the earth, while phosphorus washes away into the nearest body of water. Industry has been leading the charge to ensure keratinous waste is properly disposed of in recent years due to increased management costs and public awareness of health and environmental hazards.

2.3. Feathers degradation methods

Keratin protein is useful for several biotechnological industries because it includes essential minerals, amino acids, and peptides that are not found in synthetic forms (Verma et al., 2016). Considering keratin's indigestibility and its slow natural breakdown, proper pre-treatment and hydrolysis are required before its widespread industrial application can be achieved. The physical and chemical processes currently used to valorize keratinous waste are not only inefficient, but also harmful to the environment and cause the loss of vital biological resources (Gupta et al., 2013; Kumari and Kumar, 2020). High heat and pressure can denature or even destroy certain essential amino acids (Papadopoulos, 1985). When heated in a pressured oven, feathers may be ground into a fine powder (Jaouadi et al., 2013). Milled feathers have been proposed as a soil

conditioner and a supplementary feed for ruminants (Hadas and Kautsky, 1994). Hydrolysis of the protein keratin, which is famously difficult to digest, has been confirmed by a number of microorganisms under mild conditions (Gupta et al., 2013; Kumari and Kumar, 2020; Tamreihao et al., 2017; Verma et al., 2016), suggesting that this might be a feasible alternate for the long-term disposal of chicken waste. Hydrolysate from chicken feather waste is discussed in this study, along with its prospective uses as an organic fertilizer in agriculture, as well as the numerous methods for hydrolyzing feathers, the biological treatment of chicken feather waste, and its subsequent value addition.

2.4. Structure of chicken feathers

Chicken's feather is one of the most complex structures in the animal kingdom (Bansal & Singh, 2016), as it is used for not just flight but also to control body temperature. The rachis, or central shaft, of a feather is typically 17.78 cm in length α-helix conformations with occasional β-sheet structures, the barbs are typically 2.5 cm long, and the barbules are typically between 0.3 and 0.5 mm in length (Fig.2.1). In a research (Ramakrishna Reddy, Sathi Reddy, Ranjita Chouhan, Bee, & Reddy, 2017a; K. Saravanan & Dhurai, 2012) it has established that barbs and barbules are largely composed of α--helix conformations with occasional β-sheet structures. Seventy-eight percent of the rachis was discovered to be in a β-sheet structure, whereas just 18 percent was in a α-helical form (Schor & Krimm, 1961). Feathers have a high concentration of volatile solids, with over 90% being crude protein and 80% of that being insoluble, tough, and chemically unreactive keratin (J. C. Costa, Barbosa, & Sousa, 2012; Yanuartono et al., 2018). Some feathers include ash (0.69%) and fat (2.79%). Despite its low molecular weight (approximately 10 kDa), the α-helix (41-67%) and β-sheet (33-38%) forms of keratin afford it exceptional strength and toughness (N. Z. Jaouadi et al., 2013). The creation of tetramers and octamers, bound together by disulfide bonds, inter- or intra-molecular hydrogen bonding, and hydrophobic interactions of polypeptides, is the product of the supercoiling of keratin micro- and macro-filaments (Brandelli, 2008; J. C. Costa et al., 2012; Schor & Krimm, 1961; Yanuartono et al., 2018). The high concentration of

disulfide bonds in keratin, which may reach as high as 22% in hard keratin (Schor & Krimm, 1961), is responsible for its mechanical stability, insoluble in water, acids, solvents, and alkaline solutions, as well as resistant to the action of the majority of proteases (Yanuartono et al., 2018).

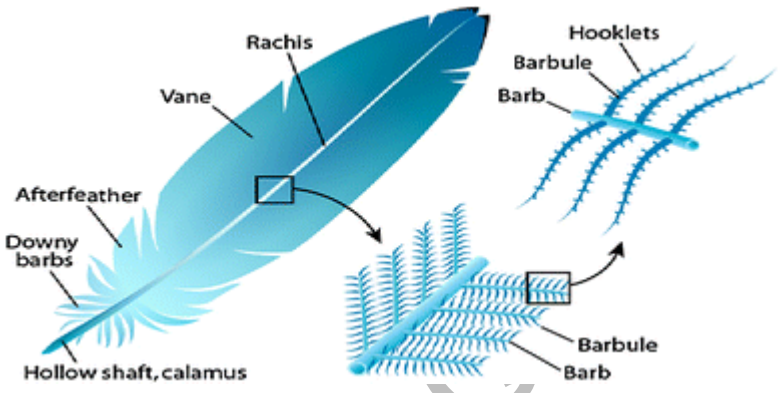

Fig 2.1: Structure of chicken feather (Tesfaye et al., 2017)

2.4.1. Amino acid content of feathers

The amino acid content in chicken feathers varies with factors such as the bird's nutrition, age, and breed (Martinez-Hernandez et al., 2005). It has been shown that feathers contain a high concentration of non-essential amino acids. It has been revealed that chicken feathers comprise beneficial macronutrients and micronutrients such as calcium, phosphorus, magnesium, iron, potassium, zinc, sodium, manganese and copper (Nurdiawati et al., 2018; Staroń et al., 2017), in addition to carbon, nitrogen, sulphur, and hydrogen.

2.4.2. Rigidity of keratin protein structure

Keratin is a structural polypeptide that is fibrous and resistant to breakdown by ordinary proteolytic mechanisms. Keratin is the third most abundant polypeptide in nature after chitin and cellulose (Ghaffar et al., 2018). Keratin's recalcitrance to degradation has been blamed on the orientation and interactions of its bonds, which make it very difficult to break down (Brandelli et al., 2010). Keratin serves a variety of physiological purposes,

including mechanical support and functional protection (Gupta and Ramnani, 2006). Mechanical stability is bestowed upon the resulting structure by the close packing into a supercoiled strand of polymer chains of α-helix or β-sheet motifs (Onifade et al., 1998). Also, the sulphur group concentration, location, and role of the polypeptide contribute to classifying keratin as either hard or soft (Su et al., 2019). As a result of their structural and defensive responsibilities against predators and abiotic elements, keratin in feathers, nails, horns, and hooves are considered tough (Yang et al., 2016). On the other hand, less disulfide bonds are present in the soft keratins of callus and skin thus allowing the elasticity of their structures (R. Tatineni et al., 2008). Agro-industrial keratinous waste from poultry and leather industry including feathers and hair, respectively that aim to fulfill the need of the increasing human population are examples a nuisance to ecosystems (Kalaikumari et al., 2019). Traditional proteases like pepsin, papain, and trypsin have a hard time breaking down these wastes (Thankaswamy et al., 2018).

Strong cross-linking of disulfide bonds, H-bonds, and hydrophobic interactions, present in the crude protein content of keratinous materials, improve conformational stability and limit protein hydrolysis (S Sangali and Brandelli, 2000). As a result, the wastes remain to be ecological issues that may threaten the wellbeing of the general population (Moridshahi et al., 2020). Historically, domestic animal diets would benefit from the addition of keratinous wastes and other animal corpses that had been roasted until they were crushed into a consistent powder The risk of prion disease, however, has prevented the widespread usage of animal and poultry waste recovering in the animal farming industry (Tsiroulnikov et al., 2004).

2.4.3. Types and sources of keratin

Keratin is the most ample fibrous protein in epithelial tissues, which are used for structural support (Toivola et al., 2015). Keratin is a kind of protein that exists in and its alpha (α) and beta (β) forms and may be distinguished by their unique structural assemblage, which has a diameter of 7 nm and a diameter of 3 nm, respectively, and is embedded in an amorphous matrix (Fig. 2.3 A and B) (B. Wang et al., 2016). Feathers, hair, horns, nails, scales, hooves, and wool are all made of keratin, which is also found in

the epidermis and appendages. Different synthetic routes are mirrored in the architectures of the α- and β-forms. However, the localized production of both types of keratins by keratinocytes requires coordinated and controlled sequential procedures involving sets of gene activation and restriction processes (Akram et al., 2020). A total of 54 conserved genes in vertebrates code for keratins, which are controlled in pairs depending on the tissue type (F. Wang et al., 2016).

2.4.4. Genetics of keratin protein

According to their evolutionary and functional similarity, the genes might be classified into several classes. Evolutionary molecular analysis of keratin encoding genes and their relationship to the other members hint at a scenario in which they emerged as a result of ancestral gene duplication (F. Wang et al., 2016). Keratin production takes place in specialized cells called keratinocytes, and the chemical steps involved are carefully orchestrated by mRNA (mRNA). The manufacture of the keratin proteins occurs in tandem with mitotic cell division, which initiates keratin creation. When keratinocytes reach maturity, they undergo a series of events that culminate in the stability of keratin and eventual cell death (B. Wang et al., 2016). There are a variety of cellular and non-cellular roles that keratins play in epithelial tissues.

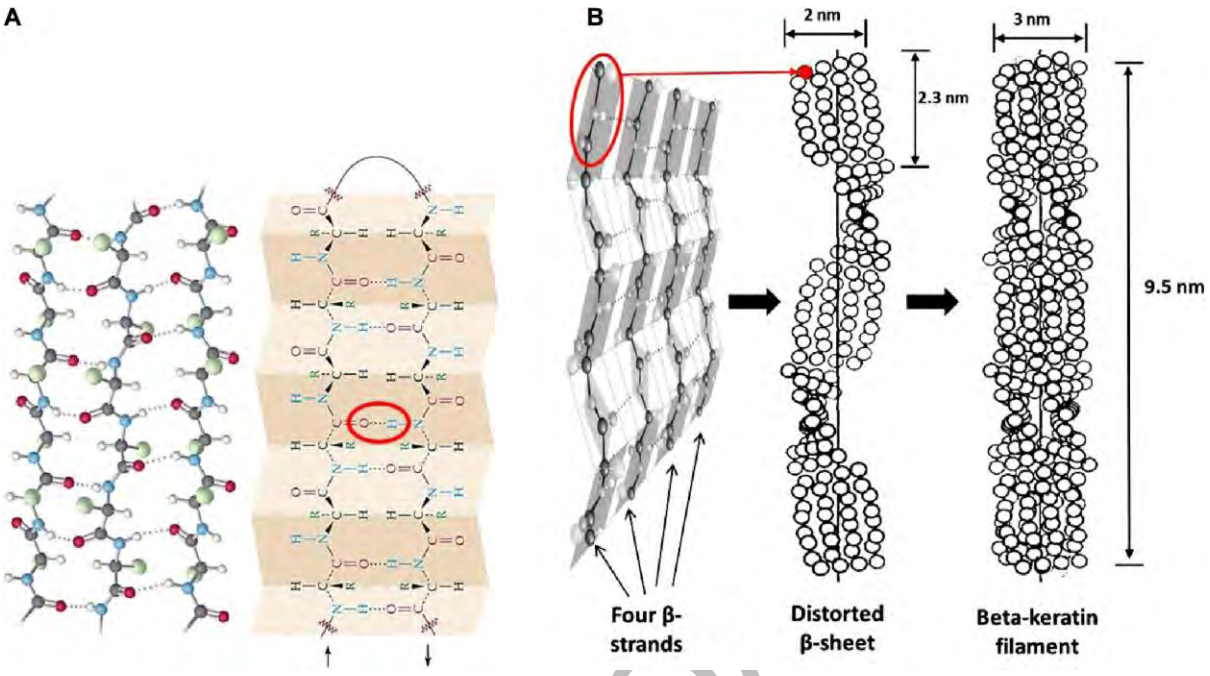

Fig. 2. 1. Ball-and-stick model (A) and schematic (B) representations of the structural components of a β-keratin filament; four lateral β-strands are formed by the folding of polypeptide chains at the center location.

2.4.5. Chemistry of keratin protein

These features, however, are bestowed through posttranslational modifications such as phosphorylation and glycosylation (Lange et al., 2016), and also by several keratin-linked proteins (Bragulla and Homberger, 2009). Similar to other proteins, keratins have a three-part structure consisting of a core -helical rod domain, an amino-terminal head domain, and a carboxy-terminal tail domain (Nafeey et al., 2016). In this capacity, proteins are distinguished by their unusual adaptability, which allows them to act as a mechanical cushion protecting cells from environmental perturbations (Feroz et al., 2020; Leitner et al., 2012). Keratin might also be generated by the assembly of antiparallel coils of coiled heterodimers, each of which is composed of an acidic and a basic component. With a diameter of 10 nm, a plane surface, and a polar properties, and roughly made up of a cross-section of 16 coiled-coil dimers, the filament is formed when two dimers stagger side by side to create a tetramer (F. Wang et al., 2016). Figures 2.4 A and B show how the molecular architecture and protein production of filaments differ between α- and β-keratins (Jones and Thornton, 1997).

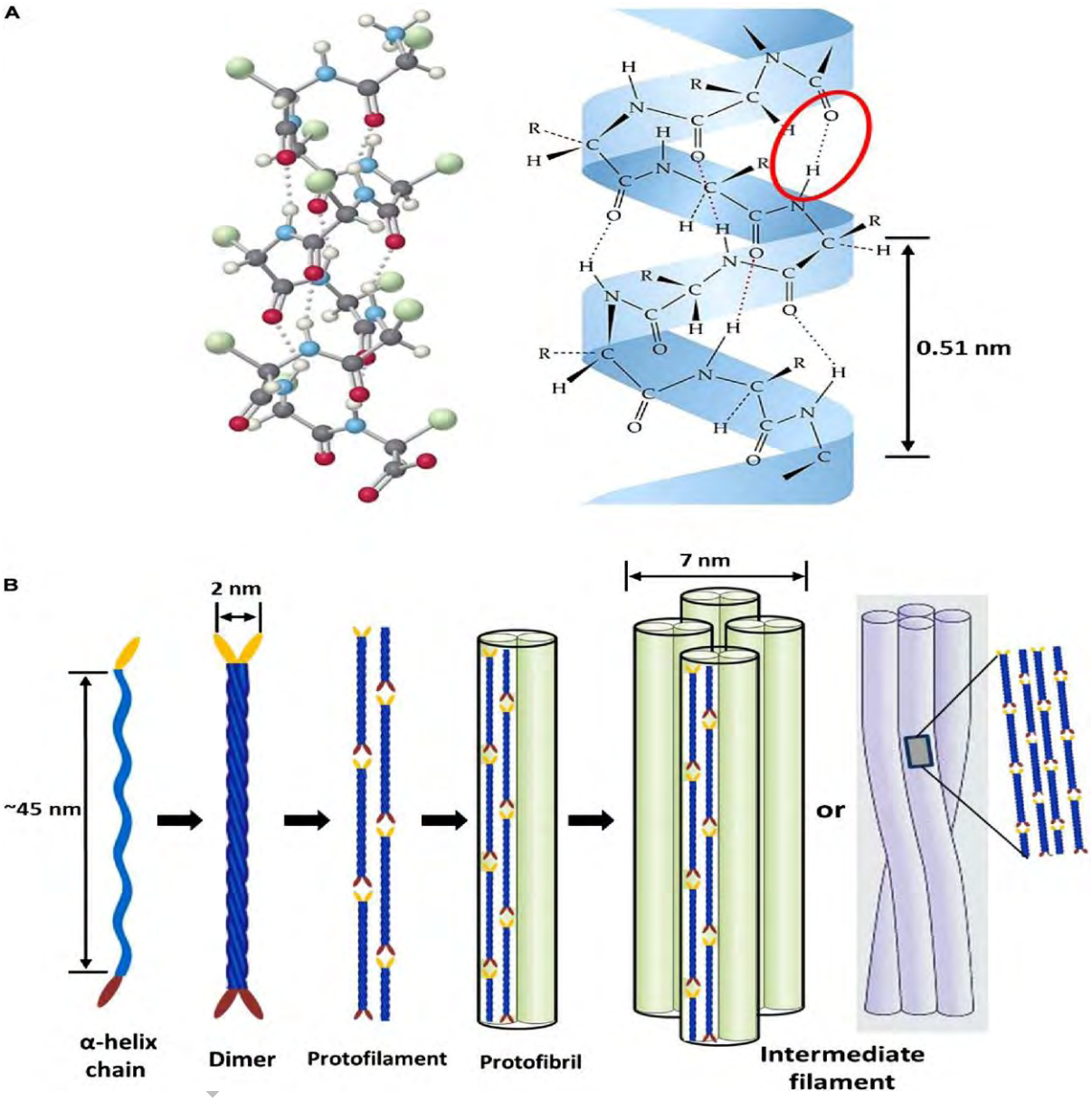

Fig. 2. 2. The a-keratin intermediate filament structure: (A) a ball-and-stick model of a typical protein chain in helical configuration, stabilized by many forces; however, the densely populated intra-molecular hydrogen bonds (red oval shape) primarily determine the helicity of the shape with a 0.5 nm pitch; (B) a schematic depiction of intermediate filament formation processes. Diameter 2 nm left-handed dimeric-coils are formed when two a-helices (45 nm long) bind together; the disulfide composition of these moieties initiates end-to-end aggregation and side-by-side staggering to form a proto-filament; two proto-filaments combine laterally to form a proto-fibril; and finally, helical intermediate filaments are formed when four proto-fibrils (Nnolim et al., 2020; B. Wang et al., 2016).

2.5. Ecological implications of keratin waste

Agro-industries are feeling the heat because in order of the exponential growth of the world's population, as they must increase output to keep up with the soaring demand of the world's burgeoning populace. Because of this, keratinous wastes from places like chicken farms, the leather industry, and slaughterhouses have expanded in quantity (Srivastava et al., 2020). It is crucial that the by-products be efficiently managed, both in terms of recycling into goods with additional value and effective disposal of the trash that cannot be reused. When the most effective methods for mitigating garbage's negative impacts on the environment are used, the price tag for disposing of the waste may go up quickly (Jaouadi et al., 2013). Environmental contamination is the result of keratinous wastes being dispersed across the environment due to a lack of waste management regulatory procedures and the non-adherence to the regulatory norm, when it exists (El-Ghonemy and Ali, 2021; Verma et al., 2017). Examples include the large amounts of hair that are discarded as waste during the leather manufacturing process and either landfilled or composted (Thankaswamy et al., 2018), despite the fact that they have a high propensity to emit noxious gases that contribute to air pollution as a result of the chemical contents and microbial activity on the nitrogen-rich biomass, they are an important source of nitrogen (Akhter et al., 2020; Moktadir and Rahman, 2022). Furthermore, leachate has the potential to pollute soil and groundwater, which poses serious health and environmental risks to the surrounding people (Ammasi et al., 2020). Equally dangerous to aquatic life is the practise of releasing badly treated or untreated wastewater with a high amount of hair particles into water bodies, which has a strong propensity toward eutrophication. Keratinaceous waste is also being subjected to degradation through microbial keratinases.

2.5.1. Potential role of keratinases for elimination of keratin waste

The bio-geo-chemical processes in the environment rely greatly on the multitude of features that bacteria exhibit. The diversity of the genome and the complexity of microbial expression systems provide the maintenance of homeostasis under harsh conditions, the utilization of all known macro- and micro-materials and elements as

energy sources and/or cellular building tools. Producing keratinases is an inducible process that occurs only in the presence of keratinous biomass and the absence of readily available nutritional reference (Nnolim et al., 2020). Keratinase exudation provides bacteria with access to a wide variety of proteinaceous substrates, which in turn promotes organic matter recycling across a wide range of ecological settings (Bohacz and Korniłłowicz-Kowalska, 2019). When keratinase is produced, it breaks down keratinous biomass into accessible and utilizable units, which is a benefit to the environment since it increases the availability of nutrients (Bohacz et al., 2020). Soil fertility is greatly increased by biodegradation because of the steady but gradual release of organic nitrogenous substances (Nafady et al., 2018). Food chain sustenance, bio-geo-ecological equilibrium, and public and animal health security as pathogens lose habitat owing to breakdown of organic matter are all potential results of the bioconversion of keratinous biomass into usable units (Spindler et al., 2018). The bacteria that produce keratinase also help reduce global pollution by recycling refractory materials that include keratin (Mi et al., 2020).

2.6. Chicken feathers waste valorization methods

Disposal and processing of poorly digested feather protein have been documented using a variety of chemical and physical approaches (Nurdiawati et al., 2018; Papadopoulos, 1985). Figure 2.2 is a summary of the existing ways for getting rid of and treating keratinous waste. Keratinous waste may be disposed of by incineration, landfilling, burning, and composting. However, their broad usage is constrained by high operating costs, high energy consumption, and the waste of vital bio-resources (Korniłłowicz-Kowalska and Bohacz, 2011a). In this part, we will go through many methods for dealing with keratinous trash.

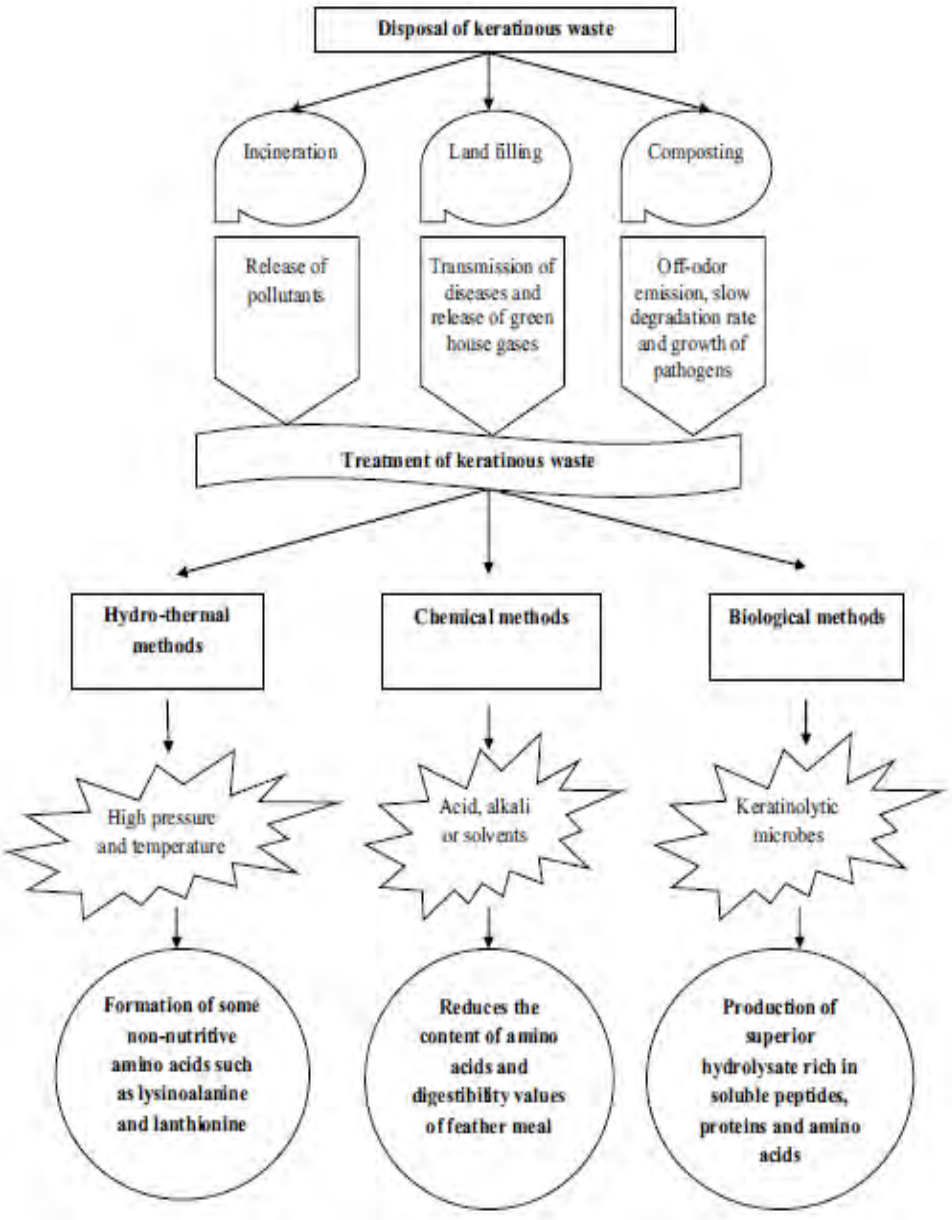

Fig. 2. 3. Waste management strategies for keratin-based materials (Korniłłowicz-Kowalska and Bohacz, 2011b)

2.6.1. Waste incineration and landfilling

Although incineration at high temperatures is one among the more traditional techniques for disposing of keratin waste, it is rarely used due to the pollution it causes (Gupta and Ramnani, 2006). Process conditions, air pollutants, and residue disposal must all be tightly regulated. The combustion of animal by-products is prohibited under European

Union Regulation (EC) No. 1774/2002. The inappropriate disposal of keratinous waste leads to landfill leachate and greenhouse gases (Dube et al., 2014), despite the fact that land dumps are the main ways of organized waste disposal in various regions across the world. Acidification of surface water can cause methemoglobinemia in babies and stomach cancer in adults (Camargo and Alonso, 2006). Leachate increases the quantity of nitrogen and phosphorus in nearby water bodies, leading to algae blooms, toxicity to aquatic species, and acidification. *Escherichia coli, Salmonella, Brucella abortus, Bacillus anthracis, Mycobacterium bovis, Erysipelothrix rhusiopathiae, Clostridium,* and other viral, bacterial, and parasite pathogens are all known to live in poultry wastes (Singh et al., 2016). Retroviruses were found in chicken feathers, according to a research by Davidson (Davidson, 2009). Pathogenicity of these microorganisms during landfilling can cause significant human and animal illnesses. Directive 1999/31/EC (Franke-Whittle and Insam, 2013). Hence, requires stringent monitoring of feather dumping to minimize virus, surface, and groundwater pollution.

2.6.2. Anaerobic digestion and composting of keratin waste

Aerobic composting with a succession of microorganisms is known to break down slaughterhouse waste (Franke-Whittle and Insam, 2013). Producing bioenergy by anaerobic digestion of keratinous waste has been documented (SHIH, 1993). When organic waste is composted, the byproduct may be utilized as a soil conditioner or fertilizer, making it a more sustainable waste management option (Yanuartono et al., 2018). The temperature achieved and the length of time the temperature is maintained to determine the microbial population that survives at the conclusion of composting. Pathogen development can be stymied by maintaining a closed composting field connected to a sewage transport system and performing routine microbiological testing in addition to diligent monitoring (Tronina and Bubel, 2008). Increased moisture sorption capacity and structural support are needed for composting high moisture, low fiber waste (Barone et al., 2006). The production of ammonia from the breakdown of proteins during anaerobic digestion hinders the development of methanogen bacteria and lowers the amount of biogas produced (Resch et al., 2011). Concerns related to product quality, cost

expertise, pollution, and environmental quality control are therefore associated with these operations (Kim and Patterson, 2000).

2.6.3. Steam pressure treatment of keratin waste

Intact feathers are treated with steam at 275-415 kPa with steady agitation for 30-60 min (El Boushy et al., 1990). Under stress, cystine and alanine are converted to lysinoalanine and lanthionine, respectively, which are not nutritional amino acids (Anitha and Palanivelu, 2013). It has been observed that the feather meal produced by high pressure and temperature treatment is low in a few amino acids, thus limiting the use of by-product for biotechnological purposes (Eggum, 1970; Papadopoulos, 1986).

2.6.4. Treatment of keratin waste with chemicals

Amino acids and soluble proteins for food and feed have been reported to be obtained by hydrolysis using alkaline, acidic, and organic solvents (Wolski, 1985). Peptide and disulfide bonds are broken by strong acids and alkalis, releasing amino acids and short-chain peptides, respectively (AL-Bahri et al., 2009). When comparing feather meals prepared with or without acid, it is known that acid hydrolysis reduces the quantity of all amino acids except cysteine, lysine, tyrosine, arginine, and tryptophan (Eggum, 1970). In reaction to sodium hydroxide treatment, amino acids react in widely varying ways. When feathers were hydrolyzed with 0.6% potassium hydroxide at 70 °C for 24 hrs., (Stiborova et al., 2016) found that 326.4 mg/l of free amino acids were produced, of which 12.8% were necessary. In a study conducted by (Gupta et al., 2012), the soluble protein content of chicken feathers was determined by hydrolysis at 30 °C, pH 10-13 for 6 hrs. using sodium sulfide, potassium cyanide, and thio-glycolic acid, yielding 53%, 29%, and 8%, respectively. When employing alkaline potassium per-sulfate to degrade feathers, (Yang and Reddy, 2013) found that the nitrogen in the feathers was released as ammonia steadily over the course of 10 days. According to research (Barone et al., 2006), formic acid vapors can dissolve feather protein by penetrating the fiber structure of feathers. Except for cysteine, methionine, lysine, and histidine, dimethyl sulfoxide was shown to enhance the concentration of all other amino acids in a study by Wolski (Wolski et al.,

1980). Chemical treatments have a number of drawbacks, including low biological value, poor digestion, and a lack of important amino acids including histidine, lysine, tryptophan and methionine.

2.6.5. Biological methods of keratin waste hydrolysis

Due to their ability to hydrolyze keratin completely under mild circumstances and produce exceptional hydrolysate rich in peptides and amino acids (Brandelli et al., 2015; Qiu et al., 2020; Schor and Krimm, 1961), keratinolytic microorganisms are attracting the attention of researchers interested in the remediation of feathers. In response to the presence of keratinous substrate, keratinolytic bacteria generate the enzymes disulfide reeducatse (EC.1.8.1.14) and keratinase (EC.3.4.99.11) (Anbu et al., 2008). Keratinolytic bacteria may be found in a wide variety of environmental samples, including those collected from chicken processing factories, feather dumping sites, and slaughterhouse/tannery effluents. They have the potential to be used in the treatment of keratinous waste since they have been shown to completely solubilize feathers in a short amount of time under neutral to alkaline conditions.

Most keratinolytic bacteria degrade keratin between 25 and 37°C (Kojima et al., 2006; Prakash et al., 2010), and fall in mesophilic range. Some keratinolytic microorganisms, however, have been shown to be active at temperatures above normal (Huang et al., 2013; Nam et al., 2002). Most effective microbes may degrade keratin after 2-5 days when stirred. Feathers may be solubilized by a small number of organisms, but only over lengthy periods of time (El-Naghy et al., 1998). It has been observed that it might take up to 30 days for *Pseudomonas microphilus* to hydrolyze feathers by up to 70% (Kani et al., 2012). The keratinolytic fungus has a significant role in keratin recycling and so play an essential ecological role. Their abundance and location appear to be influenced by the accessibility of keratin (Marchisio and others, 2000). Filamentous fungi are well-suited to degrading keratin because of their structure, which allows their mycelia to bind firmly to keratin substrates and their fungal hyphae to penetrate these substrates.

Even though the dermatophytes *Trichophyton* and *Microsporum* have been documented to have keratinolytic capability (Błyskal, 2009; Nurdiawati et al., 2018), the economic interest in these organisms has been low due to their propensity for causing disease. Hydrolysis of keratin waste has been documented in the non-dermatophytic fungus *Aspergillus, Trichoderma, Scopulariopsis, Doratomyces, Paecilomyces, Acremonium, Alternaria, Beauveria, Curvularia,* and *Penicillium* (Sharma et al., 2020). *Aspergillus flavus*, *A. fumigatus, A. niger, A. nidulans,* and *A. terreus* were used effectively for the degradation of chicken feathers, as described by Kim (Kim, 2003). In addition to chicken feathers, keratinolytic bacteria have been found to hydrolyze different types of keratinous waste. Degradation of hair from both humans and animals by microorganisms has been well-documented (Desai et al., 2010). There have also been reports of hydrolysis of sheep wool and horn meal by microorganisms (Xie et al., 2010).

Duck and pigeon feathers appear to be particularly vulnerable to keratinolytic microbes (Balaji et al., 2008). The solubilization of feathers by microorganisms is impeded by melanin (Goldstein et al., 2004). *Bacillus licheniformis* (Okoroma et al., 2012) and *Pseudochrobactrum sp.* (Yusuf et al., 2020) have been shown, however, to successfully breakdown melanized feathers. Researchers found that *Bacillus aerius* could dissolve duck feathers in 48 hrs.. In another study, Pigeon and melanized chicken feathers were degraded effectively (Bhari et al., 2018). *Bacillus megaterium*, *Bacillus thuringiensis*, and *Bacillus pumilus* were shown to degrade chicken and pigeon feathers in 5 days at pH 7.5 and 30°C by Agrahari and Wadhwa (Agrahari et al., 2010).

2.7. Sources of microbial keratinase

A wide variety of microorganisms isolated from various environmental settings have been linked to keratinolytic proteases. Bacteria and fungi are effective decomposers of keratinous substrates due to their extracellular synthesis of keratinolytic enzymes.

2.7.1. Bacterial sources

Several bacterial genera, including *Bacillus, Vibrio, Corynebacterium, Brevibacillus, Pseudomonas, Serratia, Fervidobacterium, Macrobacterium, Aeromonas, Burkholderia,*

Stenotrophomonas, Rhodococcus, Geobacillus Amycolatopsis, Meiothermus, and *Paenibacillus* have been shown to possess keratinase activity (Brandelli et al., 2015, 2010; Emran et al., 2020; Kalaikumari et al., 2019; Nnolim et al., 2020; Papadopoulos, 1985; Tamreihao et al., 2017). It has been documented that several actinobacteria, notably *Streptomyces, Arthrobacter, Bevibacterium*, and *Nocardiopsis,* may produce keratinase (Gong et al., 2020; Mitsuiki et al., 2006; Thankaswamy et al., 2018; Tronina and Bubel, 2008).

2.7.2. Fungal sources

Fungal keratinases have been found to be key players in the natural hydrolysis of keratinized tissues, and it has been claimed that these fungi are the natural colonisers of keratinous substrates (Bohacz and Korniłłowicz-Kowalska, 2020). Keratinolytic fungi have been documented to include *Aspergillus, Cladosporium, Paecilomyces, Acremonium, Doratomyces, Fusarium, Onygena, Trichoderma, MiKcrosporum, Lichtheimia, Chrysosporium, Scopulariopsis Aphanoascus,* and *Trichophyton. K*eratinolytic activity among fungi has been widely recognized within the dermatophytes. Human and animal skin mycosis has been linked to the keratinolytic feature of this group (Korniłłowicz-Kowalska and Bohacz, 2011a).

Large-scale manufacturing has been a commercial hurdle despite reports of keratinase potential for several indigenous microbial species occupying a wide variety of biological niches (Daroit and Brandelli, 2014). Significant elements that affect microbial metabolites synthesis include culture conditions, nutrition availability and innate cell precursors. The subjective selection of the fermentation conditions is impacted by the genetic variety of keratinolytic bacteria (Arokiyaraj et al., 2019). Keratinase productivity and sustainability are further affected by the lengthy fermentation period required for fabrication by most wild microbial producers (Fang et al., 2019). Therefore, it is necessary to create commercially viable strains to boost production beyond the laboratory scale. Expression of keratinase genes encoding the target metabolites is significantly influenced by the types of nutrients present in the growth medium. However, there have been reports of cell-bound and internally generated keratinases (Brandelli, 2008).

2.8. Keratinases unique biochemical characteristics

Keratinases are typically released extra-cellularly by the microbial producer in the presence of keratinous substrate. Keratinous substrates are bio converted to critical nutritional components for maintaining microbial homeostasis, and their presence is hypothesized to trigger the expression of keratinase producing genes and activate redox systems (Thankaswamy et al., 2018). One of the most important steps in breaking down keratinous biomass is alkalinizing the fermentation medium, which is expected to soften the keratinous substrate and boost sulfitolysis and proteolysis (Cai et al., 2008).

Keratinases (3.4.21/24/99) are proteolytic enzymes produced by microorganisms that efficiently degrade keratin and due to their dependability in handling difficult substrates and their remarkable durability under extreme conditions, they are particularly useful in a number of biotechnological processes (Emran et al., 2020).

Animals cannot generally digest conventionally processed protein-rich meals of keratin origin due to the absence of the redox mechanism that hydrolyzes the disulphide bonds of the polypeptides (Kang et al., 2021). To increase the nutritional properties of keratin-containing feed-products, the digestibility of protein and availability of nutrients must be improved (Nnolim et al., 2020). Solubilization of keratin by keratinases provides a means of enhancing the digestibility and nutritional value of feed-products based on keratin throughout the process of fermentation, whether it takes place in liquid or solid form. Keratin solubilization facilitated by microbial keratinases plays a significant role in the biological-economy innovation value chain. It promotes the conversion of difficult-to-use waste bio-resources into marketable goods for environmental feasibility and helps ensure the continuity of waste management practices (De Oliveira Martinez et al., 2020). Though, the precise processes by which keratinolytic proteases break down keratinous substrates to their monomeric components remain unclear. In this theory, enzymes break down keratinous substances by reducing disulfide bridges and cleaving peptide bonds (Stiborova et al., 2016). In this study keratinase uses, the environmental effect of keratinous wastes, the ecological implications of keratin hydrolysate production, and the processes used to develop value added products from feather hydrolysate are studied.

To put microbial proteases to use in the bio-industry, it is essential to fully comprehend their biochemical features. Keratinolytic proteases have unique characteristics that are organism-specific (Selvam K and Vishnupriya B, 2012). This article discusses the influence of reducing agents, inhibitors, surfactants, metal ions, and chemical solvents on the catalytic properties of keratinases, along with their ideal pH and temperature, molecular weight, and substrate specificity. The majority of keratinolytic proteases are most active between pH 7.0 to 9.0, which is in the neutral to alkaline pH range (Bach et al., 2011; Chaturvedi et al., 2014). However, certain very alkalophilic keratinases with a pH sweet spot of 10–13 have been documented in the past (Jaouadi et al., 2013). Thermophilic keratinolytic proteases already characterized function optimally between 70°C and 100°C, whereas the optimum temperature for the activity of several microbial keratinases was reported to be between 37°C and 65°C (Davidson, 2009; de Oliveira et al., 2016). Studies have shown that several keratinases, from different organisms, remain catalytically active and stable between 20°C and 100°C and pH 4 and 13, respectively.

The molecular weights of microbial keratinolytic proteases have been reported to vary from 17 to 240 kDa. So far, it has been established that keratinases from *S. maltophilia* BBE11-1 and *S. albidoflavus* have modest molecular weights of 17 and 18 kDa, respectively (Fang et al., 2013). *K. rosea's* keratinase, on the other hand, has been measured to have a molecular weight of up to 240 kDa In general most of the known keratinases are 58 kDa monomeric enzymes. On the other hand, a new dimeric keratinolytic protease of 58 kDa with constituent molecular weights of 30 and 28 kDa has been described (Tiwary and Gupta, 2010). Some microorganisms can also produce multimeric keratinases (Brandelli et al., 2015).

Keratinolytic proteases always end up being serine or metallo peptidases. Inhibition by ethylenediaminetetraacetic acid (EDTA) or 1, 10-phenanthroline reveals their type as metalloproteases, whereas inhibition by diisopropyl fluorophosphate (DFP) or phenylmethanesulfonyl fluoride (PMSF) shows their serine protease catalytic mechanism. Keratinase belongs to the metalloprotease class, and EDTA inhibits it by chelating the metal ions necessary for enzyme catalysis (Thys et al., 2004).

Previously, it has been observed that either EDTA or PMSF can partially block the keratinolytic enzymes which may be indicative of the presence of mixed keratinolytic proteases (Staroń et al., 2017; S. Tatineni et al., 2008). The role of cations as keratinase activators or stabilising agents, including Ca^{2+}, Mg^{2+}, Co^{2+}, Ba^{2+}, Fe^{2+}, Ni^{2+}, Mn^{2+}, and Li^{+}, has been well documented (Hamiche et al., 2019). Some metal ions, including as Pb, Hg, Cu, Fe, Ni, and Cd have been shown to have a detrimental effect on keratinase activity (Gegeckas et al., 2018).

Typically, a solution containing surfactants, reducing agents, or solvents will hinder, stabilise, or promote keratinase activity. The different responses of keratinases to chemical agents can be attributed to the fact that the enzymes' structural orientation and bio-catalytic effectiveness are allosteric-ally regulated by the nature of their side chains/interaction patterns (Li, 2019).

It is observed that, Tween-80, and Triton-X100 all increased the activity of *S. aureofaciens* K13 alkaline keratinase *in-vitro* (Su et al., 2017). For *B. licheniformis* ER-15 serine keratinase, the addition of 5mM mercaptoethanol resulted in a 6.25-fold increase in enzymatic activity (Tiwary and Gupta, 2010). Keratinolytic proteases have a high degree of stability in laboratory chemical agents, which suggests they might be useful as robust additives in manufacturing processes. Keratinolytic protease has a wide range of specificity for both natural and artificial protein substrates. Some differences in substrate specificity among microbial keratinases may, however, be attributable to differences in isolation method and genetic variation among their origins. The specificity of proteases to substrates is hypothesized to be group dependent, and it is largely governed by the predominated sequence residues at the C-terminal (P1) and/or N-terminal (P1‘) near to the peptide bond to be hydrolysed (Gu et al., 2016) . Some proteinaceous substrates may be insoluble because of intrinsic inter- or intramolecular tensions, such as disulphide bonds, which give structural proteins their mechanical resilience. As a result, peptide bonds in these proteins are inaccessible to traditional proteases (Navone and Speight, 2018). In contrast, keratinases have been found in nature to have hydrolytic potentials for both soluble and insoluble proteins, leading to their

widespread adoption as viable candidates in a variety of biotechnological processes (Hassan et al., 2020). Protease D-1 from *Stenotrophomonas sp.* hydrolyses N-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine 4-nitroanilide and N-succinyl-L-alanyl-L-alanyl-L-alanine 4-nitroanilide with comparable catalytic efficiencies as those reported for subtilisin and proteinase. Likewise, *Stenotrophomonas sp.* protease D-1 was not very good in hydrolysing the stiff proteins (keratins, collagen, and elastin) compared to the soluble proteins (haemoglobin, bovine serum albumin, and casein). Keratinase produced by *Vibrio sp.* strain kr2 was found to be active toward azo-keratin, azo-casein, benzoyl-arginine p-nitroanilide, and Alanyl-L-Alanine p-nitroanilide as substrates, suggesting that this enzyme may be useful in a variety of industrial settings (Sidnei Sangali and Brandelli, 2000).

2.9. Use of keratinase and keratin hydrolysate

An alternative to the traditional chemical agents currently used in industry is keratinolytic proteases, which are gaining ground slowly but steadily. Bioprocesses as diverse as feed manufacturing, organic fertilizer manufacturing, detergent formulation, leather manufacturing, cosmetics manufacture, drug development, and nanotechnology all make use of the tremendous potentials inherent in microbial keratinases.

2.9.1. Animal nutrition

For decades, feather meal has been used to augment livestock feed, however worries regarding the meal's nutritional value have been voiced due to the feed's lack of digestible protein. The keratin proteins found in feathers and other keratinous materials are indigestible by cattle and other ruminants unless their structural orientation is considerably fragmented (Mokrejs et al., 2011; Williams et al., 1995). Feather meal was typically processed by either pressurized heating or chemical treatment of feathers. Due to the high energy requirements of this processing procedure, crucial heat-labile proteins are destroyed (Dong et al., 2017; Onifade et al., 1998). As keratinolytic microorganisms and keratinases have shown promise, the bio-recycling method may also have use in the husbandry sector of the agribusiness (Gegeckas et al., 2018). Chicks fed a diet

supplemented with *B. licheniformis* PWD-1 hydrolysate, which is high in free amino acids, grew faster, much as chickens fed soybean meal (Williams et al., 1991). Broiler chicks fed a food containing feather hydrolysate generated using keratinase from *B. licheniformis* LMUB05 performed similarly to those fed a diet containing no feather hydrolysate (Adetunji et al., 2018). Decomposition occurred within 8 hrs. of incubation at 50°C and pH 8, and the degree of feather hydrolysis was related to the amount of keratinase generated by *B. licheniformis* ER-15 with strong keratinolytic potentials (Tiwary and Gupta, 2010). Hydrolysates from the biodegradation of keratinous wastes are promising for use as an alternative in the formulation of affordable and adequate diet in animal production due to their high protein contents and antioxidant potentials. This is especially true in light of the increasing demand for conventional feedstuffs like soybean meal, which is also used as human food, and the high production cost of these feeds (de Menezes et al., 2021; Fakhfakh et al., 2011).

2.9.2. Leather Industry

Many countries' economies rely heavily on the manufacture of leather, making it an important industry. The leather processing industry has been plagued by health concerns and environmental issues due to the widespread usage of chemicals that are relatively harmful (Hammami et al., 2018). To transform raw animal skin into finished leather, a number of processes—including soaking, de-hairing, bating, and tanning—are required (Hamiche et al., 2019). The de-hairing process is one of the most polluting stages of leather manufacturing (Ad\igüzel et al., 2009; Li et al., 2018) due to the use of agents that might decompose the protein-rich materials in the hides or skins. Using lime-sulphide in the traditional process of de-hairing animal skin contributes greatly to the hazardous character of the final effluents, creating disposal issues (Kalaikumari et al., 2019). There is hair loss and an increase in chemical oxygen demand (COD), biological oxygen demand (BOD), and total suspended solids (TSS) due to this operative process (Thankaswamy et al., 2018). The leather industry's need for less polluting technology drives the exploration of greener de-hairing methods.

Keratinase, a proteolytic enzyme, has been demonstrated to successfully perform the de-hairing process, which has been offered as a more practical option (Tian et al., 2021). For this reason, keratinases that lack collagen lytic activity but show modest elastin lytic activity have become an increasingly popular choice for de-hairing operations (Macedo et al., 2008; Zhu et al., 2019). The keratinous component of the follicle is degraded selectively by keratinases, allowing for the removal of the intact hairs without damaging the leather. It has been suggested that the keratinolytic protease *B. subtilis* S14, which has good de-hairing capabilities, might replace hazardous sulphide in tanneries (Macedo et al., 2008). Keratinase-based de-hairing, also known as "hair-saving de-hairing," allows for the separation of hair and decreases the massive colloidal mixture and elevated amount of organic matter in effluents brought on by the sulphide treatment process. (Jaouadi et al., 2013) provided a representative example of the de-hairing potential of keratinases by reporting the application of the enzyme (KERUS), which resulted in extraordinary de-hairing activity on the skins of sheep, goats, rabbits, and bovines. Due to the composition of the sulphide and related chemical agents, the de-hairing activities occur in an alkaline environment (Wahyuntari and Hendrawati, 2012), which is optimal for protease activity. Similar de-hairing results were achieved using keratinase from *Vibrio sp.* Kr2 at a different pH range (6-8) and temperature (30C) (Grazziotin et al., 2007). With 7 hrs. of incubation at 37°C and 220 revolutions per min, another keratinase from *Bacillus parabrevis* successfully removed all traces of hair from goat hide (Ma et al., 2017). The skin that had been dehaired enzymatically exhibited no signs of damage under SEM, and the hair pores were more easily visible. The keratinase from *Bacillus parabrevis* was shown to be collagenase-free, suggesting it might be used in environmentally friendly leather production.

Use of keratinase in the leather industry has not only mitigated environmental risks associated with traditional processing methods, but has also led to new methods that have the potential to significantly cut down on energy consumption worldwide (Jaouadi et al., 2013).

2.9.3. Formulation of detergents

Detergents made with bio-additives are currently favored over traditional synthetic detergents. As a result of their biodegradability, compatibility with low-temperature washing, stubborn filth removal characteristics, gentleness on fabric fibers, and general efficacy as cleaners, they have become more popular (Jaouadi et al., 2009). Washing medium pH, temperature, and detergent component all have a role in the general functioning of proteolytic enzymes in detergent (M. Ramakrishna Reddy et al., 2017; Zhang et al., 2020). Detergent-making proteases, ideally, would exhibit a high degree of compatibility with other detergent components like oxidizing and sequestering agents, as well as having outstanding activity and remain adequately stable throughout an expanded spectrum of pH and temperatures (Kumar and Takagi, 1999). Previously, the generally utilized detergent proteases in commercial goods were Subtilisin Carlsberg (SC), Subtilisin Novo, Alkaliser, Esperase, and Savinase (Outtrup et al., 1995). These proteases were relatively resistant to changes in temperature and pH. The proteases often exhibit a high level of inconsistency when exposed to active ingredients including peroxide agents (H_2O_2), optical fibre brighteners, non-ionic surfactants (Tween-80), and anionic surfactant (SDS) during the production of bleach-based detergent. As a result, their qualities prevent them from being used in modern detergents (Paul et al., 2016; M. Ramakrishna Reddy et al., 2017). This highlights the need of finding enzymes produced by microbes that can work in the extreme conditions required.

Since microbial keratinases are unaffected by surface-active chemicals, they can be used in the creation of both liquid and solid detergents (Benkiar et al., 2013). Keratinases may hydrolyze both insoluble and water-soluble substrates, and they are active across a wide pH and temperature range. *Bacillus tequilensis* hsTKB2 keratinase was tested on textiles stained with blood and egg yolk, and it was found to effectively remove both stains (Paul et al., 2014b). Adding hsTKB2 keratinase to detergent resulted in better cleaning performance than using detergent alone. Similarly, the reflectance and transmission readings of a blood-stained apron after washing with a detergent-keratinase combination revealed superior cleaning capability than that of detergent alone (Paul et al., 2014a).

SEM analysis confirmed that the textiles' structural integrity was not damaged by the keratinase treatment, as seen by the uniformity of the fabrics' smooth surfaces. Therefore, microbial keratinases have demonstrated useful qualities that make them a prime candidate for use as a bio-additive in detergents.

2.9.4. Fertilizers and cosmetic production

It is well-known that keratin-rich wastes have no practical use. Yet, bioconversion of these wastes with a high keratin content might be an attractive approach to valorization as a cost-effective and environmentally friendly resource with potential application as a slow-releasing nitrogen source for soil amendment (Gupta and Ramnani, 2006). The green fertilizer produced by this keratinase-mediated wastes mineralization would stimulate plant growth, increase the soil's ability to hold water, and encourage the growth of microorganisms, all of which would increase the material's phosphate solubility (Vasileva-Tonkova et al., 2009; Vidmar and Vodovnik, 2018). When compared to the control group, the germination rate of Gram seeds was significantly increased when treated with hydrolyzed feather biomass produced by a keratinolytic enzyme cocktail from a consortium of keratinolytic bacteria (Falade, 2021). For the production of indole-3-acetic acid (IAA), a phyto-hormone, keratin hydrolysate is an excellent source of the amino acid tryptophan (Tamreihao et al., 2019). Similar studies have found that keratinase-producing bacteria directly produce IAA during the decomposition of feather biomass (Jeong et al., 2010).

Keratinases are a common active ingredient in hair removal creams and other topical products in the cosmetics industry (Adelere and Lateef, 2016). Dead skin layer (hyperkeratosis) is commonly found on extremities, and keratinases have been shown to have the potential to stiffen this layer, meaning they could sustainably replace the commonly used salicylic acid (Gupta and Ramnani, 2006). Wheat protein, wool keratin, and collagen have been utilized in the synthesis of hydrolysate, which has uses in the manufacturing of hair and skin care products (Kshetri et al., 2020). Typically, they improve the skin's texture and hydration while also protecting the skin's healthy structure (Villa et al., 2013). Keratin biodegradation yields low-molecular-weight peptides that are

the product of keratinase activity (Stiborova et al., 2016). These peptides are superior to other hydrolysate in the formulation of cosmetic products like moisturizers and conditioners due to their ability to penetrate the hair or nail cuticle (Villa et al., 2013). The de-hairing efficiency of *Bacillus subtilis* DP1 keratinase was studied in 2016 by Sanghvi et al. Thus, a bio-based depilatory drug was created using excipients whose compatibility and non-reactivity of functional groups were also established. Hair removal cream produced with keratinase was more successful than its chemical cousin (Duan et al., 2020).

2.9.5. Bioenergy generation

Environmental sustainability relies on efficient and ethical waste disposal. Bioenergy is a sustainable fuel from both the economic and ecological perspectives since it can be generated from a wide variety of agro-industrial wastes with considerable saccharide contents and only requires basic building technologies. Biomass de-polymerization is the first step in the manufacturing process; therefore the fibrous and intractable keratinous leftovers that are broken down by keratinase are the raw materials for this endeavor. According to a 2018 study (Risi et al., 2018) biogas generation can be increased by treating chicken feathers with recombinant keratinase from *B. megaterium*. The amount of methane produced from treated feathers is 124% higher than the amount produced from untreated feathers when using a whole broth containing biodegradation products directed by wide-type keratinolytic *Bacillus sp.* C4 (Elsayed et al., 2022). Methane production during anaerobic co-digestion was higher in feather hydrolysate produced by the extracellular keratinase activity of *Bacillus sp.* CL18 than in untreated feathers (Schommer et al., 2020).

During methanation, the protein-rich by-products of keratinolytic digestion have been reported to provide assimilable nitrogen sources in addition to a certain proportion of saccharides (Branska et al., 2020). It's possible that keratinase could be used to treat industrial wastewater. De-colorization percentages for molasses wastewater varied from 84.7% to 90.2% when keratinase from *Meiothermus taiwanensis WR-220* was immobilised on modified bagasse cellulose, a low-cost support (Zhang et al., 2019).

When compared to other commercial immobilised enzymes, keratinase performed better in removing melanoidins, which might lead to a new avenue for a sustainable use of keratinase (Zhang et al., 2019).

2.10. Additional uses of Keratinases

Due to structural similarities between prion and keratin—both are fibrous, b-sheet-dominated proteins—keratinases have been shown to be efficient against infective prion protein (Wang et al., 2012). The disease-causing prion protein was efficiently digested by keratinase PWD-1 (Han et al., 2009). Absolute destruction of highly infectious prion protein by new alkaline keratinolytic protease (NAPase) at pH 11 and 60°C was reported (Mitsuiki et al., 2006). A research (Shiloh and Ziv, 2013) linked keratinase from keratinolytic *B. licheniformis* N22 paired with bio-surfactant in full destruction of the prion protein that causes scrapie after 10 min 65°C.

Analysis of non-protein biomolecules, such as corticosterone, using bio-digested bird feathers has demonstrated more consistent findings compared to mechanical approaches, despite the latter's limits and technical problems (Romero and Fairhurst, 2016). Consequently, glucocorticoids were more easily extracted from chicken feather biomass thanks to the presence of keratinase from *Bacillus sp.* (Alba et al., 2019). Hormone recovery using radioimmunoassay and a parallelism test both corroborated the efficacy of the enzymatic method.

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Chapter 3: Isolation, screening of keratinolytic microorganisms and optimization of culture conditions for the enhanced production of microbial keratinase

Paper from chapter 3:

Paper Title: Isolation of indigenous keratinolytic strain from chicken feathers dumping site and optimization of culture conditions for the enhanced production of keratinase and evaluation of its role for bio-active peptides extraction.

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3.1. Abstract

Feathers biomass consists of approximately 90-95% protein named keratin. Degradation of keratin into value-added products with the help of microbial keratinase is a good substitute for other physical and chemical processes. In the present study, isolation and screening were conducted to identify for the efficient keratinolytic strain. After the identification of the best keratin degrading strain, physical and nutritional parameters were optimized using multifactorial approach and one factor at time. Results showed that the C1M isolate has better hydrolysis capability for feathers keratin and is identified as *Pseudomonas aeruginosa.* After conventional optimization, Chicken feathers (Carbon source), Urea (Nitrogen source), Sodium sulfite (Sulfur source), and Zinc chloride (salts) were found to be significant media components, while 37°C temperature and pH 8 were significant physiological factors. Statistical optimization of media components through Plackett-Burman Design was carried out; chicken feathers (Carbon source) were found to be the most significant nutritional factor. Bioconversion of feathers keratin into hydrolysate was executed and bioactive peptides having antioxidant potential was purified. The study concluded that *Pseudomonas aeruginosa* – C1M has the prospective to transform keratinous waste efficiently into valuable products that can be employed in various biotechnological applications.

Key words

Keratin waste, keratinase, Optimization, Bioactive peptides,

3.2. Introduction

The poultry industry is considered an essential and diversified sector contributing to a major portion of food. However, along with its valuable aspects, this sector generates a tremendous amount of waste (Stokes, 2012). Feathers are an essential by-product wasted as feather waste consists of 8% of the live weight of chicken (Hassan et al., 2020). According to a report, 8.5 billion tons of chicken waste is produced annually. All this waste is either subjected to incineration or dumped into the soil.

Moreover, many physical and chemical remediation methods are also being investigated. Classical approaches for feathers processing have some limitations. Firstly, an asset of

nature is being lost without benefit; secondly, ecological and catastrophic issues are also being raised. Therefore, researchers are looking for less expensive and eco-friendly methods to manage waste properly and utilize the end products of feather digestate.

As feathers are composed of approximately 85-90% of keratin, a recalcitrant fibrous insoluble protein, producing valuable products, it is attracting biotechnological attention. Amorphous keratins are found in smaller proportions, while α-keratins constituting about 41-67% and β-keratin 33-38% (Lange et al., 2016). Feathers' hydrophobic nature and high resistance to a variety of proteolytic microorganisms, chemicals, enzymes, and mechanical stress are attributes of their composition. Microorganisms and their enzyme constitute a significant source for biodegradation of feathers and obtaining value-added by-products. Feathers keratin is a complex proteinaceous component; therefore, microbial degradation occurs through the mechanism of proteolysis and sulfitolysis.

Keratinase (EC 3.4.21/24/99.11), a protease, can convert chicken feathers' keratin into its sub-components by de-polymerizing peptide chains and disulfide bonds. Keratinases of several different types have been identified in various bacteria, actinomycetes, and fungal species. However, bacteria are considered as most promising for commercial applications. Based on their mechanism of action, keratinases can be categorized as exo-keratinase (cleaving of polypeptide chains from ends) and endo-keratinase (cleaving of polypeptide chains within protein). However, based on their catalytic site, they can be distributed into serine-protease, metalloprotease, and serine metal protease.

Keratinases are mainly used in leather bio-processing, the pharmaceutical industry, nitrogen fertilizer production, and feed formulation. Bio-refinery concept for bioconversion of feathers into feather hydrolysate and valuable product is also employed. When a polypeptide chain is broken, it releases a complex combination of peptides and amino acids called feathers hydrolysate.

The current study explored chicken feathers dumping soil and industrial leather tannery soil for isolating highly potent keratinolytic microorganisms. Samples were processed, and keratin hydrolyzing microbes were obtained. All keratinase producers were screened selectively on feather basal medium (FBM), and a potent strain was selected. After identifying that strain, culture conditions were optimized for enhanced keratinase

production. The optimization was performed using both a multi-factor approach and a one-factor at a time. After optimization of keratinase production, chicken feathers were converted into feathers hydrolysate (FH) with the help of keratinolytic strain.

3.3. Materials and Methodology:

3.3.1. Study area

The present research work was conducted at the Applied Environmental and Geo-microbiology lab (AEG) of the Department of Microbiology, Quaid-i-Azam University, Islamabad and Molecular biology lab of Karadeniz Technical University, Trabzon.

3.3.2. Chemicals

Most chemicals used for research purposes were mainly obtained from Merck (Germany), Sigma (USA), Acros (Belgium), Fluka (Switzerland), and BHD (UK). Raw substrates were obtained from the local markets of Pakistan.

3.3.3. Sampling and isolation of keratinase producing microorganisms

For isolation of keratinase-producing strains, two sites were explored. One sample was collected from chicken feathers dumping sites and other sample was obtained from leather industry tannery area waste soil. After checking the pH of samples, they were brought into AEG Laboratory and processed further. For prospect use, samples were kept in sterilized falcon tubes and bags. Isolation was carried out by employing a culture enrichment technique. This method could quickly isolate microorganisms for a specific purpose at a detectable range(Bhari et al., 2018). Enrichment of the samples was carried out in feathers containing medium through submerged fermentation under laboratory conditions. After that 1g from each sample was inoculated separately into a broth medium enriched with chicken feathers and kept overnight at 37°C under shaking condition. After that, 1mL samples were taken from culture broth and subjected to serial dilution. In the serial dilution technique, samples were sequentially diluted, and microbes were isolated on enriched medium plates. Keratinase producer isolates were further screened by following quantitative and qualitative screening.

3.3.4. Qualitative Screening

3.3.4.1. Primary screening

Strains obtained from serial dilution were incubated on casein or skim milk media for 24h at 37°C to obtain colonial growth of proteinase-producing strains. The composition of skim milk agar (g/l) was as follows: glucose 1.0 g, casein 5.0 g, skim milk powder 3.0 g, yeast extracts 2.5 g, and agar 15.0 g, as solidifying agent (Barman et al., 2017). According to the ratio of the clear zone of hydrolysis, the best enzyme producers were chosen and employed for further characterization.

3.3.5. Quantitative Screening

3.3.5.1. Secondary screening

Selected bacteria were transferred to FBM-containing screening agar plates (Feather Basal Media with composition (g/l); NaCl 0.5g (Sodium chloride), KH_2PO_4 0.1 (Potassium di-hydrogen phosphate), Na_2SO_3 0.2g (Sodium sulfite) K_2HPO_4 0.01g (di-Potassium hydrogen phosphate) and Chicken feathers 5g incubated for another 24h at 37°C(Yusuf et al., 2016a). Colonies were tested for their ability to manufacture keratinase based on the presence of a clear zone by FBM hydrolysis. The best keratinase producer was chosen based on colony diameter and clear zone measurements, and then employed for further characterization.

Moreover, for quantitative screening, the specific activity was determined. Specific activity (U/mg) was carried out by finding keratinase assay (U/ml) and protein concentration (mg/ml).

3.3.5.2. Keratinase Assay

The enzyme activity of crude keratinase was driven by the modified methodology of Cheng et al. (Wang et al., 2015). 5 mg of azure keratin were suspended in 0.8 ml of phosphate buffer and treated with 0.2 ml of the crude enzyme (pH 7.5). The sole components in the control experiment were the buffer and the keratin azure. In a water

bath maintained at 40 °C, the reaction mixtures were left to incubate for 45 minutes. The reaction was stopped by adding 2 ml of trichloroacetic acid (TCA) at a concentration of 10%. In order to eliminate any precipitated keratin, the reaction mixture was centrifuged at a speed of 10,000 rpm for 15 minutes while it was kept at room temperature (30 ± 2 °C). Protein release that was transformed into keratinase units(1U = 0.01 absorbance increase for 1 hour reaction time) is measured by the increase in filtrate absorbance of test samples at 400 nm compared to that of the control. Units were calculated from the tyrosine standard curve.

3.3.5.3. Protein estimation

By keeping BSA (Bovine Serum Albumin) as a reference, the Lowry's method was used to calculate the amount of protein (Kshetri et al., 2020).

3.3.6. Identification of keratinase-producing microorganisms

The selected strain was identified through Gram staining and through biochemical tests, including Citrate Utilization Test, catalase test, Triple Sugar Iron Test and molecular identification was done by using 16S ribosomal RNA sequencing. DNA extraction was done for 16S ribosomal RNA sequencing, and samples were processed for DNA sequencing in Macrogen Korea. After sequencing computational phylogenetic method was used to develop a phylogenetic tree.

3.3.7. Optimization of cultural conditions

Various parameters, either physical or nutritional (media components), were optimized to enhance keratinase production. A one-factor at-a-time optimization approach was carried out, and then, based on their results, statistical tools were applied to optimize media components for enhanced keratinase production.

3.3.7.1. One factor-at-a-time optimization:

Physical parameters selected were temperature, pH, time of incubation, and Inoculum size. While nutritional or chemical parameters include carbon, nitrogen, salts and sulfur

sources. Moreover, significant media components such as copper sulfate $CuSO_4$, calcium chloride $CaCl_2$, zinc chloride $ZnCl_2$, magnesium sulfate $MgSO_4$ and iron sulfate Fe_2SO_4 were selected by one factor at-a-time approach.

3.3.7.2. Multifactorial optimization

3.3.7.2.1. Statistical optimization by Placket-Burman design

After conventional optimization, several components at a time were optimized to obtain a high enzymatic yield. The Expert Design tool used the Placket-Burman design to optimize various media components, and their interactions were evaluated. Eleven factors were optimized, i.e., carbon sources (chicken feathers), nitrogen source (sodium nitrate), sulfur source (Na_2SO_3), salts ($ZnCl_2$ and Fe_3SO_4, K_2HPO_4 and KH_2PO_4), variable pH values, and inoculum size. Fifteen runs were provided by experimental design, and the experiment was run in duplicate. The Centre point was kept 3. Ranges for every component were selected complete experiment was conducted at around 37°C at 180 rpm. The response was measured in terms of the specific activity of keratinase. Keratinase activity was determined, as explained previously.

3.3.8. Antioxidant peptides extraction from keratin waste

3.3.8.1. Keratin hydrolysate preparation

Submerged fermentation of feathers was carried out. Briefly, 10 g of feathers were added in 500ml flask, containing 100ml of salt solutions of feathers basal medium (FBM). Flask was injected with 5% (v/v) of an overnight culture of *Pseudomonas aeruginosa*-C1M and kept at constant shaking for 72hrs.. Supernatant was extracted by filtration through gauze and centrifugation at 10,000 rpm for 15 minutes. Anti-oxidative activity of keratin hydrolysate was evaluated by using FRAP (ferric reducing ability of plasma) method.

3.3.8.2. FRAP (Ferric reducing ability of plasma) assay

Basic principle of FRAP assay was based on quantification of reduction of Fe^{+3} to Fe^{+2}. Each sample was combined with an equal volume of sodium phosphate buffer (pH 6.6), and 10 mg/l ferricyanide was added to the reaction mixture. The combination was heated

to 50°C and left to incubate for 20 minutes. Trichloroacetic acid (TCA) was used to stop the process with a concentration of 10% (w/v). The resulting solution was mixed with 10mg $FeCl_3$/l. Absorbance was recorded at 700nm by keeping water as blank.

3.3.8.3.Purification of antioxidant peptides from hydrolysate

At first, 6 M HCl (pH 2) was added to the feather hydrolysate and it was let to sit at 4°C overnight to get the desired pH level. After centrifuging at 15,5009g for 10 minutes, the pellet was extracted. Pellet was lyophilized, then dissolved in dimethyl sulfoxide (DMSO)/80% aqueous methanol (1/1, v/v) that had been equilibrated with 80% aqueous methanol. This mixture was then run through a Cation Exchange Column (Q-Sepharose). The column was eluted at a rate of 2 ml/min with 0.1 M, 0.3 M, and 0.5 M NaCl in 80% aqueous methanol. Through FRAP assay (mentioned above) antioxidant potential of fractions were evaluated.

3.3.9. Statistical Analysis

In this study, culture conditions for keratinolytic strain were optimized using Placket Burman Design; variable analysis was used to determine which factors were most important in elevating keratinase production, and their relative importance was quantified using mean±standard deviation. The optimal conditions for keratinase synthesis were determined using a Plackett-Burman experiment and one-way analysis of variance (ANOVA). In ANOVA, if the probability of factors is less than 0.05%, the results are considered significant. No transformation was desired to define the effect of variables on response.

3.4. Results and Discussion:

3.4.1. Isolation and screening of keratinase-producing microorganisms

Potent keratinolytic microorganisms were isolated from chicken feathers dumping site and leather tannery soil due to the presence of keratin rich substrate. A previous study explored feathers dumping soil to isolate potent keratinolytic microbes(Ire and Onyenama, 2017). Yusuf et al, isolated a metal-tolerant feather degrader bacteria from feather dumping soil of Malaysia, employing a culture enrichment technique (Yusuf et

al., 2016b). Zaraî Jaouadi isolated a keratinase from an isolate of leather industry waste soil(Zaraî Jaouadi et al., 2015) and used it for the leather bating process. Previously, a keratinolytic yeast was also isolated from leather tannery soil to reduce a load of chemicals in bating process of tannery(de Medeiros et al., 2016).

From both samples total of thirty-three, isolates were obtained through serial dilution. After the primary screening, carried out primarily on skim milk agar plates and on keratin agar, seven isolates were found as keratinase positive. Primary screening was done on skim milk and casein agar due to their protein content. Three isolates, T8 T9 (Leather Tannery waste soil isolates) and C1M (Chicken feathers dumping soil isolate), had a robust visible hydrolysis zone. After streaking pure colonies on skim agar, hydrolysis of casein was observed. Four strains of fungus were also obtained, but they were not used in this study. Researchers from India used casein milk agar for primary screening (Ramakrishna Reddy et al., 2017), while others used skim milk agar (Sekar et al., 2016). Of these three keratinases producing bacteria, C1M showed the best keratinase activity and was selected for further research. (Fig. 3.1)

Fig.3. 1. Zone of hydrolysis of keratinolytic strains (C1M, T8 and T9). From left to right, first photo was showing zone of hydrolysis of C1M strain while, the other two were T8 and T9.

During secondary screening, C1M strain has degraded feathers entirely in 48 hrs. Also, the specific activity of C1M strain was more significant than other strains (Fig. 3.2). Due to the reason C1M strain has been isolated from feathers dumping region and has the ability to efficiently degrade feathers protein. More likely, C1M strain has β-keratinase that was able to degrade efficiently raw substrate, feather keratin which contains β-

keratin. Therefore, C1M was selected for further studies. (Fig. 3.2 and 3.3) Chen and his colleagues used feather basal media for fermentation of feathers and isolated bacteria from spider gut (Qu et al., 2018). However, other two strains T8 and T9 were isolated from tannery soil sample and they might have α-keratinase more dominant in their system than other keratinases due to the fact they were using α-keratin of animal hair as raw substrate.

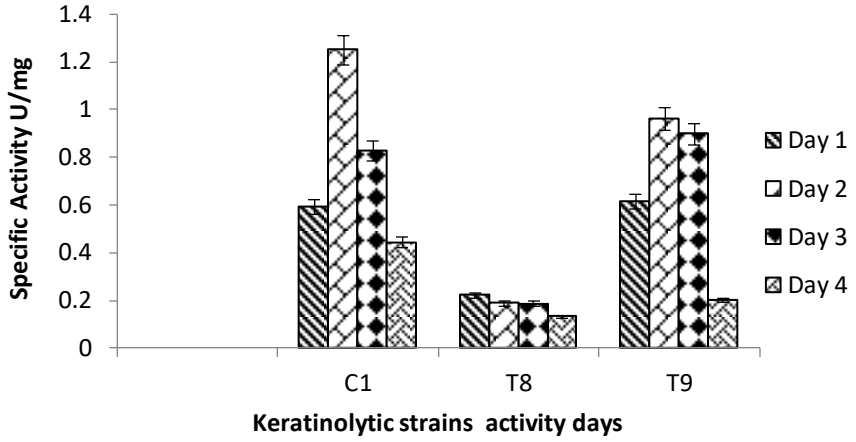

Fig.3.2. Quantitative screening of Keratinase producing strains C1M, T8 and T9

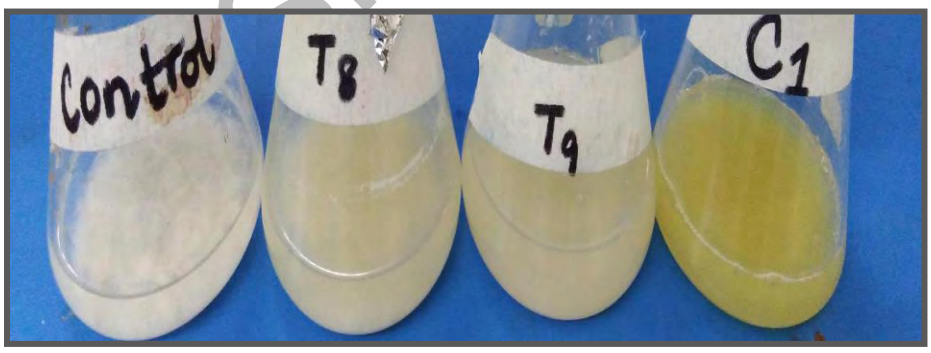

Fig.3.3. Feathers hydrolysis by keratinolytic strains C1M, T8 and T9 along with control in feathers basal medium (FBM)

3.4.2. Biochemical and molecular identification of keratin hydrolyzing strain C1M

C1M isolate of chicken feather dumping soil was identified through biochemical and molecular identification. Morphological characterization was carried out through Gram-

stain. In Gram staining, gram-negative rods were visualized in microscopy. Based on it, biochemical tests were conducted to characterize the strain before DNA sequencing.

Table 3.1. Biochemical results for strain C1M are as follow:

Biochemical Tests	Results
Triple Sugar Iron Test	Negative (-), Aerobic
Motility Test	Motile
Indole Production	Positive (+) Red color appearance after addition of KOVAC's reagent
Sulfide production	Positive (+)
Catalase Test	Positive (+)
Oxidase Test	Positive (+)

The selected *C1M* strain showed similarity to *Pseudomonas aeruginosa,* while other results were confirmed by 16srRNA sequencing, and sequence results are as follows:

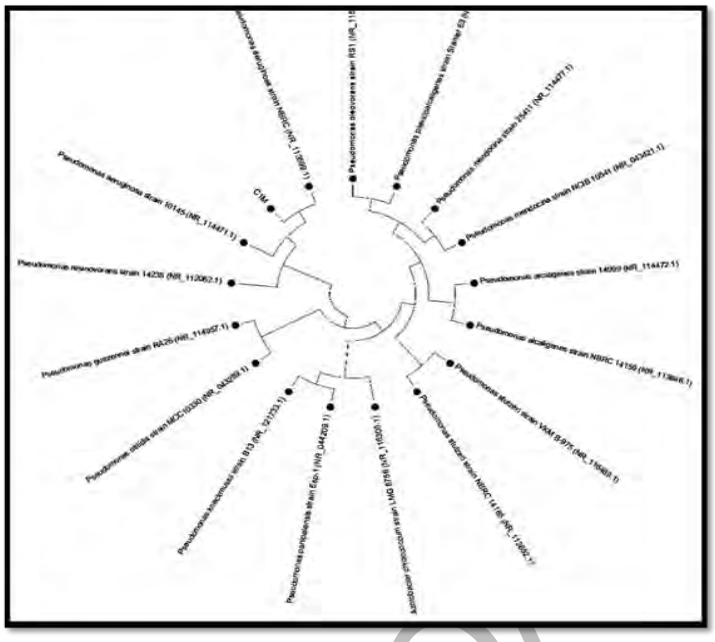

Fig.3.4. Dandogram for C1M indicates its similarity with Pseudomonas aeruginosa

3.4.3. One factor-at-a-time optimization:

Several factors were optimized by using a one factor at a time approach. Factors optimized to include the following physical factors; incubation period, temperature, pH, and chemical factors; organic carbon source, nitrogen source, sulfur source, and various salts were optimized. As a result, maximum enzyme activity was found after 48 hrs. of cultivation. Enzyme synthesis developed at 37 ^{o} C and a pH of 8. Feathers as a carbon source and urea as a nitrogen source led to the highest levels of keratinase synthesis. Sodium sulfate and zinc chloride also helped in enhancing enzyme production.

The result of each factor will be described below in detail:

3.4.3.1.Effect of time of incubation on keratinase production

Both bacterial growth and enzyme production were shown to be very sensitive to the incubation period. Keratinase production by *Pseudomonas aeruginosa*-C1M was observed by growing it in FBM and calculating its enzyme activity at 24 hrs. intervals for five days. It was seen that maximum enzyme activity was observed on the second day of incubation. After 24 hrs., bacteria primarily enter their log phase, increasing enzyme production exponentially (Fig.3.5). In another study on *Pseudomonas aeruginosa* strain, with increasing time keratinase production was increased. In another research, feathers'

weight loss percentage increased significantly after two days of cultivation by *Pseudomonas microphilus* (Sekar et al., 2016). Rapid decreases in cell number and keratinolytic activity after 72 hrs. likely indicate depletion of the growth-limiting nutrient. It is possible that feedback inhibition is responsible for the decline (Kshetri et al., 2019). This suggests that the generation of the keratinase enzyme is influenced by the quantity of cells required by Pseudomonas sp. LM19 took less time under culture to produce the most keratinase than had been previously reported.

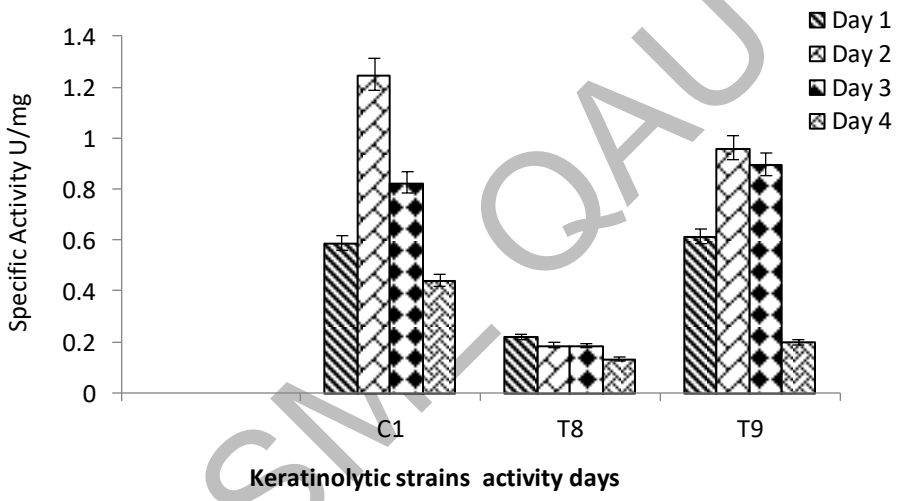

Fig.3.5. Effect of incubation time on keratinase production, For C1M strain maximum activity was observed at 48 hrs.

3.4.3.2. Effect of temperature on keratinase production

Temperature is linked with enzyme production. Temperature affects the synthesis and metabolism of enzyme. Several temperature ranges were selected to check the effect on keratinase production. Temperature ranges were 20°C, 30°C, 37°C, and 50°C. keratinase production increased with increasing temperature but after 37°C, it started decreasing. This result is linked with the fact that at higher temperature enzyme synthesis and metabolism may hinder due to mesophilic bacterium growth conditions. At 20°C, the specific activity observed was 1.32U/mg, representing that the organism does not secrete enzymes at this temperature. Quantifying the enzyme at 30°C shows that it has higher enzyme activity as compared to 20°C. At 37°C, highest keratinase activity was observed,

but it reduced on further temperature increase to 50°C. After 48 hours of incubation, 37°C was the optimum temperature for the growth of *Pseudomonas aeruginosa*-C1M. At other high temperatures enzyme was denatured, and specific activity was lowered. High heat is involved in the disruption of hydrogen and ionic bond leading to the conformational modification of enzyme – substrate complex, thus disordering specific activity of enzyme. Previous studies showed that optimal proteolytic activities were between 30°C and 37°C, keratinolytic bacteria mostly have feather-degrading activity at elevated temperatures (Balakumar et al., 2013).

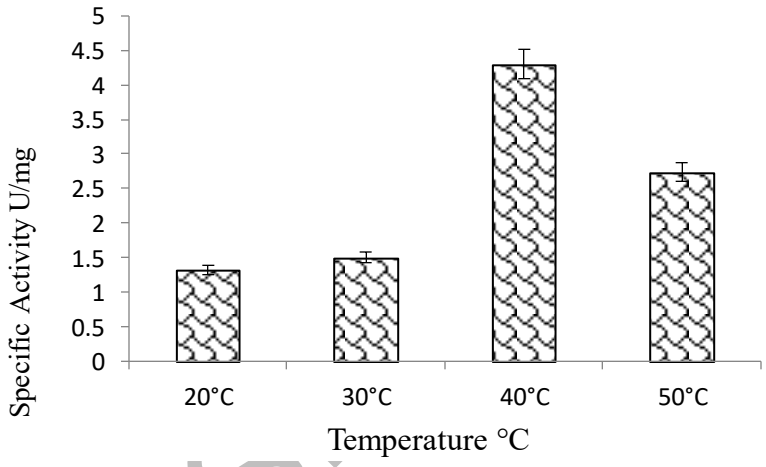

Fig.3.6. Effect of Temperature on keratinase production of Pseudomonas aeruginosa C1M

3.4.3.3.Effect of pH on keratinase production

The synthesis of keratinase was significantly impacted by the pH of the culture medium, as it has influence on the metabolic activity of enzymes. Media pH was adjusted using 1M NaOH and 0.5M H_2SO_4, and different pH values ranging from 4 to 9 were selected. The temperature was maintained at 37-40°C, for 48 hrs.. At pH 4, the specific activity while at pH 8, enzyme activity was 6.665U/mg. Maximum keratinase activity was observed at pH 8, which shows that C1M needs an alkaline environment for enhanced enzyme production. This might be due to the alkaline environment that supports the keratinase production. In a different investigation, Pseudomonas aeruginosa KS-1 was shown to be an alkaline, serine protease with a pH range of 8 to 9 (Gupta et al., 2013). In

a broad alkaline pH range (8–11), Kocuria rosea extracellular keratinase was likewise stable (Bernal et al., 2006).

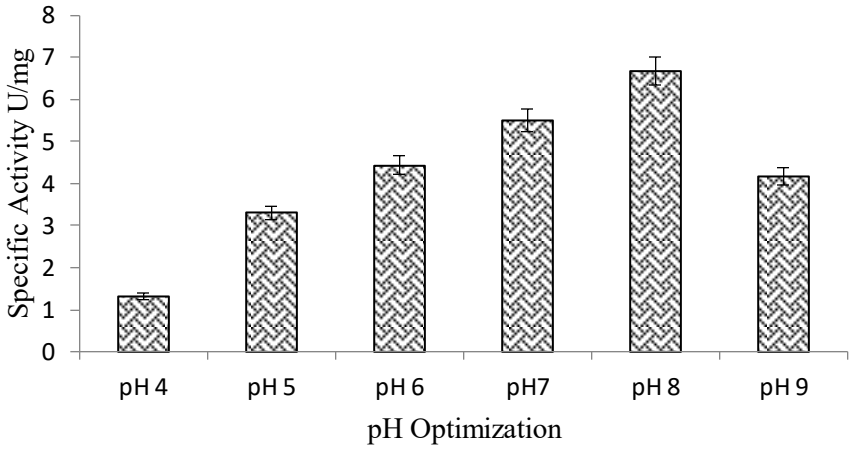

Fig.3.7. Effect of pH on keratinase production of Pseudomonas aeruginosa C1M

3.4.3.4. Effect of various carbon sources on keratinase production

Chicken feathers, human hairs, sheep wool, and nails were natural carbon sources selected to produce keratinase. Pre-treated (washed and ground) natural substrates were used. Chicken feathers showed a maximum specific activity of 8.54U/mg. While, human hair showed least keratinase activity. Results showed that human hair keratin protein varies structurally from the keratin of chicken feathers. These results are in line with *B. licheniformis* ALW1. The best substrate to stimulate keratinase (25.3 U/ml) was the feather among the various keratinous materials evaluated at 2% level. Hair also generated a significant quantity of keratinase (17.4 U/ml), indicating that it could synthesize keratinase from both α-keratin and β-keratin. A spike was used to measure the keratinase concentration that was lowest (6.7 U/ml). While it was 13U/ml and 8U/ml for wool and nails, respectively. Other studies also noted feather as the greatest source for keratinase synthesis (Hassan et al., 2020).

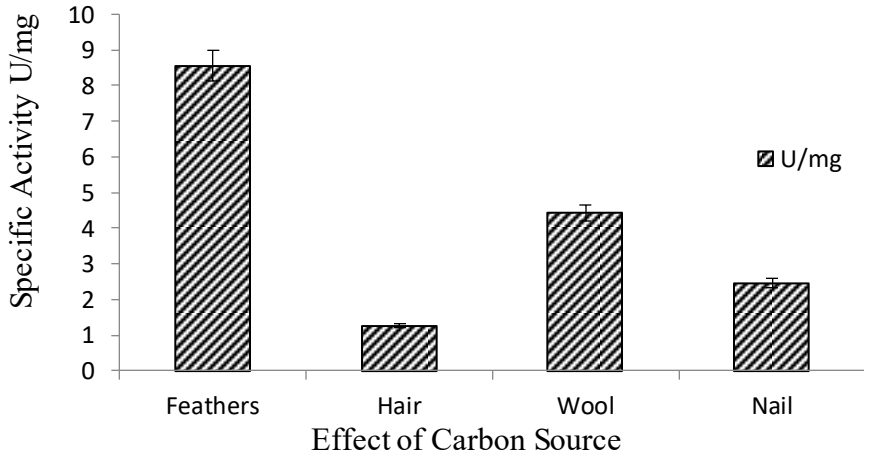

Fig.3.8. Effect of carbon supplements on keratinase production of Pseudomonas aeruginosa C1M

3.4.3.5.Effect of various nitrogen sources

Nitrogen plays vital role for the growth of micro-organisms, as proteinaceous components require nitrogen for their synthesis. Nitrogen sources used were $NaNO_3$, NH_4NO_3, $(NH_4)_2PO_4$, and urea. Maximum keratinase activity was obtained with urea, which was 9.69U/mg after 48 hrs. of incubation, while minimum activities were obtained with $(NH_4)_2PO_4$, which was 1.91U/mg. This result is in line with research on *Bacillus subtilis* strain, KD-N2, which shows an increase in the level of production of keratinase alongwith urea (Subugade Babasaheb Ambedkar et al., 2019) and *Bacillus sp*. Se-103 (일 et al., 2009) and *Scopulariopsis brevicaulis* show increased enzyme activity with $NaNO_3$ (Anbu et al., 2007). However, in contradiction with studies on *B. megaterium* F7-1, urea and inorganic nitrogen reduced enzyme production (Park and Son, 2009). Due of the catabolite suppression effect, none of the other examined nitrogen sources had a discernible impact on Pseudomonas aeroginosa-C1 keratinase production. As, feather itself has keratin which can serve as sole nitrogen source, therefore, external nitrogen supplements didn‘t effect keratinase production significantly.

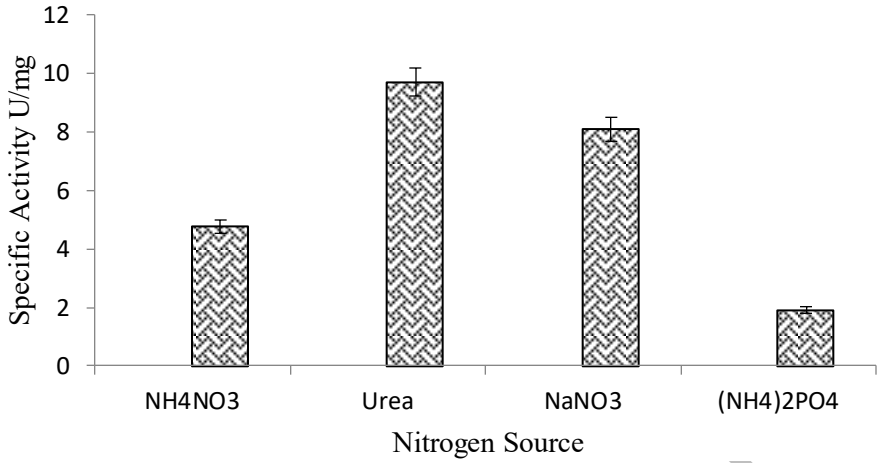

Fig.3.9. Effect of several nitrogen sources on the production of keratinase of Pseudomonas aeruginosa-C1M.

3.4.3.6.Effect of various sulfur sources on keratinase production

Various sulfur sources were used to evaluate the best sulfur source for maximum production of keratinase while maintaining previously optimized factors constant. Sulfur sources used were Na_2SO_3, Na_2SO_4, $Na_2S_2O_3$, and beta mercapto-ethanol. Maximum activity was obtained with Na_2SO_3, which was 12.06U/mg, while minimum activity was seen with Na_2SO_4, which was 3.72U/mg. Similar research results were reported by(Cheng et al., 2010). According to their study, Na2SO3 works as a powerful activator that could encourage the keratinase from Streptomyces fradiae to break down the keratin substrate. However, the other sulfur sources decrease the decomposition of keratin substrate. In another research, different results were reported by(Liang et al., 2011) showing that among the studied sulphur sources, sodium thiosulfate was the only one that increased the synthesis of the enzyme keratinase in the mold M. thermophila strain H49-1. The successful investigation with sodium sulphide could suggest that various keratinase-producing bacteria are required for sulfide's impact on degrading the keratin substrate.

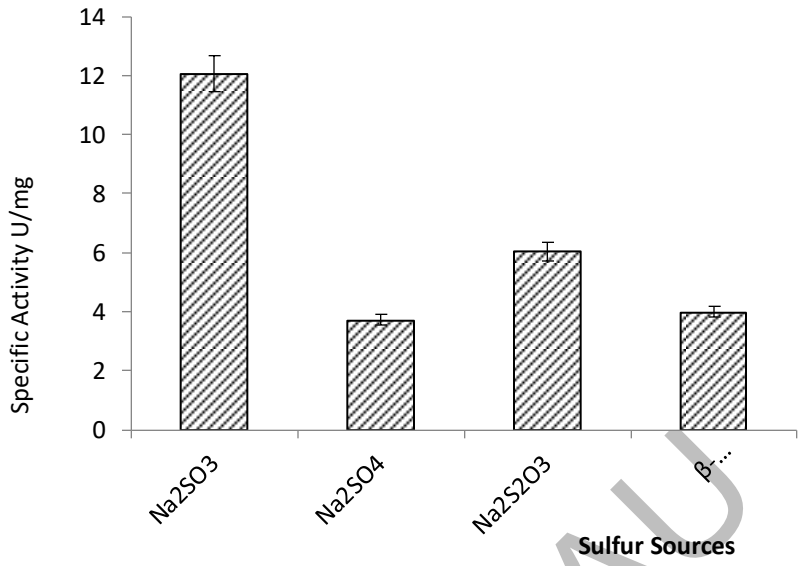

Fig.3.10. Effect of Sulfur supplements on keratinase production of Pseudomonas aeruginosa C1M

3.4.4.7. Effect of salts on keratinase production

Equal concentrations of various salts were used to evaluate the best salt for maximum production of keratinase while maintaining previously optimized factors constant. Salts used were Cu_2SO_4, $CaCl_2$, $MgSO_4$, $ZnCl_2$, and Fe_2SO_4. Almost all salts kept the enzyme production nearly constant as they are crucial for metabolic synthesis. Specific activity obtained with $ZnCl_2$ was 13.5U/mg, while the less activity was seen with Cu_2SO_4, which was 7.38U/mg. The specific action of Fe_2SO_4, $MgSO_4$, and $CaCl_2$ was 10.77U/mg, 8.15U/mg, and 9.82U/mg, respectively. Similarly, the Zn^{2+} ion positively affected keratinase production by strain H49-1.

However, some other researchers hypothesised that S. brevicaulis (Annaduraic, 2005) and *Bacillus spp* (Brandelli J. L., 2006) did not need the ion Zn2+ in order to produce keratinase. These findings suggest that the characteristics of keratinases produced by various bacteria can vary.

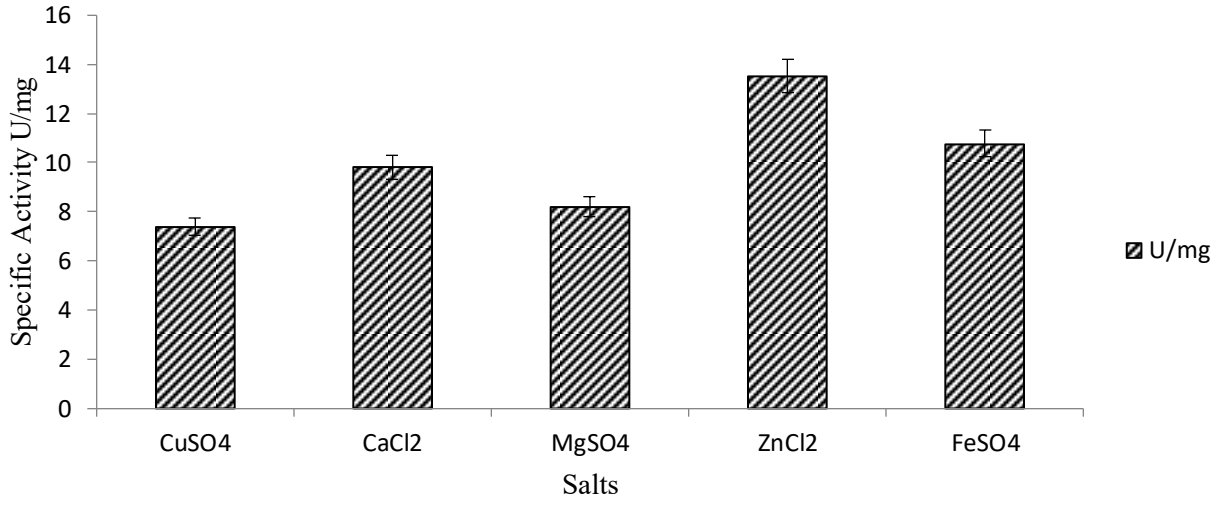

Fig.3.11. Effect of salts on keratinase production of Pseudomonas aeruginosa C1M

3.4.4. Optimization of media components by using statistical tools for keratinase production

3.4.5.1. Placket-Burman design for optimization

Placket-Burman was used for the optimization of nutritional components. Nine factors were selected and analyzed for maximum activity. Factors were selected based on previous one factor at a time optimization. Among these factors include carbon source (chicken feathers), Nitrogen source include (sodium nitrate), sulfur source (sodium sulfate), salts (potassium hydrogen phosphate, potassium dihydrogen phosphate), metals (zinc chloride, and ferrous sulfate monohydrate), inoculum size and pH. Maximum specific activity was obtained at run# 1 that was 14.64 U/m

Table.3.2. Placket-Burman Design provided value for experiment deign in the following table.

Std.	Run	Block 1	A: Feathers	B: $ZnCl_2$	C: $FeSO_4$	D: K_2HPO_4	E: KH_2PO_4	F: Na_2SO_3	G: Inoculum size	H: pH	J: $NaNO_3$	K: Dummy 1	L: Dummy 2	Specific Activity
8	1	Block 1	1.5	0.1	0.05	0.05	0.05	0.1	2.5	9	0.15	5	1	14.64
11	2	Block 1	1.5	0.05	0.1	0.1	0.1	0.05	2.5	5	0.15	5	1	9.141
3	3	Block 1	1.5	0.05	0.1	0.1	0.05	0.1	7.5	9	0.05	5	0	6.932
1	4	Block 1	1.5	0.1	0.05	0.1	0.1	0.1	2.5	5	0.05	7	0	5.358
7	5	Block 1	1.5	0.05	0.05	0.05	0.1	0.05	7.5	9	0.05	7	1	5.34
10	6	Block 1	0.5	0.1	0.1	0.1	0.05	0.05	2.5	9	0.05	7	1	7.207
12	7	Block 1	0.5	0.05	0.05	0.05	0.05	0.05	2.5	5	0.05	5	0	5.070
13	8	Block 1	1	0.08	0.08	0.08	0.08	0.08	5	7	0.1	6	0.5	4.239
14	9	Block 1	1	0.08	0.08	0.08	0.08	0.08	5	7	0.1	6	0.5	5.994
2	10	Block 1	0.5	0.1	0.1	0.05	0.1	0.1	7.5	5	0.05	5	1	4.758
6	11	Block 1	0.5	0.05	0.05	0.1	0.05	0.1	7.5	5	0.15	7	1	3.044
9	12	Block 1	1.5	0.1	0.1	0.05	0.05	0.05	7.5	5	0.15	7	0	10.30
15	13	Block 1	1	0.08	0.08	0.08	0.08	0.08	5	7	0.1	6	0.5	5.922

4	14	Block 1	0.5	0.1	0.05	0.1	0.1	0.05	7.5	9	0.15	5	0	3.668
5	15	Block	0.5	0.05	0.1	0.05	0.1	0.1	2.5	9	0.15	7	0	2.991

Table.3.3. Actual versus Predicted Value

Standard order	Actual Value	Predicted Value	Residual	Leverage	Internally studentized residuals	Externally studentized residuals	Influence on Fitted Value / DFFITS	Cook's Distance	Run Order
1	5.36	8.51	-3.16	0.5	-2.46	** -4.66	* -4.66	0.864	4
2	4.76	2.62	2.14	0.5	1.664	1.926	1.926	0.396	10
3	6.93	7.21	-0.28	0.5	-0.215	-0.202	-0.202	0.007	3
4	3.67	4.14	-0.47	0.5	-0.37	-0.349	-0.349	0.02	14
5	2.99	3.63	-0.64	0.5	-0.501	-0.476	-0.476	0.036	15
6	3.04	4.56	-1.52	0.5	-1.185	-1.22	-1.22	0.2	11
7	5.35	4.55	0.8	0.5	0.62	0.594	0.594	0.055	5
8	14.65	12.69	1.95	0.5	1.524	1.691	1.691	0.332	1
9	10.3	10.96	-0.66	0.5	-0.515	-0.49	-0.49	0.038	12
10	7.21	7.01	0.2	0.5	0.157	0.147	0.147	0.004	6
11	9.14	7.8	1.34	0.5	1.046	1.054	1.054	0.156	2
12	5.07	4.77	0.3	0.5	0.234	0.22	0.22	0.008	7
13	4.24	5.39	-1.15	0.333	-0.773	-0.752	-0.532	0.043	8
14	5.99	5.39	0.61	0.333	0.411	0.389	0.275	0.012	9
15	5.92	5.39	0.54	0.333	0.362	0.342	0.242	0.009	13

Diagnostic based statistic tool showed difference of actual to predicted value. Specific values of all diagnostic graphs are based on these values.

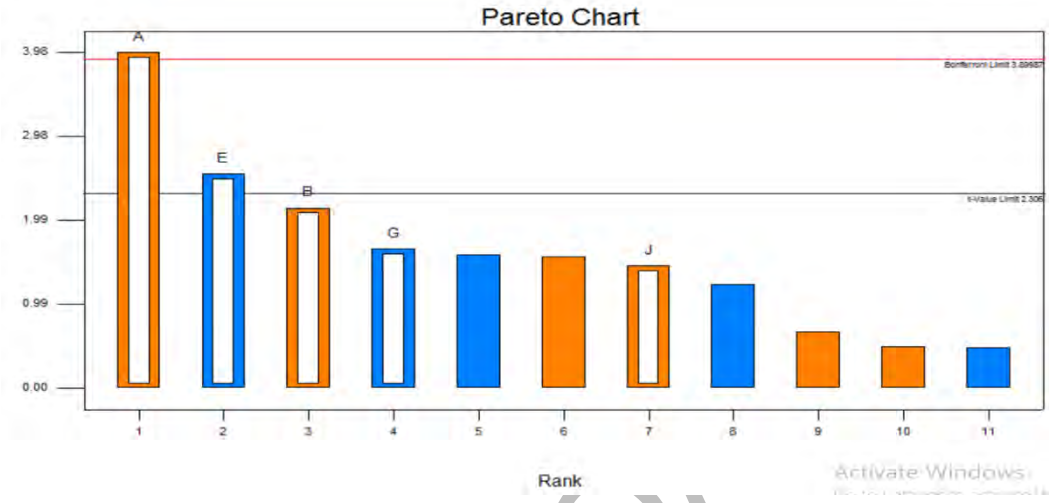

Fig.3.12. Pareto chart of Plackett-Burman optimization design

Analysis showed that two factors (chicken feathers) and bivalent cation (KH_2PO_4) were found significant. Among them include carbon source. Chicken feathers are an essential factor in all of these. The orange color shows a positive effect, while the blue color indicates a negative impact on the production of keratinase. Factor A is considered the most significant factor in placket Burman. Factor A is positively affecting as it touches the t-value line; the factors that touch the t-line are regarded as the most powerful. At the same time, factor E is negatively influencing keratinase production. The multifactorial experimental design was a valuable tool for developing optimum media and culture conditions for keratinase production by *Pseudomonas aeruginosa*-C1M. This is because it is simple to use, applicable, reliable, and accurate. Placket Burman Design was used in this research to choose the important factors required for the improved production of keratinase. Chicken feathers and KH_2PO_4 were determined to be more critical factors. Chicken feather was the most significant factor having a positive effect. It is only reasonable that substrate concentration, namely the concentration of feathers, would have the greatest influence since it is one of the most crucial elements not only for the accumulation of breakdown products but also for keratinolytic activity (Paul et al., 2014). Out of these variables, KH_2PO_4 has a negative effect. These results resemble studies upon

Bacillus pumilus A1(Fakhfakh et al., 2011) in a way that KH_2PO_4 was the significant factor having an adverse effect, but in contrast in another way that inoculum size was the critical positive factor. The production of keratinase was modestly inhibited by the addition of sodium sulphate to medium containing chicken feathers as a carbon source. It may be due to the reason that chicken feathers are an excellent carbon as well as a sulfur source for keratinase production by *Pseudomonas aeruginosa-C1M.*

3.4.6. Keratin hydrolysate preparation and antioxidant extractions

Pseudomonas aeruginosa-C1M was used to ferment chicken feather deterioration. In these circumstances, effective feather degradation results in the production of free amino acids, soluble peptides, and thiol groups. The fermentation supernatant that resulted was utilized to measure anti-oxidative activity using the FRAP assay. From the hydrolysate bioactive peptides were precipitated out due to the acid precipitation mechanism. Addition of acids in the hydrolysate decreased the pH that imparted positive charge to the protein due to the capturing of hydrogen ions by amino group. This disturbance in the hydration sphere is responsible for imbalance in structure leading to precipitation. Pellet containing bioactive peptides were purified through Q-sepharose colum. Peptide concentration of fractions was determined through spectrophotometer at 210 nm absorbance (Fig. 3.12) and antioxidant properties were evaluated by FRAP assay shown in (Fig. 3.13).

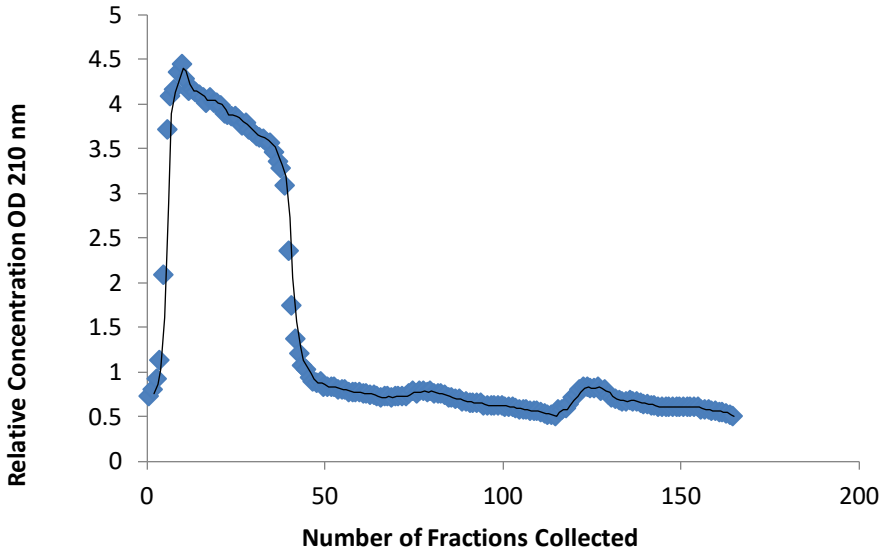

Fig.3.13. Determination of peptide concentration of fractions at 210nm

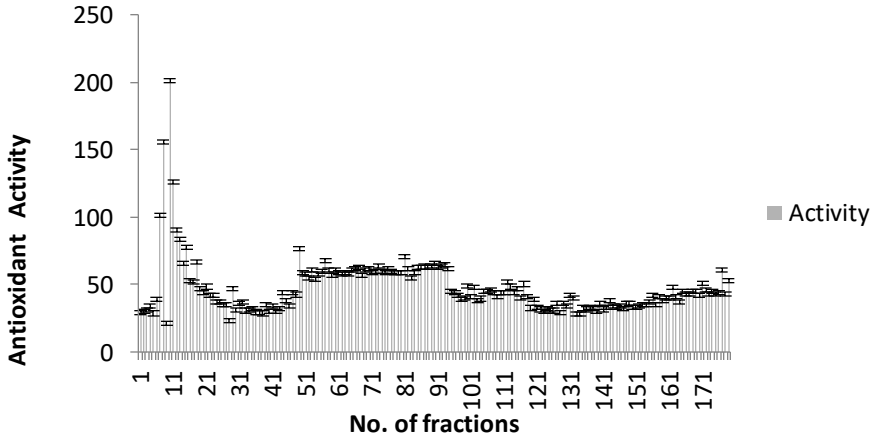

Fig.3.14. FRAP anti-oxidant elution profile of fractions obtained from keratin hydrolysate

3.5. Conclusion

According to the findings, optimising the nutritional and physical factors boosted the isolated *Pseudomonas aeruginosa*-C1M strain's keratinase production, which further resulted in a much shorter time period needed for feather disintegration. In the study, it was indicated that keratinolytic bacteria could be used to bio-convert waste into products

with value additions. Bioactive peptides having antioxidant potential were extracted from keratin hydrolysate.

3.6. Acknowledgement

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3.7. Conflict of interest

We wish to confirm that there are no known conflicts of interest associated with this publication.

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Chapter 4: Purification and characterization of microbial keratinase

Paper 2 from chapter 4:

Paper Title: Purification and characterization of keratinase of *Pseudomonas aeruginosa*-C1M and its perspective application in synergism to laundry detergents

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4.1. Abstract

Enzymes are deemed as the green chemicals of the environment due to their renowned endowments in various commercial industries. Among these great catalysts, microbial keratinolytic proteases are considered indispensable for their ability to consume agro-based waste, thus combatting pollution and adding practicality to poultry businesses. The significant role of these enzymes in various industries, such as pharmaceuticals, food, plant fertilizers, animal feed, and laundry detergents, is undeniable. In context, the present study focused on the purification of keratinase from the newly isolated strain *Pseudomonas aeruginous-C1M.* After evaluating its properties, its role was assessed as a bio-additive agent in detergents. After partial purification extracellular keratinase (32kDa) has shown 25.06 U/mg specific activity, however it increased to 74 U/mg after gel filtration chromatography. The optimum pH of purified keratinase was 8 and stable at 37-50°C. Besides feather keratin, the enzyme was active upon the soluble proteins keratin azure, bovine serum albumin (BSA), and casein; however, no conspicuous activity was noticed in the presence of elastin, gelatin, and insoluble hairs.

Keratinase displayed high compatibility and stability with detergents by efficiently removing the proteinaceous stains from fabric pieces. Keratinase enzyme was observed to be highly stable with three commercial detergents recognized as A, B, and C and interestingly 40-73% improvements in activity were observed as compared to control when treated with only commercial detergents. The enzyme increased activity with detergent A compared to detergent B and C. Furthermore, keratinase could remove potential egg yolk, blood and chocolate stains from cloth pieces without affecting the texture and strength of the cloth fiber. Thus the potential keratinase from *Pseudomonas aeruginosa* C1M was a suitable choice as a bio-additive in detergent formulations.

Key words

Keratinase, Detergents, Bio-additives, Destaining

4.2. Introduction

With the dawn of the digital industrial age, and technology evolution several environmental and health concerns lead to the development of chemical methods used to accomplish various industrial processes. Chemicals are employed to improve products; however, their toxicity made them undesirable substances. Thus, biological implications, instead of chemicals are

being used in industrial processes. Adding biological alternatives to chemical processes is the solution to cope with environmental and health issues of chemicals.

Among biological substances, specifically microbial enzymes are essential in the bio-economic arena. The global enzyme market covers almost 60% of proteolytic enzymes (Hossain et al., 2007). Keratinase is one of the most imperative proteolytic biocatalysts, which converts the recalcitrant keratin (protein) into polypeptides and amino acids (Nnolim et al., 2020). Abundant polymer, keratin exists in nature in the form of hair, feathers, horns, hoofs, scales, beaks, wool and nails (Sahni et al., 2015). Annually, a large amount of keratin-based waste is being generated from chicken farms, poultry enterprises, and slaughter houses, thus causing health and catastrophic issues (Yusuf et al., 2016).

Keratin is a ubiquitous biological entity that is robust, chemically stable, poorly soluble and sulfur-containing biopolymer found abundantly in nature (Bagewadi et al., 2018). On the basis of secondary structure, keratin is classified into α-helixes, which are found in wool and hair (Isarankura Na Ayutthaya et al., 2015), and β-sheets, the main component of feathers (McKittrick et al., 2012). Based on sulfur content, keratin is grouped into soft and hard keratin. Soft keratin has fewer disulfide bonds (callus and epidermis) (Coulombe and Omary, 2002); however, in hard keratin, disulfide content is higher (Hair, feathers, horns, claws, and hoofs), making them more resistant to degradation. Hydrogen bonding, disulfide cross-linking, and hydrophobic interactions give keratin its mechanical stability (Sahni et al., 2015). Common proteolytic enzymes trypsin, pepsin and papain cannot break down keratin (일 et al., 2009) but keratinolytic enzymes are nature‘s solution to this significant problem.

Keratinases belonging to the class of proteolytic enzymes are deemed the most promising and suitable enzymes due to their ability to be stable in the broad ranges of pH, temperature, and other harsh environmental conditions. These predominantly attack the subsequent peptide linkages within the keratin substrate structure and change it into simple conformation. Keratinases reduce disulfide bonds and proteolytic cleavage for the efficient degradation of keratin. Depending on the catalytic reaction, keratinases are often categorized as serine proteases or metalo-proteases (Nnolim et al., 2020).

Microbial keratinase (E.C. 3.4.21/24/99.11) is an exceptional biocatalyst produced by various bacteria, yeasts, fungi and actinomycetes (Gopinath et al., 2015). It has the extraordinary capacity to transform keratinaceous feather waste into useful industrial products including fertilizers, animal feed, cosmetics, and detergent supplements (Saleem et al., 2012). Physico-

chemical treatments of keratin waste are generally considered eco-destructive. However, keratinases are auspicious, eco-safe, and less expensive, converting poultry waste into value-added compounds.

Feathers keratin is converted into keratin hydrolysate by keratinase, and the hydrolysate is used as nitrogen-based fertilizers (Chew et al., 2019) for plants and nutritious feed for animals and aquaculture (Deivasigamani and Alagappan, 2008). Moreover, keratinases are employed in cosmetic and detergent formulations, feedstuff, drug delivery carriers (Brandelli, 2008), and textile and food applications. Meanwhile, several pharmaceutical and protein supplement products are manufactured by involving keratinases. Biogas and bioenergy production is also linked with keratinases.

Furthermore, microbial keratinases have been hailed due to their remarkable cleaning properties against proteinaceous stains. Supplemented commercial detergent with the enzyme acts as a bio-additive and is known to improve and restore the quality of fabrics. The cleaning action of detergents is primarily based upon the involvement of various bleaches, oxidizers, builders, perfumes, foam regulators, reducing agents, whitening agents, and many other sequestering solutions (Bhange et al., 2016). These compounds serve to be highly toxic to the environment, whether it is air, soil, or water, where these are known to produce poisonous foam. Moreover, various surfactants such as linear alkyl benzene (LAS) contaminate potable water, abolishing the lungs and other vital organs in the human body and disturbing the marine environment triggering eutrophication (Paul et al., 2016). The currently available commercial laundry detergents not only make off chemical filtrates on clothes but also serve as a source of irritability for human skin, leading to various allergies, asthma, and chronic irritation (Cavello et al., 2012). Thus, the leading solution to this situation is the employment of green technologies in the detergent industry. Keratinases are considered the most emerging additives for eco-friendly and sustainable detergents by withstanding the harsh chemical conditions. The present study focuses on purifying the keratinase from *Pseudomonas aeruginosa* C1M and characterization of enzymatic properties. The enzyme was also used in detergent formulations as a bio-additive for de-staining reasons.

4.3. Materials and methodology

4.3.1. Chemicals and reagents

Most chemicals used for research purposes were mainly obtained from Merck (Germany), Sigma (USA), Acros (Belgium), Fluka (Switzerland), and BHD (UK). Raw substrates were obtained from the local markets of Pakistan.

4.3.2. Chicken feathers processing

Chicken feathers were taken from the local poultry slaughter shops**.**

Blood stains and detritus were removed from feathers by thoroughly washing them with tap water and mild detergent to maintain their structural integrity. The feathers were then air-dried and parched in a hot oven at 50°C for 30 minutes. The obtained pre-treated feathers were bright white and were milled into small pieces for their degradation by the keratinolytic strain.

4.3.3. Keratinase production

In this study, keratinolytic strain *Pseudomonas aeruginosa* C1M (Saba et al., 2022) was used. Keratinase production was done using a Feather Basal medium (FBM) (Qu et al., 2018) under submerged fermentation. For the prospective keratinase production, the flask was seeded with *Pseudomonas aeruginosa* C1M and kept at 37°C for 48 hrs. at shaking conditions of (200 rpm). After 15 minutes of centrifugation at 3,000 g and 4 degrees Celsius, the culture supernatant was stored at -20 °C for later use as crude keratinase.

4.3.4. Purification of keratinase

After complete hydrolysis of chicken feathers at optimal culture conditions, crude supernatant was collected. By using a lyophilizer (labconco), crude supernatant was concentrated ten-fold. The supernatant was then precipitated with ammonium sulphate. Several proportions of ammonium sulfate salt 10-70% were added into the supernatant, gradually at 4°C and constant agitation for 30-45 minutes. The fractions were centrifuged at 10,000 rpm for 20 minutes after incubating them at 4°C for the night. Enzyme-containing pellets were obtained and subsequently dissolved in phosphate buffer (pH 7) monobasic disodium-hydrogen phosphate (2.2g/L) and dibasic monosodium-hydrogen phosphate (0.4g/L) and Sodium Chloride (8.5g/L). Dialyzing against the phosphate buffer (pH 7) yielded pellets with increased enzyme activity and protein content, which were then processed. Gel filtration column chromatography was then used to separate the active fractions from the clear supernatant using 0.05mM phosphate

buffer (1 ml.min-1). Each purification stage involved obtaining unbound fractions of active keratinase, checking the enzyme's purity using SDS-PAGE, and performing an activity experiment.

4.3.5. Biochemical characterization of Keratinase

4.3.5.1. Temperature optimization

Experiment was performed at temperatures ranging from 30 to 80 °C in 50mM phosphate buffer (pH 8.0) to establish the optimal temperature for keratinase activity.

4.3.5.2. pH optimization

By introducing pure enzyme to a reaction mixture including keratin azure in 50 mM phosphate buffer solution, the influence of pH on enzyme activity could be measured. The enzyme mixture's pH was changed from 3 to 10 using the following buffer systems: 0.05 M sodium acetate buffer at pH 3, 4, and 5, potassium phosphate buffer at pH 6, 7, and 8, Tris HCL buffer at pH 9, and sodium hydro-oxide/disodium hydrogen phosphate buffer (pH10).

4.3.5.3. Time optimization for activity assay

Additionally, the impact of various time periods on the pure keratinase activity was assessed. The reaction mixture was incubated for 30 minutes to 24 hrs. at the ideal temperature and pH under carefully chosen circumstances. The activity profile was used to establish the maximum incubation duration.

4.3.5.4. Substrate specificity optimization

The uniqueness of the enzyme towards various substrates was evaluated. Keratin azure, casein, gelatin, feathers and hair were used as substrates for the evaluation of keratinase activity.

4.3.6. Potential laundry detergents-enzyme compatibility and stability

The stability of keratinase was evaluated by using varying commercial laundry detergents, following crucial factors as detergent concentration; reaction‘s temperature and incubation period were quantified and optimized under the optimal standards of enzyme activity assay.

4.3.6.1. Optimization of detergent concentration

Investigations were undertaken into the stability and compatibility of pure keratinase with commercial laundry detergents produced in Pakistan. The enzyme's compatibility with three

commercial detergents was determined by dissolving each laundry detergent in tap water to form stock solutions. The enzyme was assessed with the three detergents referred to as detergent A, detergent B, and detergent C at concentrations of 2 mg/ml, 7 mg/ml, and 9 mg/ml. The controls associated with the sample mixture contained tap water, detergent, enzyme, buffer, and synthetic substrate as keratin azure.

4.3.6.2. Optimization of reaction temperature

The optimum temperature at which the enzyme-detergent combination will impart maximum activity at different temperature ranges following the standard enzyme assay. The ranges were between 40℃ to 60℃ maintained at pH 8.0. The observed activity was according to the enzyme assay, and residual activities for each detergent enzyme mixture were calculated.

4.3.6.3. Optimization of incubation time

The optimum time was determined to get the maximum detergent enzyme stability and activity. The reaction mixture was kept in incubation for 30 min, 60 min, 90 min and activity were observed after every 30 min interval.

4.3.7. De-staining application of keratinase

The goal was to utilize the potential of keratinase as a bio-additive in detergents; hence cloth washing was conducted to remove the stains of egg yolk, blood and chocolate from cotton cloth. The cotton fabric was divided into numerous pieces, each measuring 4 cm by 4 cm, and each was stained and air dried. The stained bits were then shifted to three sets of 9 petri plates to perform the spectrophotometric and visual analysis with each detergent A, B, and C in combination with enzyme and stains. Control petri plates contained only tap water (30 ml), the second plate had detergent (7 mg/ml) with tap water (30 ml), and the third plate was composed of detergent (7 mg/ml) in combination with the crude keratinase (11U/ml). Each plate was then incubated at 50℃ for 30 min in the oven (as per the conditions previously optimized for the reaction). After keeping in incubation, fabric pieces were taken out and air dried to visually analyze the extent of enzymatic de-staining (brightness obtained) when supplemented with detergents.

4.4. Results and Discussion

4.4.1. Keratinase purification from *Pseudomonas aeruginosa*-C1M

For keratinase purification, initially enzyme production was carried out using keratinolytic strain *Pseudomonas aeruginosa* C1M under optimal fermentation conditions. Crude supernatant was partially purified by using ammonium sulfate $(NH_4)_2SO_4$ salt. Maximum enzyme precipitation, with specific activity of 25.06 U/mg was found when 60% ammonium sulfate concentration was achieved. Fig.4.1. is depicting ammonium sulphate precipitation results of supernatant and pellet.

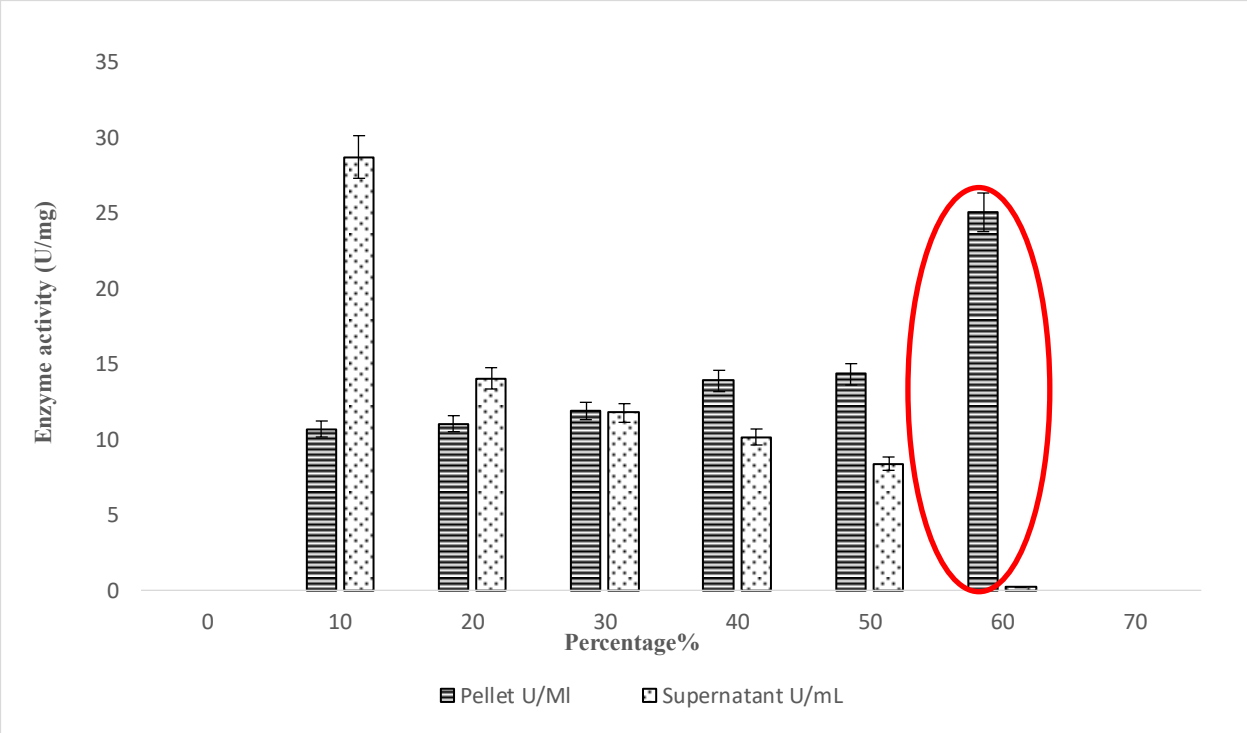

Fig.4.1. Ammonium sulphate precipitation of Pseudomonas aeurignosa C1M keratinase and evaluation of enzyme activity of pellet and supernatant at various percentage of ammonium sulphate precipitation

The findings showed similarity with the result shown by Ferrareze in which keratinase from *Bacillus subtilis* was precipitated by 60% ammonium sulphate (Ferrareze et al., 2016). Whereas Hamiche et al.also precipitated keratinase from *Bacillus amyloliquefaciens* S13 by ammonium sulphate precipitation (65%) (Hamiche et al., 2019). Dialysis was followed by gel permeation column chromatography. The total recovery yield was 43.68%, and the purification factor was around 2.98 times. The finished item had a specific activity of around 74.6U/mg (Table. 4.1). On SDS-PAGE, the keratinase's active final portion was visible as a single 32 kDa band. In another study conducted by Dhavia et al, a 56 kDa keratinase from *P.aeruginosa* was purified for valorization of feathers substrate (Wu et al., 2014). However, in the following study the active keratinase of *Pseudomonas aeruginosa*-C1M was 32-33kDa (Fig. 4.2). It has been reported previously that keratinase consist of pre-pro and mature region of protein (Su et al.,

2019). After synthesis of keratinase pro-peptide region act as chaperon and helps in folding of mature protein. Therefore, the size of mature protein reduced.

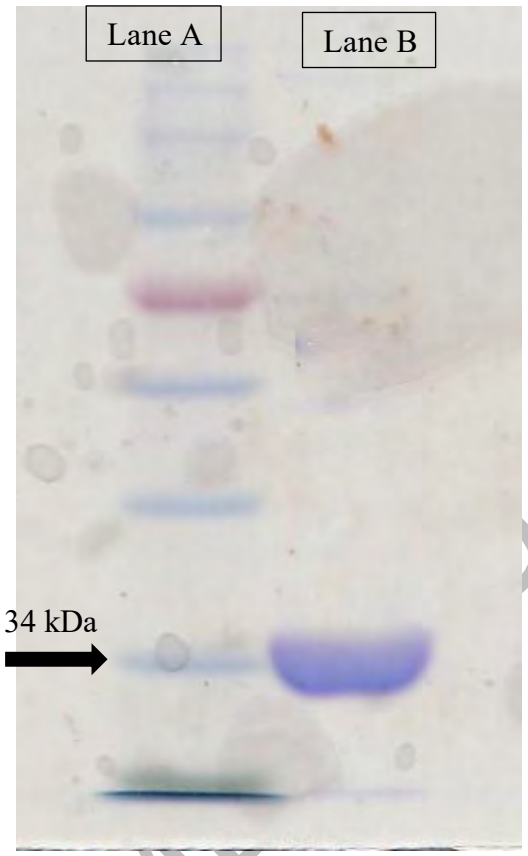

Fig.4.2. SDS-PAGE of purified keratinase from Pseudomonas aeruginosa C1M Lane A. depicting standard SDS marker (NEB p7712S) while Lane B. is showing purified keratinase from Pseudomonas aeruginosa C1M.

4.4.2. Biochemical characterization of purified keratinase

4.4.2.1. Temperature optimization

A temperature range of 30-80 °C was used to measure keratinase activity. According to research, pure keratinase functions best at 37°C, with a maximal specific activity of 74.07 U/mg. At temperature above 60 °C, the keratinase activity was measured to be decreased. Possible reason to this reduction in activity is due to the collision of substrate molecules that produces strong vibrational force of molecule at high temperature which leads to the breaking of hydrogen and ionic bond, thus reducing enzyme activity. Graph showed (Fig.4.3) that any increase or decrease in temperature initiated a strong change in enzyme activity. Deniz et al. (2019) studied that as compared to the current investigation, the pure keratinase maintained up to 95% of its activity for 1 hour at 28–45°C which is in similarity of current study (Deniz et al., 2019). Less enzyme's maximum activity was observed at lower temperature (51.6 U/mg) that is due to slow motion of enzyme molecules hence reducing the flexibility to act as catalyst. And

also substrate could thus require longer time for the enzyme to hydrolyze it, but this does not rule out the possibility of stability at high temperatures (Moridshahi et al., 2020). At higher temperatures, keratinase activity reduced due to the sensitivity of the enzyme to thermal inactivation.

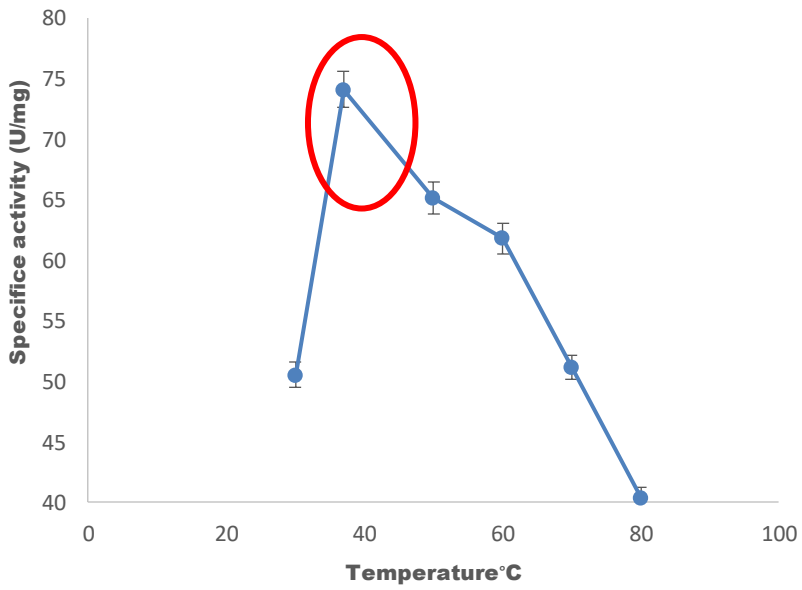

Fig.4.3. Effect of different temperatures on the activity of purified keratinase of Pseudomonas aeruginosa C1M Circular region of graph shows the maximum keratinase activity at temperature 37°C

4.4.2.2. pH optimization

Various ranges from acidic to neutral and alkali were selected for the characterization of pH. The optimal pH was found to be 8.0, with a maximal specific activity of 74.63 U/mg. The enzyme was more active between pH 6.0 and 8.0. Above pH 8, keratinase activity was decreased (Fig. 4.4).

By deprotonating and protonating polar groups, which results in the loss of active site conformation and affects pH activity, the intramolecular stability is compromised. For commercial use, the stability and activity across a wide pH range could be advantageous because precise pH control will not be required.

A considerable decrease in activity for keratinase was observed above and below pH 8. It concluded that pH is the most significant factor for maximum enzyme activity. Higher acidic

and alkaline pH levels result in a considerable reduction in activity because of the denaturation of the enzyme and modifications to its catalytic folding state. Minghai Han. (2012) reported that purified keratinase from *Pseudomonas aeruginosa C11* shows maximum activity at pH 7.5 (Minghai Han, 2012) Because of its stability and capacity to function at a variety of alkaline pH (10–11), the keratinase isolated from K. rosea enables advances in procedures with high pH requirements (Bernal et al., 2006).

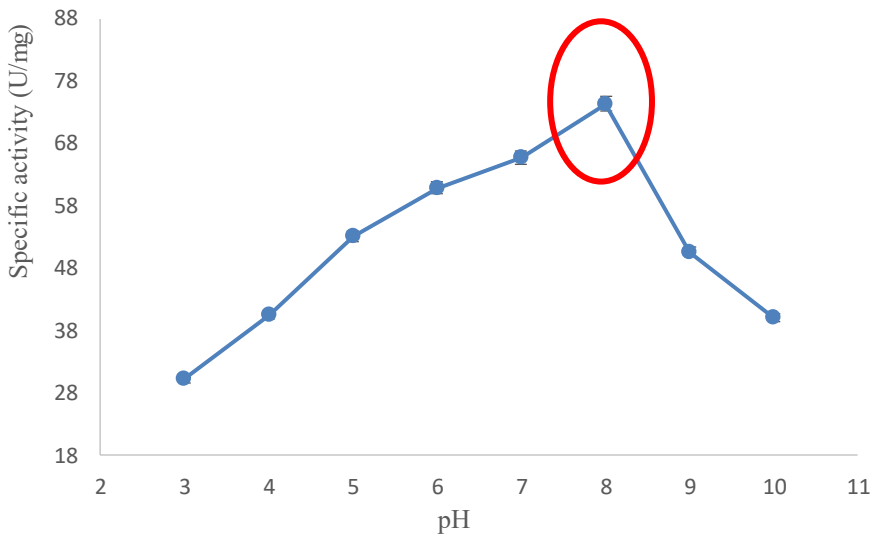

Fig.4.4. Effect of various pH on the activity of purified keratinase of Pseudomonas aeruginosa C1M Circular region of graph shows the maximum keratinase activity at pH 8.

4.4.2.3. Time optimization for activity assay

It was investigated that maximum activity of 74.91 U/mg for purified keratinase was obtained after 1hour. The reduction in the activity is due to depletion of enzyme that could convert substrate into product.

4.4.2.4. Effect of various substrates on the activity of keratinase

The enzyme has a maximum hydrolytic activity towards keratin, followed by feathers, casein, elastin, BSA, hair, and gelatin (Fig.4.5). The strong degradation activity of keratin azure and feathers is due to the presence of β-keratin. However, α-keratin in hair leads to the problematic decomposition of the helical structure. Also, cysteine bridges in hair protein hinder the β-keratinase action. Based on the previously published finding that β-keratins from feather are degraded by keratinolytic proteases at a significantly faster rate than α-keratins from hair and

wool, the results demonstrated that the purified separated protease was much more active against feather powder than hair powder (Fang et al., 2013). Moreover, natural and raw substrates are also rigid to degrade because of the presence of solid disulfide bonds, hydrophobic interactions, and cysteine groups.

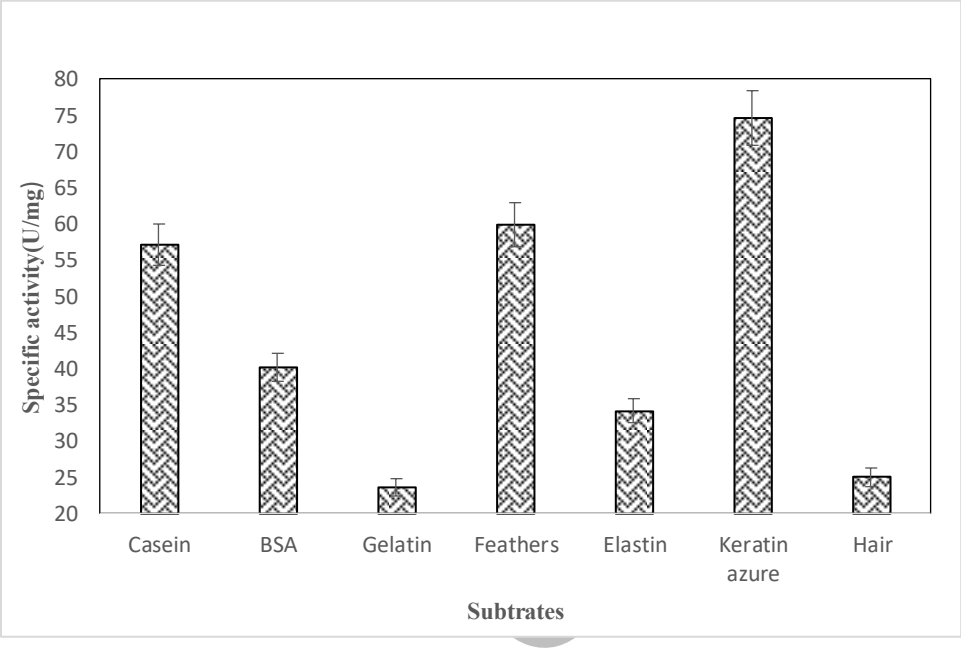

Fig.4.5. Effect of various substrates on the activity of purified keratinase of Pseudomonas aeruginosa C1M

4.4.3. Potential laundry detergents-enzyme compatibility and stability

4.4.3.1. Optimization of detergent concentration

For the determination of stability of keratinase with various commercially available detergents, various ranges of detergent from 2 to 9 mg/ml were evaluated. At the initial concentration of 2 mg/ml, the keratinase (74U/mg) displayed adequate stability with the three detergents. Similar trend was observed at 7 mg/ml concentration of detergent. Moreover, it was noticed that not only the enzyme is stable in the presence of detergents but a remarkable increase in activity was clearly observed. However, the potential activity and stability of keratinase with 9 mg/ml detergent concentration and further was observed to be highly reduced, indicating inactivation of the enzyme in the presence of the detergent Fig. 4.6.

Laundry detergents usually contain high concentrations of surfactants, such as Triton X-100, Tween-20, SDS, and Tween-80. Those surfactants and chaotropic agents have great influence on protein structure. So enzyme stability in different surfactants and chaotropic agents is an important parameter of commercial enzymes. Enzyme activity was destroyed by increasing concentrations of chaotropic compounds because these compounds at higher concentration reduced the hydrophobic effects which were essential for maintaining three dimensional

structure of keratinase, thus leading to the unfolding and denaturation of enzyme. Therefore, higher concentration of detergent reduced the stability of keratinase.

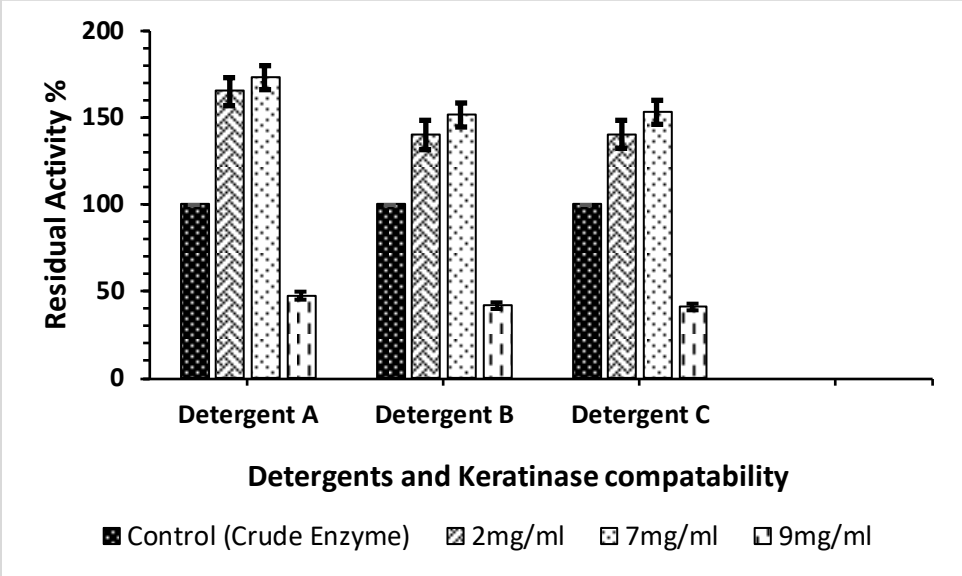

Fig.4.6.Compatability optimization of keratinase concentration with various concentrations (2,7 and 9mg/ml) of detergents

4.4.3.2. Optimization of reaction temperature

Temperature optimization for maximal enzyme-detergent activity was performed at different temperature ranges, including 40°C, 50°C and 60°C by keeping detergent concentration 7mg/ml. At 40°C residual activity observed for the keratinase with three detergents as 144% (Detergent A), 125% (Detergent B) and 129% (Detergent C) respectively. Maximum activity of enzyme was obtained at 50°C as 168% (Detergent A), 143% (Detergent B) and 147% (Detergent C) respectively. Further increase in temperature decreased the activity indicating the instability of keratinase with detergents at higher temperature. Thus, at optimized 7 mg/ml detergent concentration, 50°C was found as optimum temperature facilitating the soundness of the reaction mixture (Fig.4.7)

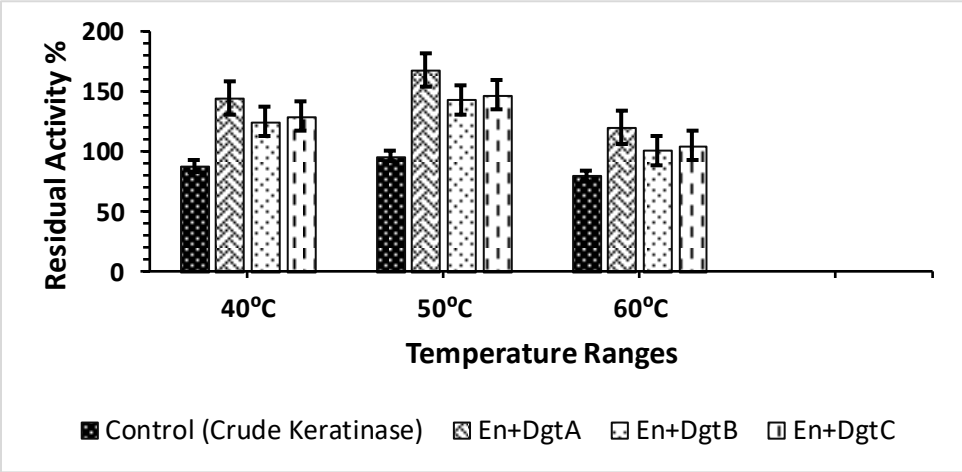

Fig.4.7.Optimization of temperature for keratinase- detergents activity at 7mg/ml detergents concentration

4.4.3.3. Optimization of incubation time

The appropriate incubation period was determined for the enzyme-detergent reaction mixture by estimating the activity at interval of 30 mins, 60 mins and 90 mins in accordance to the keratinase activity assay. Temperature and detergent concentration was maintained at optimum 50°C and at 7 mg/ml, respectively. Keratinase-detergent complex efficiently work at this optimized conditions. Maximum activity of keratinase with each detergent was observed at 30 mins of incubation as 172% (Detergent A), 156% (Detergent B) and 161% (Detergent C) respectively. Activity obtained at 60 mins and 90 mins was noted to be slightly less signifying that the enzyme is stable with detergents over a period of 90 mins. Thus, 1 hr is deemed as the optimum incubation time for the reaction mixture as this is normal washing span of clothes. At this time period the enzyme is not inactivated by the potential surfactants, metal ions and chelating agent in detergents and retains its stable structure and activity (Fig.4.8).

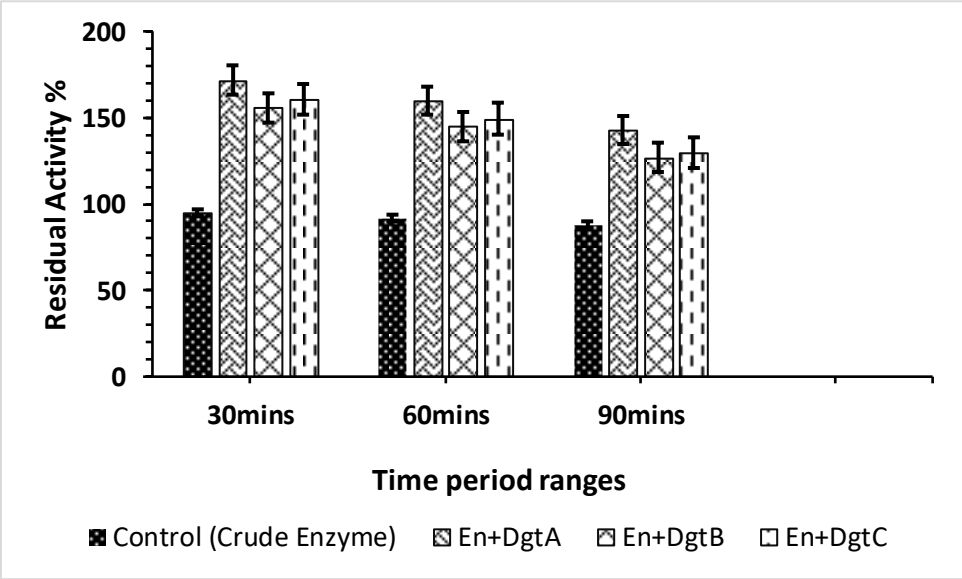

Fig.4.8. Incubation period optimization for the detergent-enzyme stability by keeping detergent concentration 7mg/ml and temperature50°C

4.4.4. De-staining application of keratinase

Evaluation of stain removal capacity of keratinase-detergent reaction mixture (for wash-performance analysis)

Cotton fabric was stained with egg yolk, blood, and chocolate smear to test the efficacy of the enzyme-detergent reaction combination in eliminating the stains and to use keratinase as a viable bio-additive in laundry detergents. The evaluation analysis was performed by determining the cloth brightness spectrophotometrically and by visual analysis.

4.4.4.1. Analysis of percentage brightness

4.4.4.2. Blood-stained cloth pieces

Maximum percentage brightness was achieved upon washing the cloth piece with keratinase and detergent composite .i.e. 198% (Detergent A), 190% (Detergent B) and 193% (Detergent C). The measure of percentage brightness with control (unstained cloth), only tap water and only detergent was determined to be less, thus, concluding that keratinase has efficient cleaning properties against the proteinaceous stains and can act as a strong bio-additive in various detergent formulations (Fig.4.9). For blood stain, 93 to 98% brightness was observed upon washing with keratinase supplemented with detergent mixture. This is because keratinase contacts with the blood haemoglobin in blood stain. Hemoglobin is a multi-globular protein that consists of four subunits with one polypeptide chain and a heme group. Within the heterocyclic ring of prophyrin, the R groups, which are the globin proteins, are cleaved by the action of the keratinolytic protease, which is the cleavage of the peptide bonds in between the proteins. This converts the R groups to the subsequent amino acids, which in turn degrades the hemoglobin structure in the blood stain and ultimately removes it (Paul et al., 2014).

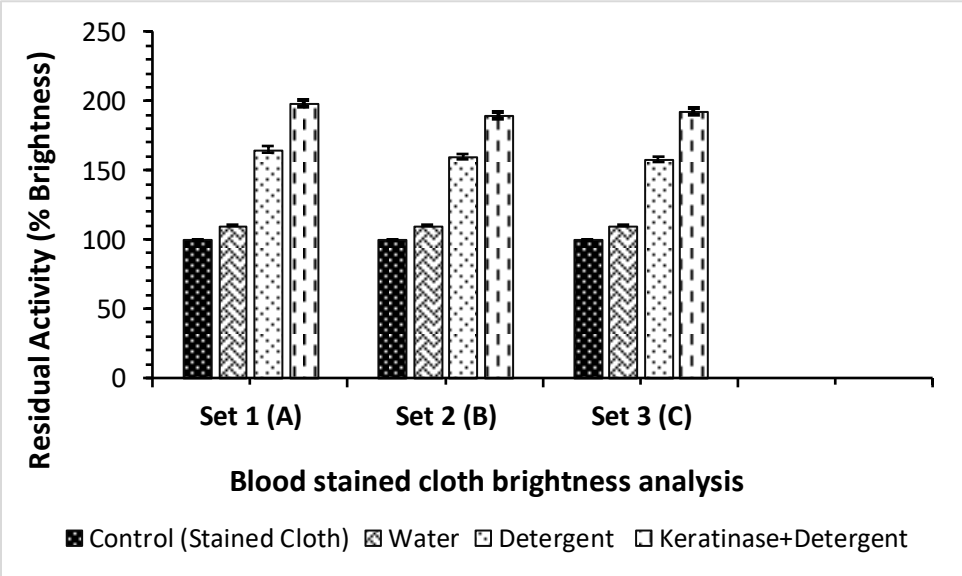

Fig.4.9. Comparative brightness analysis of blood stains with keratinase-detergents mixture by keeping water, respective detergent and stained cloth as control.

4.4.4.3. Egg yolk stained cloth pieces

It was estimated that the keratinase-detergent composite has highest activity i.e. 198% (Detergent A), 194% (Detergent B) and 196% (Detergent C) in comparison to the one observed with control (unstained cloth), tap water and detergent alone. This signifies that keratinase is a highly detergent stable enzyme (Fig. 4.10). For yolk stain, 94 to 98% brightness was observed upon washing the cloth with keratinases supplemented detergent mixture. Egg yolks are highly proteinaceous in nature consisting of albumin and lipoproteins. Therefore, the degradation of their respective protein in the presence of keratinases lead to the efficient removal of yolk stain from the cloth.

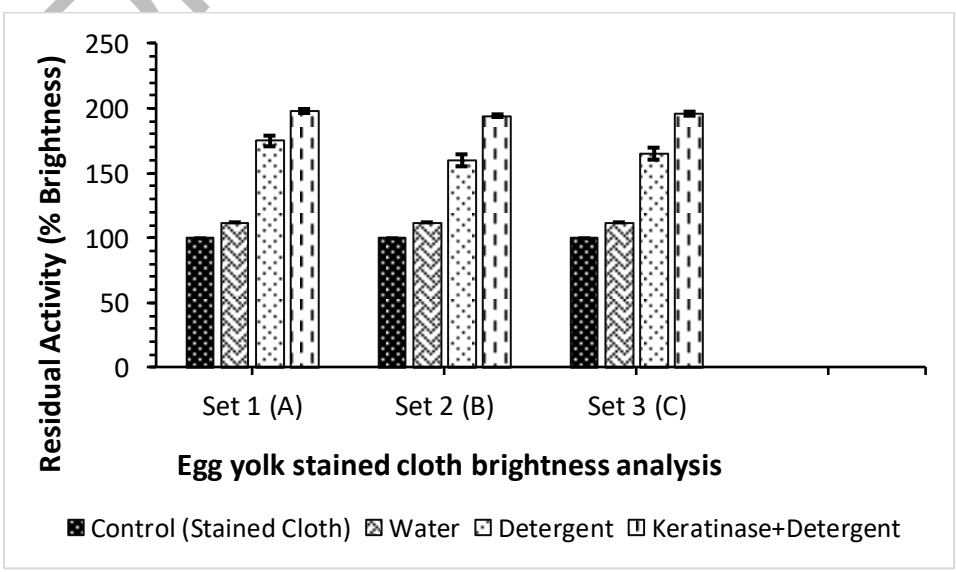

Fig.4.10. Comparative brightness analysis of egg-yolk stains with keratinase-detergents mixture by keeping water, respective detergent and stained cloth as control

4.4.4.4. Chocolate-Stained Cloth pieces

In case of chocolate smear, the stain was adamant and was not completely removed. However, the maximum degree of stain lightning was recognized when the cloth was suspended in keratinase and detergent composite i.e. 152% (Detergent A), 141% (Detergent B) and 135% (Detergent C). Only tap water and detergent alone did not much affect the stain and little to no degree of fadedness was observed (Fig. 4.11). For chocolate stain only 35 to 52% of brightness was observed, concluding that chocolate is a compound richer in carbohydrate than proteins and therefore the small protein content is degraded by the proteolytic keratinases but the carbohydrate part retains.

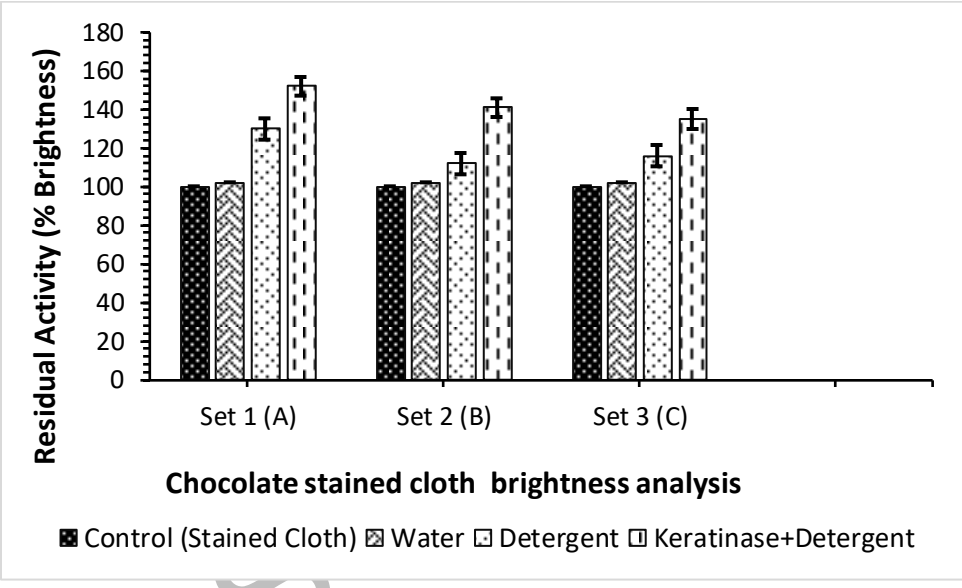

Fig.4.11. Comparative brightness analysis of blood stains with keratinase-detergents mixture by keeping water, respective detergent and stained cloth as control.

4.4.5. Visual Examination of washing performance of keratinase in combination with commercial laundry detergents

The visual inspection of stained cloth pieces was carried out after washing in order to examine the function of enzyme as a bio-additive in detergents and increasing the washing performance of detergents. The supplemented enzyme efficiently cleared the stains in comparison to the removal by water and detergent alone. However, it was visualized that among the three proteinaceous stains, chocolate stain being greasy was removed slightly. Below is the visual depiction of wash performance.

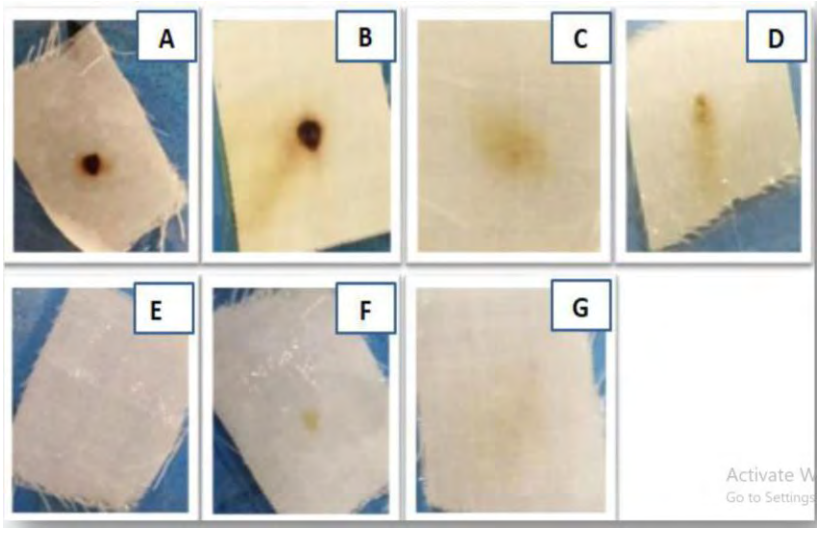

Fig.4.12. (A) Blood-stained cloth, (B) Blood-stained cloth washed with water only, (C) Blood-stained cloth washed with Detergent A, (D) Blood-stained cloth washed with Detergent B, (E) Blood-stained cloth washed with Detergent A supplemented with crude Keratinase, (F) Blood-stained cloth washed with Detergent B supplemented with crude Keratinase, (G) Blood-stained cloth washed with Detergent C supplemented with crude Keratinase.

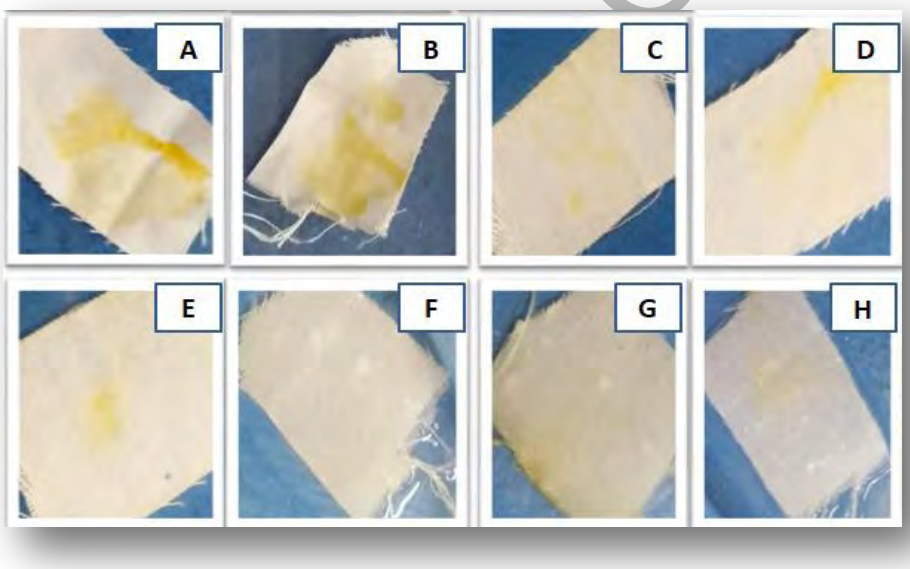

Fig.4.13. (A) Egg yolk-stained cloth, (B) Egg yolk-stained cloth washed with water only, (C) Egg yolk-stained cloth washed with Detergent A, (D) Egg yolk-stained cloth washed with Detergent B, (E) Egg yolk-stained cloth washed with Detergent C, (F) Egg yolk-stained cloth washed with Detergent A supplemented with crude Keratinase, (G) Egg yolk-stained cloth washed with Detergent B supplemented with crude Keratinase, (H) Egg yolk-stained cloth washed with Detergent C supplemented with crude Keratinase

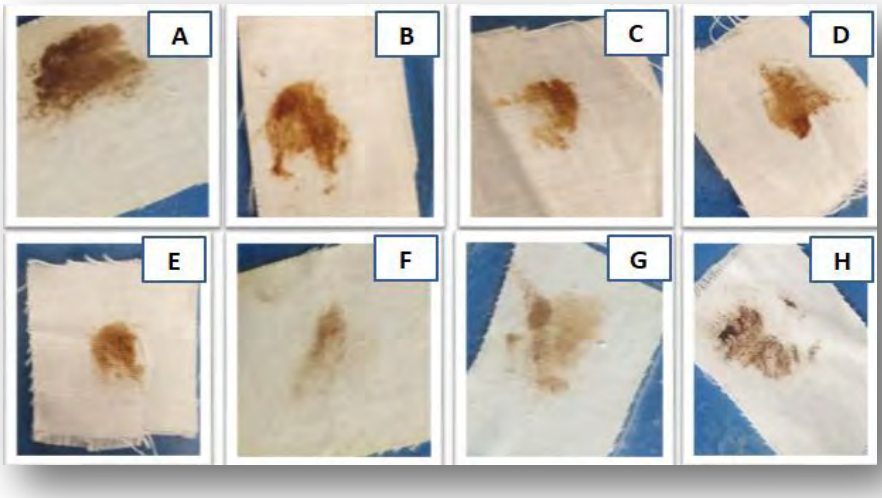

Fig.4.14.. (A) Chocolate-stained cloth, (B) Chocolate-stained cloth washed with water only, (C) Chocolate-stained cloth washed with Detergent A, (D) Chocolate-stained cloth washed with Detergent B, (E) Chocolate-stained cloth washed with Detergent C, (F) Chocolate-stained cloth washed with Detergent A supplemented with crude Keratinase, (G) Chocolate-stained cloth washed with Detergent B supplemented with crude Keratinase, (H) Chocolate-stained cloth washed with Detergent C supplemented with crude Keratinase

4.5. Conclusion

Keratinase has efficient cleaning properties against the proteinaceous stains and can act as a strong bio-additive in various detergent formulations. This bioprocessing of feathers by *Pseudomonas aeruginosa* C1M represents a technical approach for feather waste management and recycling into valuable enzyme based detergents.

4.6. Acknowledgement

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4.7. Conflict of interest

There is no conflict of interest.

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Chapter 5: Utilization of microbial keratinase for the production of value-added compounds.

Paper 3 from chapter 5:

Title: Synthesis of silver nanoparticles (AgNPs), through bio-reduction utilizing crude Keratinase from *Pseudomonas aeruginosa*-C1M as a precursor, its characterization and applications as anti-bacterial agents and in dye degradation**.**

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5.1. Abstract

Nanoparticles are considered potent agents for various industrial sectors. This study explores the biogenic synthesis of zero-valent silver nanoparticles (AgNPs) through crude keratinase of *Pseudomonas aeruginosa*-C1M. By using color visualization, which ranged from transparent to dark brown, biogenic silver nanoparticles (AgNPs) were confirmed. A single, distinctive peak of UV-Visible spectroscopy was observed at ~450nm. FTIR inference proved that silver nanoparticles were capped with proteins (crude keratinase). AgNPs produced by biogenic synthesis were found to be crystalline in nature, with face-centered cubic lattice planes, according to X-ray diffraction. Silver nanoparticles (AgNPs) were discovered as mono-dispersed spheres of various sizes, according to morphological imaging techniques such as transmission electron microscopy (TEM) and scanning electron microscopy (SEM). The diameter of AgNPs was also determined by Dynamic light scattering (DLS) measured as ~ 119 nm. High dispersion, long-term stability, and superior colloidal characteristics are supported by a high negative zeta potential value. It was discovered that green-produced silver nanoparticles have antibacterial zone of inhibition against pathogenic strains of *Staphylococcus aureus* and *E. coli.* 25mm and 33mm, respectively.

The synthesized zero-valent silver nanoparticles (AgNPs) aided in the decontamination of azo-dyes (methyl red, methyl orange, safranin O, and methyl violet) by involving sodium borohydride (NaBH4) and light catalyzed processes. *Pseudomonas aeruginosa* C1M was used for the first time to produce AgNPs. Keratinase of *Pseudomonas aeruginosa*-C1M mediated AgNPs not only have antimicrobial properties but were also involved in converting toxic dyes into a less toxic form. Based on the results, biosynthesized AgNP potential could be explored in the biomedical domain.

Keywords:

Keratinase, Biogenic synthesis, Nanoparticles, Antimicrobial, Dye decontamination

5.2. Introduction

With the convergence of nanotechnology and biology, a new field known as Nano-biotechnology has emerged, combining molecular motors, nano-bio materials, nano-crystals, and biochips with inter-disciplinary biotechnology and nanotechnology techniques. The primary goal of nanotechnology is to synthesize and stabilize various metallic and non-metallic nanoparticles to give them unique properties to be employed in various fields. The key constraints for the novel properties of nanoparticles include their shape and size (Mukherji et al., 2019). Noble metal nanoparticles are more appealing and have beneficial uses in different

industries like cancer therapy, electro-catalyst, photo-catalysts batteries, etc. (Mavaei et al., 2020). Due to increased surface area in nanoparticles that improve the characteristics of noble metals, enormous applications are possible (De Oliveira et al., 2020).

Among them are also included silver nanoparticles. They are of 100nm consist of 20-15,000 silver atoms per particle, which hold distinguished chemical, physical, and biological attributes, contrasting their primary materials. Better physicochemical properties and thermal and electrical conductivity increased AgNP's potential among other noble metals. AgNP's exclusive behavior exhibited in the biological activity makes them a promising candidate for antiviral, anti-inflammatory, antibacterial, and anti-cancer activities (Naikoo et al., 2021). Lately, synthesized silver nanoparticles have been used as an alternate replacing conventional antibiotic agents (Zhang et al., 2020).

Furthermore, AgNPs can captivate visible light and also they can regulate the electron density (Javed and Mashwani, 2020). Hence they could be used as an appropriate medium for detecting surface-enhanced Raman scattering (SERS) and could analyze sensitive molecules (Li et al., 2018). In addition, ease of design and reproducibility for the methods used in silver nanoparticle synthesis; can be used in several domains of life where novel properties are required. Many techniques, including heating, laser, and radiolysis, can synthesize silver nanoparticles (AgNPs). The problem is that these methods are hazardous and expensive due to the involvement of harmful reducing agents and toxic solvents. Therefore, biogenic synthesis has been a viable option for the last few years to fulfill the green approach and proved to be sustainable (Narayanan et al., 2021). Plants and their extracts, bacteria, fungi, and their secondary metabolites all have been employed in the past to produce silver nanoparticles (AgNPs) successfully.

The biosynthesis method using bacteria and their metabolites, especially extracellular enzymes, is a compassionate alternative to reduce the consequences of the consumption of chemicals involved in traditional methods. Furthermore, biogenic methods based on bacterial enzymes have huge potential and are more efficient and valuable than other biological substitutes because of simplicity of use, easily culturing, low cost and maintenance (Lateef et al., 2015). In this scenario, approaches to apply a single chemical to stabilize and act as reducing agent for controlling the growth process and meritoriously alter the size of the NPs are highly demanded. That is why this methodology has now shifted towards developing a sustainable method for the biosynthesis of metallic NPs according to desired applications.

In an eco-friendly manner, silver nanoparticles can be used to decontaminate organic and inorganic dyes. Generally, it is common to utilize organic dyes in sectors ranging from plastic

to paper to pharmaceuticals. Environment and aquatic bodies might be at risk from them. Organic dyes like methyl orange and congo red are frequently utilized in industries (Raj et al., 2020). Before the expulsion of industrial waste, complete removal of dyes is essential since dyes are remarkably stable in water due to their complex structure. Green techniques for eliminating dyes from the environment might be possible in the presence of sunlight and catalyst (Roy and Bharadvaja, 2019).

In addition, transition metal‘s nanoparticles, such as silver, are responsible for the photo-degrading the organic dyes. Metals have desirable photo-electronic characteristics and ions on their surface and massive surface-to-mass ratios can cause their nanoparticle photocatalytic activities to be significantly higher. Silver nanoparticles have shown significant photocatalytic activity under UV–visible irradiation (Kamat, 2002). Photocatalysis is possible with silver nanoparticles because the absorption of visible light results in energizing conduction of electrons, as a result they are excited from their ground state to higher levels of energy, allowing them to participate in chemical processes. The dispersion of silver nanoparticles on supporting materials results in reduction of agglomeration (Akpomie and Conradie, 2020). In contrast to the metal oxide nanoparticles such as TiO_2, silver nanoparticles can have a modest number of charges, especially the one formed by the reducing the metal ions. Charging nanoparticles with functional groups like amino ($–NH_2$) groups can produce coordination connections between them and their supporting materials, minimizing particle detachment.

Understanding the efficiency of noble silver nanoparticles and the outcome of hazardous dyes to the ecosystem, the current study is based on green technology for synthesizing silver nanoparticles to investigate their antimicrobial and dye degradation activity. The sustainable approach is adopted using native keratinase of *Pseudomonas aeruginosa*-C1M, and the in-situ bio-reduction processes of silver ions into silver nanoparticles are effectively achieved without externally adding any capping agent. Native keratinase of *Pseudomonas aeruginosa*-C1M, used for the proposed work, possesses keratinolytic activity and is involved in the degradation of hard-to-degrade proteinaceous substrates into peptides and amino acids. Leather bioprocessing, pharmaceuticals, nitrogen fertilizer manufacture, and feed formulation all employ keratinases to some extent (Bagewadi et al., 2018). However, keratinases potential to generate metallic nanoparticles has only been used in a few pieces of research (Jang et al., 2018). As a result, it was imperative to increase the production of keratinases for various purposes to use them as an essential reducing precursor for synthesizing silver nanoparticles. Results obtained by characterizing silver nanoparticles applying several techniques, like UV-Vis spectroscopy, XRD, SEM, TEM, FT-IR, and DLS, emphasize the novelty of keratinase *Pseudomonas*

aeruginosa-C1M for the formation of stable AgNPs. Further, antimicrobial and dye degradation properties of silver nanoparticles were also explored.

5.3. Materials and Methods

5.3.1. Biosynthesis of silver nanoparticles

Keratinase derived from *Pseudomonas aeruginosa*-C1M was used in this study. Silver nitrate solution and crude keratinase served as precursors for synthesizing silver nanoparticles (AgNPs). For this purpose, 50 ml of a solution of 2 mM AgNO3 solution was combined with 5 ml of crude keratinase. It was then stirred continuously for 24 hrs. at room temperature. A 2 mM AgNO3 solution devoid of crude keratinase was utilized as a negative control. After a change in the color was observed in the reaction mixture, purification of silver nanoparticles from the solution was accomplished when centrifuged at the speed of 10,000 rpm for 15 min at a temperature 4°C and suspending the resultant pellet in sterilized double distilled water. The following approaches were used to characterize eco-friendly silver nanoparticles;

7.3.2. Characterization of silver nanoparticles

7.3.2.1. UV-Spectrophotometry

A common method for determining the creation and properties of nanoparticles is UV-visible spectroscopy. A UV-visible spectrophotometer was used to track the generation of Ag-NPs. Using a UV-visible spectrophotometer with an accuracy of 1 nm between the wavelength ranges of 300-900 nm, the absorbance was calculated and the peak was analyzed to determine the wavelengths at which nanoparticles were created (Lateef et al., 2015).

5.3.2.2. Scanning Electron Microscopy (SEM)

Using a scanning electron microscope (SEM) (ZEISS EVO LS10) with a working voltage of 25 kV, silver nanoparticles (AgNPs) were morphologically examined. On a carbon tape-coated stub, a few drops of biosynthesized AgNPs were applied. Additionally, the stub was sputter coated with gold to provide crisp pictures (Ocsoy et al., 2017).

5.3.2.3. Transmission Electron Microscopy (TEM)

A drop of nanoparticle was placed on a copper grid with a mesh size of 200 and a 3.05mm hexagonal form for transmission electron microscopy (Agar Scientific, Essex, UK). The coating was accomplished using samples suspended in chloroform. After 305 min of imaging, the surplus liquid was swept away, and grids were air dried. The micrographs were taken with a JEM-1400 (JEOL, USA) at a voltage of 100kV.

5.3.2.4. Dynamic light scattering and Zeta potential

DLS (dynamic light scattering) and zeta potential analysis on a zeta sizer were used to assess biosynthesized silver nanoparticles' size distribution, surface charge, and hydrodynamic diameter at ambient temperature (Malvern Instruments, ZS nano, UK). For analysis, freeze-dried materials were distributed in water (Gokce et al., 2012). The set up was operated at 15V/cm of electric field. Software called Zeta Sizer was used to examine the data.

5.3.2.5. FTIR analysis

Functional groups on the surface of nanoparticles in the range 4000-400 cm-1 were identified using Fourier-transform infrared (FTIR) spectroscopy (Jasco, FTIR 6300, Japan) at a resolution of 4 cm-1. The samples were prepared by using the freeze-drying technique.

5.3.2.6. X-ray diffraction

Silver nanoparticles made by biosynthesis were examined using X-ray diffraction (XRD) to identify their crystalline structure. The XRD pattern was recorded using an EMPYREAN X-ray diffract meter running at 45kV and 40mA current strength with Cu-K/radiation (=1.5406). The diffracted intensities were measured at a scan rate of 0.5°/min from 5° to 90° 2 angles(Pasupuleti et al., 2013).

5.3.3. Applications of silver nanoparticles

5.3.3.1. Anti-bacterial activity assay

Using the traditional well diffusion approach, the antibacterial activity of the AgNPs against the pathogenic pathogens Escherichia coli and Staphylococcus aureus was assessed. Culture broths were placed onto Müller-Hinton agar plates after test strains in nutrient broth had been incubated for 24 hrs. at 37 °C and 200 rpm. Wells were drilled in the agar with a sterilized 5 mm cork borer, and 100µl of AgNP solution was decanted into each well. Additionally, antibacterial activity was determined using DMSO, crude keratinase, and sterilized distilled water as controls. The widths of the clear inhibitory zones were measured after 24 hrs. of incubation at 37°C to assess the antibacterial activity.

5.3.3.2. Dyes degradation by silver nanoparticles

5.3.3.2.1. Sodium borohydride catalyzed degradation of dye

1 m of a 100 mM sodium borohydride solution was added to 1 ml of 0.15mM methyl red (MR) and methyl orange (MO). After diluting the solutions to 10 ml with deionized water, the reaction mixture was rapidly agitated for 5 min. Solutions were then supplemented with 2 ml of silver nanoparticle and agitated for five min. Dye degradation was evidenced by the solution becoming discolored. The reaction that was not catalyzed was used as a reference. The deteriorating process was observed using a UV-visible absorption spectrophotometer.

5.3.3.2.2. Photocatalytic degradation of dyes by silver nanoparticles

In the presence of silver nanoparticles, the dyes methyl violet (MV) and safranin O (SO) were selected for photocatalytic degradation. In a flask, 10mL of dyes (0.15mM) Methyl violet and Safranin O were added separately. After adding 1 mg of AgNPs and thoroughly mixing with a magnetic stirrer, the reaction mixture was exposed to sunlight for 3 hrs.. The kinetics of photocatalytic degradation of methyl violet and safranin O was investigated by observing the change in emission spectra.

5.4. Results and Discussion

5.4.1. Biosynthesis of silver nanoparticles

As silver ions reduced to silver nanoparticles after 24 hrs., the appearance of dark brown nanoparticles demonstrated the synthesis of silver nanoparticles (Fig. 5.1).

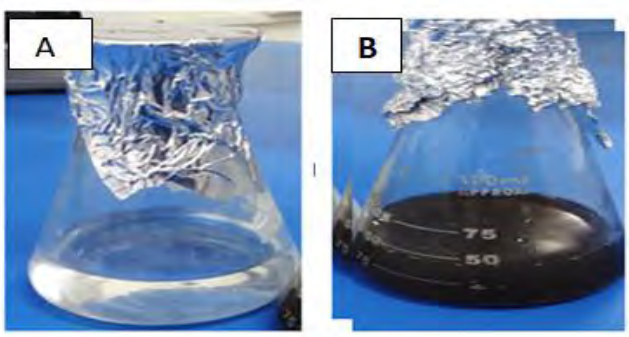

Fig. 5. 1. Visual representation of biosynthesis of silver nanoparticles (A) is representing control containing silver nitrate solution while (B) represents formation of silver nanoparticles.

However, 2 hrs. after crude keratinase was incubated with silver nitrate (AgNO3) solution, color creation started. The secondary metabolite keratinase was responsible for capturing the metal ion and acting as a reductant to create silver nanoparticles (Durán et al., 2005). Increased bio-reduction of silver ions results in an increase in color intensity and provides stability after 24 hrs.. The control solution remained clear, while the production of dark brown particles confirms the creation of silver nanoparticles. (Fig. 5.2)

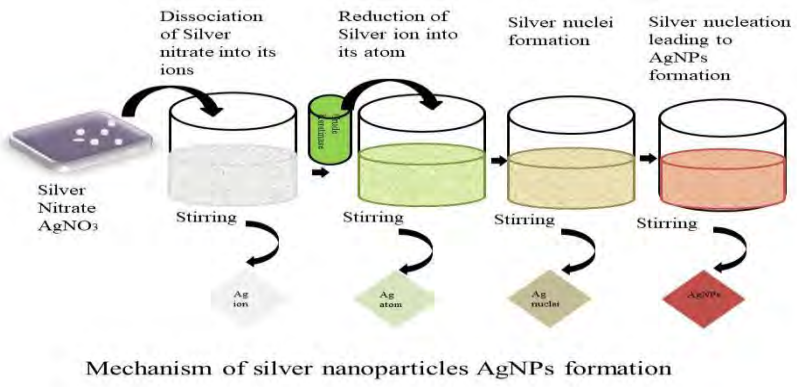

Fig.5. 2. Synthesis mechanism of crude keratinase mediated silver nanoparticles (AgNPs) by using AgNO3 precursor

Numerous investigations have demonstrated that silver nanoparticles may be formed from bacteria and their metabolites. However, just a few publications exist on the production of AgNPs using keratinase. Lateef and colleagues said that they synthesized silver nanoparticles using *Bacillus safensis* keratinase (Lateef et al., 2015). Similarly, another study revealed that keratinase was accountable for the brown hue of silver nanoparticles. (Tao et al., 2018). Similarly, the reducing agent *Stenotrophomonas maltophilia* R13 keratinase was employed to synthesize AgNPs in an environmentally suitable manner. (Jang et al., 2018). Characterization revealed more details on the structure and properties of the silver nanoparticles.

5.4.2. Characterization of silver nanoparticles

5.4.2.1. UV-Visible Spectroscopy

UV-visible spectrophotometers were used to induce localized surface plasmon resonance in metal, which results in the formation of an electric field and the excitation of electrons. (Fig. 5.3) Resonance at a certain wavelength might result in beam scattering within that wavelength range. When Ag^{+} ion is reduced from AgNP complex to Ag^{0} ion by *Pseudomonas aeruginosa*-C1M keratinase resulted in surface plasmon resonance. At 450-470nm, a robust, single, and wide resonance peak was seen, demonstrating the biogenic production of AgNPs (Vigneshwaran et al., n.d.). Furthermore, it was shown that AgNPs have a spherical shape because they only have one surface plasmon resonance peak, while anisotropic particles have several peaks (Zhang et al., 2016). The new discovery is supported by earlier research on the creation of silver nanoparticles from fungus, which indicated a peak in the UV-Visible spectrum at 442 nm (Majeed et al., 2018). Meanwhile, Parkash and colleagues reported that a

surface plasmon resonance peak at 420nm was recorded, followed by a steady rise to 445nm during a 48-hour period (Bhuyar et al., 2020).

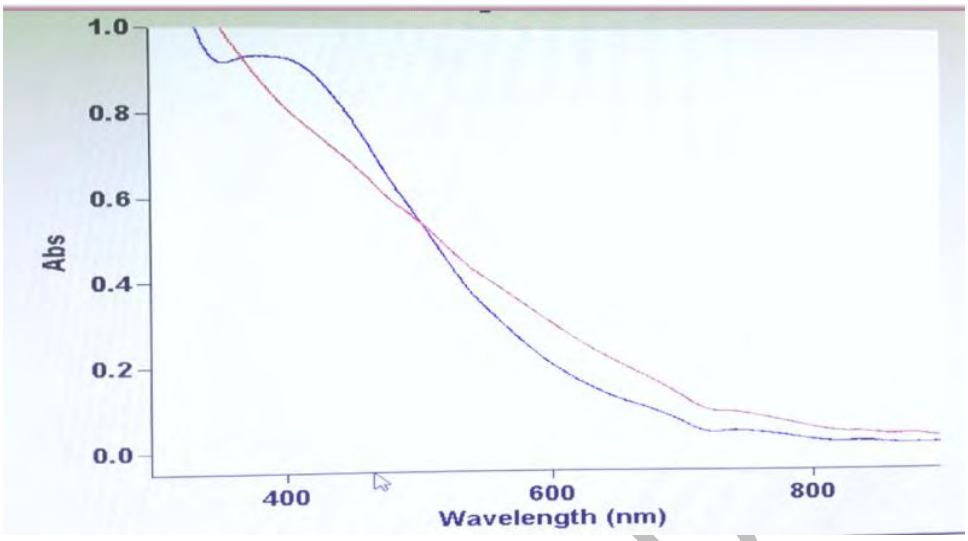

Fig.5. 3. UV-Visible spectrum of keratinase mediated silver nanoparticles at 300-600 nm wavelengths. Red line indicates control (silver nitrate solution) while blue indicates peak of silver nanoparticles at 460 nm

5.4.2.2.Scanning Electron Microscopy

AgNPs generated were examined using a SEM to assess their size and surface form. Silver nanoparticles were determined to be round, smooth, and ranging in size from 15nm to 100nm using SEM micrographs (Fig. 5.4). Although images were collected at various magnifications, aggregation was also observed, which might be due to the sample drying on the stab. However, several scientists have already discovered that AgNPs vary in size (Phull et al., 2016). Recently, in a study on the synthesis of AgNPs using the marine macroalgae Padina sp., the average particle size determined by the standard error of the mean was 33.75nm (Bhuyar et al., 2020). Meanwhile, another research discovered AgNPs to be 35nm in diameter and spherical in form (Saravanan et al., 2017).

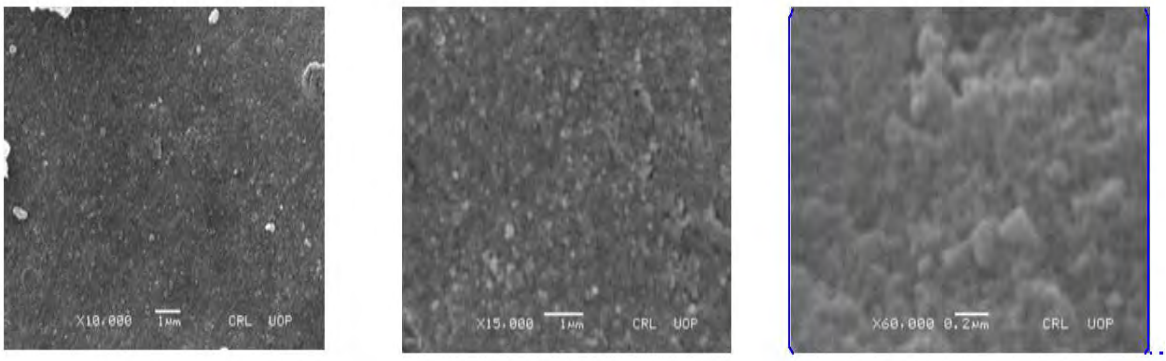

Fig. 5.4. Scanning electron micrographs of biogenic synthesized silver nanoparticles at various magnifications (Magnification of A: x 10,000 B: x 15,000 C: x 60,000)

5.4.2.3.Transmission Electron Microscopy

Transmission electron micrographs of silver nanoparticles are presented in the image below (Fig. 5.5). AgNPs were discovered to be between 15 and 35nm in average size and spherical in form. Kanan et al. estimated the size to be between 30-44nm (Kannan et al., 2013).

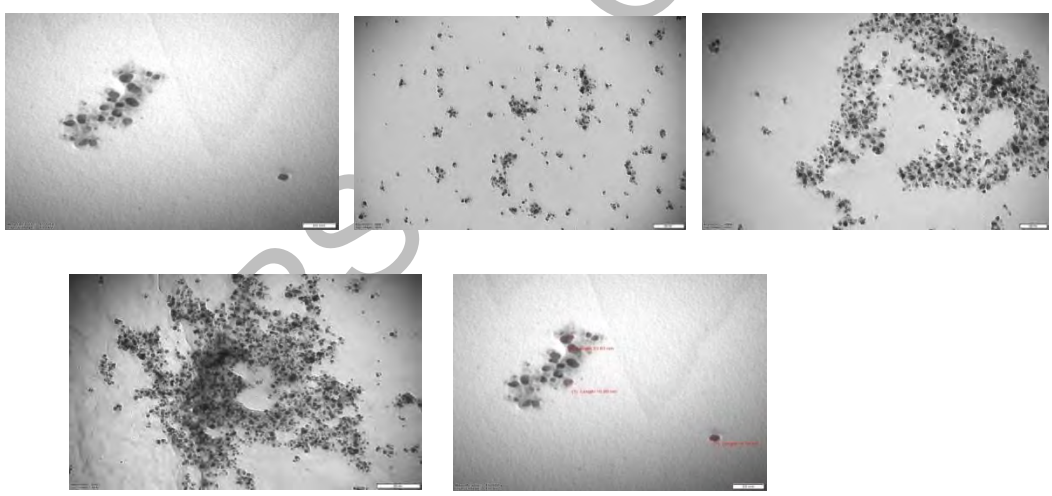

Fig.5.5. Transmission electron micrographs of biogenic synthesized silver nanoparticles, round surfaced AgNPs have diameter 15-35nm.

5.4.2.4.Dynamic Light scattering (DLS) and Zeta Potential

A fundamental aspect of nanoparticles is their size, which has a significant effect on their dissolution rate, physical stability, saturation solubility, and also their active performance. With a range of 20–400nm, the average particle size was found to be 119.8nm. AgNPs were found to have a polydispersity index (PDI) of 0.210, showing good dispersion of the nanoparticles. Additionally, charge was assessed using the zeta potential, whose negative value indicates the

presence of negatively charged capping agents that inhibit agglomeration. It is, nonetheless, implicated in the stability of colloidal systems. (Fig.5.6, Fig. 5.7)

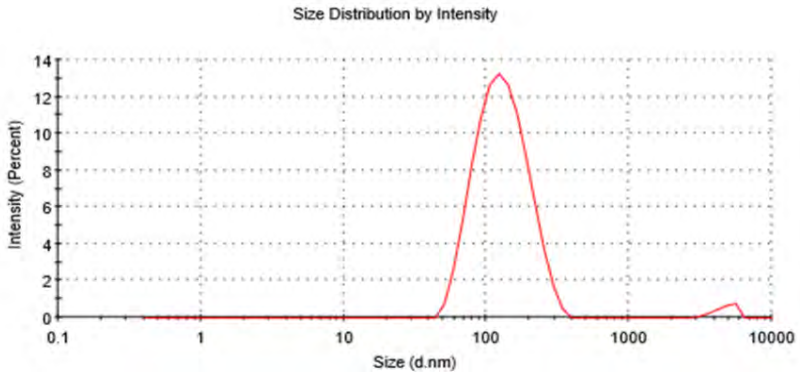

Fig.5.6. Dynamic Light scattering micrograph of silver nanoparticles synthesized from keratinase; poly dispersity index was around 0.21

Moreover, divergence in findings of TEM and DLS is owing to fluctuation in sample circumstances. For dynamic light scattering (DLS) water based hydrodynamic system was employed whereas for TEM dry samples were used (Jain et al., 2020). In the literature, it has been noted that the size of silver nanoparticles varies with the reducing agent. According to a recent research, the average size of AgNPs encapsulated in fungal chitosan was 75-80 nm; whereas AgNPs generated from Trichoderma viride had an average size assessed by zeta sizer of 242nm, but were well dispersed. (Sathiyaseelan et al., 2020),(Manikandaselvi et al., 2020).

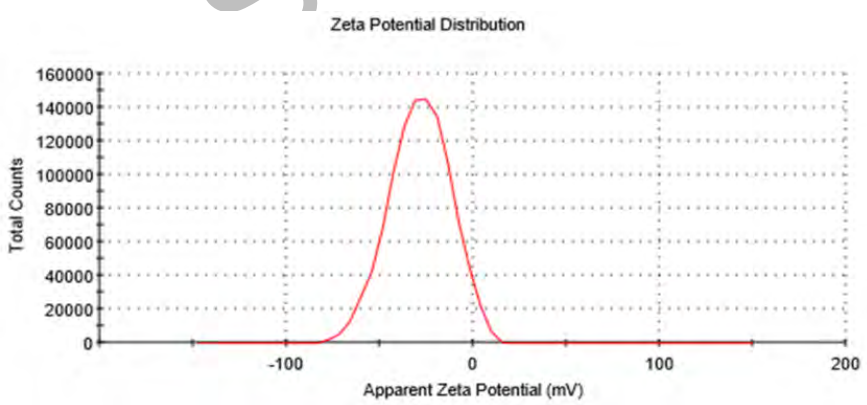

Fig.5.7. Zeta potential graph of silver nanoparticles synthesized from keratinase showing negative value is due to the presence of negatively charged capping agents.

5.4.2.5.FTIR analysis

The bio-molecular mechanism responsible for the stability of biosynthesized nanoparticles was identified using FTIR measurements. The FTIR spectrum showed a number of distinct peaks. The NH_2-amino-acidic group has a peak at 3268 cm^{-1}, which could be seen. At 2853–2963 cm-

1, side chain vibrations of symmetric and antisymmetric C–H stretching modes of aliphatic and aromatic groups were found. At a wavelength of 2500 cm^{-1}, the S-H bond was found to be present. While the peak at 1625 cm^{-1} reflected the C=O I bond, the peak at 1447 cm^{-1} demonstrated the carboxylic acid bending of the OH group. Similarly, the 1381 cm^{-1} peak revealed the aliphatic bending group of CH and CH_2. A peak at 1060 cm^{-1} indicated the presence of S=O. 623 cm^{-1}, on the other hand, was previously assumed to be N-H stretched. (Fig. 5.8)

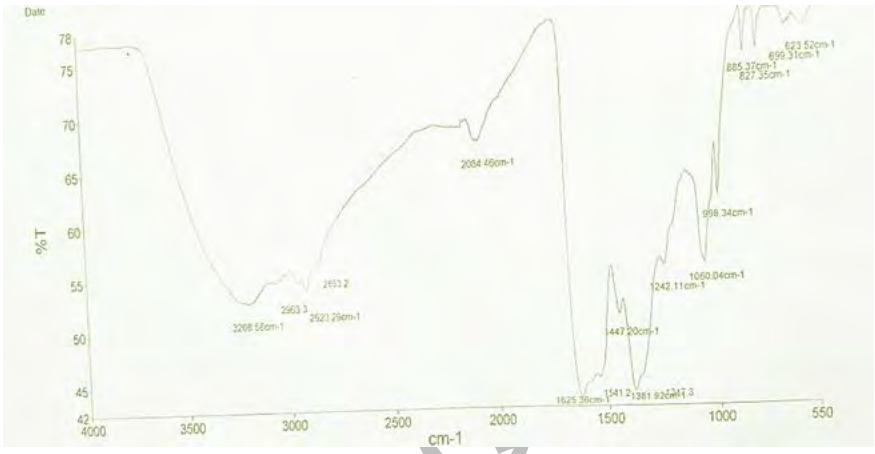

Fig.5. 8. FTIR Spectrum for biogenic silver nanoparticle, various peaks are representing the presence of amino and sulfhydryl groups, along with aromatic and aliphatic chains.

It has been demonstrated in the past that free amine groups and cysteine residues are crucial for the efficient stabilization and capping of AgNPs by interacting proteins and nanoparticles. Along with the findings previously mentioned, it seems that protein and leftover chemicals played a role in the stability and capping of AgNPs.

5.4.2.6. X-ray Diffraction

The XRD pattern analysis revealed crystalline silver nanoparticles. The five diffraction peaks at 2 values of 38.03°, 44.29°, 64.49°, 77.49°, and 81.51° indicate that the face-centered cubic structure of silver may be indexed to the (1 1 1), (2 0 0), (2 2 0), (3 1 1), and (2 2 2) reflection planes. At 38.03°, the intensity of 100% was measured at the 2 values. These peaks are attributed to the reduction of silver ions and stability of the resultant nanoparticles by the biomolecule keratinase (Vigneshwaran et al., n.d.). The generated AgNPs' face-centered cubic structure was confirmed by XRD (LewisOscar et al., 2021), (Raj et al., 2020). (Fig. 5.9)

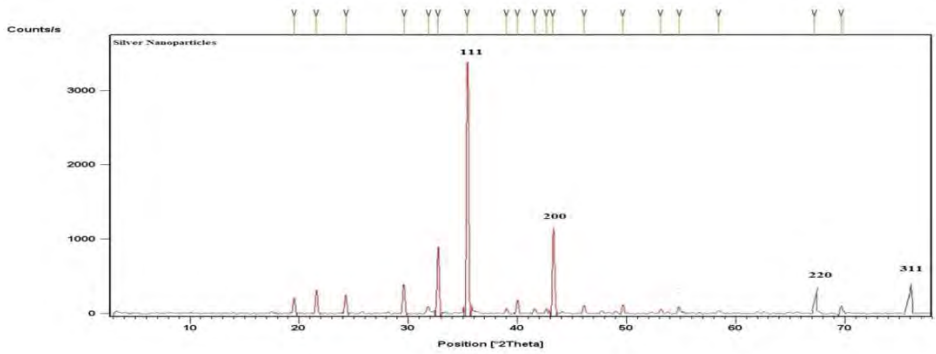

Fig.5.9. XRD pattern of biosynthesized silver nanoparticles showed crystallized, face centered and cubic structure.

5.4.3. Application of Biogenic Silver nanoparticles

5.4.3.1. Antibacterial activity of silver nanoparticles

For the purpose of testing the antibacterial properties of AgNPs produced from *Pseudomonas aeruginosa*-C1M keratinase, the well diffusion technique was used using a 100 micro liter nanoparticles solution. *E. coli*, Gram-negative and gram-positive *S. aureus* had inhibition zones of 33mm and 25mm, respectively. A class of proteases called keratinases may be responsible for the inhibitory function of keratinase, since it has been shown to limit the growth of microorganisms. (Fig.5.10)

Fig.5.10. Antibacterial activity of silver nanoparticles against pathogenic strains Escherichia coli and S. aureus

AgNPs made by biosynthesis have been shown to be effective against E. coli (Priyadarshini et al., 2013) in another study AgNPs from crude keratinase of *B. safensis* demonstrated effective inhibition against five distinct clinical isolates of *Escherichia coli* (Planavsky et al., 2014). AgNPs have a proven high surface area to volume ratio, which makes them superior than bulk silver metal in terms of their exceptional antibacterial activity since it would enable tight attachment of these nanoparticles with microbial cells, enabling their antimicrobial effect to be size dependent (Hartney et al., 2011).

5.4.3.2.1. Sodium borohydride catalyzed degradation of dye (Methyl red and methyl orange)

In today's world, environmental conservation is both a difficult challenge and a critical requirement for long-term well-being. Dye was one of the toxins that were spilled into the environment. They pose a threat to the environment since they include methyl red and methyl orange. In the presence of the reductant sodium borohydride ($NaBH_4$) and metal nanoparticles, MO, an organic sulfosalt, can be reduced to a less hazardous form. Silver nanoparticles can speed up dye degradation in the presence of $NaBH_4$ because of their large surface area and strong reactivity. 465 nm is the wavelength at which methyl orange's spectral band emerges. The enormous surface area per unit weight of nanoparticles accounts for their strong reactivity. Adsorption between MO molecules and silver nanoparticles may also be facilitated by a coating of reducing agent on the silver nanoparticles. Smaller particles have an advantage over larger ones in the oxidation-reduction reaction between active MO and $NaBH_4$. As seen in the following process, electrons from $NaBH_4$ are transferred to excited MO species and then reduced.

(1) $Ag/NaBH_4 + h \rightarrow Ag/NaBH_4 (h+-e-)$

(2) $2e- + MOH+ + H+ \rightarrow MOH_2$

(3) The 465 nm MO band began to go away over time. An electron and a hole are expected to be involved in the reaction process, that is not yet completely understood.

Methyl red is an acid-base indicator whose absorption spectra are pH-dependent. The peak shift from 442nm to 428nm was noticed after the addition of $NaBH_4$ to methyl red solution. More hydroxyl ions are present, which causes a pH change. N=N-methyl red is converted to less hazardous form, which may be seen as a degradation peak. $NaBH_4$ in the presence of nano silver catalyzes the breakdown of dyes to nontoxic and smaller molecules via an electron transfer action between nucleophilic BH_4 ions and electrophilic organic dye molecules. (Fig. 5.11)

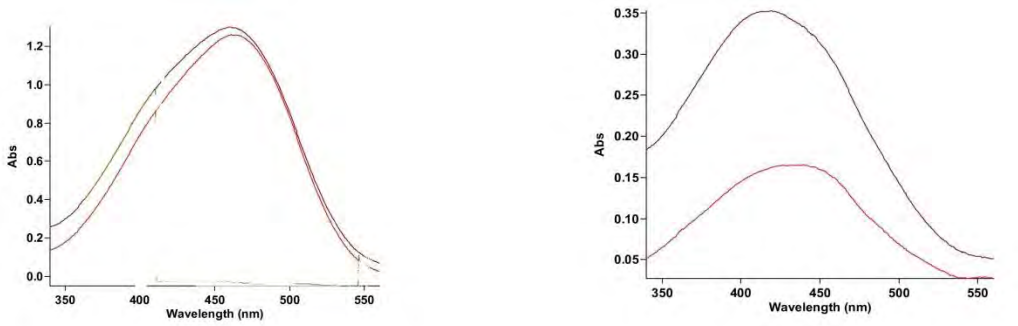

Fig.5.11. a) Methyl orange (MO) and b) Methyl red degradation by AgNP in the presence of NaBH4. Upper peak showed control while lower peak showing reaction mixture, lower peak means degradation of dye into less toxic.

5.4.3.2.2. Photo-catalytic degradation of dyes by silver nanoparticles (Safranin O and methyl violet)

SO and MV dyes degraded when silver nanoparticles were present during photolysis studies under sunlight. Irradiation for three hrs. caused the red hue of SO and MV dyes to fade. The degradation of SO and MV under used settings is indicative of a reduction in the absorption spectra of materials at the maximum wavelength of SO and MV dye. (Fig. 5.12)

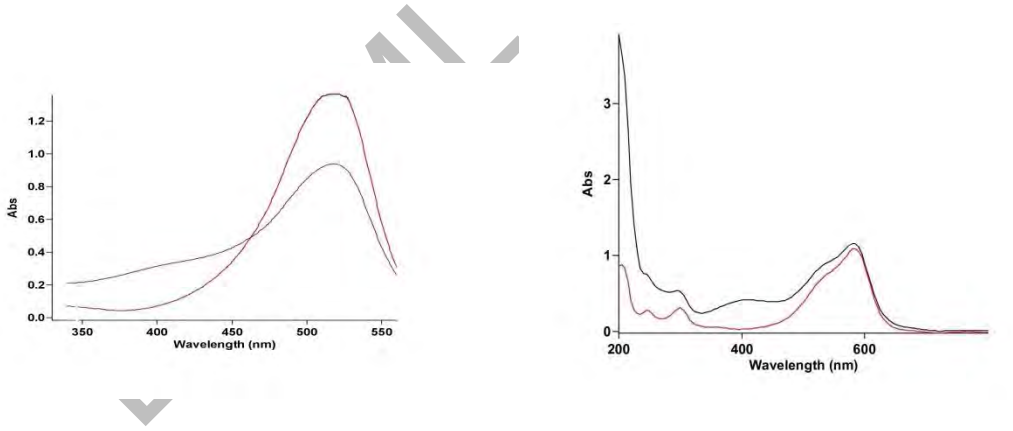

Fig.5.12. a) Photo-catalysis of safranin O and b) Methyl violet in the presence of silver nanoparticles, Upper lines are indicating control, however, lower lines in the graph depicts the conversion of dye into less toxic form.

A photon with energy of hv, which creates a hole (h+) in the valence band, stimulates an electron in the valence band (e) to go into the conduction band. Finally, hydroxyl radicals were produced by trapping holes on the surface of the hydroxyl group. When oxygen dissolved in water reacts with electrons in the conduction band, super oxide radical anions O^{2-} are formed. Hydrogen radicals (HO^2) were formed as a result of the protonation of superoxide anions O^2-. Dye degradation was ultimately caused by the super oxide anions' activity. Color changes were induced by valence band holes and conduction band electrons, respectively A photon with

energy of hv, which creates a hole (h+) in the valence band, stimulates an electron in the valence band (e) to go into the conduction band.

Because of SPR excitation, the photocatalytic activity of AgNPs may be described by their ability to absorb visible light and convert it into chemical energy, a process known as photosynthesis.

5.4. Conclusion

This study describes a straightforward, cost-effective, and environmentally friendly technique to synthesize protein-capped AgNPs utilizing the keratinase of *Pseudomonas aeruginosa*-C1M. Characterization revealed that AgNP was spherical and had a size range of nanometers. XRD measurements revealed the silver nanoparticles' crystalline structure. AgNPs were successfully bio-conjugated using FTIR. AgNPs were highly effective antibacterial against all the strains of bacteria that were examined. Before AgNPs may be employed in biological or pharmaceutical applications, more research is needed to identify whether or not they are cytotoxic.

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5.6.Conflict of Interest

We declare no conflict of interest.

5.7.References

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Chapter 6: Exploring feather hydrolysate potential for bio-gas production

Paper 4 from chapter 6:

Title: Microbial pretreatment of chicken feather and its co-digestion with greengrocery waste and rice husk in batch and continuous reactors for enhanced biogas production

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6.1. Abstract

To utilize wastes and residues sustainably and excellently, there is a need to fend for efficient methods and resources for biogas production. Use of poultry waste for biogas production represents one of the most important routes towards reaching global renewable energy targets. The current study involves microbial pretreatment of chicken feather waste, followed by its co-digestion with rice husk and green-grocery waste in batch and continuous reactors respectively. Microbial pretreatment of chicken feathers by keratinase secreting *Pseudomonas aeruginosa* was an effective and eco-friendly approach to make its recalcitrant structure available as a raw substrate for biogas production. The current study also addressed the enhancement and stability of anaerobic digestion by co-digestion. Results demonstrated that biogas production was increased by microbial pretreatment of chicken feathers and the percentage increase in biogas yield was 1.1% in microbialy pretreated feathers compared to mono-digestion (non-pretreated feathers) in batch fermentation. The highest yield of biogas was obtained in a batch reactor having co-digestion of pretreated rice husk and microbial pretreated chicken feathers. The co-digestion of chicken feathers hydrolysate with green-grocery waste in continuous fermentation mode has also enhanced the biogas yield as compared to average of mono-digestion (Chicken feather hydrolysate and green-grocery waste) and therefore improve the efficiency of the overall process.

Keywords: anaerobic digestion, biogas, *Pseudomonas aeruginosa,* microbial pretreatment, chicken feather waste.

6.2. Introduction

Bioenergy may be a significant replacement for non-renewable fossil fuels, making the path easier for sustainable development and decreasing the dependency on conventional energy sources. The world population is increasing rapidly; industrial activities are flourishing due to it. Global urbanization and industrialization lead to enhanced energy demands and more waste generation (Zhang et al., 2021). Moreover, as energy is the critical source of comfort for the modern world, most developing and underdeveloped countries have no proper access to energy services like petrol, diesel, natural gas, and electricity. Fossil fuels are the primary energy source for many countries, but their reservoirs will be scarce to meet requirements in the near future. Scientists are looking for renewable sources to overcome fuels limitations. On the other hand, globally, 17 billion tons per annum waste is being generated due to increased industrialisation and urbanisation; it will reach 27 billion tons by 2050 (Rahaman et al., 2021;

Patinvoh et al., 2016). Biogas is an emerging renewable energy source obtained through the degradation of organic matter by microorganisms under anaerobic conditions. The biogas production and applications provide a comprehensive and systematic guide to developing and deploying biogas supply chains and technology (Kanafin et al., 2021; Ogut et al., 2013).

According to the world, food demands the poultry industry is major growing strength. The necessity to cope with waste disposal needs some severe alternatives, otherwise, it will lead to global and environmental pollution (Morinval and Averous 2021). Millions of tons of organic waste are produced annually from the poultry industry (Karuppannan et al., 2021). These wastes have high capabilities to generate methane. The poultry industry generates organic waste like feathers in a vast quantity. Feathers are composed of keratin protein, having a recalcitrant structure. The cross-linking and covalent bonds added more strength to the feather's structure (Sypka et al., 2021). Different pretreatment methods are used like superheated water, strong acids, or alkalis puffing, pressurised breakdown of feathers (Chao et al., 2021), and many other physiochemical methods could be applied.

Due to high cost, sustainability, and ecological problems these pretreatment methods are seldom used. Recent research trends in cost-effective, ecological, and sustainable pretreatment methods with the help of microbial consortia (González et al., 2021). Different microorganisms like bacteria and fungi were isolated for pretreatment of chicken feathers. Chicken feathers were biologically pre-treated, and the pretreated feathers hydrolysate was used for biogas production. Production of biogas via an anaerobic digestion process effectively reduced the waste generated in large volumes. It is a sustainable and well-established process to utilise waste for energy production. The digestate that is leftover could be still used as a source of fertilisers (Awasthi et al., 2022).

Anaerobic digestion is a multistep, sensitive, and complex process with different microorganisms (Hagos et al., 2017). This process occurs in an oxygen-deficient environment. Methane and CO_2 are produced from a chain of different biochemical processes during anaerobic digestion. Anaerobic digestion has four different stages, hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Peng et al. 2021), in which the organic substances are broken down in the absence of oxygen. The organic matter that is not digested in the anaerobic digestion (AD) process is considered digestate. The vital operating parameters that must be maintained and optimised for an anaerobic digestion process are proper C/N ratio, pH, temperature, total solid contents, and volatile solid contents (Ellacuriaga et al., 2021). Pretreatment is essential to improve the biogas production and process stability. Therefore, it

will be helpful to investigate the optimal mix of substrates and the conditions of fermentation. Biological pretreatment can be carried out either by fungi or bacteria to degrade keratin waste. Bacteria use keratin as a source of carbon and energy (Chaturvedi et al., 2021). However, high nitrogen content in chicken feathers with a low C/N ratio will not suit anaerobic digestion. Therefore, the feathers, a protein-rich substrate, must be co-digested with another substrate with high carbon content to balance the C/N ratio (Mohanty et al., 2021). The rate of anaerobic digestion is affected significantly by the carbon to nitrogen ratio. A balance C/N ratio is required for microorganisms' growth and proper metabolism. The reported C/N ratio for proper digestion is 20-30:1 (Zheng et al., 2021). The biomethane potential of many wastes in batch fermentation experiments has been tested, and few studies compare batch fermentation to continuous fermentation. Batch fermentations are suitable for estimating a substrate's methane yield and biodegradability, but they do not provide information about the long-term effect of the substrate on the fermentation (Szilágyi et al., 2021).

Chicken feather contributes a significant portion to poultry waste. High content of protein in chicken feather waste is associated with the problem of ammonia inhibition. Therefore, to address this problem, the present study was designed to increase the biogas production from chicken feathers by biological pretreatment by *Pseudomonas aeruginosa* and co-digestion with carbon-rich rice husk and green-grocery waste to avoid ammonia inhibition. It will help to improve the feather waste to energy conversion through the anaerobic digestion process.

6.3. Materials and methods

6.3.1. Substrates for anaerobic digestion

Different substrates used during the current study were chicken feathers (CF), chicken feathers hydrolysate (HL) rice husk (RH), and green-grocery waste (GW). The CF was collected from a slaughterhouse (a poultry shop in Quaid-i-Azam university, Islamabad, Pakistan) and after collection, the feathers were washed with tap water to remove blood and mud attached to it. After that being sun-dried for three days and was cut into small pieces manually to increase the surface area for microbial pretreatment. Pakistan is an agricultural country, where rice is abundantly cultivated, therefore, RH was used as a co-substrate in anaerobic digestion and was collected from Swat (District in the Malakand Division of Khyber Pakhtunkhwa, Pakistan) Stones and other solid particles present in the rice husk were removed. It is then chopped in a grinder to increase its surface area. The GW was purchased from juice shops of Quaid-i-Azam University, Islamabad, Pakistan and used as a co-substrate in continuous anaerobic digestion. Solid particles present in the green-grocery waste were removed. It was then chopped in a

grinder to increase its surface area. The CF and RH were stored at room temperature, while GW was stored in plastic bags at -20 ℃ until further use. Weighed substrates were thoroughly mixed during the experiment to gain a homogenized feed before use.

6.3.2. Isolation and screening of microorganisms for pretreatment

Similar method were used that was already elaborated in chapter 3.

6.3.3. Pretreatment of chicken feathers and rice husk

For biogas production, the chicken feathers were biologically pre-treated with keratinase-producing microorganisms, i-e, *Pseudomonas aeruginosa,* to increase its biodegradability prior to anaerobic digestion (AD). For microbial pretreatment of chicken feathers (substrate), feathers basal media was prepared with composition NaCl 0.5 g/L, Na_2SO_4 0.5 g/L, KH_2PO_4 0.4 g/L, and K_2HPO_4 0.3 g/L with dried feathers 1 %, 2 %, or 3 % to the flask of media autoclaved at 121 ℃ for 20 min. 2-3 mL of inoculum per 100 mL of the feather's basal media were added and incubated for 5-8 days at 37 ℃ and 150 rpm. The negative control without the addition of microorganisms was run in parallel. The degraded feather containing media was designated as whole broth or hydrolysate of chicken feathers and stored at -20 ℃ until further use.

Prior to biogas production, RH (1.59 gVS) was pre-treated with 1 % phosphoric acid and autoclaved at 121 ℃ for 20 min.

6.3.4. Substrate characterization for anaerobic digestion

The chicken feathers, microbial pre-treated chicken feathers hydrolysate, and rice husk and green-grocery waste were used as the substrate for anaerobic digestion.

The total solids (TS) and volatile solids (VS) in all the substrates used in the experiments were determined according to National Renewable Energy Laboratory's Analytical Manual. The usual procedure was used to determine the sample's TS and VS (Pinheiro et al., 2021), and the analysis of each sample was performed in triplicate. The characteristics of the substrates are demonstrated in Table 8.1.

Table 6.1. Characteristics of substrate and inoculum

Biomass	**TS (%)**	**VS of TS (%)**
Chicken Feather	83.86 ± 7.98 a	83.47 ± 8.28 a
Chicken feather Hydrolysate (Microbial pre-treated)	1.36 ± 0.08 b	77.16 ± 5.11 ab
Rice Husk	91.36 ± 8.47 a	83.17 ± 5.97 a
Inoculum	1.18 ± 0.09 c	68.10 ± 6.43 b

6.3.5. Inoculum development

The inoculum used for the batch fermentation was collected from the running experimental biogas reactor at sustainable Bioenergy Biorefinery Laboratory, Department of Microbiology, Quaid-i-Azam University. The operational condition of the reactors were retention time 10, organic loading rates 3, co-digestion of cattle manure with fruits and vegetables, and two-stage anaerobic digestion process waste. The inoculum was developed and degassed in a 3 L volume of anaerobic reactors. The reactors were incubated at 37 °C for 20 days to allow the microbes to eliminate any organic matter in the inoculum. The inoculum for continuous anaerobic fermentation was acquired from the National Agriculture Research Council's (NARC) running experimental biogas reactor. The retention time of the digester was 57 days, and it was kept at 37 °C. The inoculum was said to be fully developed when it starts biogas production. The inoculum could be used in experiments when there is slowly decrease in biogas production was observed. The VS and TS were determined as previously explained and presented in Table 1.

6.3.6. Anaerobic digestion

The present study was conducted in batch fermentation, at 37 °C, to determine potential of chicken feathers, rice husk, and the effect of pretreatment and co-digestion on the biogas production. Continuous fermentation at 37 °C was also performed to determine the biogas potential of chicken feathers hydrolysate with green-grocery waste in co-digestion and also separately in mono-digestion.

6.3.7. Experimental setups for batch anaerobic digestion

The experimental setup for the biogas production during the batch process was carried out in 500 mL glass reactors with a working volume of 400 mL, presented in Table 2. In the present study, 8 different setups were designed for 60 days, and each setup was carried out in a triplicate. The positive and negative controls were run at the same conditions in parallel. The negative control contained only inoculum to find out the amount of biogas production in the background of the test reactors. The positive control containing cellulose ensures the inoculum activity in the reactors. In the 3rd reactor, the feasibility of non-pretreated feathers for biogas production was also studied. Dried non-pretreated feathers were cut down into a small size, and then a specific amount was added to the reactor. In this reactor, the feather is supposed to be the substrate. The 4th reactor contains hydrolysate, microbial pre-treated chicken feathers, as a substrate. The 5th reactor contains a non-pretreated rice husk in a powder form, while the other reactor has a pre-treated rice husk with inoculum. The pre-treated and non-pretreated reactors were used to check the impact of pretreatment over non-pretreatment biogas production. The 7th reactor contains the hydrolysate and its co-digestion with non-pretreated rice husk. The last setup has microbial pre-treated chicken feathers hydrolysate with pre-treated rice husk. The inoculum and substrate were added in the reactors in a ratio of 4:1 in terms of VS, and the amount of substrate required was calculated from the formula given below. The inoculum was added to the substrate to make a working volume of 400 mL.

$$\textbf{Amount of substrate} = \frac{\textit{working volume x VS of inoculum in \%}}{\textit{VS of substrate in \% x ratio of substrate in \% x VS of the sample in \%}}$$

The reactors were flushed with nitrogen and immediately closed with rubber stopper. The reactors were shaken daily manually. The pH of all setups was analyzed initially and at the end of the anaerobic digestion. The batch process was continued for 60 days and the data was recorded until the production of biogas was recoded zero for 5 consecutive days. At the end of the experiment, the accumulated biogas production and biogas yield were compared. In order to determine the increase in biogas yield due to co-digestion, the biogas yield was compared to the calculated biogas yield (calculated from the mono-digestion of each substrate).

Table 6. 2. Experimental setups for 8 different anaerobic digestion reactors

Reactor type	**Inoculum (mL)**	**Substrate (mL)**	**Distilled water (mL)**
Inoculum	290	0	110

Cellulose	290	1.2 (cellulose)	108.8
Chicken Feathers	290	1.2 (feathers)	108.8
Chicken Feathers Hydrolysate (Microbial pre-treated)	290	110 (hydrolysate)	0
Rice husk	290	1.2 (rice husk)	108.8
Rice husk (acid-pretreated)	290	1.2 (rice husk)	108.8
Co-digestion (hydrolysate + rice husk)	290	0.6 (rice husk) + 55 (hydrolysate)	54.4
Co-digestion of hydrolysate with pretreated rice husk)	290	0.6 (rice husk) + 55 (hydrolysate)	54.4

6.3.8. Experimental setup for continuous anaerobic fermentation reactors

The experimental setup for the continuous process was carried out in a 2.5 L glass reactor for 60 days with a working volume of 2 L. In the current study, three different anaerobic reactors were designed, along with two controls. In the 1st reactor, mono-digestion of the hydrolysate was carried out. In the 2nd reactor, green waste is fed as a substrate for biogas production. In the 3rd reactor, the feasibility of co-digestion of green waste and microbial pre-treated chicken feathers hydrolysate was checked. The green waste and hydrolysate were mixed in the ratio of 50:50 based on volatile solids. The inoculum (2 L) was added to all three reactors, then flushed the headspace with nitrogen to remove the oxygen and then fixed the glass reactors with cork. The corks were tightly fitted to prevent the passage of air or moisture in or out of the reactor. Two short rods were fixed in the cork; one end of the 1st rod was inside the glass reactor and other end was to the outside, to which an airtight bag was attached through a rubber pipe for biogas collection, and the 2nd rod was attached to a 60 mL syringe through a rubber pipe, through which the substrate was introduced, and the same amount of digestate was collected daily at a specific time. The reactors were daily shaken manually.

The hydraulic retention time for continuous anaerobic fermentation of the reactors was 20 days. This time was more than the doubling time of methanogens, and most organic matter could be degraded in respective time. In case of lower retention time the active cells may wash out from

the reactor resulting in lower yield of biogas and could results in process failure (Jeppu, G. P *et al.*, 2021). At the same time, to avoid process failure due to overloading, the organic loading rate was kept at 1 g VS/(L.day), while the flow rate was 100 mL/day.

The pH of all the reactors was analyzed daily throughout the incubation. All reactors were kept at 37 °C in the incubators.

6.3.9. Analytical Methods

As discussed previously, the reactor outlet is connected to a gas-tight bag to collect biogas produced in the reactor. After every 24 hrs., the biogas from the bag was measured by a 60 mL syringe. The methane content of the biogas produced in each experiment was determined by passing the gas through the scrubbing solution (3M NaOH). Along with this, VFA and alkalinity of the effluent were determined with the standard method (APHA, 1998b).

6.3.10. Statistical analysis

Data were shown as mean ± SE for three replicates of each treatment. The significance of difference among different reactors was performed using analysis of variance (ANOVA), and significance of differences among treatments means (Tukey's test) was performed using the "ggpubr" package in R. The graphs were designed using the "ggplot2" package in R software.

6.4. Results

6.4.1. Biogas production, biogas and methane yield via a batch process

The accumulative biogas and methane yield during the anaerobic batch process by co-digestion of pre-treated rich husk and feathers hydrolysate Co-P(HL+RH) was higher than all other substrates as represented in Figure 8.1a and 1b. The biogas yield was 333.6 Nml/gVS, while the methane yield was 223.5 Nml/gVS. Followed by RH-P, the biogas and methane yield was high measured in the case of HL. However, the lowest biogas and methane yield was shown by the co-digestion of hydrolysate and non-pretreated rice husk Co(HL+RH).

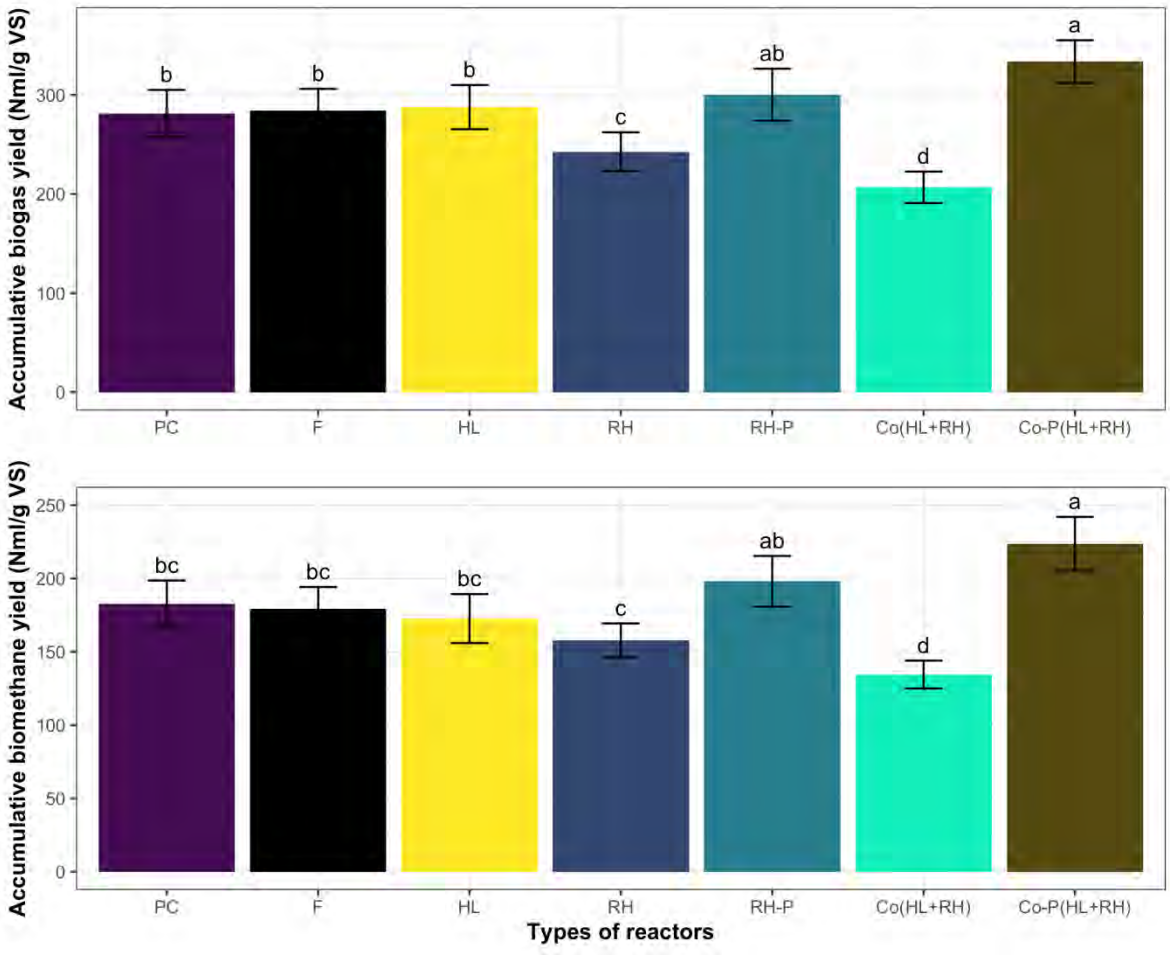

Fig.6.1 a. Accumulative biogas yield of PC (positive control containing cellulose), F(feathers), HL (feathers hydrolysate), RH (rice husk), RH-P(rice husk pretreated), Co(HL+RH){Co-digestion(hydrolysate + rice husk)}, Co-P(HL+RH){Co-digestion with pretreated(hydrolysate + rice husk). Means having the same letter are statistically similar as per the Tukey test at p < 0.05. 6. 1b. Accumulative biomethane yield from PC (positive control containing cellulose), F (feathers), HL (feathers hydrolysate), RH (rice husk), RH-P (rice husk pretreated), Co(HL+RH){Co-digestion(hydrolysate + rice husk)}, Co-P(HL+RH){Co-digestion with pretreated (hydrolysate + rice husk)}. Means having the same letter are statistically similar as per the Tukey test at p < 0.05.

In the case of non-pretreated chicken feathers, biogas yield was 284 Nml/gVS, which was increased to 287 Nml/gVS through microbial pretreatment in the hydrolysate. The increase in biogas yield in the case of microbial pretreated chicken feathers was not significant at 1.1%. On the other hand, in the case of non-pretreated rice husk (RH), the biogas yield was 242.7 Nml/gVS while increased to 300.2 Nml/gVS in pre-treated rice husk (RH-P). The chemical pretreatment of rice husk significantly increased the biogas yield by 23.75 %. To find the increase in biogas yield due to co-digestion, the actual yield from co-digestion was compared to calculated yield for co-digestion (calculated from mono-digestion of hydrolysate and rice husk). The calculated yield for co-digestion without pretreatment was 265.3 Nml/gVS, which was decreased to 206.7 Nml/gVS in co-digestion. The co-digestion of non-pretreated rich husk

and chicken feather show a 20 % decrease in methane yield. The calculated biogas yield for pre-treated co-digestion was 249 Nml/gVS, which was increased to 333.6 Nml/gVS in the case of Co-P(HL+RH) yield as represented in Figure 8.2. In comparison, the co-digestion of hydrolysate with pre-treated rice husk showed a significant increase of 34 % in biogas yield.

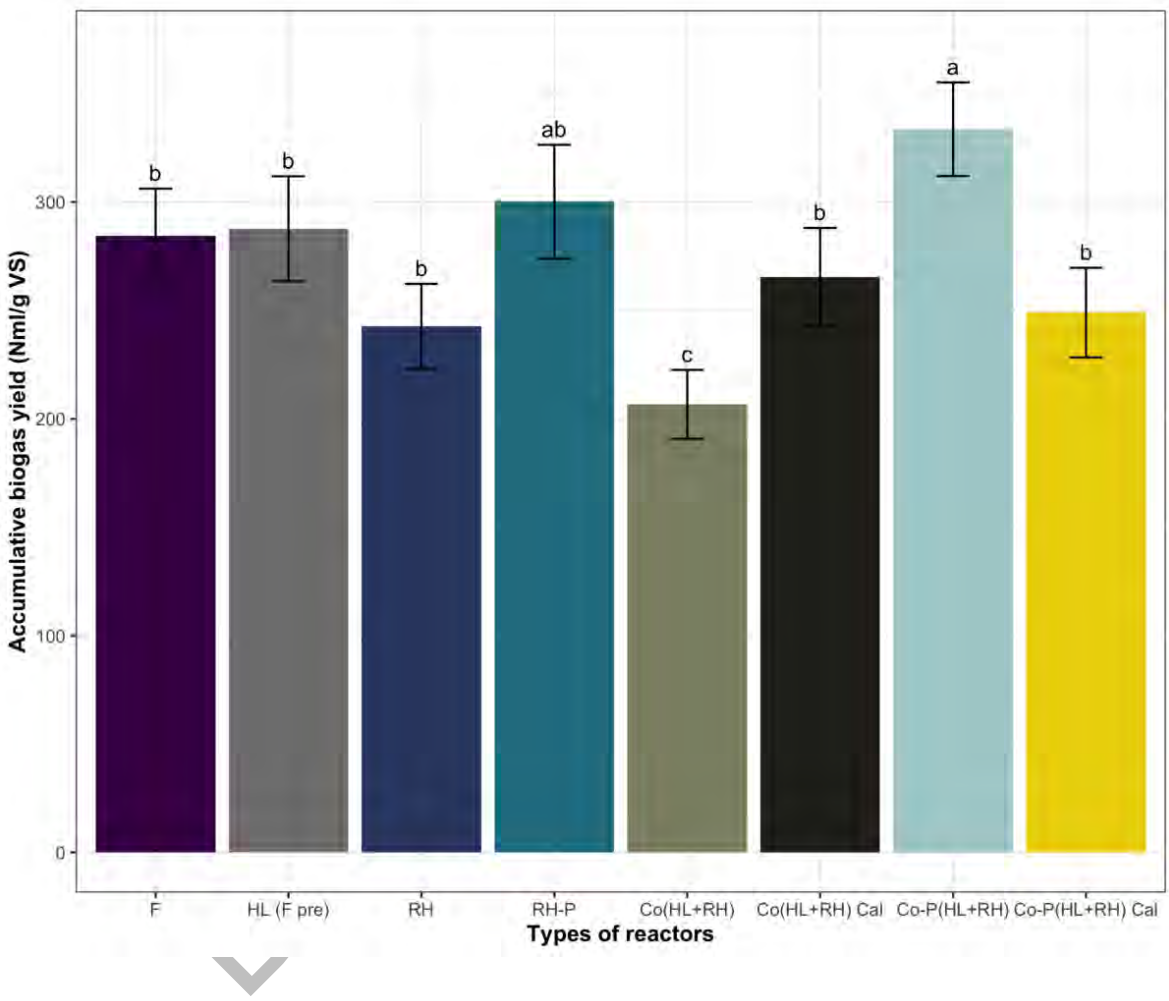

Fig.6.2. Comparison of Accumulative and Calculated biogas yield (calculated from mono-digestion of Hydrolysate and rice husk) from F(feathers), HL-(F-pre)(feathers hydrolysate biologically pre-treated), RH(rice husk), RH-P(rice husk pre-treated), Co(HL+RH){Co-digestion(hydrolysate + rice husk)}, Co(HL+RH)Cal {Co-digestion(hydrolysate + rice husk) calculated from the yield of hydrolysate and rice husk}, Co-P(HL+RH){Co-digestion with pre-treated (hydrolysate + rice husk)}, Co-P(HL+RH) calculated {Co-digestion with pre-treated (hydrolysate + rice husk) calculated from the yield of hydrolysate and rice husk}. Means having the same letter are statistically similar as per the Tukey test at $p < 0.05$.

6.4.2. Process stability parameters in anaerobic batch reactors

The pH of all the reactors was analyzed before and after the incubation. The initial pH was set as 7.2. In the case of PC, RH and RH-P, the pH was slightly decreased to 7.1, 7.14, and 7.08, respectively. However, the pH was still in the optimum range required for anaerobic digestion,

while in case of HL, CO(HL+RH), and CO-P(HL+RH), the pH was slightly increased that was 7.4, 7.6, 7.3, and 7.5, respectively. At the end of the experiment, the VFAs and alkalinity were determined and were found to be in the optimum range. The VFAs to alkalinity are shown in Fig. 8.3. The highest VFAs accumulation, 875 mg/l, was recorded in hydrolysate followed by Co(HL+RH) 450 mg/l while it was lowest in the negative control.

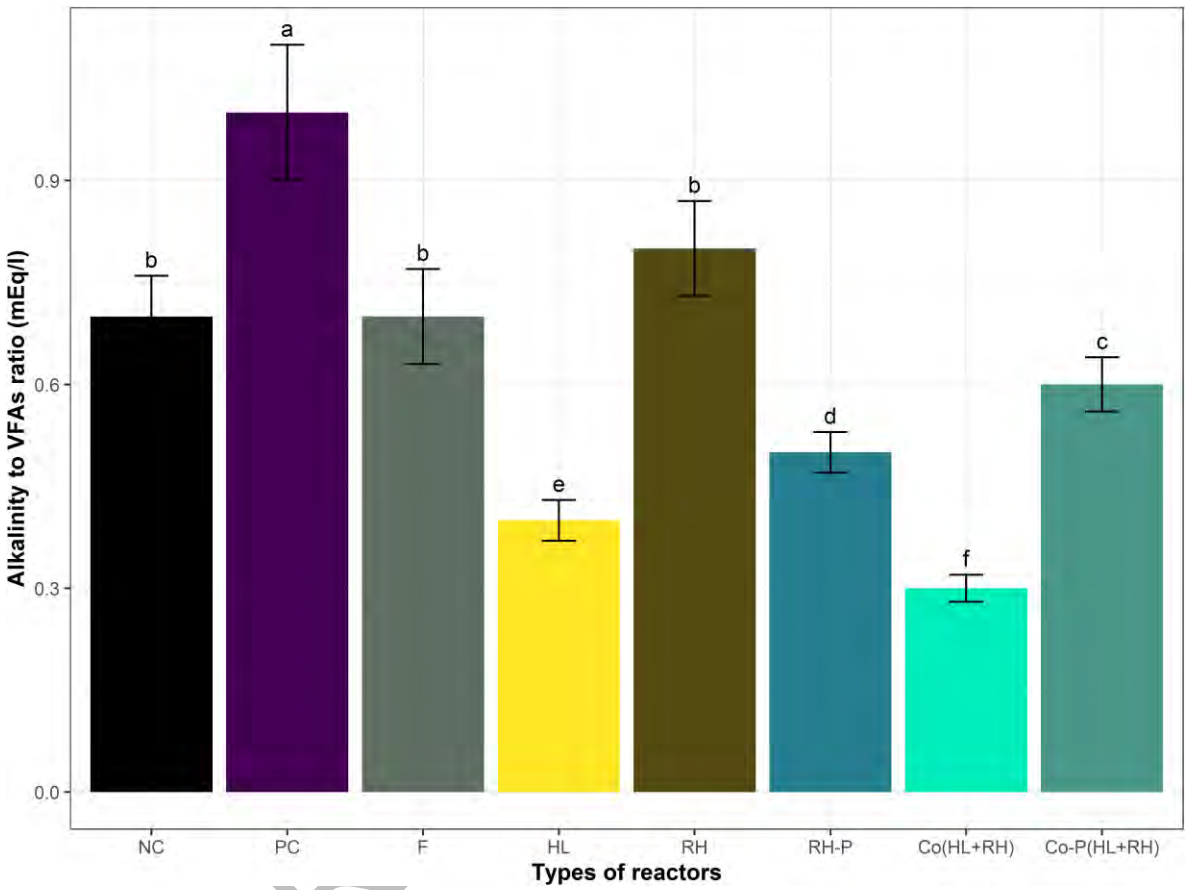

Fig.6.3. Alkalinity to VFAs ratio of different reactors. Means having the same letter are statistically similar as per the Tukey test at $p < 0.05$.

The lowest 0.3 VFAs to alkalinity ratio was in Co(HL+RH) followed by 0.4, 0.5, 0.6, 0.7 and 0.8 in feather hydrolysate, Rice husk pre-treated, Co-digestion with Co-digestion pre-treated rice husk, negative control and rice husk non-pretreated while the PC shows the highest ratio of 1.0.

From the finding during batch process, it is concluded that co-digestion of microbial free treated chicken feathers with the carbon rich pre-treated rice husk significantly increased the biogas yield. But when chicken feathers were co-digested with non-pretreated rice husk, it did not result in significant increase of biogas yield therefore, the easily degradable carbon rich green waste was used in the continuous process.

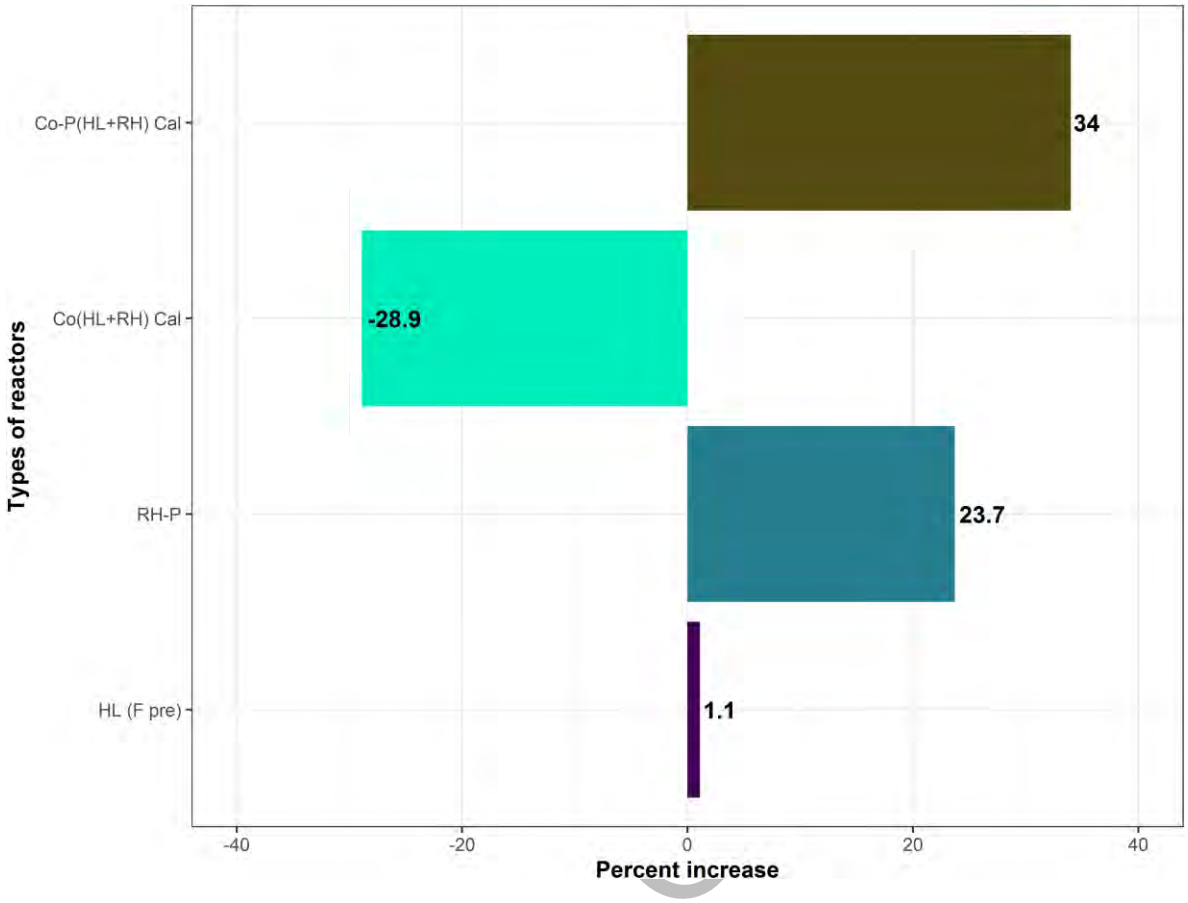

Fig.6.4. Percent increase in yield of HL (hydrolysate in comparison with feathers), RH-P (rice husk-pretreated in comparison with non-pretreated), and Co(HL+RH) co-digestion (hydrolysate + rice husk in comparison with calculated co-digestion of hydrolysate + rice husk) is decreased, Co-P (hydrolysate + rice husk incomparison with calculated co-digestion of hydrolysate + rice husk).

6.4.3. Continuous anaerobic fermentation

The hydrolysate was co-digested with green-grocery waste during continuous anaerobic digestion to enhance biogas production and process stability. The co-digestion significantly increased the biogas yield, process stability and VS removal in a continuous process.

6.4.4. Biogas and biomethane Yield

Biogas yield of continuous anaerobic mono-digestion of microbial pre-treated chicken feathers hydrolysate (H), green-grocerywaste(GW), and their co-digestion (G+H) for three retention times were determined. Daily biogas production was measured as normalised liters. The biogas yield was fluctuating daily with time initially, but at steady-state, the biogas yield for microbial pre-treated chicken feathers hydrolysate (H) becomes zero, green-grocery waste (GW) was 0.34 NL/gVS added while for co-digestion (G+H), the biogas yield was 0.281 NL/gVS measured as described in Fig. 6.5a. The biogas yield of co-digestion was compared with the average values

of both the mono-digestions, which indicated that their average was less than the biogas yield of co-digestion as represented in Fig. 6.5b.

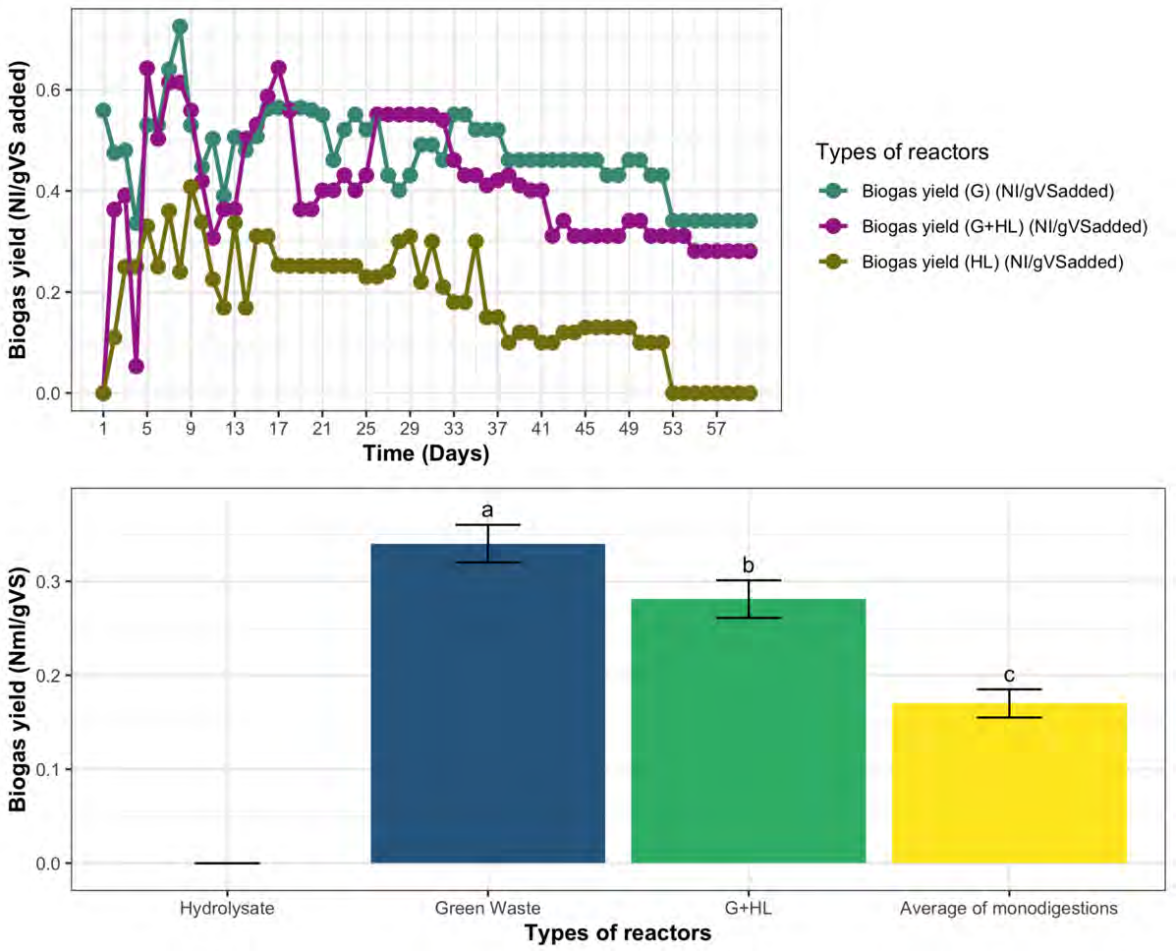

Fig.6.5 a and b. Daily Biogas yield of hydrolysate (H), green waste(G), and their co-digestion (G+H) Biogas yield of Co-digestion compared with an average of Mono-digestions for continuous anaerobic fermentation. Means having the same letter are statistically similar as per the Tukey test at $p < 0.05$.

The biomethane was measured for all the continuous anaerobic reactors; at the beginning, the biomethane fluctuated with time, but at steady-state, the biomethane for microbial pre-treated chicken feathers hydrolysate (H) reactor became zero, on addition of green-grocery waste (GW) was 0.13 NL/gVS while in case of co-digestion on addition of (G+H) it becomes 0.21 NL/g VS as represented in Fig. 6.6a.

While the biomethane yield of co-digestion was compared with the average values of both the mono-digestions, which indicated that their average was less than the biomethane yield of co-digestion (G+H) as represented in Fig. 6.6b

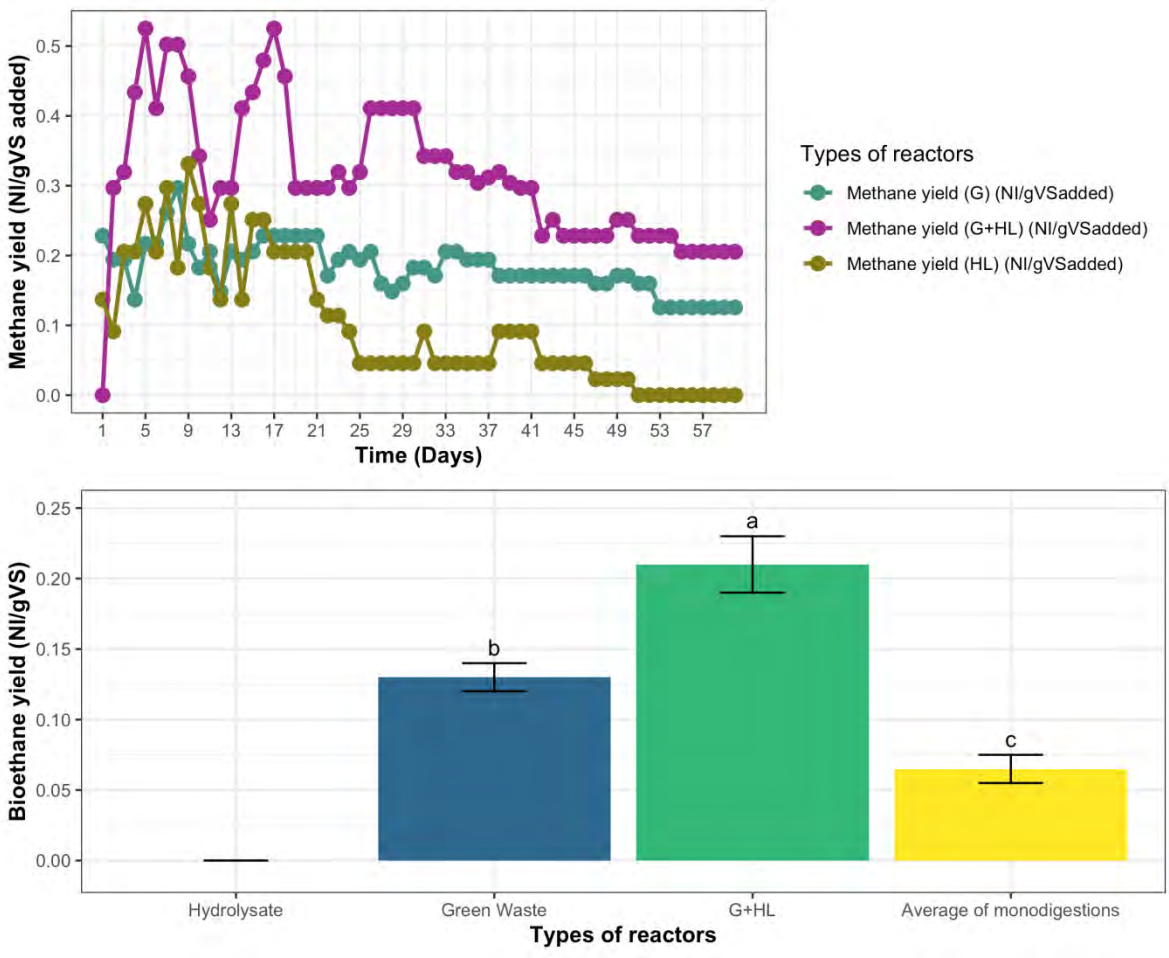

Fig.6.6 a and b. Daily Biomethane yield of hydrolysate (H), green waste (G), and their co-digestion (G+H). Biomethane yield of Co-digestion compared with an average of Mono-digestions for continuous anaerobic fermentation. Means having the same letter are statistically similar as per the Tukey test at $p < 0.05$.

6.4.5. Process stability

The pH, VFAs and alkalinity were used as parameters to monitor the stability of the process. The volatile fatty acids and alkalinity were measured for all the anaerobic reactors represented in Table 6.3

Table 6. 3. Process stability parameters during the continuous anaerobic digestion process. Means having the same letter are statistically similar as per the Tukey test at $p < 0.05$.

Reactor type	pH	VFAs (mg/l)	Alkalinity (mg/l)	VFAs to Alkalinity ratio
Mono-digestion of Hydrolysate	8.5	6250	9000	0.7
Mono-digestion of Green waste	6.8	5000	6125	0.8
Co-digestion of Hydrolysate and Green waste	7.1	5750	6250	0.9

Initially, the pH of all reactors was adjusted to 7.2, optimal for biogas production. During steady-state, the pH of hydrolysate increased to 8.5; in the case of green waste, the pH decreased to 6.8, while in the case of co-digestion, no significant change in pH was recorded. The VFA accumulation was 6250 mg/l, 5000 mg/l and 5750 mg/l in case of mono-digestion of hydrolysate, mono-digestion of green waste and co-digestion of hydrolysate and green waste, respectively. While the VFAs to alkalinity ratio was 0.7, 0.8 and 0.9 in the case of mono-digestion of hydrolysate, mono-digestion of green-grocery waste and co-digestion of hydrolysate and green waste, respectively.

6.5. Discussion

The expanding interest towards biogas generation from bio-waste through complex anaerobic digestion (AD) opened new routes in improving biogas production processes and their up-gradation. Waste generation and waste handling are the most critical issues to overcome these days. Chicken feathers is one of the major wastes, generated in a considerable amount. Feathers can be utilized for energy production by eliminating harmful environmental and catastrophic issues. In this way, waste is properly managed on one hand, and on the other hand bioenergy in the form of biogas is produced (Provin et al., 2021) from the waste. Therefore, the current study was designed to evaluate the effect of different pretreatments and co-digestion and mono-digestion by anaerobic digestion using batch and continuous fermentation and its effect on biogas yield. The volatile solids of chicken feathers, feathers hydrolysate, and rice husk were 70 %, 1.05 %, and 75.98 %, respectively. VS depends upon the type, source, and composition of the substrate.

The results of accumulative biogas production demonstrated that in the case of positive control, most of the gas was produced in the initial 15 days of the experiment, while in case of rice husk pre-treated co-digestion, it took 30 days. In the case of non-pretreated co-digestion, rice husk, and pre-treated rice, there was a gradual increase in production until the end. On the other hand, in the case of feathers, the production was low till 25 days after that, it was increased to reach the maximum level. The time taken by the process depends upon the substrate composition, whereas the positive control contains glucose that was rapidly utilized by acidogens. Different research results reported that hydrolysis depends on substrate composition and carbohydrate hydrolyzed in hrs. while complex compounds like cellulose and lignin take weeks to hydrolyze completely. Some other studies reported that some substrate has hardly accessible for the action of enzymes that ultimately took more time, and the hydrolysis became a rate-limiting step. The present study demonstrated that the microbial pretreatment was completed in 72 hrs.. of incubation. According to the research work reported by Forgács et al., 2011, with different incubation times of 1, 2, and 8 days, the biogas yield was 55 %, 94 %, and 83 %, respectively. The possible reason was the VFAs accumulation in the hydrolysate at the end of the experiment as the VFA measured in the hydrolysate was 875 mg/l and in untreated feathers was 275 mg/l. The VFA accumulation in microbial pre-treated chicken feathers hydrolysate was expected due to the accumulation of ammonia, which negatively affected the methanogenic population. Ammonia is linked to the presence of protein sources in the feed. Since chicken feathers are mainly composed of protein, hydrolysis results in ammonia accumulation (Sypka et al., 2021). During anaerobic digestion of the nitrogenous feedstock, ammonia inhibition is one of the main challenges resulting in lower biogas yield (Chen et al., 2008). At lower concentrations, ammonia acts as a buffering agent, but in a low C/N ratio, the accumulation of ammonia negatively affect the methanogenic population; hence the nitrogenous feedstock must be co-digested with the carbon-rich substrate (Christou et al., 2021). Fig. 6.1a represent the accumulative biogas yield; the microbial pre-treated feathers slightly increased the biogas yield by 1.1 % compared to non-pretreated feathers. The results followed the study reported by Forgács et al., (2011) that determined the effect of microbial pretreatment using different wild and recombinant bacterial strains from chicken feathers. Another study also reports that there was no significant increase in methane yield of chicken feathers when biological treated with *Bacillus megaterium*. However, the analysis of hydrolysate showed that the total dissolved solids were increased by 1 %. At the end of the incubation, VFAs accumulation in the pre-treated feathers was 875 mg/l, while in non-pretreated, it was 275 mg/l. This clearly indicates that the microbial pre-treatment increases the feathers' hydrolysis but results in low biogas yield

due to ammonia inhibition. In the case of the reactor fed with non-pretreated rice husk, accumulative biogas yield was lower than the pretreated rice husk and chicken feathers (Fig. 6.2). The chemical pretreatment of rice husk increases the biogas yield compared to non-pretreated rice husk. Different research results reported increased biogas yield due to acidic pretreatment at different concentrations and conditions. According to the result reported by He et al., (2017), the pretreatment of rice husk with 6 % NaOH resulted in the biogas yield of 0.53NL/gVS while non-pretreated can yield 0.36 NL/gVS. The chemical pretreatment of rice husk showed a 32 % increase in biogas yield compared to untreated rice husk in case of mono-digestion. Mu et al., (2021) also checked the effect of acid mixtures like acetic and propionic acid but was not measured any variance in the treated and untreated rice husk. In another study by Dahunsi et al., (2019), the pretreatment with 2 % sulfuric acid may reduce the hemicellulose content by 69 %. The possible reason was that the chemical pretreatment improves biodegradability, increases the biogas yield, and enhance the efficiency of the overall process. The biomass structure could be changed through chemical pretreatment methods to have more accessibility to anaerobic microorganisms, therefore enhancing the biogas production and digestion efficiency. At the end of the experiment, the VFAs accumulation was 250 mg/l and 350 mg/l in RH and RH-P, respectively, which indicates the increase in hydrolysis due to pre-treatment.

During the current study, the effect of co-digestion on biogas yield with pre-treated and non-pretreated rice husk was determined and compared with the calculated methane yield for rice husk pre-treated co-digestion and rice husk non-pretreated co-digestion. In the case of non-pretreated co-digestion, the calculated yield was determined from the yield of mono-digestion of non-pretreated rice husk and yield of hydrolysate. In the case of non-pretreated co-digestion, the accumulative biogas yield and methane yield were lower than the calculated methane yield. The co-digestion in the case of non-pretreated rice husks, the methane yield was 22.2 % decreased. The decrease in the case of non-pretreated rich husk with cattle manure is supported by Lange et al., (2016). This decrease in yield may be due to ammonia accumulation, as the feathers have high protein contents (Mohanty et al., 2021). Furthermore, rice husk has a complex lignocellulosic structure that is resistant to degradation. At the same time, chicken feathers have a high concentration of proteins, while during degradation of feathers, ammonia is produced due to microbial pretreatment. Ammonia is very toxic in higher concentrations (Christou et al., 2021), and at the end of the incubation, VFAs accumulation in the hydrolysate was 875 mg/l, while in rice husk, it was 250 mg/l. In the case of co-digestion, VFAs were lower than hydrolysate, which confirms that the ammonia in co-digestion affects the hydrolysis of

rice husk, which was the reason for a lower yield in co-digestion as compared to the calculated yield (Fig. 6). Magri (2017) reported that the yield of biogas depends upon the composition and characterisation of the substrate. Nakamura and Asada (2021) reported that rice husk contains high cellulose and lignin, resistant to enzymatic degradation, leading to low biogas yield. However, the increase in biogas yield due to co-digestion depends upon the co-substrate and pretreatment. Hamed (2015), reported a significant increase in biogas yield resulting from mixing cattle manure with food waste in different ratios.

According to the current research work, the methane yield from co-digestion of hydrolysate with pre-treated rice husk was compared to calculated methane yield compared to the yield of mono-digestion of hydrolysate and yield of mono-digestion of pretreated rice husk as represented in Fig. 6.3. The co-digestion of hydrolysate with pre-treated rice husk increases the biogas yield by 34 % compared to the calculated yield. Different studies have reported an increase in biogas yield in the case of co-digestion. Sayara et al., (2019) reported a 758 % increase in biogas yield in case of co-digestion of cattle manure with pre-treated rice husk. Dahunsi et al., (2019) reported a 52 % increase in biogas yield in co-digestion of cattle manure with cocoa husk. The increase in biogas yield was due to the pre-treated rice husk, which has more sugar than non-pretreated. It was reported previously that chemical pretreatment improves biodegradability and increases the biogas yield and enhance efficiency of the overall process (Khan and Ahring 2021). The biomass structure could be changed through chemical pretreatment methods to have more accessibility to anaerobic microorganisms, therefore enhancing the biogas production and digestion efficiency. The other reason for an increase in biogas production may be the effect of a more balance C/N ratio in case of co-digestion of hydrolysate and pretreated rice husk. The nutrients and organic content present in the feedstock affect the activity and growth of the microorganisms. The essential nutrients for microorganisms are carbon and nitrogen (Mothe and Polisetty 2021). The deficiency of any nutrient ceases the growth and activity of the microorganism. A low C/N ratio results in the formation of ammonia, while a high ratio results in the failure of the biogas plant. So in both cases, AD was failed (Nkuna et al., 2021). Appropriate carbon to nitrogen ratio/ nutrient balance is required for the proper growth of microorganisms under stable environmental conditions. Furthermore, this is why the C/N ratio greatly influences anaerobic digestion (Xiao et al., 2021). Generally, the most appropriate (C/N) ratio of 25-30 was required for the suitable development of biological processes (Sillero et al., 2021; Ramirez-Saenz et al., 2019; Liu et al., 2016). Methane production decreases because of the imbalance in the C/N ratio. Moreover, the most common problem was the inhibition by nitrogen in degrading organic wastes. So, the

problem of the C/N ratio could be overcome by co-digestion with a waste having a high C/N ratio. At the end of the experiment, the VFAs accumulation in co-digestion pre-treated rice husk and hydrolysate was lower than both mono-digestion of hydrolysate and pre-treated rice husks in Fig. 6.3. The lower VFAs accumulation in the co-digestion of hydrolysate and pre-treated rice husk means that most of the VFAs are converted to biogas, leading to high yield.

During continuous anaerobic digestion, biogas and methane yield for mono-digestion of microbial pre-treated chicken feathers hydrolysate (H), green-grocery waste (GW), and their co-digestion (G+H) was measured for three retention times. The biogas yield fluctuated daily with time initially, but at steady-state, the biogas yield for microbial pre-treated chicken feathers hydrolysate (H) becomes stable, as shown in Fig. 6.4a and 6.4b. In the case of mono-digestion of hydrolysate, the biogas yield showed a continuous decline with time, leading to process failure in the 3rd retention time. The decline in biogas yield and process failure was due to ammonia accumulation as a result of the high protein content of feathers (Mohanty et al., 2021), as during degradation of feathers, ammonia is produced due to microbial pretreatment. Ammonia was toxic in higher concentrations (Christou et al., 2021). On the other hand, the biogas yield on green waste and co-digestion was more stable during steady-state. As a consequence of co-digestion, the biogas and biomethane yield increased compared to the average of green grocery waste and hydrolysate mono-digestions shown in Figure 6.3. and Figure 6.4.

Co-digestion with substrates with high buffering capacity (alkalinity) such as manure can be good alternatives for the effective treatment of highly biodegradable materials. During the co-digestion of microbial pre-treated chicken feather hydrolysate with green-grocery waste and animal manure, the manure provides buffering capacity and various nutrients. In contrast, the plant material provides high carbon content (Ambaye et al., 2021), resulting in a high biogas yield. The result is a more balanced C/N ratio, and the co-digestion of manure and green-grocerywaste also decreases the risk of ammonia inhibition and acidification. So, it was better to consume microbial pre-treated chicken feather hydrolysate in co-digestion than mono-digestion for biogas production. As green-grocery waste has a high content of carbon and feather hydrolysate has a high amount of nitrogen so when they both were used in co-digester to balance the C: N ratio and produce more biogas and biomethane as compared to the average of their mono-digesters, thus enhanced the biogas yield and enhance the efficiency of overall process (Kiran et al., 2016).

Furthermore, at the start of the experiment, the pH of all the reactors was set to be 7.2, while at the end of the experiment, the pH of hydrolysate increased. In case of green-grocery waste, the pH was dropped down to a critical level, while in case of co-digestion there was no significant change in pH. The pH range varies for different groups of microorganisms for their growth, like for the growth of fermentative bacteria, pH was required about 4.0-8.5, and for methanogenic bacteria, the pH must be in the range of 6.8-7.2; otherwise, failure of the process may occur (Amin et al., 2021). On higher pH levels, the free ammonia concentration increased, causing more toxicity for methanogens (Wang et al., 2020). The VFAs accumulation was high in the case of hydrolysate and finely led to process failure. While in the case of co-digestion, the VFAs accumulation was lower than mono-digestion of hydrolysate and green waste. The co-digestion enhances the process stability and reduces the toxic effect of ammonia, resulting an increase in biogas production (Christou et al., 2021).

6.6. Conclusion

Non-pretreated chicken feathers were challenging to hydrolyse by anaerobic microorganisms because of their recalcitrant nature. Microbial pretreatment of chicken feathers by *Pseudomonas aeruginosa* increased its hydrolysis, but the mono-digestion of hydrolysate results in low yield of biogas due to high concentration of ammonia.

On the other hand, rice husk's pretreatment significantly increased biogas yield. Pre-treated rice husk co-digestion with hydrolysate significantly increased biogas production. However, co-digestion with non-pretreated rice husk does not enhance the biogas yield. During continuous anaerobic digestion, the hydrolysate's co-digestion with green-grocery waste significantly increases the biogas yield. These results suggest the importance of running the bioreactor for long-term in continuous fermentation mode to test the suitability of a novel biomass substrate for industrial biogas production.

6.7. Acknowledgements

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6.8. Conflict of Interest

The authors declare no conflict of interest.

6.9. Funding

Higher Education Commission of Pakistan provided financial assistance to carry out this work (No: 9904/Federal/ NRPU/R&D/HEC/ 2017).

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Chapter 7: Keratin hydrolysate utilization for biofertilizers production

Paper 5 from chapter 7:

Title: Sustainable valorization of chicken feathers and grocery waste as bio-fertilizer and its impact on yield and quality of Spinach (*Spinacia oleracea*)

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7.1. Abstract

Globally, profuse production of chicken feathers is responsible for serious environmental challenges. Feathers composition constitutes keratin protein which is resistant and hard to monomerize. It is estimated that 40 percent of the total food in Pakistan is being wasted. Utilization of feather waste resources for the production of biogas and biofertilizers may contribute to combat the environmental menace and address the crisis of energy and food deficits. In this study, chicken feathers were converted to hydrolysate with the help of indigenously isolated keratinolytic strain, *Pseudomonas aeruginosa*-C1M. Reactor digestate, a byproduct obtained after the production of biogas from hydrolysate and grocery waste also has been shown to be used as biofertilizers. A greenhouse study was carried out to examine the comparative efficacy of reactor digestate and feather hydrolysate on the growth of spinach plant. A remarkable increase in the agronomic parameters was observed with the application of keratin hydrolysate-based fertilizers. Plants treated with dry hydrolysate indicated fifty percent increase in leaf breadth. Whereas, plant height, number of leaves per plant, root length, chlorophyll content, protein content, biomass, and nutrient content in the spinach plant were also considerably enhanced with the application of keratin hydrolysate-based fertilizers. However, the use of *Pseudomonas aeruginosa*-C1M derived hydrolysate yielded more encouraging results, indicating its applicability as a possible bio-stimulant in agroindustry.

Keywords
Chicken feathers, grocery waste, keratin hydrolysate, biofertilizers, Spinach

7.2. Introduction

Worldwide four to five hundred million tons of chicken are used for different purposes in a week time and annual feather waste from the chicken sector surpasses 8.5 million tons.. During current years, broiler production witnessed a regular increase and reached 91.3 million tons (Da Silva 2018). Ninety percent of keratin constituted due to feathers and bird hair loss during processing which do not decompose easily (Verma et al. 2017). It is estimated that, 15. 95 million tons of food is wasted in Pakistan (Hamish et al. 2021). Intensive cropping, required for ever increasing population of the world depends on inorganic fertilizer application (da Costa et al. 2013). Demand for food is increasing with the growth in the population; achieving food security has become a challenge for the world. To handle the growing need for food, higher average yield and quality of crops is pre-requisite. Quality and yield of agronomic and horticultural crops mainly depends on plant available nutrients in the soil. Constant use of inorganic fertilizer led to many unexpected consequences which include increased cost of

production and nutrients. About 50% and 90% of applied N and P is lost by escaping into the atmosphere or water sources, which in return leads to greenhouse gas generation, eutrophication in aquatic system, soil salinization and environmental degradation (Ye et al. 2020).

Therefore, to meet the growing demand for food in a sustained manner, crop productivity should be improved without polluting the environment (soil and water bodies). Shifting to biofertilizer or supplementing the inorganic fertilizers by biofertilizers will not only lead to improvement in soil health but also help to eradicate the menace of environmental pollution of caused by inorganic fertilizers. Biofertilizers are an environment friendly, economical, and sustainable choice as compared inorganic fertilizers as these have a potential to enhance productivity without harming environment. It is also proven as a sustainable waste management strategy (Chew et al. 2019).

Biofertilizers prevent loss of nutrients and can supply essential nutrients for plant growth by the mineralization of organically bound nutrients within the source. Henceforth, biofertilizers employ microorganisms to promote soil fertility and are safe for soil health and product quality; nutrients are delivered to the soil through a natural method (Roychowdhury et al. 2017). It has been determined that the inclusion of renewable waste as a source of biofertilizers increases crop output and reduces nutrient loss, hence facilitating the sustainable growth of the agricultural business. In recent years, natural organic wastes have been included for crop protection and yield enhancement (Nosheen et al. 2021). For this aim, many types of organic fertilizers are produced from green waste, supermarket trash, and chicken waste. Similarly, billions of tons of valuable keratinous waste are being generated by the poultry industry in the form of feathers. Chicken feathers consist of 90% keratin that can be hydrolyzed into peptides, amino acids, and minerals (Rajput et al. 2010). It has the potential to be served as bio-fertilizer or bio-stimulant for plant growth, as it is a rich source of nitrogen. Similarly, feather hydrolysate has been utilized directly or indirectly as fertilizer for the growing of several plants. In recent decades, several scientists have investigated and produced fermented feather hydrolysate or dry hydrolysate for the growing of various plants (Tanmay et al. 2018). It is believed that bio-stimulants might be an effective means of lowering the requirement for inorganic fertilizer and pollution on agricultural grounds (Drobek and Fr 2019). Additionally, through influencing plant metabolism, feather hydrolysate might increase plant output. Studies reveal that when applied to plant leaves, hydrolysate might promote nutrient and soil water absorption (Choi and Nelson 1996). The digestate (effluent) from an anaerobic digester fed

with feather hydrolysate was utilized as crop biofertilizers. However, just a few studies have examined the influence of feathers hydrolysate and other waste on plant development, predicting higher crop growth and improved quality (Biswas et al. 2021). Therefore, more research is required to determine the impact of keratinous and other wastes on several crops by assessing their agronomic and nutritional properties.

With the restriction of inorganic fertilizers in mind, this research was done to harness the potential of an indigenously obtained, powerful keratinolytic strain, *Pseudomonas aeruginosa*-C1M, for the hydrolysis of feathers in submerged fermentation. Furthermore, biofertilizers the potential of keratin hydrolysate along with grocery waste and digestate has been studied for the growth of leafy vegetables. The influences of keratin hydrolysate independently and in combination with grocery waste on the physicochemical, agronomical, and nutritional aspects of the spinach plant were explored.

7.3. Materials and Methods

7.3.1. Preparation of Biofertilizers from Chicken Feather Hydrolysate and Grocery waste

Biofertilizers were prepared based on feather hydrolysate and grocery waste (containing fruits and vegetable waste). Chicken feathers were cleaned by washing with 10% Triton X-100, sun-dried for 1 week before microbial treatment. For the production of keratin hydrolysate, Keratinolytic strain *Pseudomonas aeruginosa-C1M*, isolated from chicken feathers dumping region was selected was used for Hydrolysate synthesis. Under submerged fermentation, chicken feathers (CF) were used as substrate and 0.1kg chicken feathers per liter were added in fermentation medium along with some salts: K_2HPO_4 (4g/L), KH_2PO_4 (3g/L), NaCl (2g/L), Na_2SO_3 (2g/L), and pH was adjusted to 8. Seeding of fermentation broth was accomplished with 5% inoculum of an overnight culture of *Pseudomonas aeruginosa-C1M.* Fermentation was carried out at 37°C, constant shaking rpm of 160 for 72 hours. After complete hydrolysis of CF, centrifugation was done to remove cell at 11,000xg for 10 minutes to obtain Hydrolysate. After the conversion of feathers into hydrolysate with the help of *Pseudomonas aeruginosa-C1M*, hydrolysate was used as biofertilizers. Digestates from mesophilic, anaerobic reactors were also used as biofertilizers. Five forms biofertilizers from reactors for biogas production were taken as following: a) Feather Hydrolysate (FH) obtained directly from bacterial strain hydrolysis of feathers (L) b) Dry Hydrolysate (DH), freeze-dried form of feather hydrolysate (FH) c) Digestate obtained from Continuous reactor utilizing feather hydrolysate as substrate referred as anaerobic digestate hydrolysate (H), d) Digestate from grocery waste operating reactor

known as GW and e) Digestate from reactor operating on co-digestion of grocery waste and feather hydrolysate mentioned as (GW+FH).

7.3.2. Green House Study

This research was conducted at the Green House of the National Agricultural Research Centre in Islamabad and continued in the Soil and Plant Nutrients Program laboratory. This study's experimental design was a Completely Randomized Design (CRD) factorial with two components, namely biofertilizer type, i.e, FH, H, GW, GW+H, DH and level of biofertilizer which consist of Control, 8% w/v, 10% w/v, 12% w/v with three replication. All treatments were applied at the time of transplantation of seedlings to pots. The samples of plant shoots and roots were gathered and tested for their chemical content. The growing medium soil was acquired from the NARC research area. It had alkaline pH (8), had low in organic matter (1%), and was a significant source of nutrition. It belonged to the Rasulpur soil series, had a sandy texture, and was a mixture of hyperthermic, udic, and usto-chreptic combocorthid. Amino acid content of feather hydrolysate was determined through amino acid analyzer (Table 7.1).

Table 7. 1. Amino acid concentration of feather hydrolysate determined through amino acid analyzer

Amino acid concentration in Hydrolysate			
S. No.	Amino acid		%
1	Glutamate	Glu	6.372
2	Aspartic Acid	Asp	4.213
3	Cysteine	Cys	3.464
4	Serine	Ser	3.075
5	Glycine	Gly	2.848
6	Leucine	Leu	2.77
7	Valine	Val	2.363
8	Threonine	Thr	2.275
9	Arginine	Arg	1.973
10	Phenylalanine	Phe	1.873
11	Alanine	Ala	1.635
12	Isoleucine	Ile	1.354
13	Lysine	Lys	1.27
14	Histidine	Hist	0.491

7.3.4. Biofertilizer, Soil and Plant Analysis

Amino acid profile of biofertilizers were determined by method described by (Ullah et al. 2005) using AA analyzer (Biochrom 30 plus, Biochrom Ltd. Cambridge, UK). Samples were oxidized

with H_2O_2-CH_2O_2, C_6H_6O in order to protect methionine and cysteine. Utilizing sodium disulphite, the surplus oxidation reagent was decomposed. The samples were then hydrolyzed for 24 hours with 6M HCl. After adjusting the pH of the hydrolysate to 2.20, it was centrifuged and filtered. Using AA analyzer at 570 nm, amino acids in solution were separated (Horowitz 2000). ECe (McLean 1983), pH (McLean 1983), organic matter (Nelson and Sommers 1983), and AB-DTPA extractable Zn, Cu, Fe, and Mn were measured in the soil (Soltanpour and Workman 1979). For the measurement of nitrogen (N), phosphorous (P), potassium (K), and micronutrient, whole-shoot samples were taken. The plant samples (leaves and fruits) were cleaned, dried at 65 degrees Celsius, and finely pulverized. The nutrients in plant material were wet digested in a 2:1 mixture of HNO3 and HClO4 (National et al. 1993). Total N was calculated using the Kjeldahl technique (Winkleman et al. 1990), and the K content in the digest was measured using a flame photometer. P was assessed using colorimetry, whereas Zn and Fe were evaluated by atomic absorption spectroscopy. SPAD 502 chlorophyll meter was used to test chlorophyll content (Konica Minolta Sensing Inc. Japan).

7.3.5. Plant growth and yield parameters

The height of the plant, the number of leaves per plant, the breadth and length of the leaves, the length of the roots, and the fresh and dry biomass of the plants and roots were recorded.

7.3.6. Statistical Analysis

Data regarding growth parameters was subjected to Analysis of Variance (ANOVA) using Statistix 8.1 software.

7.4. Results

7.4.1. Bio-chemical Composition of Prepared Hydrolysate

Results regarding amino acid analysis are summarized in (Table 7.1). Results indicated that highest concentration (6.37%) Glutamate (Glu) observed in the feather hydrolysate. It was followed by other amino acids like Aspartic acid (Asp), Cysteine (Cys), Serine (Ser), Glycine (Gly), Leucine (Leu), Valine (Val), Threonine (Thr), Arginine (Arg), Phenylalanine (Phe), Alanine (Ala), Isoleucine (Ile), Lysine (Lys) and Histidine (Hist) in descending order (Table 1). Gutamate is an essential for constituent for protein synthesis. It provides defense to plants against insects; regulate osmolality, signaling, and pH balance (Forde and Lea, 2007). Cysteine is involved in the synthesis of various metabolites for a plant's immune response (Tahir et al., 2016). Likewise, another study showed it can also stimulate the synthesis of various proteins and cofactors (Bonner et al., 2005) similarly, serine regulates biotic and abiotic stress along

with manage photorespiration (Timm et al., 2013). Results regarding chemical analyses of bio fertilizers is concerned highest nitrogen (N), phosphorus (P), and potassium (K) content were observed in dry hydrolysate (DH) in comparison to other fertilizers. (Table 7.2)

Table 7. 2. NPK (Nitrogen, phosphorus and potassium content) and pH of various types of bio-fertilizer

S. No.	Fertilizers	N (%)	P (%)	K (%)	pH
1.	FH	0.18%	0.0174%	0.14%	7.8
2.	H	0.26%	0.017%	0.13%	7.9
3.	GW	0.13%	0.017%	0.13%	7.0
4.	GW+H	0.30%	0.011%	0.11%	7.4
5.	DH	9.13%	0.065%	1.34%	7.2

7.4.2. Impact of applied biofertilizers on agronomic and nutritional composition of spinach

Our results indicated that leaf breadth was significantly influenced by applied bio-fertilizers. The highest leaf breadth (3.90 cm) was found in the plants treated with DH while the lowest (2.90 cm) in the plants fertilized with GW. Leaf breadth observed in the plants treated with fertilizers based on anaerobic digestate H, FH and combinations of GW + H was 3.25, 3.21, and 3.17 cm, respectively. The overall trend of leaf breadth was as follows: DH>H>FH>GW+H>GW (Table 7.3). Level of applied biofertilizers also significantly influenced leaf breadth, highest leaf breadth (3.81 cm) was observed in the plants treated with12% w/v, followed by other 10% w/v and 8% w/v in descending order. Increased leaf growth in terms of leaf length was also recorded with the application of biofertilizers. Highest leaf length was observed in the plants treated with DH followed by the plants treated with H, FH, GW+H, and GW showing leaf length 8.16, 7.89, 7.80, and 6.30 cm respectively (Table 7.3). An increase in leaf length of spinach plants has been seen in protein-derived fertilizers is due to the immense supply of nitrogen from roots to stem. Again, interaction between applied biofertilizers and their levels was significant, which indicated difference in growth due to differential nutrient composition of applied biofertilizers. Moreover, as dry hydrolysate contained the highest amount of nitrogen and other minerals therefore sub-sequential rise in bio-fertilizer concentration leads to distinguishable enlargement of leaf length (Table 7.3).

Table 7. 3. Leaf breadth, Number of leaves, Plant height, Fresh biomass and Dry biomass as influenced by applications of bio-fertilizers

Treatments	Leaf breadth	Number of leaves	Plant height	Fresh Biomass	Dry Biomass
----------Levels----------					
0	2.54D	5.15D	4.73D	7.95D	0.31D
8	3.30C	12.73C	8.55C	10.07C	0.40C
10	3.49B	15.0B	9.39B	11.03B	0.43B
12	3.81A	18.3A	10.8A	11.79A	0.47A
---------- Types----------					
FH Ω	3.21B	12.2B	7.89B	10.22B	0.39B
H$	3.25B	12.78B	8.16B	9.825C	0.40B
GW£	2.90C	9.12C	6.34C	9.07D	0.36C
GW+H	3.17B	12.28B	7.80B	9.8C	0.39B
DHλ	3.90A	17.62A	11.73A	12.06A	0.48A
Interaction (LxT)	S	S	S	S	S

The number of leaves serves as an important parameter in the spinach plant since these leaves are related to plant photosynthetic rate and utilized as a vegetable. Results showed that the number of leaves exhibited marked growth in feather-derived fertilizers as compared to control. Pots supplied with dry hydrolysate have around 25 leaves on average while Feather hydrolysate (FH), Hydrolysate (reactor digestate), and Grocery waste+ Hydrolysate (GW+H) had the approximately same number of leaves (around 15-18). However, pots containing green waste (GW) depicted the least number of leaves. Similarly, with an increasing percentage of the bio-fertilizer number of leaves also increase. Some leaves of leafy vegetables are directly linked with the concentration of macro (N, P, and K) and micronutrients. After harvesting, the observation made was between organic fertilizers at 8%, 10%, and 12%. Highest chlorophyll content was observed in dry hydrolysate fertilized plants :(51.16 cm) followed by H, FH, GW+H, GW with 35.78, 34.85, 34.16, and 32.49 cm respectively. The least chlorophyll content observed was in the case of grocery waste 32.49. Plants treated with12% DH has the highest chlorophyll content (73.26). As chlorophyll synthesis involves sulfur and keratin has a high content of cysteine amino acid this is a direct source of sulfur for the spinach plant. Sulfur not only reduces soil pH but also enhances root's growth. The fresh biomass of plants in the pot experiment after harvesting was measured. The result revealed a significant difference between bio-fertilizer and control. Dry hydrolysate (DH) treated plant showed higher fresh weight. (12.06g), followed by feather hydrolysate (F.H), hydrolysate (H), grocery waste+ hydrolysate (GW+H), and grocery waste (G.W) respectively in descending order; 10.22g, 9.825g, 9.8g, and

9.0g. The highest fresh weight of 88.3% (11.79g) observed was with the application 12%w/v. This high biomass may be due to the high uptake of nutrients and effective growth in the case of dry hydrolysate fertilizers. Minimum fresh weight was observed in the spinach shoot where poultry-driven fertilizer was not applied. After drying of plants, measured was shoot dry mass. The highest dry biomass of shoots (0.48g) observed was in DH treated spinach samples in comparison to other fertilizers and control. The lowest value related to dry biomass among fertilizers was found in plant samples grown with grocery waste 0.36g as a fertilizer due to lack of nutrients. The least dry mass observed was in the case of control C. Dry weight of spinach was superior for the 12% fertilizers followed by other applied levels. e, 10% w/v and 8% w/v in descending order. There is a 51% increase in dry shoot mass of 12 %w/v organic fertilizers compared with control. Our results indicate that all levels of fertilizers. Our results indicated that total K and N content in the spinach plant were significantly influenced by application of biofertilizers (Table 7. 4). Highest K content (3.75%) was observed with the application of 12% w/v level which was followed by 10, 8 and 0% in a descending order. Similar trend was observed in case of total N content; Maximum N content (3.40%) was observed with the application of 12% w/v level of biofertilizers. Interactive effect of applied levels of biofertilizers and types was significant which might due to differential initial N and K status of biofertilizers given in Table 2. Highest protein content (21.2%) was observed with the application of 12 % w/v, biofertilizer level which was statistically significant (higher) than all other applied levels of biofertilizers (Table 7.4). The means of root length of all fertilizers treated plants showed a significant difference. Dry hydrolysate treated plants have a maximum root length of 6.39cm. It was followed by H (5.51cm), FH (5.30cm), GW+H (5.15cm) GW (5.06cm). The highest root length of 6.18cm was analyzed in 12% of dry hydrolysate bio-fertilizers with a 50.4% increase from control, followed by 10% (Table 7.4).

Table 7. 4. Potassium, Nitrogen, Protein, chlorophyll content and Root length as influenced by the applications of bio-fertilizers

Treatments	K (%)	N(%)	Protein (%)	Chlorophyll content (SPAD)	Root length
----------Levels----------					
0%	1.26D	2.33D	14.6D	14.10D	4.11D
8%	3.12C	2.89C	18.1C	42.94C	5.74C
10%	3.68B	3.14B	19.6B	45.15B	5.89B
12%	3.75A	3.40A	21.2A	48.56A	6.18A
---------- Types----------					
FH Ω	3.08B	2.85B	17.8B	34.85B	5.30C
H$	2.86C	2.91B	18.2B	35.78B	5.51B
GW£	2.45D	2.58C	16.2C	32.49C	5.06D
GW+H	2.82C	2.84B	17.8B	34.16BC	5.15B
DHλ	3.53A	3.49A	21.8A	51.16A	6.39A
Interaction (LxT)	S	S	S	S	S

7.5. Discussion

It is a well-established fact that micro fauna present in the soil enhance the absorption of peptides and amino acids, therefore application of biofertilizers derived from chicken feathers has become a common practice (Genç and Atici 2019). Due to increased energy demand, scarce natural resources, biogas production using grocery wastes, poultry litter and other wastes has become a new renewable energy alternative (Khan and Martin 2016).

Solids and other wastes are discharged as a byproduct of biogas production by anaerobic digestion, which is regarded a waste and a possible environmental pollution. Use of these byproducts of biogas reactors as biofertilizers is not only considered as a source of macro and micro nutrients to plants but it also enhances the bioavailability these nutrients to crops and can increase the productivity of crops while reducing greenhouse gas emission to environment (Xu et al. 2021). Analysis of variance (ANOVA) is a robust and straight forward statistical technique employed to analyze the data regarding experiments related to agricultural research (Meftahizadeh et al. 2019).

In this study we used two CRD two factorial designs to compare the efficacy of different biofertilizer levels and types on the yield and growth of spinach. Positive responses of various crops to digestates of various reactors through which biogas is produced are well documented

(Xu et al. 2021). However, only a handful of research have examined the influence of feathers hydrolysate and other waste on plant development (Biswas et al. 2021), which projected increased crop growth and quality. This study was conducted to determine the impact of keratinous waste and other waste on numerous crops by examining their agronomic and nutritional properties. Crops obtain essential nutrients from the soils which play a vital role. Nitrogen is involved in various metabolic pathways, photosynthesis as it is a main component of Chlorophyll and growth of plants (Boussadia et al. 2010; Leghari et al. 2016). K is found to provide abiotic stress tolerance. Potassium also maintains ion homeostasis and to adjust osmotic balance. Potassium controls opening and closing of stomata and helps plants to withstand harsh climatic condition of drought. It also increases antioxidant protection safeguards from oxidative stress under environmental extremes (Hasanuzzaman et al. 2018). Deficiency these nutrients in the soils of Pakistan is well documented due to regular nutrient mining, calcareousness, alkaline pH and low organic matter content (Ahmed et al. 2014).

It is estimated that more than 90% soils of Pakistan are deficient in plant available N and P content, Similarly plant available potassium content are deficient in 50% cultivated soils of Pakistan (Rizwan et al. 2016). The observed increase in plant growth and biomass in response to the application of biofertilizers containing essential nutrients such as N, P, and K, Zn, Cu, Fe, and Mn may be attributable to the biofertilizers' positive effect on cell elongation, cell division, and increasing photosynthetic surface, resulting in increased production and accumulation of photosynthetic compounds (Ahmad et al. 2010; Ahmed et al. 2012; Malik et al. 2014).

Additionally uptake of amino acids by plant is advantageous when compared with the inorganic application of N in the form of NO_3 and NH_4 as there is no prerequisite energy demand to assimilate N and then convert it into amino acids (Teixeira et al. 2018). Application of an aqueous solution of different biofertilizers containing amino acids to the soil increases their availability in the rhizosphere due to the action of amino acid transporters in the cell membranes of root hair that are in direct contact with the amino acid-containing soil solution (Jämtgård et al. 2010).

Use of biofertilizers obtained from chicken feather and biogas reactors for spinach cultivation is an agricultural eco-circulatory system for livestock, grocery and poultry waste and organic vegetable production.

7.5. Conclusion

The human growth leads to the exacerbation of agricultural production. For enhanced crop production and yield use of agrochemicals has been increased. Excessive use of chemicals is toxic to the environment, human health, and other creatures. Hence, bio-based cost-effective fertilizers are need of the hour to meet world food requirements and to improve crop quality and yield by reducing hazardous outcomes. Feather‘s waste is a rich source of nitrogen, with the help of indigenously isolated keratinolytic strain, *Pseudomonas aeruginosa*-C1M; it was bio-converted into protein rich hydrolysate which served as a superior fertilizer in comparison, with other resources responsible for better growth of spinach plant. However, for bioconversion of feathers into hydrolysate pilot-scale production methods should be devised.

7.6. Acknowledgements

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7.7. Disclosure statement

The authors declare that they have no conflict of interest.

7.8. References

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Chapter 8: Conclusion

In this study keratinase producing microorganisms were isolated and screened from feathers dumping site and leather industry tannery area. *Pseudomonas aeruginosa* C1M was selected for this study, due to its efficient keratinolytic properties. Optimization of nutritional and physical parameters increased the production of keratinase from *Pseudomonas aeruginosa-C1M* strain, which further lead to significant increase in feather degradation. The potential of keratinolytic microorganisms were used for the bioconversion of feathers waste into value added products such as bioactive peptides having antioxidant potential extracted from keratin hydrolysate (**Paper I**).

Keratinase (32kDa) after purification was active on the soluble proteins keratin azure, casein, and bovine serum albumin (BSA); however, no conspicuous activity was observed in the presence of insoluble hair. Keratinase displayed high compatibility and stability with detergents by efficiently removing the proteinaceous stains from fabric pieces and exhibited massive activity improvements, compared with commercial detergents (**Paper II**).

Further study describes a straightforward and environmentally friendly method to synthesize protein-capped silver nanoparticles by utilizing the keratinase potential. Characterization revealed that nanoparticles were spherical and had a size range in nanometers. XRD measurements revealed the silver nanoparticles' crystalline structure. AgNPs were successfully bio-conjugated using FTIR. AgNPs were highly effective antibacterial agents against the tested strains of bacteria. Toxic dyes were converted into less toxic form by the help of silver nanoparticles (**Paper III**).

Non-pretreated chicken feathers were challenging to hydrolyze by anaerobic microorganisms because of their recalcitrant nature. Microbial pretreatment of chicken feathers by *Pseudomonas aeruginosa C1M* increased its hydrolysis, but the mono-digestion of hydrolysate results in low yield of biogas due to high concentration of ammonia. Pre-treated rice husk co-digestion with hydrolysate significantly increased biogas production. However, co-digestion with non-pretreated rice husk does not enhance the biogas yield. During continuous anaerobic digestion, the hydrolysate's co-digestion with green-grocery waste significantly increases the biogas yield. These results suggest the importance of running the bioreactor for long-term in continuous fermentation mode to test the suitability of a novel biomass substrate for industrial biogas production (**Paper IV**).

The human growth leads to the exacerbation of agricultural production. For enhanced crop production and yield use of agrochemicals has been increased. Excessive use of chemicals is toxic to the environment, human health, and other creatures. Hence, bio-based cost-effective

fertilizers are need of the hour to meet world food requirements and to improve crop quality and yield by reducing hazardous outcomes. Feather‘s waste is a rich source of nitrogen, with the help of indigenously isolated keratinolytic strain, *Pseudomonas aeruginosa*-C1M; it was bio-converted into protein rich hydrolysate which served as a superior fertilizer in comparison, with other resources responsible for better growth of spinach plant (**Paper V**).

Chapter 9: Future Prospects

Prospects to continue this in the future are as follows:

Further research could be carried out on feathers from other sources than chicken, and the degradation effect of melanin-containing feathers must be explored. The structure and properties of active antioxidant peptides from feathers hydrolysate could be studied and artificially synthesized for their applications in pharmaceutical products. The role of bioactive peptides could be investigated as encapsulating agents for the drug-delivery system.

The expression of keratinase in active form makes them valuable industrial candidate. Crystal structure determination of keratinase can enable better rational designing for further studies. Extensive characterization of the existing proteases with a particular focus on substrate specificity needs to be conducted to explore their keratinolytic potential

Nitrogen spray fertilizers could be developed for better application in the agricultural field.

The potential of other raw substrates along with chicken feathers could be explored in co-digestion process for enhanced biogas production and improved quality.

By utilizing a broad area of applications of keratinases, their utility could be enhanced by improving their better catalytic efficiency and versatile substrate specificity. Keratinase potential can be utilized in synthetic biology applications to produce other value-added compounds. A nutritionally rich protein-based feed could be prepared for aqua-cultures and other animals. However, for bioconversion of feathers into hydrolysate pilot-scale production methods should be devised.

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Microbial Pretreatment of Chicken Feather and Its Co-digestion With Rice Husk and Green Grocery Waste for Enhanced Biogas Production

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To utilize wastes and residues sustainably and excellently, there is a need to fend for efficient methods and resources for biogas production. Use of poultry waste for biogas production represents one of the most important routes toward reaching global renewable energy targets. The current study involves microbial pretreatment of chicken feather waste, followed by its co-digestion with rice husk and green grocery waste in batch and continuous reactors, respectively. Microbial pretreatment of chicken feathers by keratinase secreting *Pseudomonas aeruginosa* was an effective and eco-friendly approach to make its recalcitrant structure available as a raw substrate for biogas production. The current study also addressed the enhancement and stability of anaerobic digestion by co-digestion. Results demonstrated that biogas production was increased by microbial pretreatment of chicken feathers and that the percentage increase in biogas yield was 1.1% in microbialy pretreated feathers compared to mono-digestion (non-pretreated feathers) in batch fermentation. The highest yield of biogas was obtained in a batch reactor having co-digestion of pretreated rice husk and microbial pretreated chicken feathers. The co-digestion of chicken feathers hydrolysate with green grocery waste in continuous fermentation mode has also enhanced the

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