DURBAN UNIVERSITY OF TECHNOLOGY
INYUVESI YASETHEKWINI YEZOBUCHWEPHESHE

Biodegradation of poultry feathers using a keratinolytic enzyme to produce feather meal

NOLENE RAMALINGUM

Student Number: 19400303

Submitted in fulfilment of the requirements for the degree of

Master of Applied Science

in

Biotechnology

Department of Biotechnology and Food Science, Faculty of Applied Sciences,

Durban University of Technology, Durban, South Africa

2022

Supervisor: Prof. Kugenthiren Permaul

Co-supervisor: Prof. Santhosh Kumar Kuttan Pillai

DECLARATION

I hereby declare that this dissertation is my own, unaided work. It is being submitted for the award of the Master of Applied Science in Biotechnology, to the Durban University of Technology, Department of Biotechnology and Food Science, Faculty of Applied Sciences, Durban, South Africa. It has not been submitted before any degree or dissertation to any other institution.

Student: Date 06 April 2022

Mrs. Nolene Ramalingum (B. Tech.)

Supervisor: Date 06 April 2022

Prof. K Permaul (Ph. D)

Co Supervisor: Date 06 April 2022

Prof. S. K. Pillai (D.Tech)

DEDICATION

Dedicated to Almighty God

and

My Family

Who always picked me up and encouraged me

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ACKNOWLEDGEMENTS

First and foremost, praise and thanks to God almighty for guiding me through this research.

I cannot express enough thanks to Professor Santhosh Pillai for his invaluable guidance, encouragement and patience. I could not have imagined a better supervisor and mentor for my study. Many thanks also extended to Professor Kugenthiren Permaul for his mentorship and his valuable input. I owe a deep sense of gratitude to Dr Prashant Bhagwat. The completion of this project could not have been accomplished without his wealth of knowledge. His sincerity and motivation really inspired me. It was a great privilege to work under his guidance. I would also like to acknowledge and give my warmest thanks to Ayodeji Amobonye for his help, support and encouragement throughout the research.

Many thanks are extended to RCL Foods for the support received during my research. A special thanks to Johan Gravett and Brett Rosendaal for their permission and understanding to pursue the research.

Last but not least, to my husband and children, my deepest gratitude. Your encouragement when times were rough are much appreciated. I am extremely grateful for your love, care, prayers and sacrifices.

ABSTRACT

The application of biotechnology through the utilisation of enzymes is considered an easy and inexpensive method of producing valuable products from poultry feather wastes. The present study describes production of a keratinolytic enzyme from *Pseudomonas aeruginosa* S-04, which showed efficiency for feather biodegradation. The production of extracellular keratinase was improved 1.3-fold through one factor at a time (OFAT) optimisation of various parameters. Ammonium sulphate precipitation and DEAE-cellulose anion exchange chromatography were used to purify the keratinase produced by *P. aeruginosa* S-04 to homogeneity. Purified keratinase (35.5 kDa) showed optimal activity at 60°C and pH 9.5 and displayed stability over the pH range 7-9.5 and temperatures ranging from 4-40°C for 2 h. Catalytic activity of keratinase was enhanced in the presence of Fe^{3+} and Mn^{2+} ions, Triton X-100, Tween 20, DMSO, isopropyl alcohol and ethanol, but reduced activity was recorded in the presence of methanol and acetone. The enzyme activity was deactivated by EDTA, suggesting that this keratinase belongs to metallo-protease family. The K_m and V_{max} of the purified keratinase was found to be 7.62 mg/ml and 200 U/mg protein, respectively. The partially purified keratinase revealed great potential for feather degradation (93% in 24 h), and the nutritional content of the resulting feather hydrolysate makes it a promising candidate for application in the poultry industry.

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CHAPTER 1

Introduction and literature review

The global population is increasing at an alarming rate, and South Africa is not an exception. Recent estimates showed that South Africa has 58.8 million people (Africa, 2022), and due to the increasing population, there is a constant demand for food products, especially meat, which forms an important protein source in the diet. Furthermore, the relative affordability of poultry meat than red meat has resulted in poultry becoming the major protein source in many South Africans' diets (Bisschoff, 2017). As a result, chicken output has increased, making the poultry industry the largest section of the South African agricultural economy, accounting for more than 16% of the country's gross domestic product (Oluwatayo *et al.,* 2016).

Poultry processing for human use produces massive volumes of organic waste, such as feathers, which poses a number of environmental issues (Lemes *et al.,* 2016). Landfilling is one of the most practiced traditional strategies for feather disposal. However, landfilling demands increased disposal areas, resulting in environmental liabilities (Abdel-Shafy and Mansour, 2018). Though chicken feathers are considered a waste material, it comprises mainly keratin which has a high protein content of 80 – 90%. As a result, landfills with uncontrolled anaerobic breakdown of keratin-rich feathers and associated materials are likely to emit ammonia and hydrogen sulphide. Incineration is an alternative, that removes enormous amounts of feathers almost instantly. Nonetheless, it is a costly procedure with the potential for serious health and environmental consequences due to harmful gas emissions (Sharma and Devi, 2017).

Hydrothermal treatment is the most common method for feather reclamation, where feathers are converted into feather meal using high temperatures (80 - 180°C) and high pressures (207-690 kPa). Feather meal produced as a result could then be utilized in animal feed (Kormanjos *et al.,* 2015). However, there are some drawbacks to the hydrothermal treatment. The loss of thermolabile amino acids such as methionine, lysine and histidine occur when exposed to such high temperatures (Embaby *et al.,* 2015). In addition, lanthionine and lysinoalanine are also produced, which are potentially toxic and diminish the bioavailability of amino acids (Tamreihao *et al.,* 2019). Furthermore, the digestibility of feather meal is diminished, especially in monogastric animals, due to the resistance of

digestive tract enzymes such as pepsin and trypsin (Callegaro *et al.,* 2019). As a result, hydrothermally treated feather meal is a 'low added-value product' with poor digestibility and nutritional content.

Keratin is a fibrous structural protein that is quite robust and has a high mechanical strength thanks to disulphide bonds that form cross-links between protein-peptide chains, resulting in a dense polymeric structure when combined with hydrogen bonds and hydrophobic forces (Wang *et al.*, 2016). This recalcitrant property of keratin renders it inaccessible to degradation by common proteases such as trypsin. However, it can be efficiently degraded by various microorganisms, *viz.,* bacteria, fungi and actinomycetes, by the action of their secreted keratinases (Calin *et al.,* 2017).

Microbial keratinases are a cost-effective and environmentally beneficial way to degrade and recycle keratinous wastes into useful by-products. Microbial keratinases have a wide range of biochemical features, including activity and stability in a wide range of pH and temperature ranges, as well as broad specificity for keratins from feathers, hair, nails and other sources (Sharma and Devi, 2017). The use of keratinases to hydrolyse and degrade feathers aims to eliminate some of the limitations posed by the hydrothermal feather treatment methods (Dhoolappa *et al.,* 2016). Some of the advantages of enzymatic hydrolysis include milder reaction conditions (e.g., temperature, pH and atmospheric conditions), eliminating the need for the protection of substrate functional groups, thereby providing the product with a longer shelf life. Enzymes can also be genetically and chemically altered to improve critical features like stability, substrate specificity and specific activity (Adrio and Demain, 2014). Keratinases are considered promising biocatalysts for the preparation of animal nutrients, protein supplements, leather processing, fibre modification, detergent formulation, feather meal processing for feed and fertilizer, the pharmaceutical, cosmetic and biomedical industries, and waste management due to their diverse properties and multifarious biotechnological implications (Sharma and Devi, 2017).

1.1 Chicken feather - A waste product from the poultry industry

The poultry industry is an important and diverse component of the food sector since poultry products, including eggs, chicken and turkey meat are an important protein source in the diets of many people (Brandelli *et al.,* 2015). Moreover, being one of the cheapest and healthiest protein sources, the poultry industry is continuously expanding to meet its increasing global demand (Nur-E-Alam *et al.,* 2019). Poultry production, on the other hand, generates a large amount of organic waste, such as feathers and viscera, as a result of the processing of raw materials. Considering that feathers account for approximately 5 - 10% of the total weight of chickens, globally, an estimated 5 million tons of poultry feathers are generated from farms each year (Callegaro *et al.,* 2019).

1.2 Environmental problems associated with feather disposal

Management of feather disposal encompasses enormous challenges for the poultry industry (Callegaro *et al.,* 2019). Traditionally, methods such as incineration and landfilling are usually employed to dispose of feathers. Disadvantages associated with these processes include high operational and energy costs, loss of natural resources and detrimental effects on the environment (Wu *et al.,* 2017). This increases the disposal cost, and thus, the poultry industry seeks alternate and economical waste disposal methods. Furthermore, smoke and ash emitted by the chimneys of incinerators could contain acid gases, nitrogen oxide, heavy metals, particulates, and carcinogenic dioxin (Patil *et al.,* 2014).

Landfills occupy large area of land and the surrounding regions are often heavily polluted. It is difficult to eliminate the dangerous chemicals leaching out from landfills into the surrounding land and the groundwater system (Salami *et al.*, 2013). Landfills also add to the greenhouse effect by creating methane (from anaerobic decomposition), a gas 20 times more efficient than carbon dioxide at trapping heat in the atmosphere. In addition, many insects and rodents, e.g., mosquitoes and rats, are attracted to landfills and can spread several diseases (e.g., malaria) (Melikoglu *et al.,* 2013). Besides being cost-intensive and detrimental to the environment, the conventional methods of feather disposal ultimately lead to the loss of a potential protein source since feathers comprises mostly of keratin protein.

1.3 Structure and composition of feather keratin

Chicken feathers contain approximately 91% protein (keratin), 1% lipids, and 8% water. Keratins are scleroprotein proteins that are insoluble and extremely resistant to physical, chemical and biological activities (Femi-Ola *et al.*, 2015). Keratins can be classified as α-keratin or β-keratin based on the secondary structure (Figure 1.1). The β-keratin is rich in β-pleated sheets and is constructed from supramolecular fibril bundles, whilst α-keratin consists of α-helical-coils which are self-assembled into intermediate filaments (Navone and Speight, 2018).

Hard β-sheet keratins contain more cysteine than soft α-helix keratins, resulting in a higher number of disulphide (S–S) bonds connecting adjacent keratin proteins (Perta-Crisan *et al.*, 2021). Hard keratins (those containing up to 5% sulphur) are found in appendages like feathers, hair, hooves and nails; have high disulphide bond content and are tough and inextensible. On the other hand, soft keratins (those containing up to 1% sulphur) like skin have a low content of disulphide bonds and are more pliable (Singh and Kushwaha, 2015).

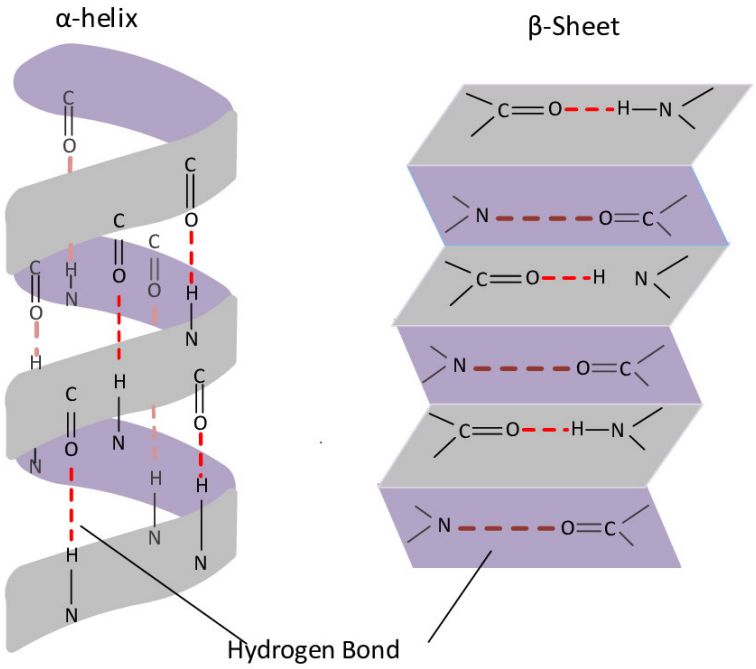

Figure 1.1 Secondary structure of feather keratin depicting α-helix and β-pleated sheets (Whitford, 2013).

Keratins comprise a tight packing of supercoiled long polypeptide chains with molecular weights of approximately 10 kDa. The chemical composition and spatial arrangement confer biological specialities to these fibrous proteins such as high resistance to diverse proteolytic microorganisms, enzymes, chemicals and mechanical stresses (Wang *et al.,* 2016). Disulphide bridges (S-S bonds between two cysteines), hydrogen bonds (the H^+ interacts with an O^-), ionic interaction (between ammonium cations and carboxylic anions), and hydrophobic interactions are the interactions that keep the protein structure stable (apolar interactions which are located at the central portions of the keratin sequence due to the abundance of hydrophobic amino acids) (Figure 1.2).

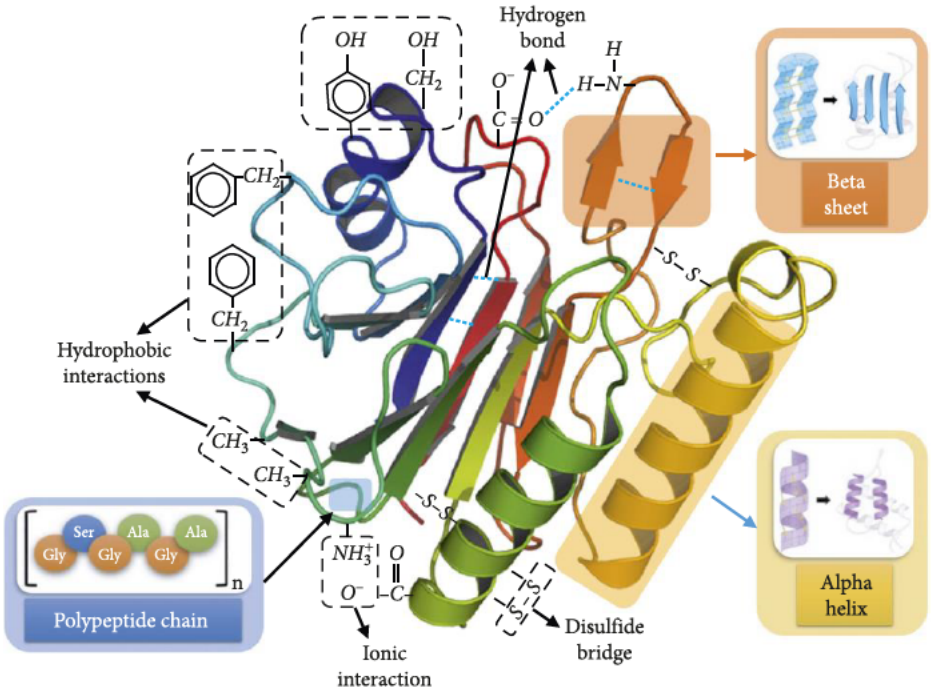

Figure 1.2 Different interactions such as hydrogen bonding, ionic interactions, hydrophobic interactions and disulfide bridging involved in maintaining the stability of keratin structures (Rodriguez-Clavel *et al.*, 2019).

The composition and molecular conformation of its constituent amino acids contribute to the stability of keratin (Wang *et al.,* 2016). A considerable majority of feather keratins is made up of the smallest amino acids, such as glycine (which has a single hydrogen atom as a side group) and alanine (with a small and non-charged methyl group). As indicated in Table 1.1, cysteine, glutamine, proline and serine make up the majority of the amino acid sequence. Serine (16%), proline (12%) and cysteine (8.85%) are the most prevalent amino acids in chicken feathers (Tesfaye *et al.,* 2017a).

Table 1.1 Amino acid content of chicken feather keratin (Tesfaye *et al.*, 2017a)

Amino Acid	Functional Group	Percent Content
Arginine	Positively charged	4.3
Aspartic acid	Negatively charged	6.0
Glutamine		7.2
Tyrosine	Hydrophobic	1.0
Leucine		2.6
Isoleucine		3.3
Valine		1.6
Cysteine		8.9
Alanine		3.4
Phenylalanine		0.9
Methionine		1.0
Threonine	Hygroscopic	4.0
Serine		16
Proline	Special	12
Asparagine		4.0

Amino acids play a vital role in the biological and chemical functioning of any living organism. The OH- group in each serine residue helps chicken feathers absorb moisture from the air, thus conferring hygroscopic properties to the feather (Sadeghi *et al.*, 2019). Proline is a key building block for the synthesis of arginine, which is required for the production of nitric oxide, which is necessary for appropriate haemodynamics (blood flow dynamics) and nutrient transport in the body (Li and Wu, 2017). Keratins are distinguished from other fibrous proteins such as collagen and elastin by a high cysteine concentration in the main sequence, which is prevalent at the protein N- and C-terminal regions (Callegaro *et al.*, 2019).

Cystine provides stiffness and thermostability to structures by acting as a location for redox reactions and the formation of covalent bonds with amino groups. It also encourages the joining of amino acids in the protein's main chain. Furthermore, depending on the cysteine concentration, cystine defines the structure of keratin in the form of α-helix or β-sheet, which plays an important role in establishing the physicochemical properties of keratin (Shavandi *et al.,* 2017). Considering the amino acid composition of feather keratin, feathers can be processed into feather meal, a feed ingredient. This overcomes the challenges associated with feather disposal and provides an alternate option for its reuse.

1.4 Feather meal as a feed ingredient

A major challenge for South African animal and poultry producers is the non-availability of alternate and cheaper feed ingredients, and competition between humans and animals for most of the conventional feed ingredients. Prices of the conventional feed ingredients such as maize for feeding chickens, escalate unpredictably. This is partly due to the alternate uses of maize (used in the biofuel industry), and its production has not been able to meet the demands for both human and animal use (Swain, 2016).

The choice of feed ingredients for poultry feed involves several economic and nutritional factors. Aside from the cost of preparation and marketing margins, the costs and availability of components are the most important factors to consider when planning poultry nutrition (Wilson, 1995). It is observed that feed cost accounts for 60-75% of the total incurred cost for poultry production (Tahir and Pesti, 2012). The nutritional component includes nutritive value, feed ingredient availability and quality, processing requirements, and diet formulation ratios (Wilson, 1995).

The main emphasis when formulating poultry diets is placed on crude protein, as protein is its critical constituent along with other nutrients such as carbohydrates, fats, vitamins and minerals (Beski *et al.,* 2015). The ability of a protein feedstuff to provide adequate essential amino acids to a bird, as well as the protein digestibility and the level of hazardous compounds connected with it, determines its utility (Akbari *et al.,* 2017). Animal protein sources originating from animal sources (e.g., meat meal) are generally regarded as high-quality protein because they have a better balance of essential amino acids, a higher biological value, and are easier to digest than plant or vegetable protein sources (Marangoni *et al.,* 2015). However, plant proteins may contain anti-nutritional factors (ANFs), limiting their use in animal feed. ANFs are substances that, when present in animal feed, either by themselves or through their metabolic products, reduce the availability of one or more nutrients (Beski *et al.,* 2015). As a result, plant proteins frequently require additional amino acids or other protein sources, such as animal protein (feather meal).

Feather meal can be considered as an ideal feed ingredient for poultry producers. Considering that poultry production is a continuous process, there will be a constant supply of feathers which overcomes the problem of shortage of raw materials that poultry producers face today. Furthermore, the use of feathers to produce feather meal will reduce feed costs and create a secondary income source for the meat and poultry sector (Woyengo *et al.,* 2014). In addition to these advantages, the nutritional composition of feather meal as described in table 1.2 meets the nutritional requirements of a feed ingredient.

Table 1.2 Composition of feather meal (Cotanch *et al.*, 2007)

Dry matter (DM), %	93
Crude protein, % of DM	80 – 90
Ether extract, % of DM	10.0
Ash, % of DM	1.9
Ca, %	0.54
P, %	0.34
Mg, %	0.03
K, %	0.13
Na, S, %	0.11
Cu, ppm	0.11
Fe, ppm	239
Mn, ppm	7
Zn, ppm	87

Furthermore, feather meal can be used as an alternative protein feed ingredient to replace a significant portion of other protein sources (e.g., fishmeal) in livestock and aquaculture diets (Ghosh *et al.,* 2016). Feather meal outperformed soybean meal in terms of total cysteine, valine, and threonine content, and may be used to substitute soybean meal at a 7% dietary level (Embaby *et al.*, 2015). In addition, feather meal can be added up to 6% of the ration for broilers, 7% for layers, and 5% for turkeys in well-balanced diets without causing harm to poultry productivity or animal health (El-Boushy *et al.,* 1990).

1.5 Techniques employed for the valorisation of keratin

Chemical (reduction, oxidation, hydrolysis, sulfitolysis, and the use of ionic liquid), physical (steam explosion and microwave irradiation), and biological (microbial and enzymatic) procedures are some of the most prevalent extraction methods for keratin valorisation (Figure 1.3).

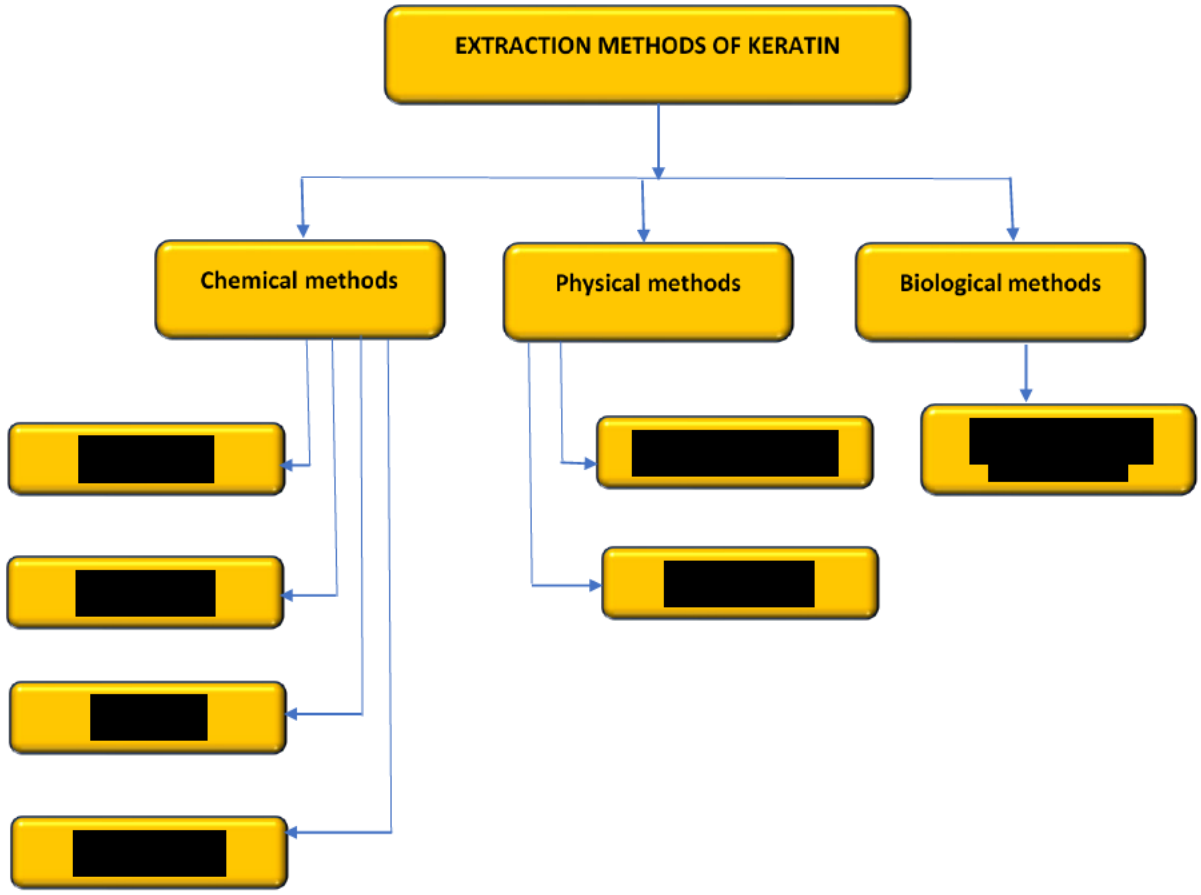

Figure 1.3 Techniques employed for the valorisation of keratin.

1.5.1 Chemical methods for keratin extraction

Chemical hydrolysis (acid, base, and catalyst) requires a high temperature and pressure to extract keratin. Different acids, such as hydrochloric acid, sulphuric acid, and peracetic acid, can be used to extract keratin in an acidic manner (Perta-Crisan *et al.*, 2021). The extraction of keratin in the presence of hydrochloric acid necessitates a high hydrochloric acid concentration (12 M), a temperature of 110°C, and a 12-hour hydrolysis period (Ding *et al.*, 2019). The use of peracetic acid to extract keratin involves a long incubation time of 24 h followed by dialysis which results in a low yield of keratin and secondary structural conformation (Agarwal *et al.*, 2019).

1.5.1.1 Oxidation method for keratin extraction

To partially dissolve the disulphide bridges in the keratin tissues and oxidize cystine to cysteic acid residues, oxidative agents such as peracetic acid, hydrogen peroxide, performic acid, or potassium permanganate are utilized (equation 1) (Vineis *et al.*, 2019).

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Keratoses are chemically modified oxidized keratins that may be extracted and separated sequentially from the keratin source into different fractions (α-, β- and ɣ-keratose) based on their solubility at various pH values (Shavandi *et al.*, 2017). Long extraction durations, huge amounts of oxidizing agents, and just a portion of keratin being solubilized are all downsides of oxidative techniques. In addition, keratoses are vulnerable to hydrolytic destruction (Rajabinejad *et al.*, 2018).

1.5.1.2 Reduction method for keratin extraction

Thiols like thioglycolic acid, 2-mercaptoethanol, and dithiothreitol can reduce the disulphide linkage in the polypeptide chain of the keratin macromolecule. In alkaline media, reducing agent thiol anions cause double nucleophilic substitution, resulting in soluble keratin forms known as kerateines (equation 2) (Khumalo *et al.*, 2020).

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However, reducing agents like mercaptoethanol have significant drawbacks due to their toxicity and negative environmental impacts (Shavandi *et al.*, 2017). Sulfitolysis can also cause keratin reduction. Sulphites and bisulphites cleave the disulphide bonds, yielding cysteine thiol and cysteine-S-sulphonate anion or Bunte salt (equation 3). (Sinkiewicz *et al.*, 2017).

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1.5.1.3 Alkaline extraction of keratin

By disrupting peptide links, primary amide bonds, and cystine disulphide bonds, heating in a concentrated alkali solution promotes severe irreversible hydrolysis of the keratin macromolecule (Bertini *et al.*, 2013). The creation of the disagreeable alkaline sulphide odor is caused by the breakage of these bonds, which results in a soluble oligopeptide fraction and a solid residue. To neutralize and precipitate the solubilized keratin component, alkaline techniques require large volumes of alkali reagents and acids (Wang *et al.*, 2021). The yield and stability of keratin hydrolysates are determined by the hydrolysis conditions (temperature, reaction time, alkali type, and concentration) (Holkar *et al.*, 2018).

1.5.1.4 Extraction of keratin with ionic liquids

Ionic liquids (ILs) are a class of salts made up of an organic cation and certain organic or inorganic anions with a low melting point (typically below 100°C) (Gough *et al.*, 2020). These are utilized for a wide range of applications due to their unique qualities such as chemical and thermal stability, miscibility with other solvents, high solvation, environmental friendliness, low vapor pressure, low volatility, and non-flammability (Han and Row, 2010). Ionic imidazole liquids, such as 1-allyl-3-methylimidazolium chloride, 1-ethyl-3-methylimidazolium chloride, and 1-butyl-3-methylimidazolium chloride, are commonly employed for protein dissolving because they have the best extraction capability (Gough *et al.*, 2020). However, the expense of ILs is a substantial barrier to their use in the industrial keratin extraction approach (Chaitanya Reddy *et al.*, 2021).

1.5.2 Physical methods for keratin extraction

1.5.2.1 Steam explosion

Steam explosion is a short-term hydrothermal treatment for biomasses that uses high-temperature saturated steam (180–240°C) under pressure (1–3.5 MPa) (Chaitanya Reddy *et al.*, 2021). The process proceeds with a quick return to atmospheric pressure, resulting in an explosive decompression that triggers keratin biomass structural rupture (Hill *et al.*, 2010). For example, steam explosion of keratin sources resulted in a mixture of low molecular mass soluble peptides and free amino acids (Perta-Crisan *et al.*, 2021). Despite the fact that the steam explosion method has a low environmental impact, a short processing time, and a low cost, the high temperature and pressure used during the process damaged cysteine, lowering the end product's quality (Shavandi *et al.*, 2017).

1.5.2.2 Microwave treatment

Microwave technology has advanced in recent years as a substitute for traditional heating methods. With the rise in temperature in the reactor, the molecules in microwave-assisted heating tend to absorb energy uniformly. As a result, the activation energy required for keratin extraction is minimized, and the keratinous mass is heated uniformly. Because homogeneous heating happens in a matter of seconds, it is an efficient method for keratin destruction and extraction (Chilakamarry *et al.*, 2021). However, a major disadvantage of this method is that high temperatures cause considerable cysteine loss, which can reach 99% at 180 °C (Zoccola *et al.*, 2012).

1.5.3 Biological methods for keratin extraction

To protect the environment from biological dangers and to make use of the retrieved valuable components, appropriate and eco-friendly handling of feather wastes employing a proper biotechnological technique is vital (Hassan *et al.*, 2020b). In addition, keratins and other refractory proteins are destroyed in the environment, demonstrating that microbes play an active and crucial role in recycling activities. As a result, microbial bioconversion is being studied as a potential alternative management strategy for converting feather waste into a nutritionally balanced and digestible feather lysate that contains free amino acids, peptides, and ammonium ions. Furthermore, the microbial process takes place in mild settings, is inexpensive, and is environmentally friendly (de Oliveira *et al.*, 2016).

1.6 Mechanism of keratin degradation by enzymes

Keratinases do not have the ability to break disulphide bonds, hence structural investigations of keratinases and feather degradation *in vitro* revealed that keratinase alone is not enough to breakdown keratin. Several processes have been hypothesized in this regard, and it is now known that the keratinolytic process involves two steps: sulfitolysis and proteolysis (Li, 2019). Sulfitolysis involves the breakdown of disulphide bridges between the polypeptide chains of keratin (Callegaro *et al.,* 2019) which causes the amino acids in the β -sheet of keratin to shift conformation, resulting in new hydrolytic sites for proteolytic attack by keratinases (Tamreihao *et al.*, 2019). During sulfitolysis (Figure 1.4), thiol groups are liberated due to the cleavage in the disulphide bond between the polypeptide keratin chains whereas, in the presence of sulphite, cleavage of the disulphide bonds form cysteine and S-sulfocysteine (Hassan *et al.,* 2020b). Sulfitolysis necessitates the existence of enzymes such as disulphide reductase (Figure 1.5) or reducing agents such as β-mercaptoethanol, thioglycolate, dithiothreitol or sulphite for the complete breakdown of keratin (Peng *et al.,* 2019a).

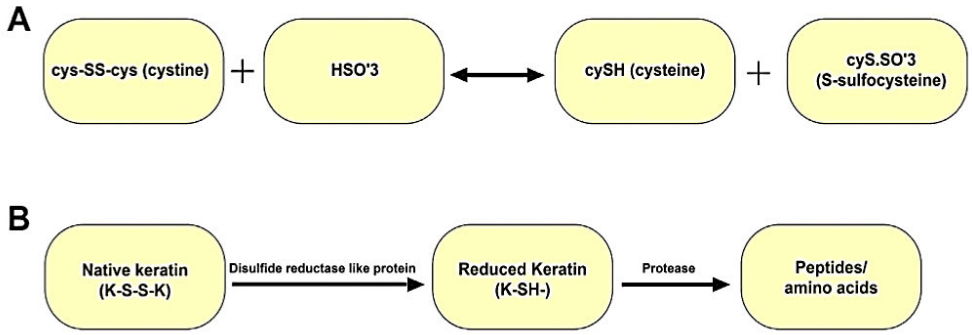


Figure 1.4 Sulfitolysis process for keratin degradation (A) Disulfide bonds between polypeptide chains are cleaved using inorganic sulfite resulting in cysteine and S-sulfocysteine and (B) Disulfide bonds are reduced by disulphide reductase and protease hydrolyse keratin (Hassan *et al*., 2020b).

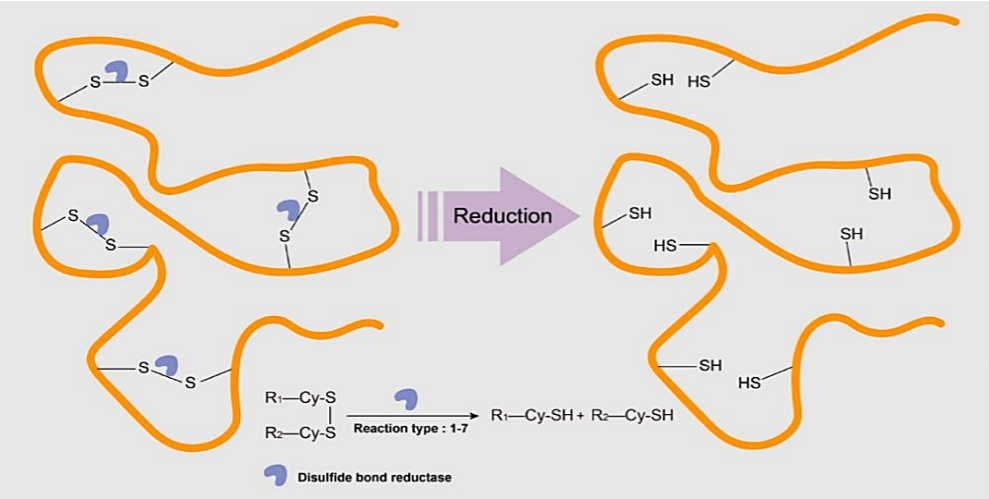


Figure 1.5 Sulfitolysis process involving action of disulphide reductase for the complete breakdown of keratin (Peng *et al.,* 2019a).

When sulfitolysis disrupts the dense structure of keratin, the keratin-degrading microbe secretes keratinase, which degrades polypeptides into essential and non-essential amino acids (Hassan *et al*., 2020b). Keratinase is a proteolytic enzyme that specifically destroys keratin, making it more specialized than other proteases for keratinolytic substrates. This enzyme can dissolve a wide range of soluble and insoluble proteins, including casein, albumin, heme protein, collagen, and globulin, as well as wool and feathers (Brandelli *et al*., 2010).

Yamamura *et al*. (2002) used a crude enzyme from the keratinolytic strain *Stenotrophomonas* sp. to demonstrate the concept that keratin breakdown requires more than one enzyme. When enzyme fractions were combined, keratinolytic activity increased by more than 50 times. A study by Rahayu *et al.* (2012) further proved this theory by showing that two enzymes (keratinase and disulphide reductase) of *Bacillus* sp. MTS are involved in keratin degradation. Keratinolytic activity of 4.5 U/mg was observed upon application of purified keratinase and keratinolytic activity of 12.8 U/mg was observed upon application of purified keratinase in the presence of reducing agent, DTT. However, when keratinase and disulphide reductase were applied, a much higher keratinolytic activity of 27.5 U/mg was observed. In the presence of suitable reducing agents, such as β-mercaptoethanol and dithiothreitol, keratinase can breakdown keratin (Peng *et al*., 2019a).

1.7 Keratin-degrading microorganisms

Keratinases are one of the most diversified enzymes in terms of their sources and applications, and they can use keratin from a variety of bacteria (Verma *et al*., 2016b). As a result, microbes are appealing sources because they can be cultured in huge quantities using proven procedures in a short amount of time, resulting in an abundant and consistent supply of enzymes (Ravi *et al*., 2015). Keratinolytic microorganisms can be divided into two types based on their keratin degradation abilities: true keratinolytic and potentially keratinolytic. True keratinolytic microorganisms degrade hard keratin and fully solubilize keratin structures. On the other hand, potentially keratinolytic microorganisms play an important role in the conversion of non-keratin proteins occurring together with hard keratin in hair, feathers, or nails and soft keratin in callus (Kornillowicz-Kowalska and Bohacz, 2011).

Keratin-degrading microorganisms mostly belong to bacteria, fungi and actinomycetes (Wronska and Cybulska, 2016). Of these, keratin-degrading bacteria are most abundant, followed by fungi and actinomycetes. Microorganisms that degrade keratin have been isolated from a variety of sources, including decomposing feathers (Nagal and Jain, 2010), penguin feathers (Pereira *et al*., 2014), poultry waste digestor (Williams *et al*., 1990), fish intestine (Daroit *et al*., 2009), and slaughterhouse polluted water (Fakhfakh-Zouari *et al*., 2010).

1.7.1 Bacteria

Most keratin-degrading bacteria utilize feather keratin as their substrate. A major proportion of feather-degrading bacteria are Gram-positive and belong to the genus *Bacillus* spp. (Yong *et al.,* 2020). Other common Gram-positive feather degrading bacteria includes *Micrococcus* (Laba *et al*., 2015)*, Kocuria* (Bernal *et al.,* 2006), *Lysobacter* (Pereira *et al*., 2015) and *Clostridium* sp. (Ionata *et al.,* 2008). A few Gram-negative keratin degrading bacteria belonging to *Pseudomonas* spp. (Mohamad *et al.,* 2017)*, Chryseobacterium* spp. (Bouacem *et al.,* 2016) and *Fervidobacterium* spp. (Lee *et al*., 2015) have also been reported. Various keratin degrading bacteria and their isolation sources are listed in Table 1.3.

Table 1.3 Various keratin-degrading bacterial strains and their sources

Microorganisms	Collection site	Reference
Azobacter chroococcum	Dump soil	Mamangkey *et al.,* 2020
Alcaligenes sp.	Soil from feather dumping sites	Yusuf *et al.,* 2016
Bacillus amyloliquefaciens	Forest soil	Manonmani *et al.,* 2015
	Marine brown alga *Zonaria tournefortii*	Hamiche *et al.,* 2019
Bacillus cereus	Soil from poultry waste	Subathra Devi *et al.,* 2018
	Soil sediment	Arokiyaraj *et al.,* 2019
Bacillus halodurans	Soil from hot spring	Kaewsalud *et al.,* 2020
Bacillus licheniformis	Keratinous wastes	Abdel-Fattah, 2018
Bacillus megaterium	Feather dumping site and cockroach gut	Sharma *et al.,* 2019
Bacillus olironius	Poultry farm soil	Parashar *et al.,* 2017
Bacillus pseudofirmus	Soil sample of poultry farm	Kojima *et al.,* 2006
Bacillus pumilus	Aquatic environment	Parvathi *et al.,* 2009
	Carbonate cave	Huang *et al.,* 2020
	Polluted water	Fakhfakh *et al.,* 2013
Bacillus sp.	Intestine of Amazon basin fish *Piaractus mesopotamicus*	Daroit *et al.,* 2009
Bacillus subtilis	Poultry waste	Mazotto *et al.,* 2017
	Hot springs	Pillai and Archana, 2008
	Gut of tarantula *Chilobrachys guangxiensis*,	Liu *et al.,* 2014
	Skin waste	Macedo *et al.,* 2005
Bacillus tequilensis	Contaminated soil of leather tannery industry	Jaouadi *et al.,* 2015
Bacillus thuringiensis	Feather dumping soil	Sivakumar *et al.,* 2012
Brevundimonos terrae	Poultry farm soil	Kulkarni and Jadhav, 2014
Brevibacillus brevis	Contaminated soil of leather tannery industry	Jaouadi *et al.,* 2013
Brevibacillus thermoruber	Hot spring	Zilda *et al.,* 2013
Caldicoprobacter algeriensis	Hydrothermal hot springs	Bouacem *et al.,* 2016
Chryseobacterium sp.	Poultry farm soil	Riffel *et al.,* 2007
Chryseobacterium indologenes	Industrial poultry waste	Wang *et al.,* 2008
Fervidobacterium islandicum	Hot spring	Lee *et al.,* 2015
Fervidobacterium pennivorans	Hot spring	Friedrich and Antranikan, 1996

Microorganisms	Collection site	Reference
Kocuria rosea	Aerobic soil	Bernal *et al.,* 2006
Kytococcus sedentarius	Varying environments including human skin and groundwater	Longshaw *et al.,* 2002
Lysobacter sp.	Decomposing-penguin feathers	Pereira *et al.*, 2015
Meiothermus sp.	Hot spring	Kuo *et al.,* 2012
Microbacterium sp.	Decomposed feathers	Thys *et al.,* 2004
Micrococcus luteus	Poultry feather waste	Laba *et al.*, 2015
Paenibacillus woosongensis	Soil from feather dumping sites	Paul *et al.,* 2013
Pseudomonas aeruginosa	Feather dumping soil	Han, 2012
Pseudomonas sp.	Poultry waste	Tork *et al.,* 2010
Serratia marcescens	Forest soil	Bach *et al.,* 2011
Stenotrophomonas maltophilia	Soil and roots of plant	Jeong *et al.,* 2010b

1.7.2 Fungi

Keratinophilic fungi are present in the environment with variable distribution patterns which may depend on the presence of humans or animals. Keratinolytic fungi belong to three genera: *Microsporium, Trichophyton* and *Epidermophyton* which are further divided into three categories according to their natural habitats. These include anthrophilic, in which humans are the natural host; zoophilic, in which animals are the natural hosts and geophilic, whose natural habitat is soil (Srivasta *et al.,* 2019). The prevalence of keratinolytic fungi depends on different factors such as the organic matter, dissolved oxygen concentration, creatinine contents in the soil, and environmental factors such as temperature, pH, and geographical location (Khan and Bhadauria, 2015). Various keratin degrading fungal species and their sources are cited in Table 1.4.

Table 1.4 Various keratin-degrading fungal strains and their sources

Microorganisms	Collection Site	Reference
Acremonium sp.	Poultry soil sample	Friedrich *et al.*, 1999
Acremonium hyalinulum	Chicken litter	Marcondes *et al.*, 2008
Alternaria tenuissima	Feather waste	Saber *et al.*, 2010
Aspergillus flavus	Poultry farm soil	Mini *et al.*, 2015
A. niger	Chicken litter	Marcondes *et al.*, 2008
	Poultry farm soil	Mini *et al.*, 2012
A. fumigatus	Poultry farm soil	Mini *et al.*, 2012
A. nidulans	Poultry farm soil	Mini *et al.*, 2012
A. terreus	Chicken litter	Koutb *et al.*, 2012
A. parasiticus	Poultry soil	Anita and Palanivelu, 2013
Beauveria bassiana	Chicken litter	Marcondes *et al.*, 2008
	Laboratory contaminant	Borgi and Gargouri, 2014
Chrysosporium georgiae	Chicken feathers	El-Naghy *et al.*, 1998
	Activated sludge	Awad and Kraume, 2011
C. indicum	Soil	Jain and Sharma, 2012b
	Activated sludge	Awad and Kraume, 2011
C. tropicum	Food pellets	Jain and Sharma, 2012a
	Soil	Bohacz *et al.*, 2020
C. keratinophilum	Waste site containing organopollutants,	Singh, 2003
	Activated sludge	Awad and Kraume, 2011
Coprinopsis sp.	Soil sample	Al-Musallam *et al.*, 2013
Cunninghamella echinulata	Poultry soil sample	More *et al.,* 2013
Curvularia brachyspora	Chicken litter	Marcondes *et al.*, 2008
C. inaequalis	Poultry soil sample	Friedrich *et al.*, 1999
Fusarium culmorum	Poultry soil sample	Friedrich *et al.*, 1999
F. solani	Soil	Rai *et al.,* 2014
Myceliophthora thermophila	Rhizosphere soil	Liang *et al.*, 2011
Myrothecium verrucaria	Poultry soil sample	Friedrich *et al.*, 1999
Paecilomyces lilacinus (Purpureocillium lilacinum)	Alkaline forest soil	Cavello *et al.*, 2013
Penicillium sp.	Poultry farm soil	Mini *et al.*, 2012
P. purpurogenum	Soil	Ogbonna and Ogbonna, 2019
Scopulariopsis brevicaulis	Marine sponges	Sankar *et al.*, 2014
Sporothrix schenckii	Soil	Jain and Sharma*,* 2012b
Trichophyton mentagrophytes	Poultry farm soil	Mini *et al.,* 2012
	Soil	Jain and Sharma, 2012b
T. simii	Soil	Jain and Sharma, 2012b
Verticillium sp.	Activated sludge	Awad and Kraume, 2011

1.7.3 Actinomycetes

Keratin-degrading actinomycetes are particularly abundant in alkaline soils rich in organic matter (Jani *et al.,* 2014). Actinomycetes from the *Streptomycete*s group, *viz.*, *Streptomycetes fradiae* (Li *et al.,* 2007)*, S. albus* (Esawy, 2007); *S. acremycini* (Selvam and Vishnupriya, 2013)*, S. coelicoflavus* (Jadhav *et al.,* 2016) and the *Thermoactinomyces* group, *viz., T. candidus* and another *Thermoactinomyces* sp. are reported as keratin degraders (Singh and Kushwaha, 2015). Table 1.5 lists some of the keratin degrading Actinomycetes and their sources.

Table 1.5 Various keratin-degrading actinomycetes and their sources

Microorganisms	Collection Site	Reference
Actinomycetes sp.	Poultry farm soil	Jayalakshmi *et al.*, 2010
Actinomadura keratinilytica	Bovine manure	Puhl *et al.*, 2009
	Poultry compost	Habbeche *et al.*, 2014
Microbispora aerata	Antarctic soil	Gushterova *et al.*, 2005
Nocardiopsis sp.	Feather dumped soil	Mitsuiki *et al.*, 2002
Streptomyces sp.	Human foot skin	Chao *et al.*, 2007
S. acremycini	Marine sediments	Selvam and Vishnupriya, 2013
S. albus	Mediterranean seashore	Esawy, 2007
	Human hair	Nyaka *et al.*, 2013
S. coelicoflavus	Soil	Jadhav *et al.*, 2016
Thermoactinomyces sp.	Soil	Gushterova *et al.*, 2005
	Rice mill waste yard effluents	Verma *et al.*, 2016a

1.8 Keratinase production

Microorganisms are a gold mine of enzymes and the ideal source for keratinases due to their rapid growth, low cultivation space requirements, and ease of genetic manipulation (Israel-Roming *et al.*, 2014). The use of keratinase to degrade feathers is preferable to microbial degradation because it eliminates the risk of users being exposed to pathogenic organisms (Wang *et al.,* 2011b). However, the utility of an enzyme at the industrial level necessitates its production in sufficient amounts. The first step in designing a commercial enzyme production process is to find a microorganism that can generate the enzyme in commercial quantities. After isolating a suitable organism, the enzyme production must be increased by optimizing the process parameters (media, pH, temperature, agitation, aeration etc.). Most commercial enzyme preparations are crude, whereas pure enzyme preparations are required for pharmaceutical and other medical uses (Kamal *et al.*, 2016).

Keratinases are mostly extracellular enzymes that are secreted into the culture medium, but cell-bound and intracellular enzymes have also been discovered (Singh and Kushwaha, 2015). Extracellular keratinases from *Aspergillus parasticus* (Anita and Palanivelu, 2013), *Bacillus pumilus* ZED17 (Talebi *et al*., 2013), and *Bacillus subtilis* CH-1 (Chen *et al.,* 2020) have been reported. Extracellular keratinases facilitate enzyme recovery by eliminating the need for cell lysis and thus reduce enzyme production costs (Brandelli *et al.,* 2015). Various researchers have also reported a few cell-bound keratinases (Friedrich and Antranikian, 1996; Nam *et al.,* 2002; Onifade *et al.,* 1998; Rissen and Antranikian, 2001). A study by Wawrzkiewicz *et al.* (1987) reported an intracellular keratinase from *Trichophyton gallinae.* Interestingly, both extracellular and intracellular keratinases was observed from *Arthroderma quadrifidium*, *A. curreyi* and *Chrysosporium pruinosum* (Kornillowicx Kowalska, 1999) and *Coprinopsis* sp. (Al-Musallam *et al.,* 2013).

1.8.1 Fermentation conditions for keratinase production

Shake flasks can be used to produce keratinases on a small scale, while large-scale fermenters could be used for industrial-scale production (Zaghloul *et al*., 2011). Moreover, at the industrial level, keratinase production could be conducted under static or submerged conditions (Cai *et al.,* 2011). Abdel Fattah *et al*. (2018) showed maximum keratinase production under static conditions for *Bacillus licheniformis* using feathers as substrate. Keratinase production of *Bacillus safensis* LAU 13 was performed in a culture medium using feathers as substrate under submerged shaking conditions (Adelere and Lateef, 2016).

Generally, submerged fermentation is preferred since the bioactive compounds (keratinase) are secreted into the fermentation medium and this technique is best suited for microorganisms such as bacteria. An additional advantage of this technique is that the purification of products is easier (Subramiyam and Vimla, 2012). During fermentation, aeration is crucial in maintaining the oxygen level in the fermentation medium for aerobic microorganisms. Thus, shaking conditions become necessary to promote oxygen solubilization from the headspace into the culture medium and proper mixing of nutrients (Fuke *et al*., 2018).

1.8.2 Microbial growth and keratinase production

Keratinase synthesis normally begins during a late exponential or stationary growth phase (Verma *et al.*, 2016b). Keratinases' proteolytic activities increase slowly during early logarithmic growth and peak during late logarithmic growth or the advent of stationary phase (Lakshmi *et al.*, 2013). *Bacillus megaterium* (Park and Son, 2009) and *Streptomyces thermoviolaceus* (Park and Son, 2009) were shown to produce the most keratinase during the late logarithmic growth phase (Syed *et al.*, 2009). For *Bacillus subtilis* (Kim *et al.*, 2001) and *Bacillus cereus*, however, maximum keratinase production was reported near the start of the stationary phase (Tamreihao *et al.*, 2019).

1.8.3. Substrate specificity for keratinase production

Keratinases are mostly synthesized in a basal media that has a keratinous substrate as a carbon source or inducer (Daroit and Brandelli, 2014). Whole chicken feathers, feather powder, wool, and skin are all examples of exogenous keratin inducers (Table 1.6). Although most reports on keratinases categorize them as inducible enzymes, there have been a few findings on constitutive keratinases (Son *et al.,* 2008). It is worth noting that most papers on constitutive keratinases describe the enzyme's caseinolytic activity rather than its keratinolytic activity (Manczinger *et al.*, 2003). As a result, it is hypothesized that keratinolytic activity is largely inducible (Kanchana and Mesta, 2013). However, a keratinous substrate is not necessarily necessary for the synthesis of keratinase. Other non-keratinous substrates that have been reported as inducers of keratinase production include soy flour (Gradisar *et al.,* 2005), soybean meal (Pillai and Archana, 2008), skim milk (Mitsuiki *et al.*, 2002), shrimp shell powder (Wang *et al.*, 2008), gelatin (Thys *et al.,* 2006), casein and cheese whey (Casarin *et al.*, 2008).

Table 1.6 Production of keratinase by various bacterial species using different keratinaceous substrates

Bacterial species	Growth substrate	Keratinase yield (U/ml)	Reference
Arthrobacter sp.	Feather meal powder	16.4	Barman *et al.,* 2017
Bacillus agaradhaerens C9	Feather waste	17.3	Liu *et al.,* 2020
B. amyloliquefaciens	Cotton	922	Hassan *et al.,* 2013
B. amyloliquefaciens	Feather	610	Bose *et al.,* 2014
B. cereus	Feather	350	Ahmadpour *et al.,* 2016
	Feather meal	390	Ahmadpour and Yakhchali, 2017
B. licheniformis	Feather	1295	Ramnani and Gupta, 2004
	Insect cell	635	Huang *et al.,* 2017
B. licheniformis ALW1	Feather	72.2	Abdel-Fattah *et al.,* 2018
B. pumilus	Feather	373	Reddy *et al.,* 2017
Bacillus sp.	Wheat bran	318	Chitturi and Lakshmi, 2015
	Chicken feather	3.5	Mariyammal *et al.,* 2018
B. subtilis	Human hair	125	Cai and Zheng, 2009
	Horn meal	15972	Kumar *et al.,* 2010
	Feather	319	Mazotto *et al.,* 2011
	Cotton	814	Fang *et al.,* 2013b
	Chukar partridge feather	913	Singh *et al.,* 2014
	Feather meal	13.7	Singh *et al.,* 2017
B. thuringiensis Bt407	Feather	94.5	Uttangi and Aruna, 2018
B. subtilis and *B. polyfermenticus (recombinant)*	Feather meal	473	Dong *et al.,* 2017
Micrococcus luteus	Feather	32.3	Laba *et al.,* 2015
Pseudochrobactrum sp. IY-BUK1	Feather	168.7	Yusuf *et al.,* 2019
Stenotrophomonas maltophilia	Wool waste	1728	Fang *et al.,* 2013b
	Goatskin	34.7	Shah and Vaidya, 2017
Stenotrophomonas sp.	Feather meal	52.8	Birari *et al.,* 2013
Streptomyces gulbargensis	Chicken feather	14.3	Syed *et al.,* 2009
Streptomyces sp.	Feather meal	8	Azeredo *et al.,* 2005
	Feather meal	70.9	Tatineni *et al.,* 2007

1.8.4 Carbon sources for keratinase production

For the growth of keratin-degrading microorganisms, most researchers use mineral media constituted by phosphates, sulphates, and chlorides necessary for their metabolism and supplemented with different carbon and nitrogen sources to enhance keratinase production (Jain and Sharma, 2012a). It must be pointed out that the main carbon source used is the chicken feather itself, which contains keratin necessary to stimulate the production of keratinase (Jain and Sharma, 2012a). Additional carbon sources such as glucose, mannitol and wheat bran are incorporated into the medium to increase keratinase production. Simple sugars, such as glucose, have been demonstrated to reduce keratinase production due to catabolite suppression, but complex carbohydrates, such as starch, have been shown to boost keratinase production (Ghaffar *et al.,* 2018). Considering the high-cost inputs required for pure carbon substrates, waste biomass could be an ideal alternative for keratinase production. Among the various biowastes, whey, a by-product of the dairy industry, has been considered a suitable substrate for synthesizing keratinases (Fuke *et al.,* 2018).

1.8.5 Nitrogen sources for keratinase production

There are conflicting reports on the requirement of nitrogen sources for enzyme production which were found to differ from organism to organism. Generally, organic and inorganic nitrogen sources are supplemented to fermentation medium for enhanced enzyme production (Puri *et al.,* 2002). Organic nitrogen sources supply cells with growth factors and amino acids required for cell metabolism and enzyme synthesis, whereas inorganic nitrogen sources supply cells with growth factors and amino acids required for cell metabolism and enzyme production (de Almeida *et al.,* 2013). Some examples of additional nitrogen sources required for keratinase production include yeast extract (Parashar *et al.,* 2017), peptone (Ramnani and Gupta, 2004), soya bean meal (Laksmi *et al.,* 2013), ammonium sulphate (Matikeviciene *et al.,* 2011) and ammonium chloride (Mousavi *et al.,* 2013).

A study by Kainoor and Naik (2010) reported increased keratinase production for *Bacillus* spp. JB-99 upon adding 0.1% (w/v) yeast extract; however, enzyme production decreased when the yeast extract concentration was increased to 1% (Kainoor and Naik*,* 2010). In another study, ammonium nitrate inhibited the degradation of feathers by *Stenotrophomonas maltophilia* CA-1, resulting in keratinase activity depletion due to catabolite repression (Qu *et al.,* 2018). The repression prolongs keratinolysis as the biosynthesis of keratinolytic enzymes was decreased when easily assimilable sources of nitrogen were used up

(Kornillowicz-Kowalska and Bohacz, 2011). As a result, the influence of various growth substrates on keratinase synthesis varies greatly depending on the microorganism, substrate, and nitrogen content, implying that medium composition should be evaluated on a case-by-case basis (Cai and Zheng, 2009).

1.8.6 Effect of pH on keratinase production

Besides the additional carbon and nitrogen sources, culture parameters used for keratinase production varies even for microorganisms within the same genus (Table 1.7), therefore, the selection of standardized production methods, needs extensive investigation. The influence of pH on enzyme production is crucial for efficient fermentation, as pH influences the transfer of various nutrients in and out of the microorganism's cell membrane (Fuke *et al.,* 2018). The increase in pH that occurs during cultivation is a key feature of keratin hydrolysis and bacteria with keratinolytic capability. It has been discovered that organisms with higher keratinolytic activity produce more alkaline medium than organisms with lower keratinolytic activity. This is due to keratin breakdown events involving deamination, which result in an elevation in pH (Kodak *et al.,* 2019).

It has been noted that most microorganisms exhibit optimal keratinase activity at pH 7-9 (Barman *et al.,* 2017). For example, strains of *Caldicoprobacter algeriensis* (Bouacem *et al.,* 2016) and *Actinomadura keratinilytica* Cpt 29 (Habbeche *et al.,* 2014) have been reported to show keratinase activity at pH 7 and 9, respectively. However, Abdel-Fattah *et al.* (2018) reported optimal keratinase production at pH 6 for *B. licheniformis* ER-15 while Reddy *et al.* (2017) reported optimal keratinase production at pH 10 for *Bacillus pumilus* GRK.

1.8.7 Effect of temperature on keratinase production

Temperature requirements for the formation of keratinase are species-specific and change depending on the microorganism. A higher temperature normally increases enzyme production, but if the temperature goes beyond a certain threshold, enzyme production levels out and rapidly drops, because most microbes cannot survive at such high temperatures (Ire and Onyenama, 2017). The temperature at which most bacteria produce keratinase ranges from 30 to 55°C. For example, Yusuf *et al.* (2019) reported optimum keratinase production for *Pseudochrobactrum* IY-BUK1 at 30°C, whilst Kuo *et al.* (2012) reported optimum keratinase production for *Meiothermus* sp. 140 at 55°C. Certain actinomycetes and fungi also prefer temperatures as high as 70°C for keratinase production.

Optimal temperature of 70°C was reported for *Thermoanaerobacter* (Rissen and Antranikian, 2001) and *Fervidobacterium* spp. (Nam *et al*., 2002).

Mesophilic bacteria have a few benefits over thermophilic bacteria from an industrial standpoint. Mesophilic bacteria are also more resilient and adaptive to changing environmental circumstances than other bacteria. Furthermore, mesophilic systems are less expensive to operate in large-scale industrial applications since they need less energy. Furthermore, high temperatures may induce important amino acid loss during the generation of amino acids from feather hydrolysis. As a result, the mesophilic bacteria are a better choice for producing keratinase for applications such as chicken feather hydrolysis and amino acid synthesis (Mohamad *et al.,* 2017).

1.8.8 Effect of inoculum size on keratinase production

Optimum inoculum size is essential for the maximum enzyme production in any fermentation. A higher inoculum (concentration of culture) during keratinase fermentation is beneficial for higher enzyme activity and faster solubilization of feather waste (Fuke *et al.,* 2018). A study by Subathra Devi *et al*. (2018) reported optimum keratinase production at 2% inoculum for *Bacillus cereus*. However, Sivakumar *et al.* (2013) found that increasing the inoculum concentration to 4% resulted in an increase in keratinase synthesis for *B. cereus*, with little or no improvement in activity at higher inoculum levels. Thus, inoculum levels above certain levels lead to lower enzyme activity and it is important that the inoculum concentrations be optimized for higher keratinase production.

Table 1.7 Culture parameters for keratinase production using feather as the main carbon source

Organism	Temp (°C)	pH	Time	Keratinase Yield (U/ml)	Reference
Aphanoascus fulvescens	28.7	7.5	-	-	Bohacz, 2017
Bacillus sp.	37	8	48 h	-	Janaranjani *et al.,* 2015
Bacillus sp.	40	9	72 h	-	Ire and Onyenama, 2017
Bacillus sp. MBRL 575	30	-	-	305	Kshetri and Ningthoujam, 2016
Bacillus sp. CL18	30	7.5	6 d	-	Sobucki *et al.,* 2017
B. aerius	35	7.5	-	127.63	Calin *et al.,* 2017
B. licheniformis ALW1	40	9	45 h	-	Kazzaz *et al.,* 2015
B. licheniformis ER-15	42	6	4 d	50.97	Abdel-Fattah *et al.,* 2018
B. licheniformis	35	-	96 h	-	Peng *et al.,* 2019b
B. paralicheniformis	-	9	72 h	-	Santha Kalaikumari *et al.,* 2019
B. pumilus GRK	37	10	-	373	Reddy *et al.,* 2017
B. pumilus ZED17	37	10	72 h	-	Talebi *et al.,* 2013
B. subtilis	30	8	4 d	53.3	Jeong *et al.,* 2010a
B. subtilis AMR	26	8	144 h	360	Mazotto *et al.,* 2017
B. thuringiensis strain Bt407	37	7	48 h	-	Uttangi and Aruna, 2018
B. weihenstephanensis PKD5	37	7	36 h	-	Tiwary and Gupta, 2010
B. weihenstephanensis	40	7	-	-	Sahoo *et al.,* 2012
Microsporum fulvum	30	6.5	-	-	Darah *et al.,* 2013
Micrococcus luteus	-	-	15 d	32.3	Laba *et al.,* 2015
Nocardiopsis sp. SD5	45-50	9	4 d	64.6	Saha *et al.,* 2012
Pseudochrobactrum IY-BUK1	30	7.5	168 h	95.25	Yusuf *et al.,* 2019
Pseudomonas sp.	30	8	60 h	127	Mohamad *et al.,* 2017
Stenotrophomonas maltophilia	23	-	96 h	-	Peng *et al.,* 2019b
Streptomyces sp. 2M21	27.5	8	5.5 d	405.5	Demir *et al.,* 2015

1.9 Biochemical properties of keratinase

Keratinases have a wide range of characteristics depending on the substrate and producer organism. Keratinases attack the peptide (hydrophobic amino acids) linkages in the keratin structure to convert them to simplified forms, releasing free amino acids (Bhari *et al.,* 2018). However, the biochemical characteristics of keratinases (Table 1.8) are diverse and depend on the type of keratinase-producing microbes involved (Brandelli *et al.,* 2015). Some of the biochemical characteristics of keratinases are described below.

Table 1.8 Biochemical characteristics of selected keratinases

Microorganisms	Catalytic type	Mol. Mass (kDa)	Optimum pH	Optimum temp (°C)	Reference
BACTERIA					
Actinomadura keratinilytica strain Cpt29	Serine protease	29.23	10	70	Habbeche *et al.,* 2014
Bacillus sp.	Serine	32	8	50	Deivasigamani and Alagappan, 2008
Bacillus sp. JB 99	Serine protease	66	10	65	Kainoor and Naik, 2010
Bacillus sp. DZ100	Serine protease	32	12.5	85	Benkiar *et al.,* 2013
B. amyloliquefaciens S13 KERZT A	Serine	28	6.5	50	Hamiche *et al.,* 2019
B. amyloliquefaciens S13 KERZT B	Serine	47	8	60	Hamiche *et al.,* 2019
B. cereus DCUW	Serine	80	8.5	50	Ghosh *et al.,* 2008
B. halodurans PPKS-2 Keratinase – 1	Disulphide reductase	30	11	60-70	Prakash *et al.,* 2010
B. halodurans PPKS-2 Keratinase – 2	Serine protease	66	11	70	Prakash *et al.,* 2010
B. licheniformis FK14	Serine	35	8.5	60	Suntornsuk *et al.,* 2005
B. licheniformis K-508	Thiol	42	8.5	52	Rozs *et al.,* 2001
B. licheniformis MSK103	Serine	26	9.0-10.0	60-70	Yoshioka *et al.,* 2007
B. licheniformis RPk	Serine	32	9	60	Fakhfakh *et al.,* 2009
B. licheniformis ALW1	-		8	65	Abdel-Fattah *et al.,* 2018
B. licheniformis PWD-1	-	38 and 30	8	45	Huang *et al.,* 2017
B. pumilus	Serine	65	8	65	Kumar *et al.,* 2008
B. pumilus	Metalloprotease	30	7.5	50	Tork *et al.,* 2016
B. pumilus FH9	Metalloprotease	50	9	60	Abdel-Naby *et al.,* 2017
B. pumilus K9	Serine-metallo	32	9	60	Gong *et al.,* 2015
B. pumilus ZED17	-	50	8	40	Talebi *et al.,* 2013
B. subtilis DP1	Serine protease	97.4	10	37	Sanghvi *et al.,* 2016
B. subtilis KD-N2	Serine	30.5	8.5	55	Cai and Zheng, 2009
B. subtilis KS-1	Serine	25.4	7.5	-	Suh and Lee, 2001

Microorganisms	Catalytic type	Mol. Mass (kDa)	Optimum pH	Optimum temp (°C)	Reference
B. subtilis MTCC (9102)	Metallo	69	6	40	Balaji *et al.,* 2008
B. subtilis RM-01	Serine	20.1	9	45	Rai *et al.,* 2009
B. subtilis S14	Serine protease		5.5-10.5	50	Silva *et al.,* 2013
B. tequilensis hsTKB2	Serine protease	59.89	10.5	70	Paul *et al.,* 2014c
B. thuringiensis serovar israelensis H4 (IPS-82)	Serine protease	40	7	30	Poopathi *et al.,* 2014
B. thuringiensis SH-II- 1A	Serine protease	67	10-11	45	Sunil *et al.,* 2018
B. weihenstephanensis PKD5	-		8	40	Sahoo *et al.,* 2012
Brevibacillus brevis strain US575	Serine protease	29.12	8	40	Jaouadi *et al.,* 2013
Chryseobacterium sp. strain Kr 6	-	20	-	50-60	Silveira *et al.,* 2010
C. gleum	Metalloprotease	36	8	30	Chaudhari *et al.,* 2013
C. indologenes TKU014 P1	Metallo	56	10	30-50	Wang *et al.,* 2008
C. indologenes TKU014 P2	Metallo	40	7-8	40	Wang *et al.,* 2008
C. indologenes TKU014 P3	Metallo	40	8-9	40-50	Wang *et al.,* 2008
Clostridium sporogenes	-	28.7	8	55	Ionata *et al.,* 2008
Fervidobacterium islandicum AW-1	Serine	>200	9	100	Nam *et al.,* 2002
F. pennavorans	Serine	130	10	80	Friedrich and Antanikan, 1996
Geobacillus stearothermophilus AD-11	Serine protease	57	9	60	Gegeckas *et al.,* 2015
Gibberella intermedia CA3-1	Serine protease		9	60	Zhang *et al.,* 2016
Kocuria rosea	Serine	240	10	40	Bernal *et al.,* 2006
Kytococcus sedentarius	Serine	30-50	7.0-7.5	40-50	Longshaw *et al.,* 2002
Lysobacter sp. NCIMB 9497	Metallo	148	-	50	Allpress *et al.,* 2002
Microbacterium sp. kr10	Metallo	42	7.5	50	Thys *et al.,* 2006
Nesterenkonia sp. AL-20	Serine	23	10	70	Gessesse *et al.,* 2003
Nocardiopsis sp. TOA-1	Serine	20	>12.5	60	Mitsuiki *et al.,* 2004
Paenibacillus woosongensis TKB2	Serine protease	190.2	9	50	Paul *et al.,* 2014a

Microorganisms	Catalytic type	Mol. Mass (kDa)	Optimum pH	Optimum temp (°C)	Reference
Pseudomonas sp., MS 21	Serine	30	8	37	Tork *et al.,* 2010
P. aeruginosa	Serine	33	7	50	Sharma and Gupta, 2010
*P. aeruginos*a C11	Metalloprotease	33	7	50	Han, 2012
Serratia marcescens P3	Metalloprotease	53	6.5	40-45	Bach *et al.,* 2011
Stenotrophomonas maltophilia	Serine	35.2	7.8	40	Cao *et al.,* 2009
Streptomyces strain BA 7	Serine	44	8.5	50	Korkmaz *et al.,* 2003
Streptomyces sp. 57	Serine-metallo	44	11	45	Tatineni *et al.,* 2007
Streptomyces sp. strain 16 KI	Serine	203.2	9	50	Xi*e et al.,* 2010
Streptomyces sp. strain 16 KII	Serine	100.8	9	50	
Streptomyces sp. strain 16 KII	Serine	31.8	9	50	
Streptomyces sp. strain 16 KIV	Serine	19.2	9	60	
S. albidoflavus	Serine	18	6.0-9.5	40-70	Bressollier *et al.,* 1999
S. gulbargensis DAS 131	-	46	9	45	Syed *et al.,* 2009
S. minutiscleroticus	-	29	9	50	Allure *et al.,* 2015
S. pactum	Serine	30	7.0-10.0	40-75	Bockle *et al.,* 1995
S. sclerotialus	-	46	9	55	Yadav *et al.,* 2011
Thermoanaerobacter sp. 1004-09	Serine	150	9.3	60	Kublanov *et al.,* 2009
T. keratinophilus	Serine	135	8	85	Riessen and Antranikan, 2001
Vibrio sp. Kr 2	Serine	30	8	55	De Toni *et al.,* 2002
Xanthomonas maltophilia	Serine	36	8	60	De Toni *et al.,* 2002
FUNGI					
Aspergillus fumigatus	Serine	-	6.5-9.0	45	Santos *et al.,* 1996
A. oryzae	Metallo	60	8	50	Farag and Hassan, 2004
A. oryzae NRRL-447	-	39.7	7	70	Thanaa *et al.,* 2011
Doratomyces microsporus	-	45-70	9	-	Friedrich and Kern, 2003

Microorganisms	**Catalytic type**	**Mol. Mass (kDa)**	**Optimum pH**	**Optimum temp (°C)**	**Reference**
Myrothecium verrucaria	Serine	22	8	37	Da Gioppo *et al.,* 2009
Paecilomyces marquandii	Serine	33	8	60-65	Gradisar *et al.,* 2005
Penicillium sp. Ahm 1	Metallo	19	8	50	El-Gendy, 2010
Penicillium sp. Ahm 2	Protease	40	6.0-11.0	60-65	El-Gendy, 2010
Scopulariopsis brevicaulis	Serine	36-39	8	40	Anbu *et al.,* 2005
Trichoderma atrvoviride F6	Serine	21	8.0-9.0	50-60	Cao *et al.,* 2008
Trichophyton mentagrophytes	Serine	38-41	4	-	Tsuboi *et al.,* 1989
T. schoenleinii	-	38	5	50	Qin *et al.,* 1992
T. vanbreuseghemii	Serine	37	8	-	Moallaei *et al.,* 2006
T. sp. HA-2	Serine	34	7	40	Anbu *et al.,* 2008

1.9.1 Molecular weight of keratinase

The molecular weights of many keratinases have been reported, ranging from 16-240 kDa (Srivasta *et al.,* 2019). Inacio *et al.* (2018) reported keratinase with a molecular weight of 16 kDa from *Pseudomonas pulmonarius*, whilst Bernal *et al.* (2006) reported keratinase with a molecular weight of 240 kDa from *Kocuria rosea.* Most keratinases are monomeric enzymes; however, multimeric keratinases are also reported (Xie *et al.,* 2010). A study by Hamiche *et al.* (2019) revealed that molecular weights of KERZT-A and B (different keratinases from the strain) from *Bacillus amyloliquefaciens* S13 were 28 and 47 kDa, respectively. Higher molecular weights are usually detected for metallo-keratinases and keratinases from thermophiles (Hassan *et al.,* 2020b).

1.9.2 Effect of pH on keratinase activity

Keratinases from most organisms are best active within the neutral and alkaline pH range (Sanghvi *et al.,* 2016). From an industrial standpoint, the ability of keratinase to retain its activity over a wide pH range is critical, as it eliminates the need for a pH control system (Brandelli *et al.*, 2015). Keratinases are most active in alkaline environments, with pH optima ranging from 8.0 to 10.0. However, acidic pH optima have also been reported and are usually typical for keratinases from fungal sources (Verma *et al.*, 2016b). For example, a study by Tsuboi *et al.* (1989) reported an optimum pH of 4 for the keratinase of *Trichophyton mentagrophytes* while Benkiar *et al.* (2013) reported an optimum pH of 12.5 for keratinase of *Bacillus* sp. DZ100. However, a study by Mitsuiki *et al.* (2004) reported that keratinase from *Nocardiopsis* TOA-1 was stable over a wide pH range of 1.5 to 12.

1.9.3 Effect of temperature on keratinase activity

The temperature optima of keratinases may also be variable, often depending on the isolate's source and origin (Tamreihao *et al.,* 2019). For example, the keratinase of the thermophilic *Fervidobacterium pennavorans* has an optimum temperature of 80ºC (Friedrich and Antanikan, 1996), while keratinase from mesophilic *B. thuringiensis serovar israelensis* H4 showed maximum activity at 30ºC (Poopathi *et al.,* 2014). However, Nam *et al*. (2002) observed an optimal temperature of 100°C for keratinase from *Fervidobacterium islandicum* AW-1, which is one of the most thermostable keratinases yet discovered.

1.9.4 Effect of inhibitors on keratinase activity

Keratinases are characterized by determining their activity to various protein inhibitors. Based on the catalytic action and nature of their active sites, keratinases are usually classified into different groups *viz*., aspartic, serine and metallo keratinases (Hamiche *et al.,* 2019). Keratinases from yeast strains are usually identified as aspartic proteases, while keratinases from bacteria are usually reported as serine or metallo proteases (Sivakumar *et al.,* 2013). Keratinases are generally serine or metalloproteases, according to studies with specific substrates and inhibitors (Tamreihao *et al*., 2019). By studying their protein sequences, which revealed a serine residue at their catalytic site and demonstrated characteristic phenylmethanesulfonyl fluoride (PMSF) suppression and/or benzamidine inhibition, some keratinases have been linked to the subtilisin family of serine-type proteases (Jaouadi *et al*., 2013). Ethylene diamine tetra acetic acid (EDTA) inhibits the metalloproteases, and both PMSF and EDTA inhibit the serine metalloprotease keratinases (Okoroma, 2012).

1.9.5 Effect of metal ions on keratinase activity

Enzymes often require metal ions for structural stabilization and optimal substrate binding at the active site, as well as transition state stabilization. The increase in enzyme activity is aided by divalent metal ions, particularly Ca^{2+} and Mg^{2+} (Sivakumar *et al*., 2013). At 60°C, Fakhfakh-Zouari *et al*. (2010) investigated the effect of $CaCl_2$ on *B. pumilus* crude keratinase. The half-life of keratinase was 43 minutes in the presence of 5 mM $CaCl_2$ but was reduced to 20 minutes in the absence of Ca^{2+}. This beneficial effect can be attributed to the stability of the keratinase-substrate complex as well as the preservation of enzyme structure (Hassan *et al*., 2020b). Furthermore, metal ions may shield the enzyme from heat denaturation, which gives a benefit. Furthermore, metal ions may protect the enzyme from thermal denaturation, resulting in a high level of enzyme stability at high temperatures (Kojima *et al*., 2006). For example, native keratinase (nKBALT) from *B. altitudinis* RBDV1 and recombinant keratinase (rKBALT) from *Bacillus altitudinis* RBDV1 showed high activities at 85°C and pH 8.0, especially when metal ions such as Mg^{2+}, Mn^{2+}, Zn^{2+}, Ba^{2+}, and Fe^{3+} were present, as these ions maintain the conformation (Pawar *et al*., 2018). Heavy metals such as Cu^{2+}, Ag^{2+}, Hg^{2+}, and Pb^{3+}, on the other hand, impede or significantly deplete keratinase activity when present in high amounts (Ghaffar *et al*., 2018).

1.9.6 Effect of other chemicals on keratinase activity

Other compounds, including as detergents, organic solvents, inhibitors, reducing agents, and others, have an effect on keratinase activity depending on their microbiological origin (Verma *et al*., 2016b). The enhancement of keratinolytic activity by reducing substances is a regular occurrence. Metallokeratinase from *Chryseobacterium aquifrigidense* FANN1 showed remarkable stability in the presence of chemical agents, with residual activity 141 ± 10.38%, 98 ± 0.43%, 111 ± 1.73%, 124 ± 0.87%, 104 ± 3.89%, 107 ± 7.79%, and 112 ± 0.86% against DTT, H_2O_2, DMSO, acetonitrile, triton X-100, tween-80, and SDS, respectively (Bokveld *et al*., 2021). Rather than a direct influence on the enzyme, such effects are usually attributed to the decrease of cysteine bridges in the keratinous substrate (Tiwary and Gupta, 2010). Keratinases can usually tolerate inhibitors and stay active in the presence of bleaching chemicals, demonstrating its utility in a variety of industrial applications (Ghaffar *et al*., 2018). For example, keratinase from *Bacillus mojavensis* SA was stable with anionic surfactants (SDS) retaining more than 88% of its activity. Keratinase was strongly stable in the presence of Tex'tile and Carrefour detergents retaining more than 92% of its activity at 50°C while 100% activity was retained in the case of Shames and Dixan powder (Hammami *et al*., 2018).

1.10 Keratinase assays

Considering that not all proteases can degrade keratin, a thorough examination of the enzyme activity of putative keratinase manufacturers is essential. The heterogeneity in assays used to evaluate keratinase activity has made comparing keratinase activity of the same or various keratinase producing bacteria within or between laboratories impractical. Different assays are used to calculate keratinase activity, as shown in Table 1.9. Although the necessity for a uniform test to allow quantitative comparison of keratinase activity has been proposed previously (Gradisar *et al*., 2005), multiple assay methods are currently used by different researchers.

Several key parameters, including as the homogeneity of the test substrate and the consistency of the assay technique, are required for a repeatable and reliable keratinase activity assessment. Keratin azure, a pure keratin produced from wool coloured with azo dyes, is the most often used test for keratinase activity. Keratin azure is highly resistant to denaturation and degradation by ordinary proteases; however keratinase degradation remains a risk (Navone and Speight, 2018). Enzymatic breakdown of keratin bonds releases

azure-bound amino acids or peptides into solution, resulting in a blue-colored product that may be quantified spectrophotometrically (Hamiche *et al.*, 2019).

Table 1.9 Various assay methods employed for keratinolytic activity determination

Assay substrate	Enzyme unit definition	Reference
Azokeratin	Increase of 0.01 absorbance unit at 450 nm after 15 min in test reaction compared with control	Riffel *et al.,* 2003
Azokeratin	An increase of 0.001 absorbance unit at 450 nm at optimum temperature	Kim *et al.,* 2001
Azokeratin	An increase of 0.01 absorbance unit at 420 nm after 30 min at 45°C	Thys *et al.,* 2004
Azokeratin	An increase of 0.01 absorbance unit at 450 nm ml^{-1} h^{-1}	El-Refai *et al.,* 2005
Azokeratin	An increase of 0.01 absorbance unit at 440 nm after 15 min at 50°C	Riffel and Brandelli, 2006
Chicken feather	Increase in absorbance unit of 0.1 at 280 nm after 1 h at 37°C	Anbu *et al.,* 2008
Cow horn	An increase of 0.01 absorbance unit at 280 nm ml^{-1} h^{-1}	Dozie *et al.,* 1994
Feather	Amount of enzyme required to liberate 354 µg of tyrosine at 584 nm	Chitte *et al.,* 1999
Feather	Increase in absorbance unit of 0.01 at 280 nm after 1 h at 60°C	Tiwary and Gupta, 2010
Hooves and horns	Increase in absorbance unit of 0.1 at 280 nm after 1 h at 60°C	Haddar *et al.,* 2011
Keratin	Increase in absorbance unit of 0.01 at 280 nm after 10 min at 50°C	Cai and Zheng, 2009
Keratin	Increase in absorbance unit of 0.01 at 280 nm after 3 h at 30°C	Jeong *et al.,* 2010b
Keratin	Release of 1 µg of tyrosine after 30 min at 40°C under experimental conditions	Sahoo *et al.,* 2012
Keratin	Increase in absorbance unit of 0.001 at 280 nm after 10 min at 37°C	Gurav and Jadhav, 2013
Keratin Azure	An increase of 0.1 absorbance unit at 595 nm h^{-1}	Suntornsuk and Suntornsuk, 2003
Human hair	An increase of 0.01 absorbance unit at 660 nm h^{-1}	Yamamura *et al.,* 2002
Soluble keratin	An increase in absorbance unit of 0.01 at 280 nm after 3 h at 30°C	Park and Son, 2009
Wool powder	Release of 1 µmole of tyrosine liberated per hour at 50°C	Fang *et al.,* 2013b

Although keratin azure is considered a good substrate for keratinase activity, there is an underlying problem with the commercial keratin azure (K8500). Due to the dye's uneven dispersion, measurements between sample replicates are inconsistent, irreproducible, and untrustworthy. The fluctuating release of azure dye into the reaction mixture during spectrophotometric measurements demonstrates this. To alleviate this issue, commercial keratin azure is standardized prior to the keratinase assay by washing it with distilled water and then drying it overnight at 30°C. This method eliminates loosely attached blue dye as well as dye particles stuck to keratin fibres, leaving a uniform substrate. In addition, this process assures that the dye released during a reaction is entirely due to keratin azure hydrolysis (Okoroma *et al.,* 2012).

1.11 Keratinase purification

Crude protease preparations are used for most commercial uses, whereas pure protease preparations are required for pharmaceutical and other medical applications, as well as the assessment of biochemical properties (Lakshmi *et al.*, 2018). As a result, keratinase purification techniques typically start with ammonium sulphate or acetone precipitation, followed by dialysis and ultrafiltration. However, the choice of purification methods such as ammonium sulphate precipitation or acetone precipitation is generally dependent on the property of the enzymes (Kamal *et al.,* 2016). The final techniques typically include multiple chromatographic steps, including ion-exchange and gel filtration columns. The advantages and disadvantages associated with each purification step are outlined in Table 1.10.

Table 1.10 Advantages and disadvantages of keratinase purification (Brandelli *et al*., 2015)

Purification Technique	Advantages	Disadvantages
Precipitation with salts/organic solvents	Simple technique	Some organic solvents promote enzyme denaturation
	Able to scale up	Require a step of dialysis (desalting) after salt precipitation
	Low cost	Generally low resolution
	Easy labour	
Ultrafiltration	Low pressure and temperature minimize the enzyme denaturation	Membranes are costly
	Simple operation	Membrane fouling and polarisation reducing permeate flux
	Concentration of protein	Low resolution
	Desalination	
	Easy scale-up	
Gel filtration	High resolution	Diluted sample at the end of the process
	Desalination	Slow process
		Difficult to scale-up
		Costly process
		Lower throughput when compared with a non-chromatography process
Ion-exchange chromatography	High resolution	Costly process
	High capacity for protein binding	Lower throughput when compared with a non-chromatography process
	Decreased sample volume	
	Easy scale-up	

The amount of enzyme present in the cell-free filtrate is typically low, thus, the elimination of water from the crude broth is a primary objective during purification. For the concentration of culture filtrate, several procedures are used, including salt or solvent precipitation or ultra-filtration via membranes with different cut-offs (Kamal *et al.,* 2016). Precipitation is the most often used approach for separating and recovering proteins from crude biological mixtures because it allows for both purification and concentration. The addition of reagents such as salt (ammonium sulphate) or an organic solvent (acetone),

which reduce the solubility of the target proteins in an aqueous solution, has a significant impact (Bhunia *et al.,* 2012). Although precipitation is a simple technique that allows scale-up of the enzyme of interest, some solvents can denature enzymes depending on the enzyme's characteristics. Therefore, dialysis or desalting is a critical step which needs to be performed after precipitation.

Various membrane separation processes, i.e., ultrafiltration (UF), diafiltration, and temperature-sensitive hydrogel ultrafiltration have been extensively used for enzyme purification. Ultrafiltration is a low-cost pressure-driven separation method that provides purification and concentration while causing little loss of enzyme activity. The most significant disadvantage of ultrafiltration is the gradual decrease in permeate flux through the membrane as a result of membrane fouling (Vardanega *et al.,* 2013). Diafiltration is used to remove or change salt composition (Kamal *et al.,* 2016).

Ion exchange chromatography is used to purify proteins and other charged particles. This procedure offers high resolution with a smaller sample volume; however, it is a costly process. To further concentrate the enzyme, gel filtration can be applied. This method also offers a high resolution as with ion exchange, but it is a slow, costly process which is difficult to scale-up (Gopinath *et al*., 2015).

Initial purification methods such as acetone or ammonium sulphate precipitation result in a high enzymatic recovery rate of 76.1–91%. Chromatographic procedures like ion exchange in a fixed bed and gel filtration, on the other hand, provide great purification (Rai and Mukherjee, 2011). The type of keratinase purification technique to use is determined by the enzyme's final application. Purification procedures such as ion-exchange chromatography in fixed bed and gel filtration can be utilized if the goal is to explore biochemical features of purified keratinase. These methods offer a high level of purification (Brandelli *et al*., 2015).

Allure *et al.* (2015) proposed a purification protocol for the crude extract of *Streptomyces minutiscleroticus* DNA38, which involved ammonium sulphate precipitation (60 – 90% saturation), ion exchange on DEAE cellulose and gel filtration on Sephadex G-75. The keratinase was purified 113.45-fold with an enzyme recovery of 3.8%. For purification of keratinase from *B. subtilis* SCK6, 50% ammonium sulphate precipitation followed by SP-Sepharose fast flow chromatography was used, where the keratinase was purified 9.34-fold

and the enzyme recovery was 14.6% (Tian *et al.,* 2019). Some of the commonly used purification protocols, purification factors and recoveries are shown in Table 1.11.

Table 1.10 Purification factor, enzymatic recovery and specific activity of keratinases purified by different protocols

Source	Purification protocol	Purification factor	Enzyme recovery (%)	Specific Activity (U/mg)	Reference
Actinomadura keratinilytica Cpt29	ASP, heat treatment and GFC	48.1	11.6	70000	Habbeche *et al.,* 2014
Aspergillus fumigatus TKF1	ASP and GFC	2	1.9	77	Paul *et al.,*2014b
A. oryzae	ASP, GFC and IEC	11.4	33.3	304	Farag and Hassan, 2004
A. parasiticus	Acetone precipitation, dialysis, ASP and IEC	2.19	2.47	106	Anitha and Palanivelu, 2013
Bacillus megaterium	ASP and IEC	655	12.7	545	Agrahari and Wadhwa, 2012
B. pumilus KS12	UF and thermal precipitation	3.2	89.1	35886	Rajput and Gupta, 2013
B. sp. P7	ASP, GFC and two steps IEC	29.8	27	12967	Correa *et al.,* 2010
B. subtilis MTCC (9102)	ASP, IEC and GFC	45.9	27.6	4182	Balaji *et al.,* 2008
B. subtilis NRC 3	ASP, IEC and GFC	31	20	5233	Tork *et al.,* 2013
B. subtilis 8	ASP and IEC	2.2	34.2	513	He *et al.,* 2018
B. subtilis SCK6	ASP and IEC	9.34	14.6	9813	Tian *et al.,* 2019
Brevibacillus sp. strain AS-S10-11	Acetone precipitation and GFC	18	58.5	216000	Rai and Mukherjee, 2011
Chryseobacterium sp. strain kr6	ASP, GFC and IEC	40.2	7.1	21466	Silveira *et al.,* 2010
Cunninghamella echinulata	Acetone precipitation and affinity chromatography	13.7	39.7	35	More *et al.,* 2013
Meiothermus sp. 140	ASP, hydroxyapatite IEC and GFC	30.2	45	35365	Kuo *et al.,* 2012
Purpureocillium lilacinum LPS#876	ASP, GFC, two steps IEC and GFC	19.9	1.3	1433	Cavello *et al.,* 2013
Streptomyces sp. strain AB1	ASP, heat treatment, GFC and IEC	86	24	67000	Jouadi *et al.,* 2010
S. minutiscleroticus DNA38	ASP, IEC and GFC	113.45	3.8	2487	Allure *et al.,* 2015

1.12 Major milestones in keratinase research

Keratin degradation is a long-standing phenomenon, with Molyneux (1959) being the first to isolate bacteria capable of doing so. Following that, a report on the isolation and characterisation of a feather-degrading bacterium *B. licheniformis* PWD-1 shed information on the biotechnological and environmental significance of keratin degradation (Williams *et al.,* 1990). The study focused mostly on feather recycling and feather meal synthesis, and it discovered a possible keratinase, KerA, from *B. licheniformis*. Following that, KerA was thoroughly studied, including its sequence and expression in a variety of heterologous hosts (Gupta et al., 2012).

Lin *et al*. (1992) were the first to purify and describe keratinase from the *B. licheniformis* strain, which belongs to the S8 protease family. By chromosomal integration of the KerA gene and producing asporogenic *B. licheniformis* strains, efforts were made to optimize keratinase production (Wang *et al.,* 2004). As a result, KerA has been the subject of multiple articles and patents (Gupta *et al*., 2012). Researchers began looking into different subtilisins for their keratinolytic potential when Shih and Williams (1992) identified KerA as a mutant of subtilisin Carlsberg. The functions of cell redox, disulphide reductase, and substrate selectivity were investigated in order to better understand the mechanism of keratin breakdown (Ramnani and Gupta, 2007).

However, keratinase research did not take off until the discovery of KerA's prion degrading capability in 2003, when Mad Cow disease broke out in Europe, culminating in a beef ban across the UK (Gupta *et al*., 2012). Around the same time, Shih's group reported in 2003 that keratinase from *B. licheniformis* PWD-1 (KerA) could degrade prion protein in brain tissue infected with scrapie, demonstrating for the first time that keratinase from B. licheniformis PWD-1 (KerA) could degrade prion protein in brain tissue infected with scrapie (Langeveld *et al*., 2003). Since then, the number of findings on keratinases from various bacteria that can successfully destroy prion has increased dramatically (Mitsuiki *et al*., 2006). Keratinase is currently thought to be a potential green approach for prion decontamination.

Researchers, nutritionists, and feed producers have been drawn to the PWD-1 keratinase because of its dexterity in degrading resistant keratinous wastes and enhancing their nutritional value. As a result, Shih and colleagues at BioResource International (BRI)

developed two patented products, Versazyme (launched 2005) and Valkerase (released 2006), based on this thermostable keratinase (Potera 2013). In 2008, the Shih-founded BRI expanded the market for keratinase-based products outside of the United States by teaming with Novus International, a Chinese based animal nutrition company. This collaboration aided in the dissemination of these keratinase-based formulations in the Asian market, resulting in significant revenue (Potera 2013).

In addition to NOVUS and BRI, PROTEOS Biotech's products were created by microencapsulating *B. licheniformis* keratinase. These benign natural formulations can be used as a substitute for synthetic alpha hydroxyl acids (AHAs), urea, and thioglycolates, which are commonly used in cosmetics as cell renewal, moisturizing, and anti-hair growth agents. The commercialization of keratinase–based formulations have the potential to revolutionize numerous sectors of the bioeconomy, while also reducing pollution that could have been exacerbated by traditional chemicals (Nnolim and Nwodo, 2021).

Further research by Paul and collaborators has advanced the use of microbial keratinase for a variety of purposes, including the valorization of keratinous wastes into high-value functional peptides and essential amino acids (Paul et al. 2014b), the cleaning properties of keratinase with a detergent admixture (Paul *et al.*, 2014a, c), plant growth promotion using keratinase derived organic (Paul *et al.*, 2014d). The discovery of novel keratinases from *Stenotrophomas maltophilia* (Fang et al. 2013a), the keratinase production process optimum construction (Fang et al. 2013b), and the biotechnological development of the bacterial strain for the overproduction of keratinase have all demonstrated Fang and co-workers' expertise (Fang et al. 2014). They have also employed various protein engineering approaches to improve keratinase biotechnological and industrial values which were detailed in studies including truncation of keratinase PPC domain for catalytic efficiency improvement (Fang et al. 2016a), keratinase domain exchange for an improved catalytic efficiency (Fang et al. 2016b), keratinase substrate specificity alteration (Fang et al. 2015), thermostability improvement through rational protein engineering approaches (Fang et al. 2017b), and cloning and overexpression of keratinase in an heterologous industrial host (Fang et al. 2019). These rigorous investigations therefore underpin the industrial and biotechnological potentials of *S. maltophilia* keratinolytic protease.

In the last two decades, there has been an upsurge in reports mentioning keratinases, owing to developments in enzyme technology. According to the Web of Science Core

Collection, more than 70% of the research published in the topic "keratinases or keratinolytic enzymes" were published in 2010–2020. This demonstrates the growing interest in the topic as well as technologies that aid in the advancement of understanding about these enzymes (de Menezes *et al*. 2021).

Following these studies, numerous patents and commercial enzymes used to degrade keratin, in addition to Versazyme and Valkerase (BRI), have been described (Table 1.12). Other commercial enzymes include CIBENZA DP100 (Novus International, Inc), NATE-0853 and FEED-0001 (Creative Enzymes) and PURE100 KERATINASE (PROTEOS Biotech) (Nnolim and Nwodo, 2021).

Table 1.12 Keratinase-based formulation in commercial use (Nnolim and Nwodo, 2021)

Enzyme	**Microbial producer**	**Formulation**	**Uses**	**Manufacturer /Supplier**
Keratinase	*B. licheniformis*	Keratoclean Sensitive PB	Skin care products	PROTEOS Biotech
Keratinase	*B. licheniformis*	Versazyme	Animal feed preparation, keratin and collagen degradation	BioResource International, Inc
Keratinase	*B. licheniformis*	Valkerase	Feather meal processing	BioResource International, Inc
Keratinolytic protease	*B. licheniformis*	Cibenza DP 100	Poultry and swine feed ingredient digestion	Novus International
Keratinase	*B. licheniformis*	Keratoclean Hydra PB	Skin care products	PROTEOS Biotech
Keratinase	*B. licheniformis*	Keratoclean PB	Skin care products	PROTEOS Biotech
Keratinase	*B. licheniformis*	Prionzyme M	Biodecontamination	Genencor International, Inc
Keratinase	*B. licheniformis*	FEED-0001	Insoluble keratin degradation	Creative Enzymes
Keratinase	Recombinant *Escherichia coli* BL21	NATE-0853	Enzymatic treatment of EB, GAGs and cells	Creative Enzymes
Keratinase	*B. licheniformis*	PURE 100 KERATINASE	Biomedical, pharmaceutical and cosmetic applications	PROTEOS Biotech

1.13 Biotechnological applications of keratinases

Keratinases are regarded as attractive biocatalysts for a variety of sectors due to their broad substrate specificity, resilience, and various biochemical characteristics (Gupta *et al.*, 2012). Keratinases and keratinolytic microbes, for example, are extremely useful in the recycling of feathers, leather, textiles, feed, fertilizers, and cosmetics (Verma *et al.*, 2016b). Furthermore, keratinases have the ability to hydrolyze a wide range of substrates, implying that it could be used to bioconvert waste into value-added goods (Sahni *et al.*, 2015).

1.13.1 Significance of keratinase in prion management

Prions are non-nucleotide-containing protein particles with unknown functions in brain cells (Lathe and Darlix, 2020). These protein particles are rich in an α-helix form in their normal form, i.e. PrPc, but are rich in β-sheets in their disease-causing form, i.e. PrPsc (Langeveld *et al.*, 2003). This simple conformational change causes fatal neurodegenerative disorders such as Bovine Spongiform Encephalopathy (BSE), often known as "Mad Cow Disease," Scrapie, and Creutzfeldt-Jakob Disease, which are all referred to as Transmissible Spongiform Encephalopathies (TSE) (Terry and Wadsworth, 2019).

The cattle sector suffered significant losses as a result of BSE epidemics, and enormous investments were necessary to properly handle affected tissues (Verma *et al.*, 2016b). Furthermore, an increase in reports of horizontal prion transmission from animals to people, as well as iatrogenic transmission through infected surgical tools, has encouraged the development of more stringent prion decontamination techniques. The common methods of prion decontamination used high temperature, extreme pH, and harsh chemicals in an energy-extensive process (Taylor, 2000), and scientists turned to microbial enzymes as a greener solution to the problem (Gupta *et al.,* 2012).

Because prion proteins have a structure similar to keratin proteins (rich in β-sheets) and are resistant to proteases, 'keratinases' that rapidly destroy the α-keratin of feathers were thought to be attractive candidates for prion cleanup (Verma *et al.*, 2016b). Yoshioka *et al.* (2007) investigated the effectiveness of *B. licheniformis*-produced keratinase in decomposing PrPsc in infected brain homogenate from mice and cattle. They discovered that the enzyme could breakdown PrPsc in BSE-infected brain homogenate, but that it

needed to be pretreated at 115°C for 40 minutes in the presence of a surfactant. The search for catalytically more specific keratinases that can degrade PrPSc without denaturing treatment continued because such harsh treatments were not environmentally feasible. The first protease capable of digesting PrPsc was keratinase from *B. licheniformis* PWD 1. (Sahni *et al.*, 2015). This discovery prompted a flurry of research on the use of bacterial proteases with keratinolytic activity for the treatment and breakdown of PrPSc.

1.13.2 Importance of keratinase in the animal feed industry

The hydrothermal technique, or steam pressure cooking, was used to turn feathers into feather meal, which took a lot of energy (Sahni *et al.*, 2015). Because it damaged certain critical amino acids including methionine, lysine, histidine, and tryptophan, the hydrothermal treatment lowered nutritional value (Adelere and Lateef, 2016) As a result, because it was low in key amino acids, feather meal was only employed as an element in animal feed on a limited basis. Enzymatic hydrolysis of feather by keratinases was an alternative to steam pressure boiling to make feather meal (Sahni *et al.*, 2015).

Many studies have shown that feather hydrolysates can be used in animal feed, and these studies have also shown that keratinase can be used to convert feather and keratinous wastes to animal feed instead of the hydrothermal process (Verma *et al.*, 2016b). Keratinases could potentially be introduced to feed products to help animals digest their food better. Cafe *et al.* (2002) found that using keratinase improved the digestibility of animal feed and resulted in significantly higher animal body weights.

Furthermore, the use of keratinase-supplemented diets has resulted in a lower feed demand (Wang *et al.*, 2008). In addition, dietary keratinase supplementation has been shown to improve immunological response, weight gain, nutrient digestibility, intestinal morphology, and ecology in growing nursery pigs (Wang *et al.*, 2011b), as well as have a positive impact on broiler growth and feed consumption (Stark *et al.,* 2009).

1.13.3 The value of keratinase in the fertilizer industry

Keratinases are useful in the manufacturing of environmentally friendly fertilizers because the hydrolysates they create are a rich source of nitrogen and amino acids that may be utilized to improve soil quality (Brandelli *et al.*, 2010). Feather meal, for example, was used as a slow-release nitrogen fertilizer in organic farming (Adelere and Lateef, 2016),

and has proven to be one of the safest and most cost-effective technologies available (Verma *et al.*, 2016b). The utilization of feather hydrolyzate and keratinolytic bacteria with antagonistic and plant growth promoting (PGP) activities as a superior organic fertilizer, biocontrol, and biofertilizing agent could provide a number of economic and environmental benefits over chemical-based alternatives. Organic additions including N and/or C-rich feather hydrolyzate can improve plant development while also increasing beneficial microbial activity in the soil. By producing indole-3-acetic acid (IAA), siderophore, solubilizing inorganic P, and other significant growth promotion qualities, keratinolytic bacteria with biocontrol and/or PGP activities can protect pathogen-caused illnesses and boost plant growth by producing IAA, siderophore, and other essential growth promotion traits. As a result, using feather hydrolyzate and keratinolytic bacteria with PGP and/or biocontrol activities as a sustainable and alternative tool to promote and improve organic farming, agro-ecosystems, environment, human health, soil biological activities, and, at the same time, enhance sustainable agricultural productions, could be a viable option (Tamreihao *et al.,* 2019).

Feather hydrolysate from *Paenibacillus woosongensis* TKB2 was found to induce nodule formation, improve seed germination, and increase Bengal gram (*Cicer arietinum*) development (Paul *et al*., 2013). It also boosted mineral elements and microbial activities in the soil, with a 1.2-fold rise in N, P, K, and the C/N ratio. Furthermore, the quantity of free-living nitrogen fixers and phosphate solubilizers were increased by 2 and 5.8-fold, respectively. In another study, a keratinase-producing *B. subtilis* B8 demonstrated plant growth-promotion by releasing indoleacetic acid (IAA) and also displayed broad-spectrum antimicrobial activities. Thus, *B. subtilis* S8 could act as a potential biofertilizer or biocontrol agent to crop plants (Jeong *et al.,* 2010a). Kornillowicz-Kowalska and Bohacz (2002) demonstrated that compost from poultry feather and plant waste improved soil conditions and limited the growth of phytopathogenic fungi, *Fusarium*. Furthermore, because keratinous wastes are nitrogen-rich, they can be used as an appealing and cost-effective co-substrate for compost to support plant growth, promote plant-microbe interactions, improve soil texture, improve soil health, and prevent soil erosion (Verma *et al.,* 2016b).

1.13.4 Implication of keratinase in the leather industry

Leather processing mainly involves three steps; beam house or pre-tanning process (clean hides or skins); tanning (permanently stabilize skins or hides) and post tanning and finishing (aesthetic value-added). Various chemicals such as sodium sulphide, sodium hydroxide, and lime are utilized at each stage (Sahni *et al.*, 2015). These compounds, together with the release of solid wastes as a result of various treatments, are powerful water pollutants that cause ecological disturbance in water bodies by increasing parameters such as biochemical oxygen demand (BOD), chemical oxygen demand (COD), and total dissolved solids (TDS). Leather of lower quality is also a result of the chemical treatment (Verma *et al.*, 2016b). The use of biocatalysts to replace inorganic sulfide demonstrated significant benefits in terms of environmental protection and unhairing efficiency. Keratinolytic protease is a good biocatalyst for hydrolyzing disulfide bond-rich proteins in hair with no damage to the leather. The volume and toxicity of wastewater effluent from the leather industry could be greatly reduced by biological treatment with keratinolytic proteases. However, these enzymes' low thermostability and substrate specificity or particular activity hampered their practical utility. In order to improve the keratinolytic enzyme, current advances in protein engineering technologies (site-directed mutagenesis, protein fusion, N/C-terminus truncation, and domain swapping) have been applied (Fang *et al.*, 2017a).

Microbial keratinases have been reported to dehair animal hides in many cases. For example, keratinase from *B. safensis* was shown to be effective in dehairing goatskin in 12 hours without harming the skin, whereas chemicals caused significant skin damage (Lateef *et al.,* 2014). Proteolytic enzymes, as well as modest amounts of sulphide and lime, are commonly used in enzymatic dehairing. For example, Gehring (2002) reported that bovine hides were dehaired using a mixture of *Streptomyces griseus* proteolytic enzymes and carbonate buffer 7 surfactants. Thanikaivelan *et al.* (2004) found that 0.5% sodium sulphide and 1% enzyme (bacterial alkaline protease) were utilized to completely remove hair from cow skin. The application of enzyme-assisted dehairing was found to lower sulphide levels by 85%. In addition, when compared to traditional leather processing, effluent loads of COD and total solids were reduced by 45% and 20%, respectively. Furthermore, total dry sludge was reduced from 152 kg per 1000 kg of rawhide treated to 12 kg per 1000 kg (Thanikaivelan *et al.,* 2002). This shows that keratinases have the ability

to turn environmentally hazardous leather production into an environmentally friendly leather treatment procedure. Furthermore, by substituting less expensive microbial keratinases for more expensive chemicals used in leather treatment, high-quality items can be produced at lower costs (Verma *et al*., 2016b).

1.13.5 Significance of keratinase in the detergent industry

Keratinases are valuable in detergent formulation due to their ability to degrade insoluble keratin and properties such as stability at high temperatures and pH, activity over a wide temperature and pH range, stability in the presence of surfactants, oxidizing and bleaching agents, chelating agents, and compatibility with some commercial laundry detergents (Gupta and Ramnani, 2006). Furthermore, keratinases are commonly used in the detergent sector as a safe substitute for toxic chemicals like caustic soda (Verma *et al*., 2016b).

Several investigations have shown that keratinases have detergent stability and compatibility, as well as the capacity to clear various stains. Keratinase produced by *B. pumilus* KS12, for example, showed detergent stability and oxidation resistance and might be employed as a detergent addition (Rajput *et al.*, 2010). In another study, *Paenibacillus woosongensis* TKB2 keratinase was coupled with detergent and successfully removed blood, fruit juice, and turmeric stains from fabric (Paul *et al.,* 2013). *B. keratinase* has also been observed to secrete keratinase which was more effective at removing blood stains from cotton material than detergent, which only partially cleaned blood spots (Singh *et al*., 2014). As a result, keratinases are significant enzymes that may be utilized as additions in detergent formulations to effectively remove keratinous wastes while also being environmentally benign.

1.13.6 Relevance of keratinase in the textile industry

Keratinases' capacity to change silk and wool pointed to its possible use in the textile processing industry. Cai *et al*. (2011) used crude keratinase from a *Pseudomonas* strain to treat wool and polyester-blend materials and found that the enzyme increased shrink resistance and tensile strength. Cutinase, keratinase, and protease have been shown to work together to improve the wettability and anti-felting properties of wool fabrics, resulting in a 66° contact angle, 5.2% area shrinkage, and a 14% acceptable strength loss (Wang *et al.,* 2011a). In another study, free keratinase (from *B. licheniformis* expressed in *E.*

coli) showed the potential to improve the quality of woollen fabrics (Srivastava *et al.*, 2020). It was observed that the loss of tensile strength was reduced from 12.5 (untreated wool fabric) to 6.8 at 2.5% keratinase concentration. The keratinase breaks down the disulphide bonds, and thereby increases the performance of keratinase treated wool fibres. Furthermore, the application of keratinase improved the hydrophilicity of wool fibres by removing the fatty acids on the wool surface. Gunes *et al.* (2018) also reported improved hydrophilicity of wool after treatment with keratinase from *Streptomyces* sp. 2M21.

1.13.7 Essence of keratinase in the pharmaceutical industry

In the pharmaceutical and cosmetic industries, non-collagenolytic keratinases are attractive biocatalysts. They've been described as a skin lightening agent and a component in depilatory formulations for hair shaving (Yang, 2012). Furthermore, keratinases have been proven to have the ability to destroy thickened layers of dead skin (hyperkeratosis) seen in the toes and fingers, making them a feasible alternative to salicylic acid (Gupta and Ramnani, 2006). Similarly, keratinases are capable of skin peeling to eradicate acne, which is caused by keratins blocking the sebaceous gland (Selvam and Vishnupriya, 2013). Keratinase has also been utilized to improve medication distribution via topical treatment. Drug penetration through the nail plate has been reported to be aided by the presence of keratinase, which may be a barrier to other medications' penetration (Mohorcic *et al.*, 2007). Previously, docking studies suggested that native keratinase from *B. subtilis* RSE163 and recombinant keratinase from *E. coli* could be used to treat psoriasis by evaluating keratinase binding to pharmaceutical medicines. This assumption opens up a new avenue for keratinase use in the pharmaceutical industry to meet unmet needs (Pawar *et al.*, 2018). Donkey hair hydrolysates stimulated vitamin B-complex synthesis by *Saccharomyces cerevisiae* after being treated with metallokeratinase from *B. thuringiensis* MT1 (Hassan *et al.*, 2020b).

1.13.8 Application of keratinase in X-ray film reutilization

X-ray films are made of polyterephthalate (PET) with an emulsion-based coating on both sides that responds to light and are frequently used in research and medical services. Metallic silver deposited in a gelatin layer coats the X-ray films (Khunpraset *et al.*, 2008). The used photographic/X-ray films produce solid waste issues after radiography because the majority of them still contain metallic silver and the plastic PET base. X-ray film wastes

can be found in large quantities in the backyards of many hospitals, dispensaries, and pathological laboratories, as well as a minor component in municipal waste (Nakiboglu *et al.*, 2000).

Burning the films directly or oxidizing metallic silver followed by electrolysis are two traditional methods for recovering silver from discarded X-ray films. However, the traditional process of incineration does not recover the PET base and is inefficient because it is expensive and produces secondary pollutants like smoke and soot. Similarly, the acid treatment includes leaching a gelatin layer with a strong acid solution, resulting in an acid effluent problem following silver recovery (Cavello *et al.,* 2013). Keratinolytic enzymes, on the other hand, provide an environmentally benign silver recovery approach from X-ray/photographic films since they can use gelatin as a substrate, releasing metallic silver in the reaction solution while leaving the PET base intact (Verma *et al.*, 2016b).

1.13.9 Relevance of keratinase in other applications

In addition to the aforementioned, keratinolytic microbes or their keratinase (crude/purified) can be used to partially hydrolyze keratin for biofilms, coatings, and glue manufacturing (Adelere and Lateef, 2016). Keratinous waste can be used as a raw material in the creation of biodegradable agrotextiles such as fruit coverings, crop covers, and edible films (Abdel-Fattah *et al.*, 2013). To use keratinous wastes for these applications, the waste material's keratin structure must be altered using keratinases or keratinolytic bacteria.

Revathi *et al*. (2013) used a green chemistry technique to find microbial keratinase's ability to generate silver nanoparticles (AgNPs). Furthermore, the particles demonstrated significant inhibitory efficacy against clinical *E. coli* and *S. aureus* isolates. As a result, the strain's keratinase might be exploited to produce an environmentally benign technique for producing AgNPs quickly.

Another potential use of keratinases is for biofuel synthesis and energy generation by anaerobic digestion of biowastes (Brandelli *et al.*, 2010). Keratinases can also be used as biocontrol agents because of their pesticidal and insecticidal properties (Yue *et al.*, 2011). Keratinolytic fungi could also be employed to bioremediate settings damaged by crude oil (Ulfig *et al.*, 2003). Keratin hydrolysates are also being used to regulate haemostasis and nerve regeneration as well as to clean contact lenses. Keratinases can hydrolyze gelatin, which makes them useful in the food processing industry (Adelere and Lateef, 2016). As

a result, keratinases offer a wide range of potential applications that will require more investigation.

1.14 Rationale of the study

The accumulation of feathers in the environment poses a serious threat, if not treated. A major component of feathers is a resilient protein, keratin. The current methods employed for the waste management of poultry feathers include hydrothermal treatment, which involves high temperature and pressure resulting in the conversion of indigestible poultry feathers to an easily metabolizable feather-meal. This treatment results in the loss of certain valuable amino acids due to high temperatures. The presence of keratinolytic microorganisms in nature provides an alternative method for the treatment of poultry feather waste. Microorganisms possessing the enzyme keratinase are capable of utilizing keratin, thus carrying out the degradation of poultry feathers. Microbial treatment of poultry feather waste would be an eco-friendly approach to combat the problem of poultry waste. This treatment would result in the biodegradation of poultry feathers and generate amino acids, which are useful by-products of keratin degradation. The feather hydrolysate obtained at the end of microbial treatment would be enriched with amino acids and have the potential to be used as animal feed.

The aim of this study was to enzymatically produce feather meal using a keratinolytic enzyme. The first step in this investigation was to screen for feather degrading microorganisms from various poultry samples, which involved selective enrichment media. Subsequently, the highest keratinase producer was identified using 16S rDNA sequencing. Process parameters and media components was optimised to attain maximum keratinase production. Purification of the crude enzyme using ammonium sulphate precipitation and DEAE-Cellulose column chromatography was applied and the characteristics of purified enzyme was determined. The potential of the keratinase to biodegrade feathers into feather meal (feed ingredient) was evaluated by determining the proximate analysis and amino acid content of the biodegraded feather meal.

CHAPTER 2

Optimisation of keratinase production from bacteria isolated from poultry waste

2.1 Introduction

Chicken is the cheapest and most widely available source of animal protein, with per capita consumption far exceeding that of beef, lamb, mutton, and veal (Bisschoff, 2017). This has led to an increase in chicken farming, resulting in increased chicken waste production and accumulation. Though regarded as waste, feathers are the largest by-product generated by the chicken industry, which accounts for approximately 7% of a chicken's live weight (Tamreihao *et al.,* 2019). Commonly, feather wastes are disposed of in dumps landfills or incinerators. However, these methods are expensive and environmentally unsustainable (Godheja *et al.*, 2014). The incineration of feather wastes release greenhouse gases (carbon dioxide, methane, nitrous oxide and fluorinated gases), contributing to environmental pollution. On the other hand, landfilled feather wastes take a long time (5 – 7 years) to decay, which reduces the availability of landfill space (Sharma and Devi, 2017). Furthermore, the environmental laws in South Africa (Gazette no. 37083) urge the waste generators to recycle, reutilize, minimize, treat, and dispose of waste only as the last alternative (Ningthoujam *et al.,* 2018). Owing to the various problems associated with feather disposal, many studies have looked into the idea of using biological and chemical approaches to transform feather waste into usable end-products (Jin *et al.,* 2017).

The structural protein in chicken feathers, keratin, is insoluble and has a high stability. Keratin's structural stiffness is ensured by the composition and molecular configurations of its constituent amino acids (Savita and Archana, 2014). Furthermore, the keratin chain is densely packed into a supercoiled polypeptide chain in the α-helix (α-keratin) or β-sheet (β-keratin), resulting in mechanical stability. Furthermore, the use of cysteine bridges to crosslink protein chains confers exceptional mechanical stability and resistance to keratin breakdown (Kodak *et al*., 2019).

Considering that feather wastes are protein-rich sources that possess many essential amino acids, it can be utilized to manufacture various agricultural commodities such as animal feed (Hassan *et al.,* 2020a). Chemical and mechanical methods can be employed to degrade

keratin; however, such methods may result in the destruction of amino acids (Qiu *et al.,* 2020). Hence, biodegradation of feather has been identified as an efficient, cost-effective, and environmentally friendly alternative for the bioconversion of feather waste into useful products. In addition, bioconversion of feathers allows for the recycling of feather waste and eliminates the use of chemicals (Kodak *et al.,* 2019). Bioconversion involves using keratinolytic microorganisms that produce keratinases and degrade feathers more efficiently than other proteases (Mini *et al.,* 2015).

Keratinases are ubiquitous in nature and are predominantly found in microbes isolated from slaughterhouses or poultry wastes. The majority of the keratinases are produced from bacteria (e.g. *Bacillus licheniformis*, *B. paralicheniformis*, *B. subtilis*, *Pseudomonas* sp., *Microbacterium* sp., *Streptomyces* sp. and *Thermoactinomyces candidus*), filamentous fungi (e.g. *Trichoderma*, *Aspergillus*, *Fusarium*, *Doratomyces*, *Penicillium*, *Epidermophyton floccosum* and *Scopulariopsis* sp.) and yeasts (e.g. *Candida* sp., and *Trichosporon*) (Abdel-Fattah *et al.,* 2018). These microbes possess the capability to utilize keratin as the sole source of carbon and nitrogen (Srivastava *et al.,* 2020). Microbial keratinases are mostly extracellular enzymes that are inducible, however, some have also been observed to be membrane linked (cell-bound) and intracellular (Srivasta *et al.*, 2019).

The biodegradation process usually demands that certain biological parameters like temperature and pH be maintained at their optimum range throughout the process to achieve high specific growth rates and enzyme activity. For enzyme production, liquid fermentation is quite preferred for bacterial keratinase as aeration of the media is optimum under submerged fermentation, whereas solid-state fermentation is best suited for fungal keratinases, owing to their proliferation mechanism (Srivastava *et al.,* 2020).

Optimisation of physiological conditions of culture medium are also important for optimal keratinase production. The majority of keratinolytic bacteria perform well in neutral to alkaline environments, with pH levels ranging from 7–9 (Yusuf *et al.,* 2019). Temperature is also quite important for keratinase production and ranges from 28°C to 50°C for most keratinases from bacteria, actinomycetes and fungi. Some thermophiles, such as *Thermoanaerobacter* and *Fervidobacterium* spp., had optimal temperatures of up to 70°C (Gopinath *et al.*, 2015). The substrate also plays an important regulatory role, as the presence of substrate, such as feather, usually triggers the synthesis of microbial keratinase. However, because feathers are difficult to decompose, they are insufficient as a sole

substrate for optimal keratinase synthesis. Thus, carbon and nitrogen additions are important in the creation of keratinase since they can aid in the organism's growth and enzyme production.

This chapter describes the isolation and identification of keratinase-producing organisms from poultry waste. Focus has also been placed on the optimization of enzyme production, specifically, the effects of nutritional and physical parameters on keratinase production using the one-factor-at-a-time (OFAT) approach. The optimized conditions were used for subsequent experiments.

2.2 Materials and methods

2.2.1 Collection and processing of samples

To isolate potential feather-degrading bacteria, environmental samples were collected from a poultry farm in Cato Ridge, KwaZulu Natal, South Africa, and a poultry-processing plant in Hammarsdale, KwaZulu Natal, South Africa. The samples included wood shavings and wastes from the poultry farm as well as sludge and feathers from the poultry processing plant. Samples were collected in sterilized sampling bottles and brought to the laboratory on the same day for immediate processing.

The chicken feathers used in fermentation media were collected from a local poultry farm in Hammarsdale, KwaZulu Natal and washed thoroughly with running tap water to remove surface impurities, followed by rinsing with distilled water. The feathers were then dried in a ventilated oven at 50ºC for 48 h. Subsequently, the feathers were cut into small pieces and milled using a ZM200 Retsch mill equipped with a 1.0 mm sieve screen and stored at 4ºC until further use (Sharma and Devi, 2019).

2.2.2 Enrichment technique for the isolation of keratinolytic microorganisms

The collected samples were processed for the isolation of keratinolytic microorganisms via an enrichment technique. Each sample was processed for three successive enrichments of five days each. For the first enrichment, 10 g of sample was added separately to 250 ml Erlenmeyer flasks containing 100 ml feather meal medium comprising of the following ingredients (g/l): powdered chicken feathers (10), yeast extract (0.1), K_2HPO_4 (0.3), KH_2PO_4 (0.4), NaCl (0.5), $MgSO_4.7H_2O$ (0.1), and NH_4Cl (0.5) at pH 7.5 (Ahmadpour *et al.,* 2016). For the subsequent enrichments, 10 ml supernatant from the previous enrichment was taken aseptically and transferred into 90 ml fresh enrichment medium. The enrichments were carried out at 37ºC (Memmert IN 110 incubator) at 100 rpm (Janaranjani *et al.,* 2015).

2.2.3 Isolation and screening of proteolytic microorganisms

One ml of the tertiary enrichment medium was transferred into 9 ml sterilized deionized water and mixed properly. Serial dilutions were performed, and 100 µl of 10^{-4} - 10^{-8} dilutions were plated on nutrient agar. The plates were incubated at 37ºC for 24 h and morphologically dissimilar colonies were selected, inoculated onto 10% skim milk agar plates and incubated at 37ºC for 24 h for the primary screening of keratinase producers. The isolates demonstrating clear zones of hydrolysis on milk agar plates were selected for further studies (Patil and Jadhav, 2017).

2.2.4 Qualitative screening for keratinase activity

Overnight bacterial cultures were spot-plated in duplicate on modified feather agar medium comprising of (g/l): powdered chicken feathers (10), yeast extract (0.1), agar (15), K_2HPO_4 (0.3), KH_2PO_4 (0.4), NaCl (0.5), $MgSO_4.7H_2O$ (0.1) and NH_4Cl (0.5) at pH 7.5. Following incubation at 37ºC for 48-72 h, the plates were examined for growth and one plate was flooded with Gram's iodine (1.0 g iodine dissolved in 100 ml of 2% potassium iodide solution) and observed for hydrolysis zones (Sharma and Devi, 2019). Isolates demonstrating good keratinolytic properties were maintained as 30% glycerol stocks at -20ºC.

2.2.5 Quantitative screening for keratinase activity

2.2.5.1 Inoculum preparation for keratinase production

Nutrient broth was prepared, autoclaved at 121ºC for 20 min and cooled to room temperature. A freshly grown single colony was aseptically inoculated into 50 ml nutrient broth and incubated at 37ºC with gentle agitation (100 rpm) until an OD_{600} of 1 was attained after spectrophotometric analysis (Barman *et al.,* 2017).

2.2.5.2 Determination of keratinase activity

To identify the most potent keratinase producer, a 2% inoculum was added to 50 ml of sterile feather meal medium in 250 ml Erlenmeyer flasks and incubated at 37ºC, 120 rpm, for 5 days. After incubation, the fermented medium was centrifuged at 7000 ×*g* for 10 min at 4ºC and the cell-free supernatant was assayed for keratinase activity. The isolates showing good keratin degradation potential were used for further experiments (Ahmadpour *et al.,* 2016).

2.2.5.3 Keratinase assay

Keratin azure (Sigma-Aldrich) was used as a non-specific chromogenic substrate for the keratinase assay. Upon proteolysis, soluble peptide fragments (blue in colour due to azo dye-impregnation) released were detected spectrophotometrically (Cary 50 UV-Vis spectrophotometer) at 595 nm. The keratin azure substrate was treated prior to the assay to ensure the uniform release of the dye and reliable absorbance measurements. Briefly, keratin azure was incubated with five times the displaceable volume of distilled water for 1 h at 50ºC and 250 rpm in a rotary incubator. The resulting keratin azure was washed three times with sterile distilled water by shaking vigorously for 1 min and allowed to dry overnight at 30ºC in an oven (Okoroma *et al.,* 2012).

Keratinase activity was measured using 5 mg of keratin azure suspended in 1 ml of 50 mM Tris HCl buffer (pH 7.5) and 1 ml of the crude enzyme extract. After 1 h of incubation at 37ºC, the reaction was terminated by placing the tubes on ice for 5 min, filtered using 0.45 µm filters and absorbance read at 595 nm. All assays were performed in triplicate and a control was prepared in the same manner except that the enzyme was added after incubation and filtered immediately. One unit (U) of keratinase activity was defined as the amount of enzyme causing 0.01 absorbance increase between the sample and control at 595 nm under the standard assay conditions (Uttangi and Aruna, 2018).

2.2.6 Identification of a keratin-degrading microorganism

The isolate showing highest keratin-degrading potential was identified by 16S rDNA sequencing. Genomic DNA was extracted using the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research). Subsequently, the 16S rDNA target region was amplified using OneTaq Quick-Load 2X Master Mix (NEB) with 27F (forward primer- 5′ AGAGTTTGATCMTGGCTCAG 3′) and 1492R (reverse primer- 5′ CGGTTACCTTGTTACGACTT 3′) (Alaylar *et al.*, 2019).

The PCR products were purified using Zymoclean Gel DNA Recovery Kit (Zymo Research) and sent to Inqaba Biotec for sequencing. The basic local alignment search tool (BLAST) was used to compare the sequences to the database's reference sequences (Altschul *et al.*, 1990). The nucleotide sequence was deposited at the National Biotechnology Information Centre (NCBI) Genebank. The evolutionary history was inferred using the Neighbor-Joining method (Bryant and Moulton, 2004). The

phylogenetic tree was drawn to scale, with branch lengths in the same units as the evolutionary distances used to infer the tree. The evolutionary distances were calculated using the Maximum Composite Likelihood approach (Tamura *et al.*, 2013), and are in base substitutions per site units. The 1st+2nd+3rd+noncoding codon positions were included, and any positions with gaps or incomplete data were removed. Evolutionary analyses were conducted in MEGA6 (Lee *et al.*, 2015).

2.2.7 Optimization of media and cultural conditions for keratinase production

To achieve maximum keratinase production from the selected isolate, the culture conditions and media components were optimised using the OFAT approach, i.e., in each experiment, the variation of a single factor was carried out while keeping the others constant. The optimized parameter was thereafter incorporated in the subsequent experiments. Fermentation was carried out in 50 ml sterile feather meal medium in 250 ml shaking flasks and inoculated with 2% bacterial culture, unless otherwise stated. All the experiments were done in triplicate and the mean values were calculated.

2.2.7.1 Effect of incubation period on keratinase production

The effect of the incubation period on keratinase production was examined by varying the fermentation periods from 24 to 120 h at 37°C, pH 7.5 and 100 rpm (Barman *et al.,* 2017).

2.2.7.2 Effect of pH of culture medium on keratinase production

The optimal initial pH for keratinase production was determined by adjusting pH of the culture medium from pH 7 to 10 using 0.1 N HCl or 0.1 N NaOH (Barman *et al.,* 2017).

2.2.7.3 Effect of temperature of culture medium on keratinase production

For temperature optimization studies, cultures were grown between 30 and 40°C in a shaking incubator at 100 rpm, using previously optimised conditions (Barman *et al.,* 2017).

2.2.7.4 Effect of substrate (feather) concentration on keratinase production

The effect of substrate concentration for keratinase production was studied at 1–5% (w/v), keeping the other factors at optimum conditions (Barman *et al.,* 2017).

2.2.7.5 Effect of inoculum concentration on keratinase production

Different inoculum concentrations (1–5%, v/v) were also investigated to determine the appropriate inoculum required for maximizing the enzyme production (Barman *et al.,* 2017).

2.2.7.6 Effect of additional carbon sources on keratinase production

The effect of additional carbon sources on keratinase production was assessed by adding different substrates (0.5% w/v) to the feather meal medium. The additional carbon sources used were glucose, maltose, sucrose, yeast, lactose, starch and molasses (Mohamad *et al*., 2017).

2.2.7.7 Effect of additional nitrogen sources on keratinase production

Similarly, the effect of additional nitrogen sources (0.5% w/v) on keratinase production was also assessed. The nitrogen sources used were urea, tryptone, peptone, casein and ammonium chloride. All other parameters were kept at their optimum levels (Mohamad *et al*., 2017).

2.3 Results and discussion

2.3.1 Enrichment technique for the isolation of keratinolytic microorganisms

To induce growth of the most productive strains for keratinase production, samples were enriched in basal minimal media with autoclaved feather as the main carbon source under submerged conditions. By making the growth conditions favourable for the organism of interest, the enrichment culture technique helps to increase the number of desired organisms at a rapid rate than others (Madhuri *et al.*, 2019). The submerged (liquid medium) method was selected because this method was easy to control and optimize the environmental and nutritional parameters. Considering that the culture medium contained feather as the sole carbon source and a low concentration of yeast extract, only those microorganisms capable of using the feather as a source of carbon would grow in such medium conditions. Subsequent dilution of the enrichment media and plating on nutrient agar plates displayed an appreciable growth of microbes (especially at higher dilution), indicating that the enrichment media could be a vital source of potential keratinolytic bacteria.

After 21 days of the enrichment protocol, a total of 43 colonies were isolated from eight different poultry waste samples based on their morphological difference on nutrient agar plates, as outlined in Table 2.1. The bacterial colonies obtained were mostly round and predominantly cream in colour, whilst two of the isolates produced green pigments. Among these 43 isolates, 11 different colonies were isolated from waste feathers and 14 colonies were isolated from sludge samples. Samples from poultry waste yielded eight different isolates and shaving samples yielded 10 different isolates. Based on the number of different colonies obtained from the different sampling sites, the processing plant reflects a slightly higher diversity of keratinase-producing microorganisms than the poultry farm.

Table 2.1 Macroscopic evaluation of bacterial isolates after enrichment

Source	Isolate	Colony shape	Colony characteristics
Waste feathers	1	round, entire, raised	small, clear
Waste feathers	2	round, entire, umbonate	opaque
Waste feathers	3	round, entire, umbonate	matt
Waste feathers	4	round, entire, umbonate	light yellow, shiny
Waste feathers	5	round, entire, umbonate	greenish in colour
Waste feathers	6	round, entire, umbonate	opaque, matt
Waste feathers	7	irregular, entire, flat	opaque
Waste feathers	8	round, entire, umbonate	opaque
Waste feathers	9	round, entire, raised	mucoid
Waste feathers	10	round, entire, umbonate	opaque, matt
Waste feathers	11	round, entire, slightly raised	green, shiny
Sludge	12	round, entire, raised	small
Sludge	13	irregular, rhizoid, flat	opaque
Sludge	14	round, entire, raised	cream
Sludge	15	round, entire, raised	clear
Sludge	16	round, entire, raised	shiny
Sludge	17	round, entire, raised	medium, clear
Sludge	18	round, entire, raised	mucoid, shiny
Sludge	19	round, entire, flat	cream
Sludge	20	round, entire, raised	cream, shiny
Sludge	21	round, entire, flat	dull
Sludge	22	round, irregular, concave	opaque
Sludge	23	round, entire, raised	shiny
Sludge	24	irregular, entire, raised	shiny
Sludge	25	round, entire, raised	cream, small
Poultry waste	26	round, filamentous, umbonate	light yellow
Poultry waste	27	round, entire, umbonate	shiny
Poultry waste	28	round, entire, raised	shiny
Poultry waste	29	round, entire, raised	clear
Poultry waste	30	round, entire, raised	cream
Poultry waste	31	round, entire, umbonate	opaque
Poultry waste	32	round, entire, raised	mucoid
Poultry waste	33	round, entire, raised	matt
Wood shavings	34	round, entire, raised	shiny
Wood shavings	35	round, entire, raised	light green fluorescent
Wood shavings	36	round, entire, raised	shiny
Wood shavings	37	round, entire, raised	medium
Wood shavings	38	round, entire, umbonate	opaque
Wood shavings	39	round, entire, umbonate	dull
Wood shavings	40	round, entire, raised	shiny
Wood shavings	41	round, entire, raised	small
Wood shavings	42	round, entire, raised	clear
Wood shavings	43	round, entire, raised	shiny

A similar enrichment procedure was followed by other researchers to successfully isolate keratinolytic microorganisms using feathers as a sole carbon and nitrogen source (Godbole *et al.,* 2017; Ire and Onyenama 2017; Jadhav *et al.,* 2016; Laba *et al.,* 2018; Uttangi and Aruna, 2018). In addition, there are also reports of isolating keratinase-producing microorganisms from poultry soil (Jadhav *et al.,* 2016), poultry wastes (Murthy *et al.,* 2019) and feather (Dada and Wakil, 2019).

2.3.2 Screening for proteolytic microorganisms

The skim milk agar tests are cost-effective and time-efficient preliminary approaches for the detection of proteolytic microbes (Vijayaraghavan and Vincent, 2012). Proteolytic microbes convert casein, a highly stable, hydrophobic, and exceedingly slow-digesting non-fibrous protein found in milk, into nitrogen compounds. The proteolytic activity of the microorganism is indicated by a clear area (zone of hydrolysis) surrounding the colony (Wibowo *et al.,* 2017). Following enrichment, the bacterial isolates were screened for proteolytic potential and zone of hydrolysis (inclusive of colony diameter) was measured. Protease activity was observed for 21 isolates with hydrolysis zones ranging from 8 to 42 mm, with isolate 35 showing the largest zone of hydrolysis on skim milk agar (Table 2.2). This isolate also had the largest colony size (24 mm) but the reference size of the hydrolysis zone was also the largest among all proteolytic isolates. Large clearance zones (30–35 mm) were also observed for other isolates and are shown in Figure 2.1. The highest relative caseinolytic activities of 4.5 and 4.0 were detected for isolates 1 and 3, respectively. The lowest relative caseinolytic activity of 1.2 was observed for isolate 22.

Table 2.2 Zone diameters of selected strains on skim milk agar plates

Isolate	Clearance in proteolytic zone (mm)	Colony diameter (mm)	Relative Caseinolytic Activity*
1	18	4	4.5
2	32	15	2.1
3	20	5	4.0
4	10	7	1.4
6	30	14	2.1
7	35	20	1.8
8	20	6	3.3
10	31	14	2.2
13	28	12	2.3
14	12	8	1.5
15	13	8	1.6
18	15	12	1.3
21	12	9	1.3
22	16	13	1.2
30	27	15	1.8
31	25	12	2.1
34	10	8	1.3
35	42	24	1.8
38	30	14	2.1
39	28	14	2.0
40	8	4	2.0

*Relative caseinolytic activity = Proteolytic zone (mm)／colony diameter (mm)

Proteolytic screening of keratinolytic microorganisms have been documented in earlier reports. Ahmad *et al*. (2019) reported hydrolysis zones of 6 to 12 mm for 6 bacteria isolated from soil samples and Jagadeesan *et al.* (2020) reported the largest zone of 19 mm for an isolate from slaughterhouse soil. However, Nnolim *et al*. (2020) reported zones of 21.5 to 37 mm for 18 isolates from soil samples. Thus, the hydrolysis zones are highly variable among proteolytic isolates and necessitates the need for individual screening.

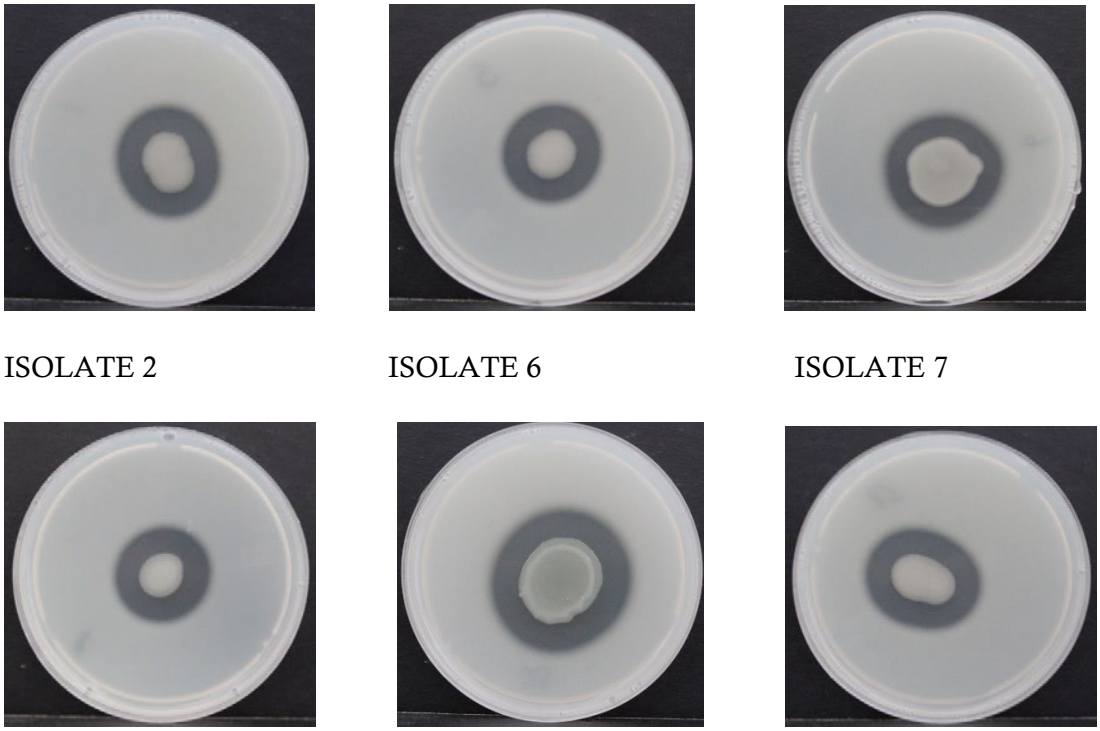


Figure 2.1 Bacterial isolates cultured on milk agar plates at 37°C for 24 h depicting distinct and large clearance zones.

2.3.3 Qualitative assay for keratinolytic isolates

All keratinases are proteases; however, not all proteases are keratinolytic (Vidmar and Vodovnik, 2018). Hence, isolates that tested positive for proteolytic activity using the qualitative assays were explicitly screened for the keratinase activity. Routinely, qualitative screening for keratinolytic activity exhibited by microbes is carried out on feather agar medium. In the present study, the formation of a clear zone was observed after the addition of Gram's iodine on feather agar medium (Figure 2.2). Only 19 of the 21 isolates displayed clear zones on feather agar medium suggesting that these isolates can produce keratinase (Table 2.3). Keratinolytic zones ranged from 2 to 11 mm, with the largest keratinolytic zone observed for isolate 35. A similar protocol for the screening of keratinolytic microorganisms was followed by Barman *et al.* (2017), Siddharthan *et al.* (2019) and Yusuf *et al.* (2019).

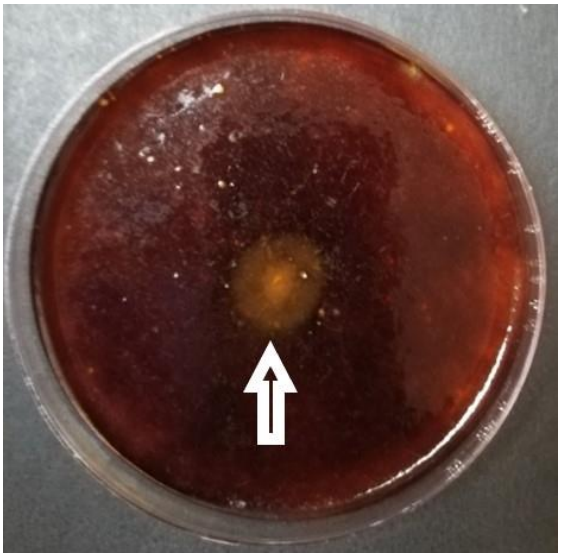


Figure 2.2 Halo around proteolytic-positive colony for isolate 35, confirming keratinolytic activity of the organism on a feather meal agar plate incubated at 37°C for 24 h.

Table 2.3 Zone diameters of selected strains on feather meal agar plates

Isolate	Clearance in keratinolytic zone (mm)	Colony diameter (mm)
1	3	2
4	4	2
6	4	4
7	7	4
8	3	3
10	3	2
13	3	2
14	2	1
15	4	4
18	8	4
21	5	3
22	9	6
30	8	4
31	7	4
34	3	2
35	11	5
38	8	4
39	3	2
40	5	3

2.3.4 Quantitative assay of keratinolytic isolates

All promising feather degrading isolates were selected for the quantitative determination of enzyme activity using raw feather medium. Keratinase activity was observed for all 19 isolates ranging from 0.28 to 6.03 U/ml (Figure 2.3). The highest keratinolytic activity of 6.03 U/ml was demonstrated by isolate 35, followed by isolate 22 which showed 4.03 U/ml. The lowest keratinolytic activity of 0.28 U/ml was produced by isolate 14. The results of this study indicate that keratinolytic activity was not correlated to the source of isolation.

A similar maximum keratinolytic activity of 5 U/ml was reported by Sultana and Saha (2017) for *Stenotrophomonas* sp. strain Norja -1. However, Uttangi and Aruna (2018) reported 63 U/ml for *Bacillus thuringiensis* BT 407. Conversely, Gumilar *et al*. (2015) reported 2.87 U/ml for *Exiguobacterium* sp. DG1. Apart from the different production levels by bacteria, the difference in keratinase activity levels recorded by the different studies could also arise from the different assay conditions used to determine keratinase activity. Sultana and Saha (2017) used a buffer at pH 9 while Uttangi and Aruna (2018) used a temperature of 55ºC and a pH 8.5 buffer to determine keratinolytic activity. Conversely, Gumilar *et al.* (2015) performed the assay at 30ºC for 30 min, followed by 10% TCA to stop reaction and centrifuged at 13 000 g for 5 min. The assay conditions used in this study for screening purposes were a pH of 7.5 at 37ºC for 1 hour as per the method stipulated in the keratinase substrate quality control test procedure (Sigma Aldrich). Thus, there is a need to standardise a keratinase assay procedure to ensure consistency and reliability of reported results.

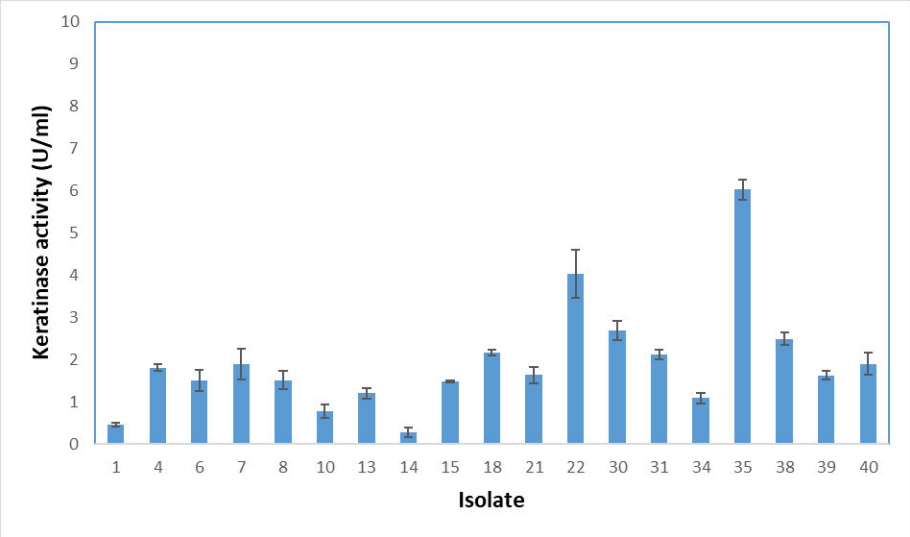


Figure 2.3 Bacterial isolates showing keratinase activity after 5 days of growth on feather meal media at 37°C, pH 7.5 and 100 rpm. Each value represents a mean of three replicate determinations with standard deviation (± SD).

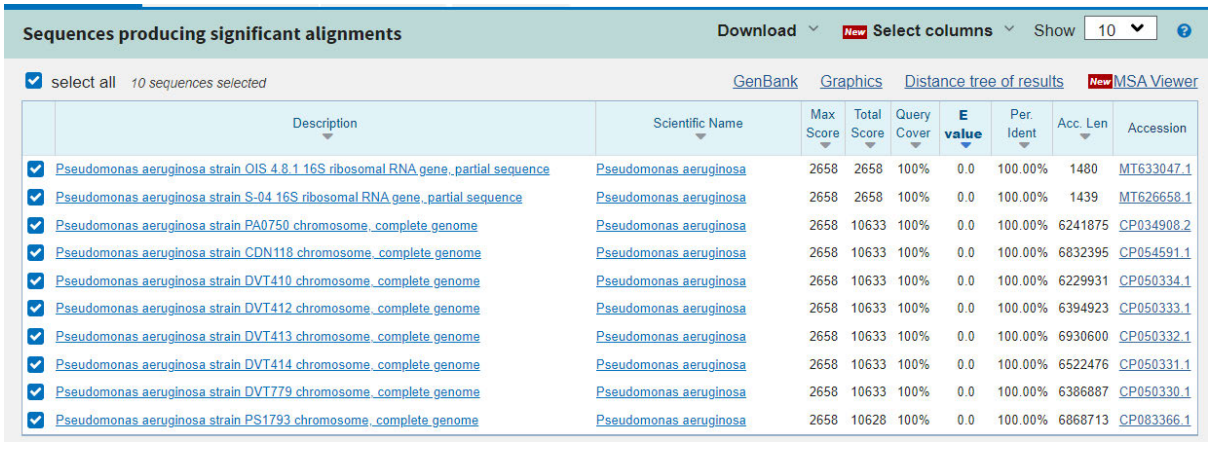
In this study, isolate 35 produced the largest hydrolysis zone on skim milk agar and showed the highest keratinolytic activity during the keratinase assay. This qualitative and quantitative similarity was also observed for *Bacillus* species, demonstrating a large hydrolysis zone on proteolytic medium and exhibiting high keratinolytic activity (Yasmeen *et al.*, 2015). In contrast, a study by Vijayvaraghavan and Vincent (2012) reported smaller zones of hydrolysis on skimmed milk agar plates but high keratinase levels for *B. licheniformis*. Similarly, Wakil *et al.* (2011) reported low proteolytic activity and high keratinolytic activity for *Pseudomonas putida* B2. Thus, proteolytic and keratinolytic activity must be determined for each isolate to correctly determine the feather-degrading potential of the organism. Since bacterial isolate 35 demonstrated highest keratinase activity, it was selected as the feather-degrading microorganism for further investigation.

2.3.5 Identification of keratin-degrading microorganism

The accurate identification of bacterial isolates and the discovery of novel bacteria have both been aided by 16S rDNA sequencing (Woo *et al.*, 2008). Partial 16S rDNA gene of 1439 bp of the approximately 1500 bp gene was obtained after sequencing and shown below in Figure 2.4.

Figure 2.4 Sequence of the 16S rDNA gene from isolate 35. The size of the region amplified by the 27F and 1492R primers was 1439 bp.

The 1439 bp partial 16S rDNA sequence was converted to FASTA format and subjected for BLAST search in GenBank (Figure 2.5). The BLAST results corresponded to the similarity between the sequence queried and the biological sequences within the NCBI database and confirmed the organism as *Pseudomonas aeruginosa* with 100% similarity and E value of 0.0 (Figure 2.5). The sequence was then deposited on NCBI as *Pseudomonas aeruginosa* strain S-04 with the GenBank Accession number MT626658.



Sequences producing significant alignments Download New Select columns Show 10

select all *10 sequences selected* GenBank Graphics Distance tree of results New MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Pseudomonas aeruginosa strain OIS 4.8.1 16S ribosomal RNA gene, partial sequence	Pseudomonas aeruginosa	2658	2658	100%	0.0	100.00%	1480	MT633047.1
Pseudomonas aeruginosa strain S-04 16S ribosomal RNA gene, partial sequence	Pseudomonas aeruginosa	2658	2658	100%	0.0	100.00%	1439	MT626658.1
Pseudomonas aeruginosa strain PA0750 chromosome, complete genome	Pseudomonas aeruginosa	2658	10633	100%	0.0	100.00%	6241875	CP034908.2
Pseudomonas aeruginosa strain CDN118 chromosome, complete genome	Pseudomonas aeruginosa	2658	10633	100%	0.0	100.00%	6832395	CP054591.1
Pseudomonas aeruginosa strain DVT410 chromosome, complete genome	Pseudomonas aeruginosa	2658	10633	100%	0.0	100.00%	6229931	CP050334.1
Pseudomonas aeruginosa strain DVT412 chromosome, complete genome	Pseudomonas aeruginosa	2658	10633	100%	0.0	100.00%	6394923	CP050333.1
Pseudomonas aeruginosa strain DVT413 chromosome, complete genome	Pseudomonas aeruginosa	2658	10633	100%	0.0	100.00%	6930600	CP050332.1
Pseudomonas aeruginosa strain DVT414 chromosome, complete genome	Pseudomonas aeruginosa	2658	10633	100%	0.0	100.00%	6522476	CP050331.1
Pseudomonas aeruginosa strain DVT779 chromosome, complete genome	Pseudomonas aeruginosa	2658	10633	100%	0.0	100.00%	6386887	CP050330.1
Pseudomonas aeruginosa strain PS1793 chromosome, complete genome	Pseudomonas aeruginosa	2658	10628	100%	0.0	100.00%	6868713	CP083366.1

Figure 2.5 BLAST score, query coverage and E-value for the query sequence, showing highest hits to *Pseudomonas aeruginosa*.

The phylogenetic relationship of the test organism was accessed by comparison with other partial 16S rDNA sequences of microorganisms belonging to the same genus. The sequences of closely related *Pseudomonas* sp. were matched under Maximum Composite Likelihood method using MEGA6. Phylogenetic tree generated by using the neighbour-joining method (Figure 2.6). Finally, it was found that the test organism was under the group of *Pseudomonas* sp. and closely related to identified organism *Pseudomonas aeruginosa*.

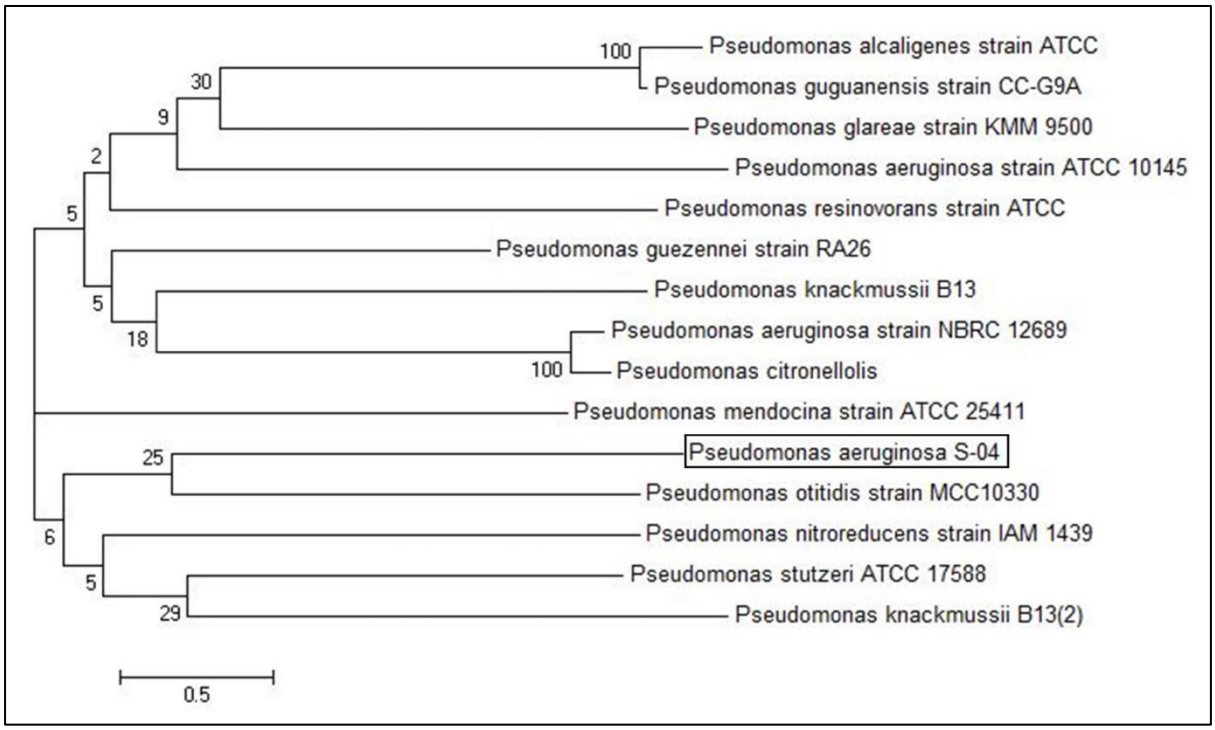


Figure 2.6 Phylogenetic tree of the *Pseudomonas aeruginosa* S-04 and other closely related *Pseudomonas* species based on 16S rRNA sequences. The tree was generated by using the neighbour-joining method. Bar = 0.5 substitutions per site.

2.3.6 Optimization of cultural conditions for keratinase production

The biotechnological application of keratinases necessitates the manufacture of a large volume of these enzymes for commercial use. The optimization of medium composition is used in commercial fermentations to balance various medium components, reducing the amount of unutilized substrates at the end of the fermentation. Multiple factors influence cell growth, survival, and the level of protease synthesis in a microbial culture, including temperature, pH, substrate concentration, nature and composition of carbon and nitrogen

sources, and inoculum condition. As a result, optimizing these variables is critical for optimal keratinase synthesis (Okoroma *et al.,* 2012). However, such parameters are species-specific and thus vary with respect to the organisms used for keratinase production (Jani *et al.,* 2014). Therefore, in this study, a variation of one growth factor at a time approach was used while holding the conditions for other components constant. This was performed as a preliminary screening method to investigate the most suitable nutritional and culture conditions required for the maximum production of keratinase by *P. aeruginosa* S-04.

2.3.6.1 Effect of the incubation period on keratinase production

The maximal enzyme yield is always used in commercial enzyme manufacturing. Keratinase was produced utilizing submerged state cultivation with basal media supplemented with 1% (w/v) chicken feather meal. The presence of a keratinous substrate, such as chicken feather, altered the intensity of keratinase release by the bacteria. From monitoring the keratinase activity of *P. aeruginosa* every 24 h, it was observed that there was a steady increase in keratinase production from 4.3 to 6.1 U/ml over a 5-day period, with maximum keratinase production peaking at 6.8 U/ml after 72 h of incubation (Figure 2.7). A 16% increase in keratinase production was observed for the 24-to-48-h incubation period and a 21% increase in keratinase production for the 48-to-72-h incubation period. This increase in keratinase production suggests that *P. aeruginosa* produces keratinolytic enzyme as a primary response to utilising feather as a source of nutrients in the medium. Beyond 72 h, enzyme activity declined slightly, reaching 6.1 U/ml at 120 h. The observed decline in keratinase production after 72 h could be either due to a decrease in nutrient availability in the medium or catabolite repression of the enzyme.

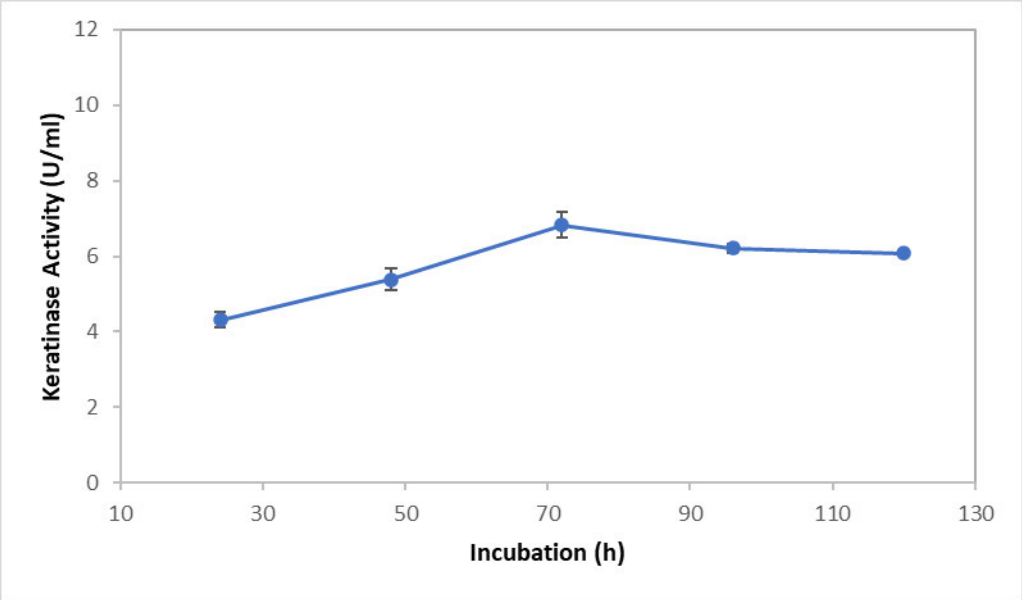


Figure 2.7 Effect of incubation period on keratinase production of *Pseudomonas aeruginosa* S-04 using feather meal media at 37°C, pH 7.5, 100 rpm, 1% feather and 2% inoculum. Each value represents a mean of three replicate determinations with standard deviation (± SD).

The incubation period is quite variable for different organisms. For example, in bacteria, keratinase production can take from hours to several days, whereas in fungi, it can take several weeks. A study by Mohamad *et al*. (2017) reported highest keratinase production of ±73 U/ml by *Pseudomonas* sp. LM 19 after 60 h of incubation. Azokeratin was used as substrate for determination of keratinase activity which could be the reason for the variance in enzyme activity levels between this study and Mohamad *et al.* (2017).Optimum keratinase production after 72 h of incubation was also reported for other bacteria, *viz. Bacillus* sp. P45 (Daroit *et al.,* 2009), *B. megaterium* (Saibabu *et al.,* 2013), *B. pseudofirmus* FA 30-01 (Kojima *et al.,* 2006), *Vibrio* sp. strain kr 2 (Sangali and Brandelli, 2000) and *Alcaligenes* sp. AQ 05-001 (Nam *et al.,* 2002). However, a shorter incubation period of 48 h was recorded by Uttangi and Aruna (2018) for *B. thuringiensis* strain Bt407 whilst Calin *et al.* (2017) recorded an incubation period of 3 weeks for *Fusarium* sp. 1A. Thus, time of incubation depends on various characteristics of the microbial culture, its multiplication rate and enzyme production (Salman *et al*., 2016).

2.3.6.2 Effect of pH of culture medium on keratinase production

The pH of the growth media appears to affect bacterial growth as well as the activity, stability, and generation of their metabolites (Akram *et al.*, 2020). Furthermore, the pH of the media has an impact on the reaction mixture and nutrient transfer across the cell membrane of bacteria (Akhter *et al.*, 2020). Significant activity was exhibited between pH 7.0 and 10.0, showing 5.5 to 7.6 U/ml keratinase activity within that pH range (Figure 2.8) Optimal keratinase production of 7.8 U/ml was observed at pH 9.5, thus classifying *P. aeruginosa* S-04 as a facultative alkaliphile. There was no discernible difference in keratinase production (0.2 U/ml) from pH 9.5 to pH 10.0. It can be deduced that at lower pH ranges (7.0–8.0), bacteria grew slowly due to plasma membrane instability, which resulted in partial suppression of membrane enzymes and transport proteins.

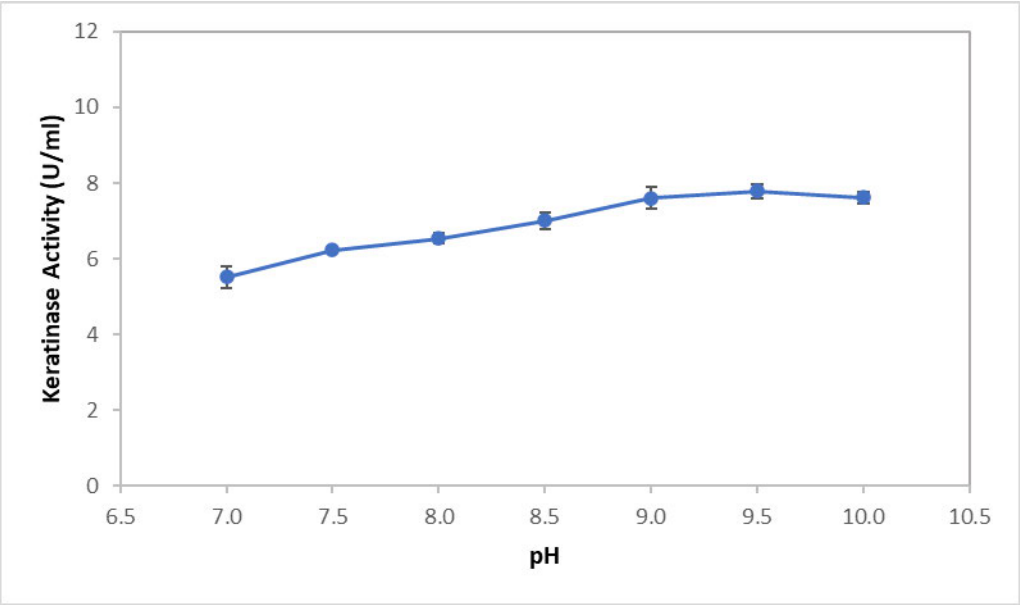


Figure 2.8 Effect of pH on keratinase production of *Pseudomonas aeruginosa* S-04 using feather meal media at 37°C, 72 h, 100 rpm, 1% feather and 2% inoculum. Each value represents a mean of three replicate determinations with standard deviation (± SD).

A similar study by Akhter *et al*. (2020) reported optimal keratinase production for *B. cereus* (56.33 U/ml) and *Pseudomonas* sp. at pH 9.5 (43.33 U/ml). However, previous studies observed optimal keratinase production for *P. aeruginosa* using feather meal media at pH 7 and pH 8 for, respectively (Dhiva *et al.,* 2020; Mohamad *et al.,* 2017). The difference in optimum pH for keratinase production by these *P. aeruginosa* may be attributed to the source of isolation. The different strains of *P. aeruginosa* were isolated from soil and feather dumping site and these sites could have been neutral to slightly alkaline thus organism thrives at that pH range. The isolate in this study, however, was isolated from shavings (material used on floor in chicken house) which is alkaline in nature. Each microorganism has an optimum pH for its growth and activity and thus varies significantly with different organisms and source of isolation (Sanchez-Clemente *et al.,* 2018).

Alkaliphiles are of interest because they produce valuable, stable enzymes, and the cells can be employed for biotechnological and other high-pH applications including leather tanning and wastewater treatment (Preiss *et al.*, 2015). In alkaline circumstances, alkaliphilic bacteria have much better external and internal cytoplasmic buffering capacities than non-alkaliphilic bacteria. High amounts of basic proteins or polyamines in alkaliphilic cells are thought to impart this feature (Borkar *et al.*, 2015). Adaptations are required to allow secreted or partially exposed enzymes or protein complexes to function at the high external pH. To protect cytoplasmic components from the external alkaline pH, alkaliphiles must maintain a cytoplasmic pH that is substantially lower than the pH of the surrounding medium (Preiss *et al.*, 2015).

2.3.6.3 Effect of temperature on keratinase production

The temperature at which submerged fermentation incubation are conducted is a key property that has a significant impact on the yield and duration of the enzyme production phase (Akram *et al.,* 2020). The correlation between keratinase production and temperature is shown in Figure 2.9. The results showed that maximal keratinase production of 9.6 U/ml was attained at incubation temperature of 35ºC and gradually decreased beyond 35ºC. The results revealed that keratinase production increased with increasing temperature until maximum production of 9.6 U/ml was reached at 35°C. However, the enzyme production declined to 7.3 U/ml at 37°C and continued to decline even further to 6.4 U/ml at 40°C. Considering the decline in enzyme activity, higher temperatures were not tested as it was deemed unnecessary.

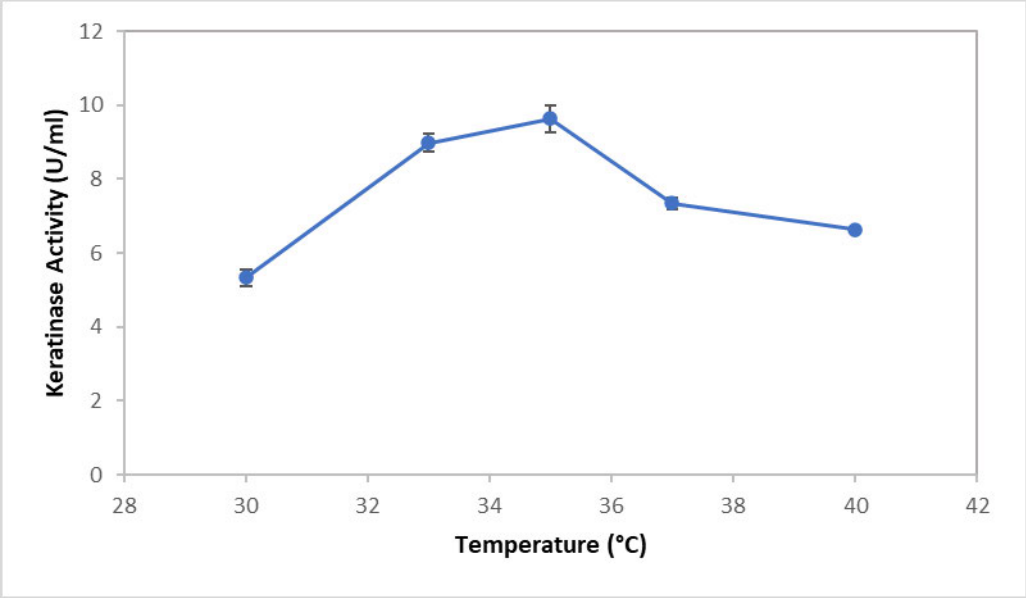


Figure 2.9 Effect of temperature on keratinase production of *Pseudomonas aeruginosa* S-04 using feather meal media at pH 9.5, 100 rpm, 1% feather and 2% inoculum for 72 h. Each value represents a mean of three replicate determinations with standard deviation (± SD).

Temperature strongly affects the synthesis of keratinases by either inducing or repressing their production (Adelere and Lateef, 2016). Temperature has been observed to impact the release of microbial extracellular enzymes, which could be linked to changes in the physical properties of the cell membrane (Verma *et al.,* 2016b). Considering the optimum temperature of 35°C for *Pseudomonas aeruginosa* S-04 in this study, a relatively low temperature is required for inducing the production and secretion of keratinases. However, low keratinase production was recorded at 30°C (below the temperature optima) possibly due to stiffening of the lipids in the membrane leading to decreased efficiency of transport proteins embedded in the membrane. On the other hand, an increase in temperature above 35°C shows a decline in keratinase production due to denaturation of proteins.

A similar temperature optimum of 35°C for maximum keratinolytic enzyme production was reported by Bhuyar *et al*. (2018) for *Pseudomonas* species. Other microorganisms that exhibited optimal keratinase production at 35°C include *Arthrobacter* sp. A08 (Pereira *et al.,* 2014), *B. megaterium* (Saibabu *et al.,* 2013), *B. aerius* (Calin *et al.,* 2017), *B. licheniformis* (Peng *et al.,* 2019b) and *Trichophyton* (Anbu *et al.,* 2008). In general, these isolates tended to grow better at 30 - 37°C, reflecting the environmental conditions from where they were isolated. In the current study, the optimum temperature of 35°C for *P. aeruginosa* S-04 indicates the

mesophilic nature of the microorganism. Mesophilic bacteria are more resilient and adaptable to changing environmental conditions than other bacteria. Mesophilic temperatures for enzyme production would be desirable as this would require a lower production temperature resulting in less process energy, thereby, reducing production costs (Darwesh and Ghoname, 2021).

2.3.6.4 Effect of substrate concentration on keratinase production

Keratinase is an inducible enzyme that is best produced when a sufficient amount of keratin substrate is present (Akram *et al.*, 2020). In this study, the results demonstrated that as feather concentration increased, keratinase production increased and reached maximum production of 9.8 U/ml at 1% (w/v) feather (Figure 2.10). However, further increments in substrate concentration to 2% (w/v) were observed to substantially reduce keratinase production to 8.6 U/ml. At higher feather concentrations of 3 to 5%, keratinase production decreased to 7.4 U/ml.

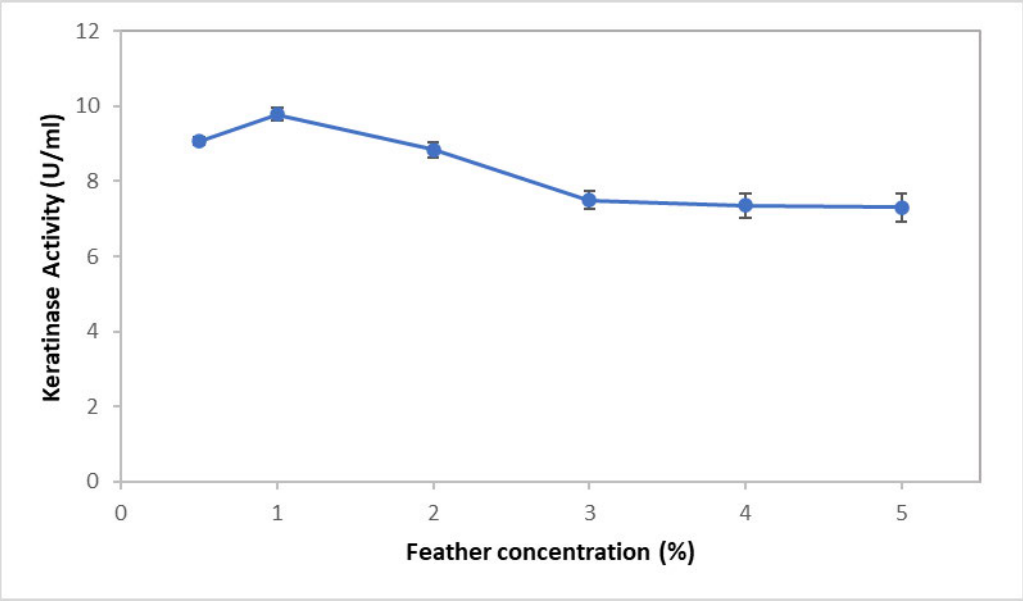


Figure 2.10 Effect of substrate (feather) concentration on enzyme production of *Pseudomonas aeruginosa* S-04 using feather meal media at 35°C, pH 9.5, 100 rpm, 2% inoculum for 72 h. Each value represents a mean of three replicate determinations with standard deviation (± SD).

Feather concentration is an essential factor for the production of keratinases. The medium viscosity increased at high feather concentrations, potentially limiting bacterial growth by limiting oxygen availability. Higher feather concentrations also hinder the homogenization of the media, resulting in substrate inhibition which adversely affects bacterial growth and keratinase production (Mohamad *et al.*, 2017). Similar studies by Mohamad *et al.* (2017) and Abdel-Fattah *et al.* (2018) also observed optimum keratinase production at 1% (w/v) feather concentration for *Pseudomonas* sp. LM19 and *B. licheniformis* ALW1, respectively.

2.3.6.5 Effect of inoculum size on keratinase production

The inoculum volume (size) causes a distinct effect on the production of the enzyme. In this investigation, keratinase production of 8.5 to 7.1 U/ml was observed for inoculum concentrations of 1 to 5% (v/v), optimal keratinase production of 9.5 U/ml was observed for inoculum concentrations of 2 and 3% (v/v) (Figure 2.11). At higher inoculum concentration of 4 to 5% (v/v), keratinase production decreased from 8.8 to 7.1 U/ml, respectively.

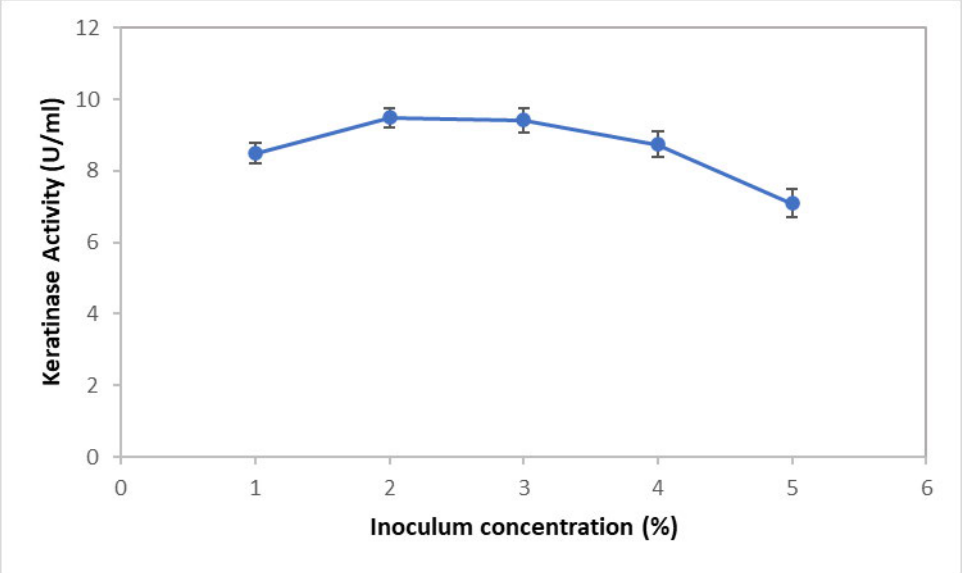


Figure 2.11 Effect of inoculum concentration on keratinase production of *Pseudomonas aeruginosa* S-04 using feather meal media at 35°C, pH 9.5, 100 rpm, 1% feather for 72 h. Each value represents a mean of three replicate determinations with standard deviation (± SD).

In this study, the 1% inoculum is less dense compared to the optimum inoculum (2%), thus decreases the number of bacterial cells in the culture during the production phase and results in lower keratinase production. In contrast, a higher inoculum (4 to 5% inoculum) has a higher density of cells compared to optimum inoculum. This increases the medium's viscosity and competition for nutrients, reducing bacteria's ability to reach the production phase and, as a result, lowering enzyme output (Akram *et al.,* 2020). Other studies reported optimum keratinase production using 2% and 3% (v/v) inoculum of *Thermoactinomycetes* sp. and *Pseudomonas* sp., respectively (Verma *et al.,* 2016b and Akhter *et al*., 2020).

2.3.6.6 Effect of additional carbon sources on keratinase production

The effects of different monosaccharides, disaccharides or polysaccharides as additional carbon sources on keratinase production from *P. aeruginosa* S-04 were examined (Figure 2.12). To maintain feathers as the primary source of carbon (which was included at 1%, w/v), additional carbon sources were included at a lower concentration (0.5%, w/v), individually. The fermentation medium without additional carbon sources was used as the control. The addition of molasses to the growth medium favoured keratinase production while the other carbon sources, *viz*. starch, maltose, glucose, sucrose and lactose reduced keratinase production. Keratinase production was not affected by the addition of yeast extract to fermentation medium. Keratinase production increased 1.3-fold upon the addition of molasses with an activity of 12 U/ml recorded. However, the addition of sucrose and glucose decreased keratinase production by 0.9-fold to 8.5 U/ml and 8.0 U/ml, respectively. A further decrease in keratinase production by 0.8-fold was noted after the addition of lactose (7.8 U/ml) and maltose (7.0 U/ml). The addition of starch strongly repressed keratinase production by 0.2-fold and recorded only 2.2 U/ml activity. Gurav and Jadhav (2013) reported a 2-fold increase in keratinase production for *Chryseobacterium* sp. RBT upon addition of molasses (1%, w/v) to the culture medium. A study by Mohamad *et al.* (2017) demonstrated that the keratinase production by *Pseudomonas* sp. LM 19 was also repressed by approximately 2-fold by the addition of starch to the culture medium. The decrease in keratinase activity could be attributed to catabolite repression.

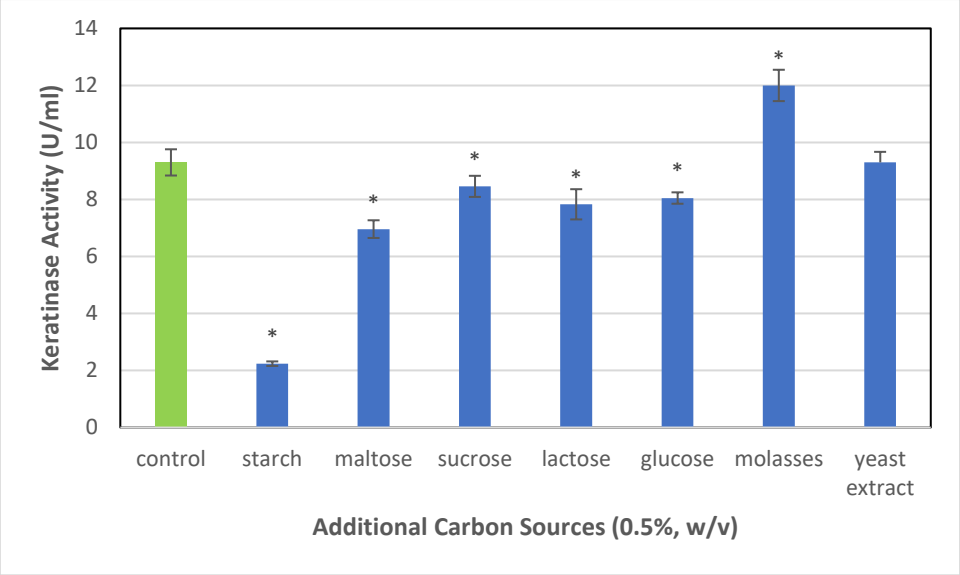


Figure 2.12 Effect of additional carbon sources on keratinase production of *Pseudomonas aeruginosa* S-04 using feather meal media at 35°C, pH 9.5 for 72 h. Each value represents a mean of three replicate determinations with standard deviation (± SD). * Significant p-values at $P < 0.05$ when compared to the control.

The carbon source in the culture medium plays a vital role in the growth of cells, the generation of metabolites, and the availability of energy to drive endergonic reactions during microorganism growth (Kazzaz *et al.,* 2015). There is much variation in the degree of utilization of carbon sources in bacteria; hence investigating different carbon sources is essential and the medium composition should be determined on a case-by-case basis (Brandelli *et al.*, 2010). Evaluation of the effect of external carbon sources in this study showed that molasses acted as the most efficient keratinase activator, which could be due to the presence of certain vitamins and minerals (Gurav and Jadhav, 2013). However, further investigation is required to define the exact nature of molasses to cause an increase in keratinase production.

The cultivation profile of a microorganism can be influenced by the forms of the carbon sources (e.g. simple versus complex carbon sources), which can have different effects on the production of the end product (e.g. keratinase production) because of their variable composition. The addition of glucose, sucrose, lactose, maltose and starch had a catabolic repression effect on *P. aeruginosa* S-04. This is most common when microorganisms are cultivated on a medium with many utilizable growth substrates (Akram *et al.,* 2020). Considering that carbon sources which caused a reduction of keratinase production in this

experiment were much simpler compared to feather, it can be deduced that *P. aeruginosa* S-04 utilized the alternate carbon source as a source of energy instead of feather. This resulted in a decrease in the keratinase production of the organism.

2.3.6.7 Effect of additional nitrogen sources on keratinase production

In addition to carbon, the nitrogen source is the medium constituent that has the greatest impact on enzyme production by bacteria. The amount of nitrogen supplemented for enzyme production varies depending on the concentration, medium composition, and microorganism type. Nitrogen is one of the non-metallic elements that bacteria require for their structural and functional purposes (Sahoo *et al.*, 2015).

To maintain feather as the main source of nitrogen (which was included at 1%, w/v), additional nitrogen sources were included individually at a concentration of 0.5% (w/v). The fermentation medium without an additional nitrogen source was used as the control. There was a 1.1-fold increase in keratinase production when the media was supplemented with casein (10.8 U/ml) when compared to control (9.7 U/mL) (Figure 2.13). Supplementation of tryptone to the culture medium resulted in 9.8 U/ml, which was quite similar to the control result. Keratinase production decreased to 0.9-fold upon the addition of peptone (8.3 U/ml) and urea (8.2 U/ml). The addition of ammonium chloride resulted in a 0.4-fold decrease in keratinase production to 4 U/ml. Barman *et al.* (2017) reported a 58% decrease in keratinase production for *Arthrobacter* sp. NFH5 upon the addition of ammonium chloride in the fermentation medium. However, the addition of peptone to the culture medium increased keratinase production by 1.6-fold for *Pseudomonas* sp. LM19 (Mohamad *et al.,* 2017).

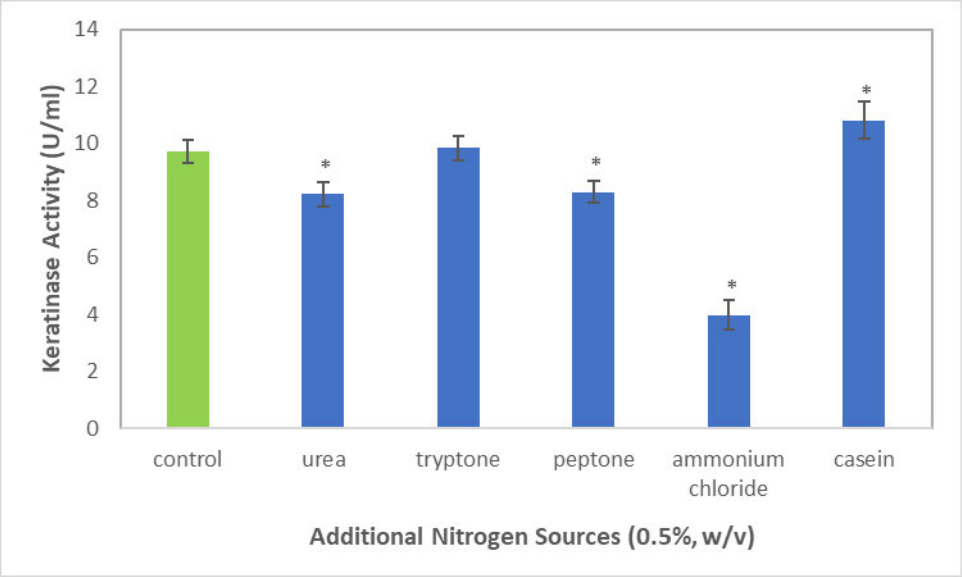


Figure 2.13 Effect of additional nitrogen sources on keratinase production of *Pseudomonas aeruginosa* S-04 using feather meal media at 35°C, pH 9.5 for 72 h. Each value represents a mean of three replicate determinations with standard deviation (± SD). * Significant p-values at $P < 0.05$ when compared to the control.

There are conflicting reports on the requirement of nitrogen sources for enzyme production which were found to differ from organism to organism with both organic nitrogen sources (casein and urea) as well as inorganic nitrogen sources (ammonium chloride) being supplemented into fermentation medium for enhanced enzyme production (Puri *et al.,* 2002). Supplementing the culture media with nitrogen sources can suppress keratinase activity and lead to catabolite repression, a mechanism in which easily digestible nitrogen sources are preferred for microbial development over complicated substrates like feathers (Sobucki *et al.,* 2017). Even though Finlay *et al.* (1992) claimed that organic forms of nitrogen are more readily utilized by microorganisms than inorganic forms of nitrogen, in this study, the inorganic form of nitrogen (ammonium chloride) had a catabolic repression effect on *P. aeruginosa* S-04. It is assumed that amino acids and short peptides present in the nitrogen source exert a stimulatory effect on keratinase production (Chaturvedi and Verma, 2014) and this can be inferred from the addition of casein to the culture medium in this study. Therefore, the supplementation of nitrogen for enzyme production is variable and it depends on the concentration, medium composition and type of microorganisms.

2.4 Conclusion

The use of keratinolytic bacteria to decompose poultry feathers has emerged as a viable and environmentally friendly solution to this problem. Microorganisms are the most important and practical sources of keratinases because they can create large amounts of them under the right conditions. The chosen keratin-degrading microbe should produce the most keratinase in the shortest period of time while producing the least amount of other metabolites. The culture medium chosen should have all of the nutrients necessary to sustain appropriate microbe growth, which will result in adequate keratinase synthesis. The medium's elements should be easily accessible, low-cost, and nutritionally sound. Because the yields are better and the chances of contamination are lower, most enzymes are produced under immersed liquid conditions. By optimizing the fermentation conditions, maximum keratinase production can be attained (pH, temperature, etc.). A thorough understanding of the genetic regulation of keratinase synthesis is essential for this aim. It is also feasible that statistical keratinase production optimization could boost yield even further.

As a result, *P. aeruginosa* S-04's potentials are indicative of comparable activity to other keratinase producers. *P. aeruginosa* S-04 produces keratinase in a simple and scalable manner; the bacterium develops on basic media with feather-meal as its only carbon, nitrogen, and energy source. As a result, an organism with high economic potential can be cultured on a low-cost substrate, resulting in reduced production costs. An alkaline starting fermentation medium, a mesophilic temperature condition, and moderate agitation speed were shown to provide the best keratinase production. The synthesis of the enzyme of interest was increased by spiking the fermentation medium with molasses. Keratinase production increased by twofold as compared to initial production when the physicochemical parameters of fermentation conditions and medium composition were optimised. *P. aeruginosa* S-04's ability to effectively use chicken feathers as a sole source of carbon and nitrogen indicates that keratinase is created to effectively deconstruct the complex pertinacious polymer. In the following chapter, more work on purification, enzyme characterization, and a study on field application logistics is discussed.

CHAPTER 3

Purification and characterization of keratinase from *Pseudomonas aeruginosa* S-04

3.1 Introduction

Microbial enzymes account for a major share of industrial catalysts, with hydrolases accounting for over 75% of the industry. Among the hydrolases, there is a particularly important group of proteases *viz*., keratinases with a wide range of applications in various industries such as animal feed and leather industry (Vidmar and Vodovnik, 2018). Microorganisms are an infinite source due to their natural diversity, simplicity of cultivation, safety at work, and amenability to genetic manipulation, which has sparked an increase in commercial interest in microbial enzymes. Microbial enzymes also have a number of advantages over chemical molecules and animal enzymes, including high activity, broad substrate specificity, and the ability to digest waste products (Thapa *et al.,* 2019). For industrial applications, purification of enzymes is necessary to hasten the efficiency of enzymatic action. Purification not only isolates the enzyme from contaminants but also improves their activity, stability and shelf life (Javed *et al*., 2017). However, the procedures used to purify enzymes should be modest, and the protein's original structure should not alter after purification (Allure *et al*., 2015).

Purification of an enzyme is generally a multi-step process. Commonly, the first step in enzyme purification is ammonium sulphate precipitation (fractionation of proteins based on solubility) followed by ion exchange and gel-filtration chromatography (Gopinath *et al.*, 2015). In addition, a well-known electrophoretic technique, polyacrylamide gel electrophoresis (PAGE) is also employed to determine the purity and molecular weight of the enzyme (Allure *et al*., 2015). In addition to purification, characterization of the enzyme is crucial for its application by providing a more detailed analysis of their mode of action and also to develop a satisfactory assay method. Characterization of an enzyme or protein involves determining its physical properties like native molecular weight and the study of chemical properties like optimum temperature and pH, temperature and pH stability, substrate specificity, etc. However, the optimal properties of various enzymes vary and should be studied independently (Gupta *et al*., 2013). Furthermore, the characteristics of enzymes determine the possibility of their further application.

Keratinases have prospective applications in a variety of industries, including chicken feather treatment, leather depilation, and laundry detergents, due to their distinct properties (Ire and Onyenama, 2017). Furthermore, keratinolytic organisms and their keratinases provide a more visible, environmentally friendly, and cost-effective method for the correct decomposition and recycling of keratinous waste materials into valuable by-products (Sharma and Devi, 2017). For example, upon feather degradation, the keratin hydrolysates can be readily used as biofertilizers and animal feed supplements which possess biological properties such as antioxidant and antimicrobial activity. Additionally, keratinases are used as additives in detergent formulations. The biochemical action of these enzymes on various proteins and their stability at alkaline pH offers an important component of formulations that can be readily used for removing protein stains from clothes (Duffeck *et al.,* 2020). Besides these applications, keratinase research has gained momentum for its versatile industrial and biotechnological applications in leather and fertilizer industries, in the production of biohydrogen, in the hydrolysis of prion proteins, in medicine and cosmetics for drug delivery, as well as for silver recovery from X-ray film (Jagadeesan *et al*., 2020)

This chapter describes the purification of keratinase from the crude cell-free supernatant of *Pseudomonas aeruginosa* S-04 using ammonium sulphate precipitation and dialysis, followed by ion-exchange chromatography. This was followed by characterization of the purified enzyme and finally evaluation of its potential to degrade chicken feathers.

3.2 Materials and methods

3.2.1 Keratinase production medium and growth conditions

Pseudomonas aeruginosa S-04 was grown in an optimized feather medium containing (g/l): chicken feathers (10); NaCl (0.5), K_2HPO_4 (0.3), KH_2PO_4 (0.4), $MgSO_4.7H_2O$ (0.1), and molasses (5) at pH 9.5. An 8-h old, 2% inoculum (prepared in nutrient broth, OD~1.0 at absorbance 600 nm) was added to the feather medium and incubated at optimum conditions as described in Chapter 2.

3.2.2. Keratinase recovery

At the end of the fermentation period, the feather residues and cells were removed by filtration using Whatman No. 1 filter paper followed by centrifugation at 7000 ×*g* for 15 min at 4ºC. The cell-free supernatant was used for further purification steps (Ahmadpour *et al.,* 2016).

3.2.2.1 Assessing enzyme activity and the protein content

The keratinase activity of the supernatant was assessed by performing keratinase assays as described in Chapter 2. The protein content was determined by the Lowry *et al*. (1951) method, using bovine serum albumin (BSA) as the protein standard.

3.2.3. Purification of keratinase

3.2.3.1 Partial purification of keratinase using ammonium sulphate precipitation and dialysis

All enzyme purification steps were performed at 4ºC unless otherwise stated. Approximately 1000 ml of the crude enzyme was subjected to ammonium sulphate precipitation at different saturations levels (30 – 90%) to determine the optimum level of saturation. An online calculator (www. encorbio.com) was used to determine the amount of ammonium sulphate required to achieve the desired saturation level. The ammonium sulphate was added slowly with constant stirring to avoid frothing and the crude enzyme was allowed to precipitate overnight. The precipitated proteins were recovered by centrifugation at 7000 ×*g* for 20 min at 4ºC and dissolved in 0.05 M Tris buffer, pH 7.5.

The dialysis membrane (12 kDa cut off, Sigma Aldrich) was immersed in warm deionized water for 20 min to remove the coating followed by rinsing with warm deionized water, and finally with cold deionized water. The dialysis bags were sealed at one end and filled with the reconstituted fractions without trapping any air bubbles. The membranes were dialyzed against 1000 ml of 0.05 M Tris buffer (pH 7.5) overnight with gentle stirring. The dialysates were collected, concentrated using a vacuum concentrator (Eppendorf Concentrator Plus) and subjected to ion-exchange chromatography (Ire and Onyenama, 2017).

3.2.3.2 Purification of keratinase by ion-exchange chromatography

Purification of keratinase was carried out by ion-exchange chromatography using DEAE-cellulose, a weak anion exchanger. The concentrated enzyme was filtered using a 0.2 µm filter and run through a fast protein liquid chromatography (FPLC) system (AKTA purifier 100, GE Healthcare Bio-Sciences) using a HiTrap DEAE FF column (Hiprep DEAE FF 16/10, GE Healthcare Bio-Sciences). The column was washed with 0.05 M Tris-HCl buffer (pH 8), prior to elution of the bound protein with a linear salt gradient of 0.1 – 0.5 M NaCl in Tris-HCl buffer (0.05 M, pH 8) at 1.0 ml/min. Fractions of 2 ml each were collected for each salt concentration and the absorbance was measured online using the AKTA FPLC UV monitor. The peak fractions were analyzed for keratinolytic activity and protein content, as described earlier (Zhang *et al.,* 2016).

3.2.4 Electrophoretic methods

3.2.4.1 Sodium dodecyl sulphate – polyacrylamide gel electrophoresis

The protein purity and molecular mass of the enzyme were estimated by sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS – PAGE) using 0.75 mm thick slab gels comprising a 12% (w/v) polyacrylamide resolving gel and a 5% stacking gel (Laemmli, 1970). Fifteen microlitres of purified protein sample was mixed with 5 µl Laemmli buffer and boiled for 10 min (to denature proteins) prior to loading into the polymerized gel. The electrophoresis unit was operated at 30 mA for 60 min in Tris-glycine buffer, pH 8.8 to separate the protein sample. After electrophoresis, the gel was stained for 2 h with Coomassie Brilliant Blue R-250 and decolourized with acetic acid:ethanol:water (10:30:60 v/v) until the bands were visible. The molecular mass standards containing porcine heart mycosin, *E. coli* β-galactosidase, rabbit muscle phosphorylase B, bovine

serum albumin, bovine liver glutamic dehydrogenase, chicken egg ovalbumin, rabbit muscle glyceraldehyde 3-phosphate dehydrogenase, bovine erythrocytes carbonic anhydrase, bovine pancreas trypsinogen, soybean trypsin inhibitor, bovine milk α-lactoalbumin and bovine lung aprotinin (M3913, Sigma Aldrich) were used to plot a standard curve of log molecular weight against relative mobility in the gel.

3.2.4.2 Zymography

Zymography is an SDS-PAGE-based electrophoretic technique that uses a substrate copolymerized with a polyacrylamide gel to measure enzyme activity. Gelatin SDS-PAGE was carried out by adding 0.5% (w/v) gelatin to regular ingredients of separating gel (10%) before polymerization and running the samples under non-reducing conditions (i.e. no heating and reducing agents applied to samples). The electrophoresis unit was operated at 30 mA for 60 min. Following electrophoresis, the gel was washed twice with 2.5% (v/v) Triton X-100 for 30 min at room temperature to remove SDS from the gel. The gel was subsequently incubated in activation buffer (0.05 M Tris-HCl buffer, pH 7.5 containing 5 mM $CaCl_2$ and 1 µM $ZnCl_2$) overnight at 37ºC. The gel was stained with Coomassie Brilliant Blue R-250 and the areas of digestion appeared as clear bands/zones against a darkly stained background, indicating where the substrate (gelatin) was degraded by the enzyme (Jagadeesan *et al.*, 2020).

3.2.5. Biochemical characterization of keratinase

3.2.5.1 Effect of pH and temperature on the activity and stability of keratinase

The optimum pH of the purified enzyme was studied by carrying out the keratinase assay in the pH range of 4 – 11 using 0.05 M buffers: pH 4 – 6 (sodium acetate buffer), pH 6 – 7 (potassium phosphate buffer), pH 7 – 9 (Tris – HCl buffer) and pH 9 – 10.5 (glycine NaOH buffer). Similarly, the optimum temperature for keratinase activity was determined by incubating the enzyme at temperatures ranging from 30 – 80ºC at optimum pH as determined above (Gupta *et al.*, 2015).

The pH stability was determined by pre-incubating the purified keratinase in buffers of varying pH (4 – 11) without the substrate for 2 h at room temperature. Samples were taken every 30 min and assayed for keratinase activity using standard assay conditions. Similarly, the thermostability of the enzyme was determined by pre-incubating the enzyme

sample lacking substrate at various temperatures ranging from 4 – 80ºC for 2 h. Samples were taken every 30 min and assayed for keratinase activity using standard assay conditions (Gupta *et al.*, 2015).

3.2.5.2 Effect of inhibitors and metal ions on the activity of keratinase

The effect of various inhibitors *viz.,* phenylmethylsulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), iodoacetamide, β-mercaptoethanol and dithiothreitol (Sigma-Aldrich) on keratinase activity was carried out by incubating the enzyme with the inhibitors at concentrations of 1 – 10 mM at room temperature for 15 min. Similarly, the effect of metal ions *viz.*, $CaCl_2$, NaCl, KCl, $MgCl_2$, $CuCl_2$, $ZnCl_2$ and $FeCl_3$ on keratinase activity was carried out by incubating the enzyme with metal ions at concentrations of 1 – 10 mM at room temperature for 15 min and then determining the residual activity at standard assay conditions. The enzyme activity was expressed as percent residual activity against the control without inhibitors which were taken as 100% activity (Verma *et al.,* 2016b).

3.2.5.3 Effect of various chemical reagents on the activity of keratinase

The effect of various chemical reagents *viz.,* Triton X-100, Tween 20, DMSO, isopropyl alcohol, methanol, ethanol and acetone (Sigma-Aldrich) on keratinase activity was carried out by incubating the enzyme with each chemical reagent at concentrations of 0.1 – 1.0% at room temperature for 15 min (Verma *et al.,* 2016b), followed by enzyme activity assays.

3.2.5.4 Determination of V_{max} and K_m

Kinetic parameters of the enzyme were determined by measuring the enzyme activity using different substrate (keratin) concentrations (5 – 20 mg) at optimum assay conditions. The K_m (Michaelis-Menten constant) and V_{max} (maximum velocity) values were determined by the Lineweaver-Burk plot, which involved plotting the values of 1/V as a function of 1/[S] (Embaby *et al.,* 2015).

3.2.6 Enzymatic degradation of chicken feather

3.2.6.1 Optimisation of enzyme dosage for feather degradation

The enzyme concentration was standardized for optimum degradation of the chicken feather. Prior to feather degradation, 100 mg of the feather was added to 50 ml glycine-

NaOH buffer (0.05 M, pH 9.5) and autoclaved at 121ºC and 105 kPa for 15 min. Estimation of feather degradation by weight loss was conducted with different purified keratinase doses (250 – 3000 U) at 40°C for 24 h at 100 rpm (selection based on previous literature). The reaction mixture was filtered through pre-weighed Whatman No. 1 filter paper and dried at 80ºC for 24 h (or until a constant weight was achieved) to determine the residual feather weight. A control experiment was conducted in the same manner except that the enzyme was inactivated by autoclaving (Tiwary and Gupta, 2010).

3.2.6.2 Amino acid analyses

A Waters Acquity Ultra Performance Liquid Chromatograph with a photodiode array detector was used to separate and detect amino acids (UPLC-PDA). Using acid hydrolysis extraction, 100 mg of freeze-dried feather hydrolysate was utilized to extract amino acids (0.5 ml of 6 M HCl). L-Norvaline was employed as the standard amino acid. The amino acids were derivatized with the AccQ Fluor reagent Kit from Waters (En Yvelines Cedex). For derivatization, 10 µl of standard/sample was mixed in vials with 70 µl buffer solution (0.2 M borate buffer) and 20 µl of derivatization reagent (2 mg/ml AQC). To obtain stable derivatives, the closed vials were placed in a 55°C oven for 10 minutes. Next, a volume of 1 µl of standard/ sample solution was injected into the mobile phase of the Waters UltraTag C18 column (2.1 × 50 mm × 1.7 µm) at 60°C. Analytes eluting off the column were detected by the PDA detector, with each amino acid coming off the column at a unique retention time. Data acquisition was performed by MassLynx V4.1 2011 software (Waters). The peak areas and retention times were used to plot calibration curves and subsequent quantification of amino acid concentration (Chikwanha *et al.*, 2018). All analyses were performed in triplicate.

3.2.6.3 Proximate analysis comparison of biodegraded feather meal and industrially produced feather meal

The efficacy of enzymatically treated feather meal was assayed by comparing its proximate composition to that of industrially produced feather meal. For enzymatic treatment, 50 000 U of enzyme was added to 10 g of feather dissolved in 1000 ml of sodium glycine buffer (pH 9.5) and incubated at 40°C for 24 h. Nitrogen content of the treated feather was analyzed by the AOAC Dumas 990.03 method using a LECO NS928 protein analyzer. A precise feather sample (0.2 g) was combusted in a combustion tube at high temperatures (700-1000°C) with a flow of pure oxygen. This led to the release of CO_2, H_2O, N_2 and

nitrogen oxides. The nitrogen oxides were reduced to N_2 in a copper reduction column at a high temperature (600°C). The total nitrogen emitted was carried by pure helium gas and measured using a thermal conductivity detector (Leco Truspec N). The total nitrogen value was multiplied by the Total Kjeldahl Nitrogen value (6.25) to evaluate protein content in the sample.

Ash was determined by the AOAC method 942.05. One gram of each sample was ignited in a muffle furnace at a temperature of 550°C until it was carbonized. Percentage ash content was calculated from difference in weight of the samples before and after ignition.

Fibre was determined by the AOAC method 978.10 using a Gerhardt Fibretherm. Samples was treated initially with 100 ml of 1.25% sulphuric acid followed by 100 ml of 1.25% sodium hydroxide for 20 min. The undissolved residue was dried, weighed and then incinerated. Percentage ash content was calculated from the difference in weight of samples before and after ignition.

Fat was determined by extracting the residue from 1 g of sample with petroleum ether for 90 min using a Soxtec 8000 apparatus according to the AOAC method 2003.05. The percentage fat content was calculated by recording weights of the flask after solvent evaporation (Oranusi *et al.,* 2014).

Mineral content was determined by the AOAC method 985.01. Samples were ashed for 6 h at 550°C in a muffle furnace. Aqua regia (1:3 HNO_3 to HCl) was added to samples prior to digestion on a heating block at 100°C to dryness. Dilute nitric acid (0.1 N) was added to samples and mineral content was assayed using inductively coupled plasma optical emission spectroscopy technique (ICP-OES).

The bioavailability of the amino acids was determined using Poultry Complete IDEA kits (Novus International). This *in vitro* assay quantifies protein and amino acid digestibility of feather meal. Samples (weight was determined based on quantity of protein present) were solubilized using 40 ml solubilisation solution for 30 min prior to incubation with 11 µl of enzyme (as specified by the manufacturer) for 2 h at 37°C. The reaction was stopped with TCA and centrifuged at 7000 ×*g* for 10 min. One ml of o-phthaldialdehyde was added to 10 µl of sample and absorbance was measured at 340 nm.

3.3 Results and discussion

3.3.1 Purification of keratinase

3.3.1.1 Partial purification of keratinase using ammonium sulphate precipitation and dialysis

When compared to other methods of protein concentration, such as ultrafiltration, protein precipitation by ammonium sulphate has a high throughput. By progressively raising the quantity of ammonium sulphate, proteins in a mixture can be sorted efficiently depending on their relative hydrophilicity. Because some proteins with few hydrophilic regions precipitate at lower ammonium sulphate saturation levels, while others with more hydrophilic regions precipitate at higher ammonium sulphate saturation levels, the amount of protein precipitated from the clarified homogenate increases as the saturation level of ammonium sulphate increases (Nooralabettu, 2014).

In this study, fractional precipitation with ammonium sulphate allowed a high degree of purification of the keratinolytic enzyme of *P. aeruginosa* S-04. Most of the enzyme activity was found in the 80-90% fraction, which retained 39% of the original activity and 17% of the starting protein, representing an overall purification of 2.2-fold. However, the enzyme activity was not restricted to this fraction. Fractions containing low yield (30-80%) were of sufficient specific activity to suggest that the enzyme was soluble over a wide range of ammonium sulphate concentrations. Because ammonium sulphate is inexpensive, readily accessible, soluble in water, and can become more hydrated (interact with more water molecules) than practically any other ionic solvent, it was chosen to precipitate the enzyme. Furthermore, because the high salt content limits microbial growth and protease activity, the recovered fraction could be stored in the salt solution for long periods of time without fear of bacterial contamination (Mukherjee, 2019).

There have not been many studies which reported ammonium sulphate precipitation as high as 90% for keratinase purification. However, Moridshahi *et al.* (2020) obtained 1.2-fold of purification and 6.6% keratinase yield from B*acillus* sp. BK111 after ammonium sulphate precipitation reached 85% saturation level. In another study, an 80% ammonium sulphate precipitation level for keratinase of *Streptomyces matensis* MPLS-1 resulted in a 1.6-fold purification and 30% yield (Pavani *et al.,* 2016). However, another keratinase of *Pseudomonas* sp. yielded a 3.2- fold increase after a lower ammonium saturation level of

50% (Han, 2012). The difference in saturation levels obtained in this study and that from Han (2012) could arise from the different hydrophilic regions of the protein. According to Nooralabettu (2014), proteins with few hydrophilic areas are precipitated at lower saturation values, while proteins with more hydrophilic regions are precipitated at greater saturation levels without affecting the natural shape of the precipitate. Contrarily, Akhter *et al.* (2020) reported 50% saturation levels for keratinase of *Pseudomonas* sp. with a 1.67-fold increase after precipitation using acetone (50% v/v). Although acetone precipitation eliminates interfering substances in the supernatant, it is not commonly used for purification due to its protein denaturing tendency (Akhter *et al.*, 2020). Ammonium sulphate precipitation is a crude purification step and usually precipitates contaminants along with the protein of interest (Javed *et al.,* 2017). Therefore, another purification step was performed to further purify the enzyme.

3.3.1.2 Purification of keratinase by ion-exchange chromatography

To further purify keratinase after ammonium sulphate precipitation and dialysis, ion exchange chromatography was used. Results showed a protein peak in the washing fraction (not shown) while other peaks appeared after elution by gradient concentration of sodium chloride (Figure 3.1). Enzyme assays showed that keratinase activity occurred in the fractions of the eluting step and was confined between fractions C12 to fraction E5. The data also showed that the protein concentration, keratinase activity and specific activity in this step were 10.05 mg/ml, 87.35 U/ml and 8.69 U/mg respectively (Table 3.1), with a 2.4-fold purification and enzyme yield of 29%.

DEAE cellulose was chosen in this study because it is a weak anion exchanger and it works well within the alkaline pH range of 9. Considering the 10% loss after ion-exchange chromatography and the 60% loss after ammonium sulphate precipitation, ion-exchange using DEAE cellulose worked quite well. The yield decreased, thus decreasing total activity with each purification step. This observation is quite common with purification as some protein is lost during each step of purification. Purification fold increased due to increase in specific activity. Specific activity is usually the activity of the enzyme itself and more of the enzyme should be obtained with each purification step.

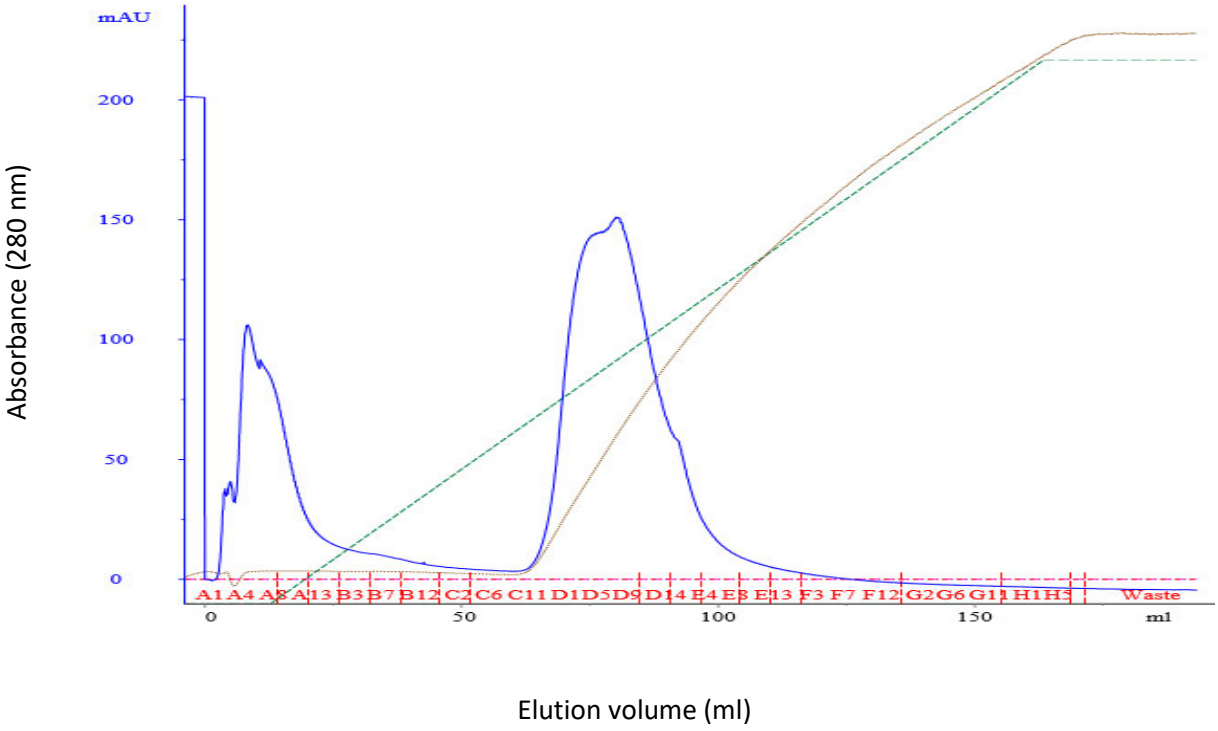


Figure 3.1 Elution profile of protein fractions from keratinase of *Pseudomonas aeruginosa* S-04 on DEAE cellulose anion exchanger column.

Table 3.1 Purification table for the keratinase isolated from *Pseudomonas aeruginosa* S-04

Stages of purification	**Total Enzyme Activity (U)**	**Total Protein (mg)**	**Specific Activity (U/mg)**	**Purification Fold**	**Yield (%)**
Crude enzyme	12020	3380	3.56	1	100
Ammonium sulphate	4664	586	7.95	2.23	38.8
Ion-exchange	3494	402	8.69	2.44	29.1

Attachment of enzyme to ion-exchange column is considered to be a simple, inexpensive and promising method to purify enzymes. Several investigators have used different methods for the purification of keratinase, resulting in variable yields and purification folds. Tork *et al*. (2010) used a CM cellulose column followed by Sephadex-76 gel filtration to obtain a 12% recovery and 4.5-fold purification fold for keratinase of *Pseudomonas* sp. In another study, a 17.4-fold purification was achieved for keratinase of *P. aeruginosa* after using G-75 Sephadex and DEAE Sepharose fast flow columns (Han, 2012). The number and type of protein impurities varies from one enzyme source to another and thus purification methods are selected to achieve purity of enzyme with the least purification steps.

3.3.2 Electrophoretic methods

3.3.2.1 Sodium dodecyl sulphate – polyacrylamide electrophoresis

Active fractions were analyzed under denaturing conditions using 12% SDS-PAGE and single bands were observed demonstrating the successful purification of keratinase to homogeneity (Figure 3.2a). The keratinase's molecular mass was calculated by comparing the enzyme's electrophoretic mobility to the electrophoretic mobilities of marker proteins. Extrapolation from a linear semi-logarithmic plot of relative molecular mass versus the Rf value (relative mobility) affirmed the molecular weight of the protein band at ~35 kDa. In general, molecular masses of keratinases range from 18 kDa for *Streptomyces albidoflavus* (Bressollier *et al.,* 1999) to 240 kDa in *K. rosea* (Bernal *et al*., 2006). However, the molecular weight of the purified keratinase obtained in this study was slightly higher than the previously reported strains such as *Pseudomonas* sp. (30 kDa) and *P. aeruginosa* (33 kDa) (Tork *et al*., 2010; Han, 2012). Other researchers have observed the molecular weight of keratinases produced by *Brevibacillus* sp. and *B. subtilis* DP1 at 83.2 and 97.4 kDa (Rai *et al.,* 2011; Sanghvi *et al.,* 2016). Dhiva *et al*. (2020) reported molecular weight of 52 kDa for keratinase of *B. pumilus* following ion-exchange chromatography using DEAE Sephadex A-50. Molecular weight of keratinase for *B. thuringiensis* MT 1 was recorded at 80 kDa (Hassan *et al.,* 2020a). The variation in keratinase molecular weight could be related to carbohydrate content in the protein, which increases its molecular weight (Hamdan and Jasim, 2018), or it could simply be owing to differing evolutionary origins of the two genes (Soliman *et al*., 2013).

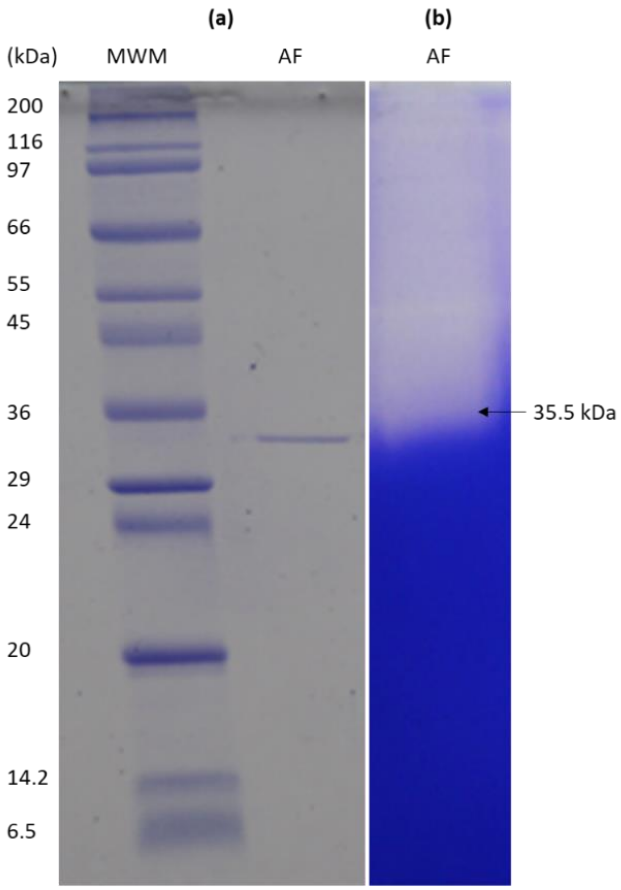


Figure 3.2 Purified keratinase of *Pseudomonas aeruginosa* S-04 on 12% SDS-PAGE gel. (a) Lane MWM: low range protein marker (Sigma M3913), AF: Active fraction and substrate gel (b).

3.3.2.2 Zymography

The zymogram produced a translucent zone against a blue background, indicating the area of substrate degradation. The zymogram showed a prominent activity zone up to the 35 kDa region, demonstrating the keratinolytic activity of the purified enzyme (Figure 3.2b). This large zone was created while the enzyme was migrating down the lane during electrophoresis. This suggests that the enzyme was gel-retarded during the zymogram and this can be seen by the smaller zone around the 35 kDa region. Similar studies displayed translucent zones on the zymogram for keratinases of *B. subtilis* strain RSE163 (Gupta *et al.,* 2015), *Micrococcus luteus* B1pz (Laba *et al.,* 2015), *Acinetobacte*r sp. (Shah, 2015), *Streptomyces* sp. strain 16 (Xie *et al.,* 2010), *Lysobacter* sp. A03 (Pereira *et al.* 2014), *Brevibacillus brevis* US575 (Jaouadi *et al*., 2013) and *Onygena corvina* (Huang *et al.,* 2015). Sharma and Gupta (2010) observed similar zones on zymogram for keratinase activity of

P. aeruginosa KS-1, using Native PAGE (10% polyacrylamide) gel followed by overlaying the gel on a 1% casein agar plate.

Typical substrates for gel zymography are gelatin, casein and fibrin, which allow for the detection of enzymatic activity in cell or tissue homogenates. A study Choi *et al*. (2001) compared these three substrates (casein, fibrin and gelatin) for zymography analysis and concluded that the gelatin gel revealed the highest enzymatic sensitivity to gel for the protease of *Bacillus* sp. Therefore, following the outcome of Choi's research, gelatin gel was used for zymographic analysis in this research. Furthermore, the use of inexpensive materials and relatively short assaying times also favoured the use of zymography for the detection of keratinase. In future, a modification involving overlays with gelatin after electrophoresis can be considered to obtain a discrete zone surrounding the enzyme only.

3.3.3 Biochemical characterization of keratinase

3.3.3.1 Effect of pH and temperature on the activity and stability of keratinase

pH is a critical factor in many enzyme-driven reactions and each enzyme has a characteristic pH optimum, since enzymes consist of amino acids containing ionic groups. The dissociation state of amino acid residues significantly affects the structure of the active site or the affinity to substrates and as a result, the catalytic activity. Due to the difficulty in characterising the active sites of enzyme, the optimum pH is usually determined experimentally (Miyanaga and Unno, 2011).

The pH assays revealed that the keratinase from *P. aeruginosa* S-04 was optimally active at pH 9.5, while it also exhibited significant activity between pH 8 and 9, showing more than 60% activity within that pH range (Figure 3.3). Beyond this range, the enzyme lost almost all its activity (97%) at pH 4 and about 80% of its optimal activity at pH 10.5. The activity also sharply dropped (60%) within 0.5 pH units, from its optimum at pH 9.5 to pH 10. This decrease is keratinase activity could be attributed to the distortion of the active site of the enzyme resulting in lower keratinase activity. The keratinase activity gradually increased from neutral to alkaline pH (pH 7 to 9.5), thus classifying the keratinase as alkaliphilic. After initial optimum pH assays, the experiment was modified to include additional assays at 0.5 pH units between pH 8 and pH 11. This resulted in a more accurate optimum pH value of 9.5, as opposed to pH 9 in the initial experiments.

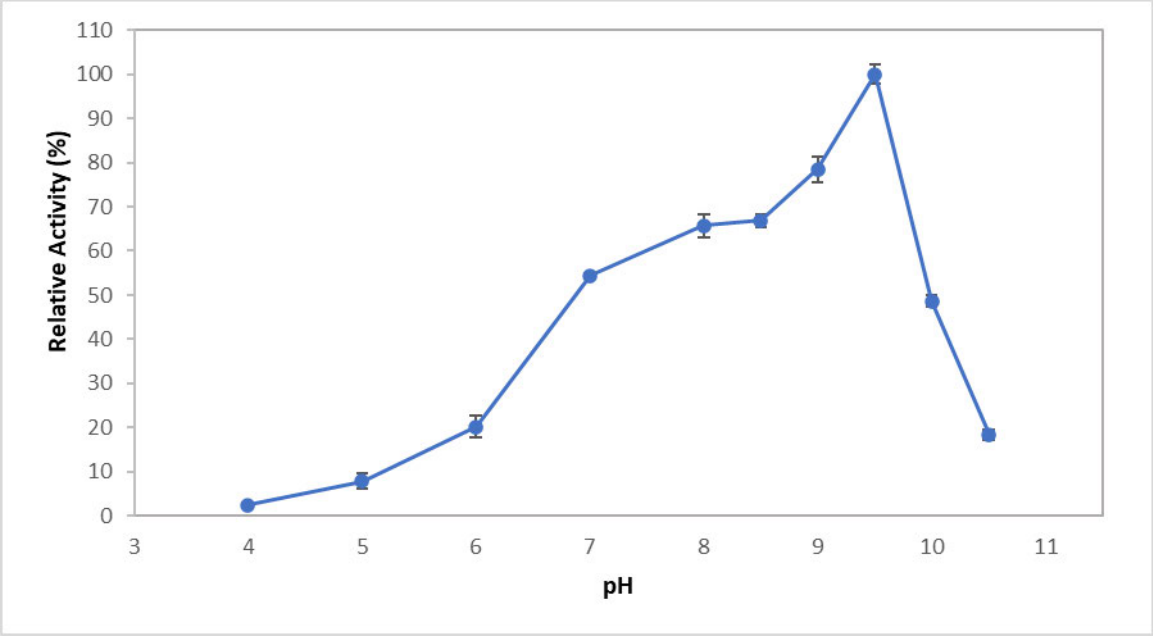


Figure 3.3 Influence of pH on keratinase activity from *Pseudomonas aeruginosa* S-04. Each point represents the mean (n=3) ±SD. pH profile was determined in different buffers by varying pH at 37°C using keratin azure as the substrate. The results are statistically significant with p-value ≤ 0.05.

Results from this study are consistent with previous reports which indicated an optimal pH of 9.5 for the keratinase from *Bacillus licheniformis* (Kazzaz *et al.,* 2015) and *B. zhangahouensis* (Moridshahi *et al.,* 2020). However, optimal activity for keratinases from other *Pseudomonas* sp. were recorded at pH 8 (Tork *et al.*, 2010) and 7.5 (Han, 2012). There are some exceptional cases where keratinases are optimally active at extreme alkaline or slightly acidic pH. For example, keratinase of *B. subtilis* MTCC (9102) showed optimal activity at pH 6.0 (Balaji *et al.*, 2008) and keratinase of *Nocardiopsis* sp. TOA-1 recorded optimal activity at pH 12.5 (Jouadi *et al.*, 2010). Thus, the optimal properties of microbial keratinases are diverse and depends on the producer microorganism.

In general, alkaline pH favors keratinase activity because it converts cysteine residues to lanthionine, allowing keratinase to hydrolyze the stubborn keratin (Hamiche *et al.*, 2019). According to Tork *et al.* (2016), the alkaline condition aids in the disulphide bond breakdown, which speeds up keratin degradation. As a result of the alkaline character of the industrial process, alkaline keratinases are more suitable for biotechnological applications, such as in the leather industry and laundry detergent manufacturing (Abdel-

Naby *et al.,* 2017). Denaturation of the enzyme will be avoided as the enzyme operates within the same alkaline conditions as the industrial process.

The stability of enzymes is a critical aspect in determining whether biocatalysis will be commercially effective. When enzymes are exposed to excessive pH, they lose some of their activity. It is noteworthy that the keratinase displayed remarkable stability, retaining more than 95% relative activity over a pH range of 7.0–9.5, after 2 h of preincubation (Figure 3.4). At pH 10, stability was the same for the first 30 minutes, then declined to 70% relative activity after 2 h. Due to the negligible difference in relative activity at pH 7.0 to pH 9.5, the optimum pH range for keratinase stability can be deduced as pH 7.0 to 9.5. However, the stability of keratinase declined sharply to less than 35% at pH 4, 5 and 11. This reveals the unstable nature of this keratinase in acidic and more alkaline solutions. The structure of the enzyme would be destabilized and altered at different pHs, according to Akram *et al.* (2018), because the protein's free energy is affected by the net charge and can be deactivated.

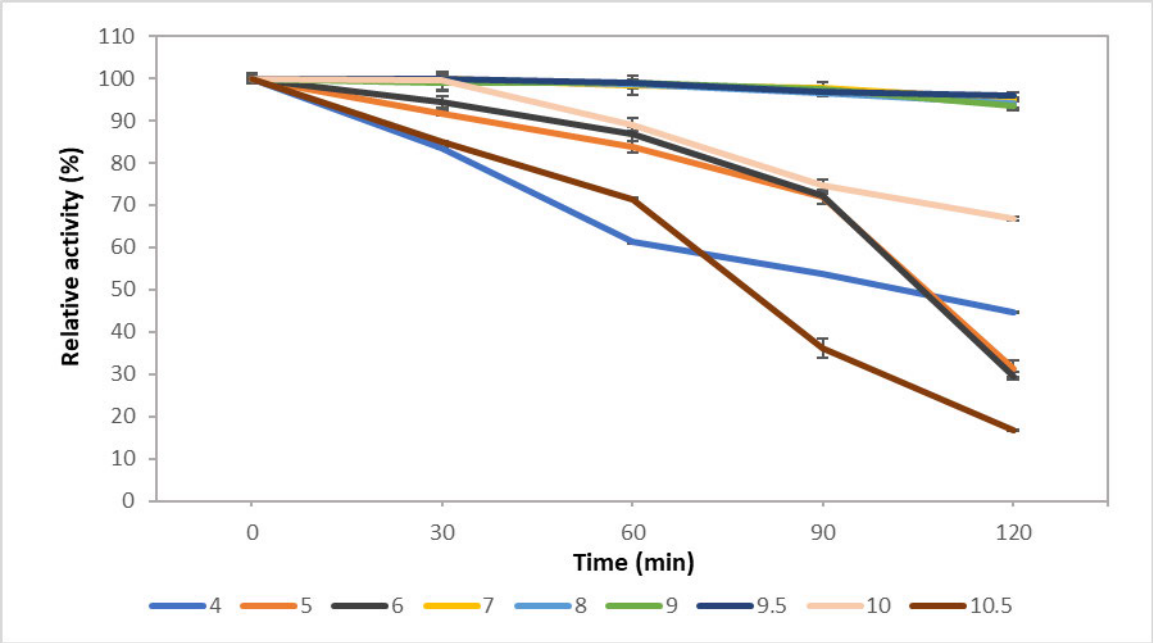


Figure 3.4 Influence of pH on keratinase stability of *Pseudomonas aeruginosa* S-04. Each point represents the mean (n=3) ±SD. The pH stability was determined by incubating keratinase in different buffers for 2 h and measuring keratinolytic activity.

With regards to the temperature and temperature stability, high temperatures can damage the intramolecular bonds of the keratinases, which sustains the tertiary structure of the protein. These alterations significantly affect the affinity of the catalytic and binding sites between enzymes and substrates, and consequently inhibit enzyme activity. The temperature profile of *P. aeruginosa* S-04 keratinase is presented in Figure 3.5. Optimal keratinolytic activity was recorded at 60°C; however, it exhibited remarkable activity at 30 to 70°C retaining more than 60% of its optimal activity. Beyond the optimum temperature range, keratinase activity decreased with increasing temperature, retaining only 37% of its optimal activity at 80°C. This massive decline in enzyme activity could be assigned to the denaturation of the enzyme as a result of the high temperature.

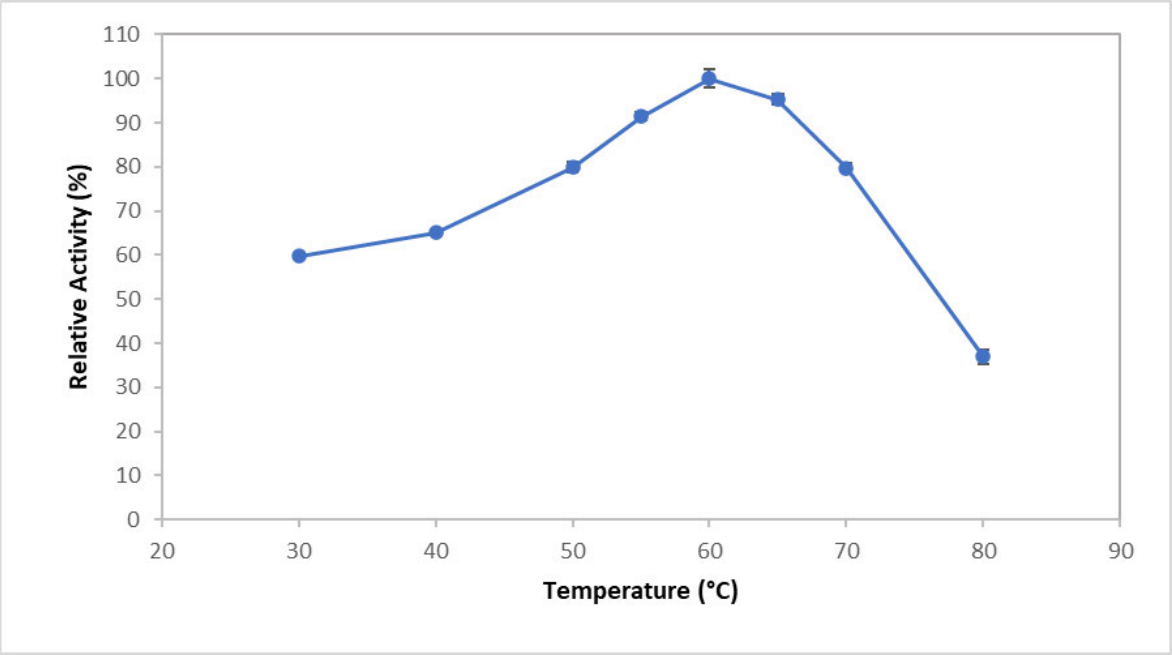


Figure 3.5 Effect of temperature on keratinase activity from *Pseudomonas aeruginosa* S-04. Each point represents the mean (n=3) ±SD. The temperature profile was determined by assaying keratinase activity at different temperatures from 30 to 80°C at pH 9.5 using keratin azure as substrate. The results are statistically significant with p-value ≤ 0.05.

The optimum temperature of 60°C observed in this study classifies the keratinase as thermophilic. Similar optimal temperature of 60ºC was reported for keratinase of *P. aeruginosa* (Han, 2012) and keratinase of *B. zhangzhouensis* (Moridshahi *et al.,* 2020). In general, microbial keratinases display optimal temperature in the range of 45-60°C (Sivakumar *et al*., 2013). Nonetheless, Nam *et al*. (2002) discovered the maximum optimum temperature (100°C) in a serine keratinase generated by *Fervidobacterium islandicum* AW1 isolated from a geothermal hot spring. In contrast, keratinase generated by *B. tequilensis* Q7 has the lowest optimal temperature of 30ºC (Jaouadi *et al*., 2015).

However, the highest activity of an enzyme is frequently reported at a temperature greater than the optimum temperature for microbe development (Fakhfakh *et al*., 2009). This phenomenon was observed in this study, the optimum temperature for *P. aeruginosa* S-04 was 35°C but the optimum temperature for keratinase activity was 60°C. A study by Elhoul *et al.* (2016) reported growth of *Acinomadura viridilutea* DZ50 at 45ºC whilst optimal keratinase activity was observed at a temperature of 80ºC. Extracellular enzymes (e.g., keratinase) are most active at temperatures higher than the host organism's optimum development temperature and are often very stable (Brandelli and Riffel, 2005).

Keratinases that are active at high temperatures may provide a cost benefit since they are more tolerant of the conditions found in industrial operations. The ability to conduct biotechnological activities at elevated temperatures, lowering the risk of contamination by mesophilic microbes, is one of the advantages of using thermostable enzymes. Furthermore, when the temperature rises, the viscosity of the medium decreases, boosting organic component bioavailability and solubility. Substrates and products' diffusion coefficients will both rise, resulting in faster reaction rates (Gomes *et al*., 2016). As a result, thermostable keratinases can be used in the detergent and feed industries.

Temperature effects on enzyme stability is also another key variable in a biocatalytic process. The purified keratinase was quite stable between 4 and 70°C retaining more than 50% of its activity after 120 min of incubation (Figure 3.6). The keratinase was most stable at a temperature range of 4 to 30ºC; retaining 90% of its initial activity after 120 min of incubation. However, keratinase activity decreased to 60% of the original activity after incubation for 120 min at 70ºC. High temperatures have been shown to impair the intramolecular structure of keratinase by diminishing the affinity of the enzyme's catalytic and binding sites for the substrate, resulting in a decrease in enzyme activity (Hassan *et al*.,

2020a). Although the enzyme recorded optimal keratinase activity at 60°C, the relative activity after incubation for 120 min at 60°C was only 67%. This agreed with the fact that the incubation of enzymes at high temperature without the substrate decreases their observed stability (Abdel-Fattah *et al.,* 2018).

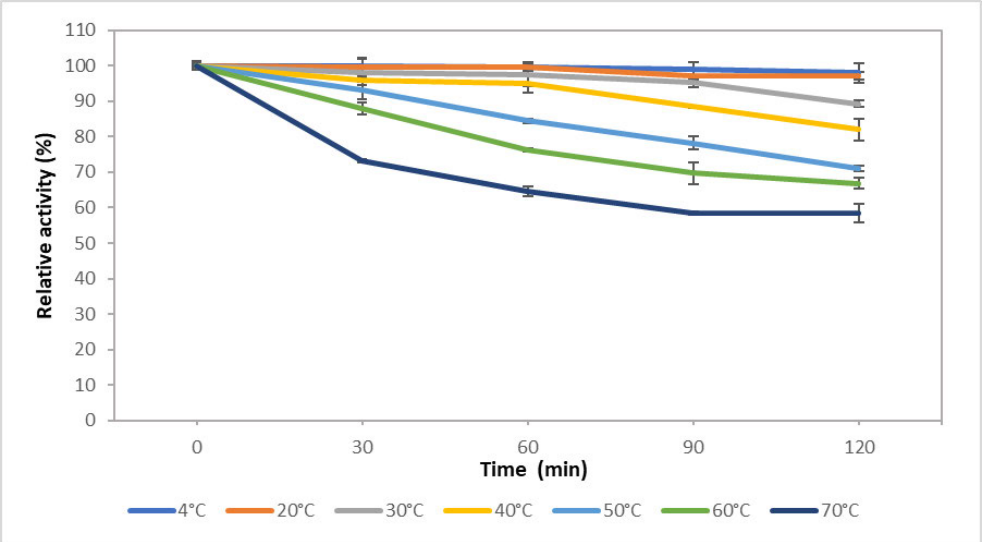


Figure 3.6 Effect of temperature on keratinase stability of *Pseudomonas aeruginosa* S-04. Each point represents the mean (n=3) ±SD. The temperature stability was determined by incubating the keratinase at different temperature for a period of 2 h.

3.3.3.2 Effect of inhibitors and metal ions on the activity of keratinase

Because protease groups are sensitive to the presence of certain inhibitors, inhibitor assays are used to detect the keratinase type. Except for the yeast group that produces aspartic proteases, microbial keratinase was previously thought to be largely related to serine proteases and metalloproteases. Among the various inhibitors, EDTA inhibited keratinase activity by 80%, however, PMSF inhibited keratinase activity only by 43%. In contrast, the presence of iodoacetamide showed 60% keratinase inhibition. However, no effect on enzyme activity was recorded in the presence of reducing agent, DTT (104%), but 75% relative activity was recorded in the presence of β-mercaptoethanol (Table 3.2).

Table 3.2 Effect of inhibitors on the activity of keratinase from *Pseudomonas aeruginosa* S-04

Reagent	Concentration (mM)	Class of inhibitor	Relative activity (%)	Fold
PMSF	10	Serine	57.24 ±2.82*	0.6
EDTA	10	Metal chelator	20.69 ±1.02*	0.2
Iodoacetamide	10	Cysteine	40.38 ±1.92*	0.4
Dithiothreitol	10	Reducing agent	103.72 ±2.82*	1.0
β-Mercaptoethanol	1	Reducing agent	75.10 ±3.22*	0.8
None			100.00 ±0.55	

* The results are statistically significant with p-value ≤ 0.**05.**

Due to its susceptibility to protease inhibitors, the enzyme under examination belongs to the metallo-class of keratinase since 80% of the enzyme activity is inhibited when incubated with EDTA. Furthermore, chelating chemicals disrupt the activity of the proteolytic enzyme by disrupting the catalytic conformation, which impacts enzyme-substrate interaction and product production. These results are in accordance with Han (2012) who also classified their keratinase from *P. aeruginosa* as a metalloprotease, showing 80% inhibition in the presence of EDTA. In general, keratin degradation depends on a sulfitolysis process that can be catalyzed by disulphide reductase, and thus EDTA can potentially compromise this reaction, and consequently, inhibit the degradation of keratin (Duffeck *et al.,* 2020).

The inhibition by iodoacetamide suggests that cysteine residues are involved in the catalytic mechanism. Iodoacetamide binds with thiol group of cysteine preventing enzymes from forming disulphide bonds, resulting in enzyme inhibition (Tusar *et al.*, 2021). Reducing agents, DTT and β-mercaptoethanol, which assist in the breakdown of disulphide bonds had variable effects on keratinase activity. Keratinase activity was adversely affected upon pretreatment with β-mercaptoethanol which suggests that the disulphide bonds present in the enzyme were denatured. Lowering chemicals are thought to be involved in the pertubation of keratinase stability by reducing the intramolecular disulphide bonds that are involved in structural stabilization (Saber *et al.*, 2010). However, the addition of DTT showed a slight increase in keratinase activity. Besides the reducing function of DTT, it is also known to protect free SH-groups from oxidation during

biochemical procedures (de Almeida and Saldanha, 2010). Hence, the difference in keratinase activity upon incubation with different reducing agents. Han (2012) reported no change in keratinase activity upon addition of DTT and β-mercaptoethanol for *P. aeruginosa*. In contrast, keratinase from *Stenotrophomonas maltophilia* K279a showed slight inhibition by reducing agents β-mercaptoethanol and DTT (Shah and Vaidya, 2017).

To provide information on possible action of specific metal ions as positive or negative modulators of the purified enzyme, its keratinolytic activity was investigated after preincubation with the specific metallic ions outlined in Table 3.3. Keratinase activity was enhanced by the addition of Mn^{2+} (180.31%) and Fe^{3+} (128.04%), with Mn^{2+} exhibiting the most increase in keratinase activity. However, the addition of Cu^{2+} (100.72%), Ca^{2+} (96.80) and K^{+} (95.05%) did not have significant effect on keratinase activity. In contrast, keratinase inhibition was apparent upon the addition of metal ions Mg^{2+} (49.90%), Na^{+} (65.67%) and Zn^{2+} (77.94%). In certain studies, enhanced keratinase activity has also been recorded for keratinases of *P. aeruginosa* C11 (121%) (Han, 2012), *Actinomyces* sp. RM4 (117%) (Verma *et al.,* 2016a) *and B. subtilis* RSE163 (120%) (Gupta *et al*., 2015) following preincubation with Mn^{2+} ions.

Table 3.3 Effect of metal ions on keratinase activity of *Pseudomonas aeruginosa* S-04

Metal Ion	Residual Activity (%)	Fold
Ca^{2+}	96.80 ±4.27	1.0
Na^{+}	65.67 ±3.06*	0.7
K^{+}	95.05 ±4.44	1.0
Mg^{2+}	49.90 ±2.29*	0.5
Cu^{2+}	100.72 ±4.58	1.0
Zn^{2+}	77.94 ±0.87*	0.8
Fe^{3+}	128.04 ±1.17*	1.3
Mn^{2+}	180.31 ±0.87*	1.8
None	100 ±3.79	

* The results are statistically significant with p-value ≤ 0.**05.**

Metallic ions tolerance has been demonstrated for metallo-keratinases. Enzymes often require metal ions for structural stabilization as well as optimal substrate binding at the active site and transition state stabilization (Verma *et al.*, 2016b). Metallic ions, on the other hand, can influence a protein's activity through allosteric regulation (the regulation of an enzyme by binding an effector molecule at a site other than the enzyme's active site) via complexation with essential residues, which causes disorientation of the proper enzyme conformation, allowing or disallowing substrate binding (Nnolim *et al.,* 2020). The negative effect of metals (Mg^{2+}, Zn^{2+}) on keratinase activity may be attributed to the disruption of the folding state by replacing the sulphur of disulphide bonds, causing enzyme denaturation. In addition, the formation of bridges between metal monohydroxide (Na^{+} and K^{+}) and catalytic ions at the active site could also occur resulting in lower keratinase activity (Jagadeesan *et al.*, 2020).

The stimulation of keratinase in the presence of a metal ion such as Mn^{2+} could be due to the development of a salt or an ion bridge that maintains the enzyme-substrate complex (Balaji *et al.,* 2008). Also, Mn^{2+} ions were reported to act as protecting agents against thermal denaturation of most microbial metalloproteases, thus, it plays an important role in maintaining active confirmation at higher temperatures (Benkiar *et al.*, 2013). The enzyme displayed an increase in activity by preincubating with Fe^{3+} confirming the metal's positive effect on stabilizing the enzyme tertiary structure and catalytic site. It is therefore likely that the EDTA inhibition (Table 3.2) is due to the sequestration of Fe^{3+} and Mn^{2+} ions.

3.3.3.3 Effect of chemical reagents on the activity of keratinase

In a solution containing surfactants, reducing agents, or solvents, keratinase activities are usually inhibited, stabilized, or boosted. The keratinase showed stimulation after pre-treatment with non-ionic surfactants, Tween 20 (152.09 ±7.63) and Triton X-100 (108.77 ±2.77). The organic solvents impacted variably on the stability of the keratinase with a stimulatory effect demonstrated after DMSO pre-treatment (114.84 ±6.49) and an inhibitory effect demonstrated after acetone pre-treatment (84.62 ±2.1). Among all the alcohols tested, isopropyl alcohol and ethanol had a stimulatory effect on the keratinase stability with relative activities of 190.69 ±8.78 and 113.36 ±4.68, respectively. In contrast, methanol had a negligible effect on the keratinase with relative activity of 96.76 ±2.1. The type of keratinases' side chains or interaction patterns, which allosterically affect the

structural orientation and biocatalytic effectiveness of the enzymes, is responsible for their diverse responses to chemical agents (Fang *et al.,* 2016a).

Table 3.4 Effect of chemical reagents on keratinase activity of *Pseudomonas aeruginosa* S-04

Chemical reagent	Concentration (%)	Relative activity (%)
Triton X-100	1	108.77 ±2.77*
Tween 20	1	152.09 ±7.63*
DMSO	1	114.84 ±6.49*
Isopropanol	0.1	190.69 ±8.78*
Methanol	0.1	96.76 ±2.1
Ethanol	1	113.36 ±4.68*
Acetone	1	84.62 ±2.1*
Control	None	100%±2.83

* The results are statistically significant with p-value ≤ 0.**05.**

Furthermore, solvent tolerance is strain-specific, and a solvent's toxicity is proportional to the logarithm of its partition coefficient in n-octanol and water (log P_{ow}) (Anbu, 2016). High log P of solvent values, i.e. the logarithm of the partition coefficient P of the solvent between octanol and water, result in increased protease stability in that solvent. Ethanol has a log P value of -0.31 while that of methanol is -0.81, hence it can be deduced that the higher the partition coefficient (ethanol), the more stable the keratinase activity of *P. aeruginosa* S-04. Acetone had a denaturation effect on the enzyme due to the disruption of intra non-covalent interactions. The electronegative oxygen present in the ketone group (C=O) of acetone can interrupt the intra hydrogen bonds that stabilizes the tertiary structure of enzymes (cause denaturation). That is why salts are preferred instead of organic solvents for protein purification (Wingfield, 2001).

Organic solvent-resistant bacteria are a category of extremophile microorganisms that, according to their adaptation abilities, can survive in harsh environments in the presence of organic solvents. The use of naturally existing organic solvent-resistant proteases eliminates the need for costly and time-consuming protein engineering procedures. The *P. aeruginosa* S-04 keratinase was naturally stable in diverse solvents and exhibited high activity thus making it a suitable candidate for industrial applications such as in leather processing.

3.3.3.4 Kinetic parameters of keratinase

Kinetic studies on enzymes aim to measure the affinity with which the enzyme binds to substrate and the turnover rate. The effect of substrate (keratin azure) concentration on the activity of pure *P. aeruginosa* S-04 keratinase was investigated at optimal conditions (pH 9.5 and 60°C). The K_m value for certain enzyme indicates the binding strength of that enzyme to its substrate. Thus, a low K_m value indicates a higher affinity for the substrate. Lineweaver-Burk plot of the pure *P. aeruginosa* S-04 keratinase gave a K_m of 7.62 mg/ml (Figure 3.7) which is lower than the reported value for *B. pumilus* keratinase (8.74 mg/ml) (Abdel-Naby *et al.*, 2017). The kinetic parameter is a measure of how quickly something happens. When the complete amount of enzyme participates in the reaction, V_{max} is defined as the maximal velocity (Abdel-Naby *et al.*, 2017). Because all enzyme molecules must be linked to their substrates at the same time, this measurement is theoretical and usually has an estimated value. The calculated value of *P. aeruginosa* S-04 keratinase V_{max} was 200 U/mg/ml protein.

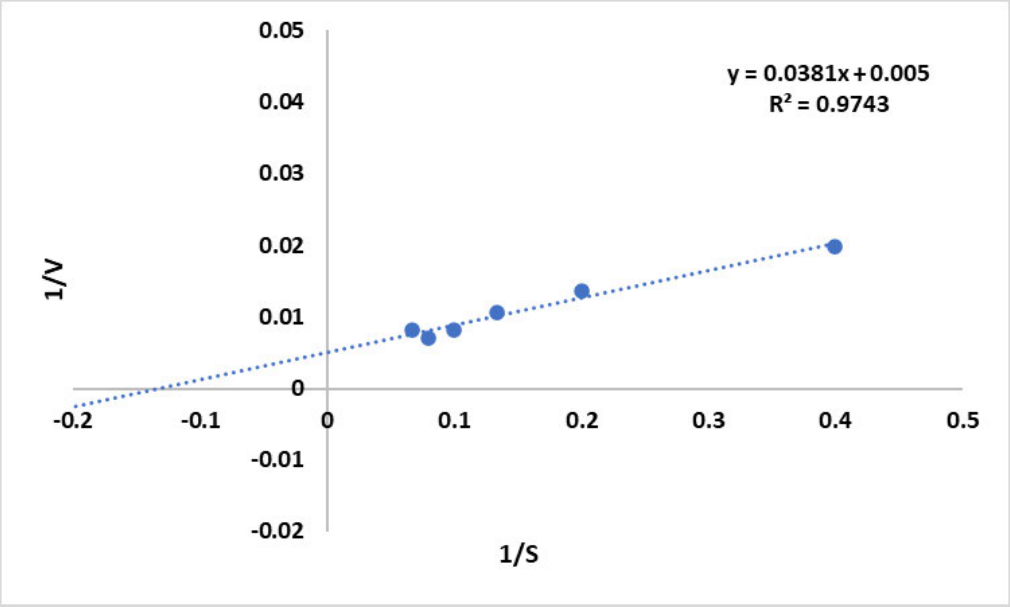


Figure 3.7 Lineweaver-Burk plot of keratinase for *Pseudomonas aeruginosa* S-04 using keratin azure as substrate.

Many researchers have reported the kinetic parameters of different keratinases on different substrates. Using raw feathers as substrate, *B. subtilis* keratinase has a K_m value of 6.6 mg/ ml and V_{max} of 5 U/ml/min (Gupta *et al.*, 2015). Pawar *et al.* (2018) reported a lower K_m and higher V_{max} values of 0.61 mg/ ml and 1673 U/mg/min, respectively for a keratinase purified from *B. altitudinis* RBDV1 when keratin azure was used as substrate. Kinetic parameters (K_m and V_{max}) depend on different substrate ranges used for kinetic parameter assay in which a lower range of substrate results in lower values of K_m and V_{max}. For example, protease from *B. licheniformis* A10 had K_m and V_{max} of 0.033 mg/ml and 8.17 µmol/ml/min, respectively, when using casein in the range of 0.2–6.0 mg/ml (Yilmaz *et al.*, 2016). Meanwhile, higher values of K_m and V_{max} of protease from *B. licheniformis* UV-9 were 5.0 mg/ml and 61.58 µmol/ml/min when casein ranging from 2.5 to 20 mg/ml was used (Nadeem *et al.*, 2013).

3.3.4 Enzymatic degradation of chicken feather

3.3.4.1 Optimization of enzyme dosage for feather degradation

The use of enzymes for feather degradation is a highly efficient, eco-friendly, and cost-effective method as it produces feather meal with high nutritional values (Tamreihao *et al.,* 2018). In addition, the biodegraded feather meal retains the released amino acids, which increase their industrial and biotechnological applications. In this study, keratinase from *P. aeruginosa* S-04 exhibited maximum activity at pH 9.5 and 60°C. Although an alkaline pH and high temperature would facilitate quick feather breakdown by lowering disulphide bonds, the loss of some important amino acids makes it unsuitable for direct use in feed. Therefore, feather degradation was studied at 40°C where the keratinase used in the study exhibited >60% activity.

Degradation of feather was optimal (93%) when 500 U of partially purified keratinase was applied (Figure 3.8). A decrease to 89% in feather degradation was observed when enzyme concentration was increased from 500 to 1000 U. Further increases in enzyme concentration (2000 U to 3000 U) resulted in negligible differences in feather degradation (88 to 87%). Thus, it can be inferred that 500 U of keratinase is optimum for optimal feather degradation. The use of an enzymatic process for feather meal production is preferred over bacterial fermentation as the latter requires several controls during the bioreactor run (Tiwary and Gupta, 2010). Furthermore, to reduce production costs,

concentrated keratinase (ammonium sulphate precipitated) was employed for feather degradation. The purification process for keratinases could be costly and time-consuming, hence, ammonium sulphate-concentrated enzyme was used instead.

In the presence of reducing substances such as dithiothretol or glutathione, or in the presence of live cells that produce a reducing environment, feather degradation using keratinase has been described. The majority of feather meal is made by fermenting it with keratinolytic bacteria, and the resulting fermentation broth is referred to as feather meal. Feathers are intended to be degraded during fermentation by a combination of proteases and cell redox processes (Tiwary and Gupta, 2010). In this regard, the current procedure is superior to previous ones because neither reducing chemicals or fermentation are required for the decomposition of feather meal.

Other studies performed to optimise feather degradation revealed variable degradation rates. For example, crude keratinase of *B. subtilis* exhibited 36.1% degradation for 1 g feather in 12 h (Gong *et al.*, 2020). However, crude keratinase of *B. licheniformis* ALW1 reported only 64% feather degradation for 1 g feather in 24 h using 480 U of the enzyme (Abdel-fattah *et al.,* 2018). In contrast, Tiwary and Gupta (2010) reported complete degradation of 1.5 g feather in 12 h using 1200 U dimeric keratinase of *B. licheniformis* ER-15. Thus, feather degradation must be investigated on a case-by-case basis to determine the optimum level of feather degradation.

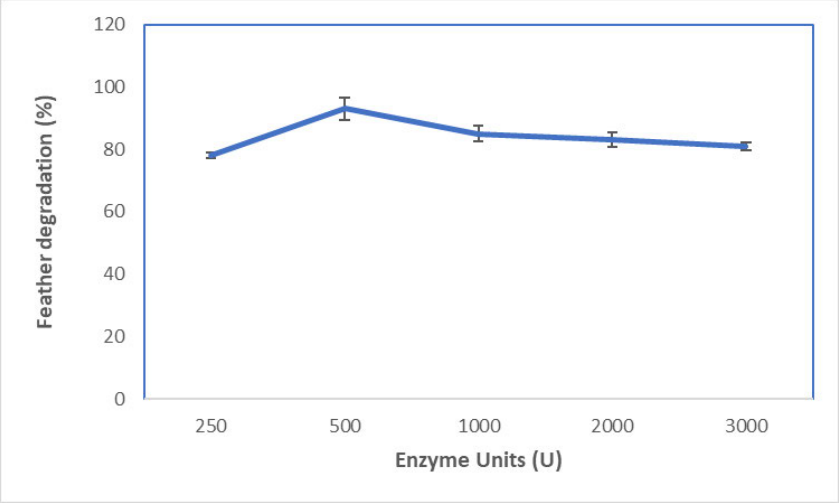


Figure 3.8 Effect of different enzyme concentrations on feather degradation by keratinase of *Pseudomonas aeruginosa* S-04 at 40°C for 24 h. Each point represents the mean (n=3) ±SD.

3.3.4.2 Amino acid analyses

Traditionally, animals and people were classified as nutritionally essential or nonessential based on nitrogen balance or growth. All non-essential amino acids were thought to be sufficiently synthesized in the body to meet protein synthesis requirements as substrates. Animal diets must contain an adequate amount of all essential amino acids to improve animal performance. If the content of one amino acid in the diet is too low, other amino acids may not be utilized as well (Wu, 2010). The nutrient composition of the feed influences animal voluntary intake, which affects growth rate, productivity, and efficiency. Feed consumption is reduced by a deficiency in the limiting amino acid or an oversupply of some essential amino acids. The maintenance and protein accretion demands of animals dictate their amino acid requirements (Millet, 2012).

From the HPLC chromatogram (Figure 3.9), a total of 17 free amino acids were identified at variable concentrations from the feather hydrolysate. The amino acid profile of feather hydrolysate in this study is shown in Table 3.5. The hydrolysate was found to possess essential amino acids such as glycine (36.4%), proline (8.5%), serine (8.1%), glutamic acid (8%), valine (5.9%) and leucine (5.54) as its major amino acid residues. However, lower levels of histidine (0.07%), methionine (0.45%), lysine (0.94%) and hydroxyproline (0.24%) were noted. Overall, the feather hydrolysate produced by keratinase of *P. aeruginosa* S-04 is rich in essential amino acids. Thus, the feather hydrolysate has a real possibility of application in the feed industry and in the production of amino acids.

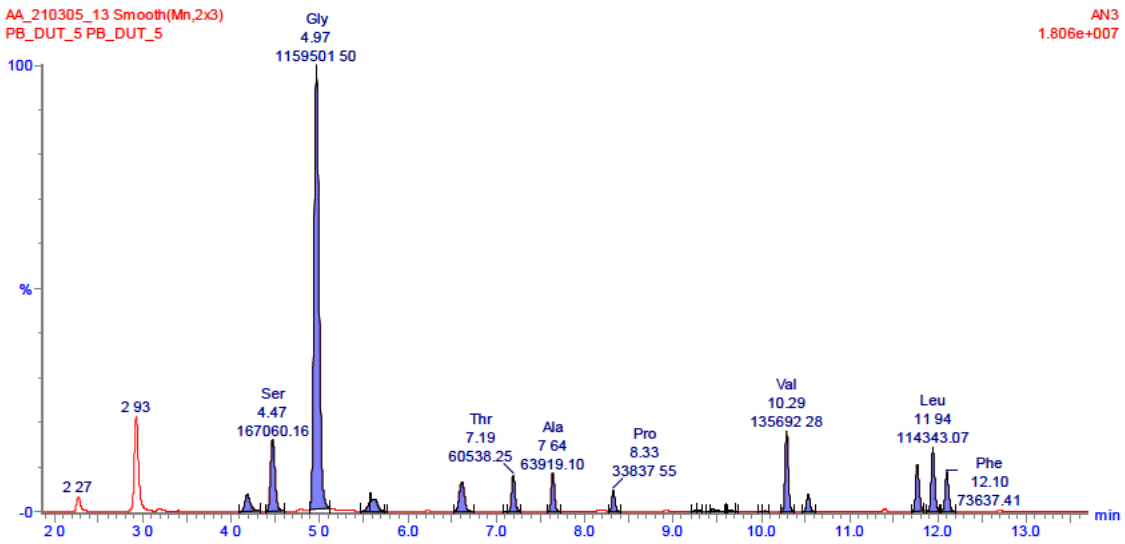


Figure 3.9 HPLC analysis of the amino acid profile of feather hydrolysates. The chromatogram depicts the presence of 17 free amino acids in the feather hydrolysate.

Table 3.5 Amino acid composition of feather meal hydrolysate produced with keratinase from *Pseudomonas aeruginosa* S-04

Amino Acids	%
Alanine	3.15
Arginine	4.92
Aspartic acid	4.50
Glutamic acid	8.00
Glycine	36.45
Histidine	0.07
Isoleucine	3.37
Leucine	5.54
Lysine	0.94
Methionine	0.45
Phenylalanine	3.98
Proline	8.51
Serine	8.14
Threonine	3.09
Tyrosine	2.74
Valine	5.91
Hydroxyproline	0.24

Stiborova *et al.* (2016) reported higher amino acid content arginine (7.4%), aspartic acid (8.0%), glutamic acid (10%), histidine (6%), isoleucine and leucine (21.9%), lysine (5.8%), methionine (1.9%), phenylalanine (7.1%), tyrosine (6.2%) and valine (11.1%) after treatment with keratinase from *Pseudomonas* sp. P5. However, lower amino acid concentrations were recorded for alanine (1.8%), proline (2.5%), serine (1.6%), threonine (1.1%) and hydroxyproline was not detected. The difference in results obtained in this study compared to Stiborova *et al.* could be attributed to the difference in volume of feather used in hydrolysis. The current study used 10 g of dry feather whereas Stiborova *et al.* used 31.9 g of dry feather.

Interestingly, Eaksuree *et al*. (2016) prepared feather meal using *B. licheniformis* KUB-K0006 keratinase, *B. pumilus* KUB-K0082 keratinase, mixture of both keratinases (5:1) and a commercial enzyme, respectively. It was observed that the amino acid profile of the different treatments were similar, however, the total amino acids were higher with *B. pumilus* KUB-K0082 keratinase treatment. Furthermore, serine (13%), glutamic acid (11.4%) and proline (11.1%) were the major amino acid residues observed. Other amino acid residues such as glycine (8.8%), leucine (7.9%), arginine (7.1%), aspartic acid (6.4%), valine (5.3%), alanine (4.9%), threonine (4.9%), phenylalanine (4.7%) and isoleucine (4.0%) also observed in appreciable amounts. However, lysine (2.2%), tyrosine (1.0%), histidine (0.9%), methionine (0.7%) and hydroxyproline (0.1%) were present at very low concentrations. The variable concentrations of the amino acid residues found in feather hydrolysate from different studies could be due to the variation of chicken feathers used in the different studies. The variation of chicken feathers arises from the growth conditions, e.g. diet of poultry, age of bird, etc., which ultimately influences the different amino acid profile of feathers.

Proteins are made up of 20 distinct amino acids, nine of which cannot be synthesized by most animal species. As a result, a steady supply of these nine amino acids, as well as enough nitrogen to synthesize the other amino acids, is required for maintenance and growth (Wu, 2010). The nine essential amino acids are: arginine, lysine, methionine, tryptophan, isoleucine, leucine, histidine, phenylalanine and valine. Arginine is an essential amino acid for many animal species e.g. birds and fish. However, mammals, e.g. pigs and ruminants, synthesize arginine in the urea cycle where urea is produced from surplus nitrogen. On the other hand, most arginine produced in the urea cycle is broken

down and some animals (e.g. young pigs) need a dietary supply (Boisen *et al.*, 2000). Therefore, arginine is considered to be a semi-essential amino acid.

Cysteine and tyrosine are also considered as semi-essential amino acids as they can only be synthesized by animals from methionine and phenylalanine, respectively. Therefore, the amino acid requirements must include the sum of methionine and cysteine (sulphur amino acids) and phenylalanine and tyrosine (aromatic amino acids), respectively. The remaining eight amino acids, including alanine, aspartic acid, asparagine, glutamic acid, glutamine, serine, glycine and proline, can all be synthesized from metabolites produced during the oxidation of glucose (Boisen *et al.,* 2000). Piglets have been proven to consume more feed when given tryptophan. A low tryptophan diet has been linked to decreased feed intake, which affects serotonin levels in the brain (Sterndale *et al*., 2020). Low valine levels, on the other hand, lead to reduced feed intake and daily gain. Somatostatin has been implicated as a cause of anorexia brought on by valine-deficient diets (Zhao *et al*., 2019). Supplementing valine or tryptophan to a diet deficient in both amino acids, on the other hand, has been shown to increase pig performance (Millet, 2012).

Glycine is a major amino acid in mammals and other animals. The high level of glycine and proline detected in this study in the feather hydrolysate makes the feather meal a suitable ingredient to be used in animal feed. Glycine, on the other hand, is not sufficiently produced in birds or other animals under regular eating conditions, especially in ill states. Although minor glycine deficiency is not life threatening, a chronic deficiency can lead to stunted growth, weakened immune responses, and other health and nutrient metabolism problems (Wang *et al*., 2013). Dietary glycine is especially important for foetal and neonatal development in birds (e.g., chickens), because foetuses and neonates cannot synthesize enough glycine to meet the optimum requirement. To enable optimum growth, glycine should be considered a conditionally necessary amino acid for mammals (Wu*,* 2010).

Depending on the animal species, proline can be produced in a variety of methods. Mammals, birds, and fish, for example, may synthesis proline from arginine in cell and tissue-specific ways. Furthermore, livestock (e.g., pigs, cattle, and sheep) manufacture proline in the small intestine from glutamine and glutamate, although birds and possibly most fish species lack this pathway (Jing *et al*., 2019). In pigs, chickens, and fish, however, endogenous proline synthesis is insufficient for optimal growth, collagen formation, or

feed efficiency. To achieve optimal growth, proline should be incorporated in the meal. Although proline can be utilized as a feed supplement in animal diets, it is an expensive substance that makes it difficult to include in practical rations (Li and Wu, 2017). Hydrolyzed feather meal (HFM), which contains 8% proline, has a lot of promise as a low-cost, high-proline nutritional source for both ruminants and nonruminants.

3.3.4.3 Proximate analysis of biodegraded feather meal and industrially produced feather meal

Feather wastes could be used as an alternate ingredient in animal feed, helping to alleviate the protein deficit in food and feed. Chicken feathers can be used for animal feed because they have a high protein level of 80–90% dry matter, which is higher than the raw protein content of soybean meal (42.5%) and fish meal (66.5%) (Nursinatrio and Nugroho, 2019). Although untreated feathers are high in crude protein, keratin, which contains a lot of cystine, makes them difficult to digest (approx. 10%). The indigestibility of the crude protein fraction is aided by cystine cross linking. Anti-nutritional agents such as tannin, lectin saponin, glucosinolates, and trypsin-inhibiting factor have no effect on the crude protein of feather (Belewu *et al.*, 2008). Crude protein is comprised of true protein and non-protein nitrogen. True protein is sometimes called natural protein and is either degradable or not degradable.

The data in Table 3.6 reveals that the crude protein was significantly higher in enzyme treated feather meal (88%) compared to raw feather (69%) and slightly higher than industrially produced feather meal (83%). A study by Zhou *et al*. (2020) also observed similar results when feathers was enzymatically treated with a cocktail of enzymes (keratinase, oligopeptidase, lipase and disulphide reductase from *B. amyloliquefaciens* 3–2). Eaksuree *et al.* (2016) also reported protein content of 80.6, 88.6, 84.9 and 81.4% for feathers treated with *B. licheniformis* KUBK0006 keratinase, *B. pumilus* keratinase, 2:1 *B. licheniformis* KUBK0006 keratinase and *B. pumilus* keratinase and commercial enzyme, respectively. It is worth noting that a higher protein content usually means a higher-quality protein source. As a result, the evidence shows that feathers may be used as a suitable supply of protein material. Furthermore, the enzymatic treatment of feathers involved milder reaction conditions than the industrially produced feather meal resulting in slightly less damage to the nutritional content of feathers. Apart from indications that enzymatically produced feather meal is a better protein source than industrially produced

feather meal, this increase could also be due to the enzymes themselves contributing to the higher protein content.

Table 3.6 Proximate composition of enzyme treated and industrially produced feather meal

Parameters	Raw feather (%)	Enzyme treated feather meal (%)	Industrially produced feather meal (%)
Crude protein	68.7 ±2.5	87.6 ±1.4	83 ±1.8
Crude fat	25.69 ±1.3	8.19 ±0.4	9.48 ±0.9
Crude fibre	1.17 ±0.3	1.28 ±0.02	1.78 ±0.4
Ash	1.42 ±0.2	0.74 ±0.07	2.42 ±0.1
Calcium	0.06 ±0.01	0.08 ±0.01	0.13 ±0.01
Sodium	0.03 ±0.01	0.24 ±0.02	0.09 ±0.01
Potassium	0.07 ±0.02	0.08 ±0.01	0.12 ±0.02
Magnesium	0.04 ±0.01	0.03 ±0.01	0.04 ±0.01
Phosphorus	0.1 ±0.02	0.05 ±0.01	0.04 ±0.03
Iron	0.01 ±0.0	0.01 ±0	0.01 ±0
Zinc	0.01 ±0	-	-
Manganese	-	-	-
Sulphur	1.22 ±0.02	1.96 ±0.04	1.22 ±0.7
Copper	-	-	-

Crude fat, an estimate of total fat content, includes true fat (triglycerides) as well as alcohol, waxes, terpenes, steroids, pigments, esters, aldehydes and other lipids. Fat is a high energy source after carbohydrates (Zainuddin *et al.*, 2014). The significant fat content in feathers (about 8%) shown in this study is indicative of a potential route for beneficiation of feathers. Eaksuree *et al.* (2016) reported crude fat content from 7.4 to 9.55% for feather treatments with different keratinases and a much higher fat content of 11.55% was recorded for feather treated with commercial enzyme. Industries can modify the percentage of components (especially ether extract) used to produce feather meals, justifying the difference between industrially produced feather meal and enzyme treated feather meal.

Fibre plays a role in binding water, cellulose and pectin and is essentially a feed material that digestive enzymes cannot hydrolyze (Ahmad *et al.*, 2019). The data in Table 3.6 indicate that chicken feathers contain negligible amounts of crude fibre (1.3%). This is to be expected considering that feather biomass, unlike cellulosic biomass, does not contain

cellulose, hemicelluoses and lignin. Tesfaye *et al*. (2017b) reported 2.5% fibre content for untreated feather, which is higher than the fibre content for raw feather observed in the current study. The difference in fibre content could arise from the source and variable composition of feathers. Feed intake of chicken could impact the fibre content of feathers and thus result in variable fibre content.

Ash is inorganic residue obtained after biomass combustion and is an approximate measure of the mineral salts and inorganic matter in feathers (Tesfaye *et al.,* 2017b). A much lower ash content (0.7%) was achieved with enzyme treated feather than industrially produced feather meal (2.4%), indicating that the enzyme treated feather meal is more easily digested. Zhou *et al*. (2020) reported a much higher level of 7.1% ash content for biodegraded feather meal using a cocktail of enzymes. This high ash content was attributed to the alkaline hydrolysis treatment, resulting in a large amount of salt. However, this excessive salt present in feather meal could endanger the health of the animals when included as feed ingredient. Despite using alkaline conditions in this study, a high ash content was not observed as compared to study by Zhou *et al*. (2020). Comparatively, Ajayi and Iyayi (2014) reported that feather meal contained 0.3 to 0.6% ash.

Mineral ions are inorganic substances, necessary for maintenance of certain physicochemical processes which are essential for life. Depending on the mineral, it is usually required in small amounts from less than 1 mg to 2500 mg per day (Soetan *et al*., 2010). Mineral ions, calcium, potassium, magnesium, phosphorus, iron, zinc, manganese, and copper of enzyme treated feather meal was similar to industrially produced feather meal (Table 3.6). However, enzyme treated feather meal contained a much higher sodium level (0.24%) than industrially produced feather meals (0.09%). This could be due to the alkaline conditions used for the biodegradation of feathers. In addition, sulphur content of biodegraded feather meal was 0.8% higher than industrially produced feather meal. This could be due to the high proportion of sulphur amino acids.

With the development of digestive physiology in monogastric animals, methods of evaluating feed nutritive value have been improved continuously. Seeing that the animal digestive experiments are tedious and expensive, the simulating technique of *in vitro* digestion of monogastric animals with the advantage of simple, fast and good repeatability was concerned. As shown in Table 3.7, *in vitro* protein digestibility was consistently higher for industrially produced feather meal than biodegraded feather meal. However, all amino

acids showed an increase in digestibility after enzymatic treatment compared to the raw untreated feathers.

Table 3.7 Amino acid digestibility of biodegraded and industrially produced feather meal

Digestible amino acids	Raw feather (%)	Biodegraded feather meal (%)	Industrially produced feather meal (%)
Alanine	59.62	69.58	74.41
Arginine	70.05	76.97	80.32
Aspartic	21.35	39.96	48.99
Cystine	-	15.01	32.89
Glutamine	49.96	63.02	69.35
Glycine	64.46	72.66	76.64
Histidine	40.46	55.60	62.95
Isoleucine	30.90	58.10	68.75
Leucine	38.84	62.24	71.55
Lysine	-	36.33	54.92
Methionine	28.13	55.96	67.02
Phenylalanine	64.09	73.88	78.63
Proline	57.83	68.16	73.17
Serine	47.58	60.62	66.96
Threonine	29.68	55.05	65.22
Tryptophan	64.59	77.75	82.69
Tyrosine	68.79	76.98	80.95
Valine	47.85	62.06	68.95

A similar study by Lakshmi and Lakshmi (2015) reported that the *in vitro* digestibility of raw feather, industrially produced feather meal and biodegraded feather meal was 24%, 32% and 72%, respectively. There was an approximately 2-3-fold increase in amino acids like proline and glycine followed by an approximately 2-fold increase in lysine, cystine, methionine and histidine content in biodegraded feather meal as compared to raw feather. Even though the digestibility values of biodegraded feather meal in this study were lower than industrially produced feather meal, there is potential for application of biodegraded feather meal. However, more research is required in this area to improve the digestibility values.

3.4 Conclusion

Novel keratinolytic enzymes that can degrade tough proteins like keratins and allow bioconversion of keratin-rich waste have been the subject of extensive investigation. Even though keratinases from various microorganisms have been extensively studied, there is still a great demand for new keratinases with improved industrial features such as greater catalytic effectiveness on keratinous substrates, increased durability at elevated temperatures, and increased pH values. The kinetics of feather degradation by the *P. aeruginosa* S-04 strain obtained from poultry waste were investigated in this study. The keratinase's biochemical properties were noteworthy, and the enzyme was discovered to be a solvent-stable alkali metalloprotease. The keratinase enzyme was active throughout a wide temperature and pH range. Furthermore, metal ions, surfactants, and high solvent stability can boost keratinase activity, indicating its potential for use in biotechnological applications such as feather degradation.

Estimation of various parameters of keratinase treated feather meal in comparison to traditional treatments of feather meal revealed that keratinase treated feather meal has particularly good potential to be used as a feed supplement. Enzymatic hydrolysis revealed that keratinase could effectively hydrolyze feather waste and maintain the nutritional value in terms of conserving the crude protein. Furthermore, *in vitro* protein digestibility and amino acid content was also maintained during enzymatic hydrolysis. Thus, this research served the dual purpose of controlling organic pollution as well as an alternative source for feed ingredients in animal feed. Further studies are recommended to assess the effect of this biodegraded feather meal on animal's performance and digestibility by using an *in vivo* approach.

4. CONCLUDING REMARKS

Chicken feather waste, a by-product of poultry processing plants, has become an interference as a result of its high rate of accumulation in the environment, especially in industrialized and urbanised societies. The common disposal methods of landfilling and incineration of poultry waste have many disadvantages such as loss of land space, environmental pollution, spread of infectious diseases, as well as the high cost incurred for these processes. Hydrothermal treatment of the feathers for the production of feather meal which can be used in animal feed has since been found to be a more environmentally sustainable approach. However, the nutritional value of the resulting feather meal is usually limited due to the harsh processing conditions used in hydrothermal treatment. These limitations include the loss of amino acids such as methionine and lysine, as well as the synthesis of non-essential amino acids e.g., lanthionine and lysinoalanine which causes undesirable nutritional effects such as decreased protein digestibility and availability of (essential) amino acids, as well as the possibility of severe side effects. Interestingly, keratinases are unique proteases that attack the recalcitrant keratin, a structural protein, that make up the majority of the feather structure. The utilization of microbial keratinase to convert the feather wastes into low-cost, high-value animal feed nutrients has, therefore, been identified to be a better alternative. Considering the beneficial attributes of the microbial keratinase, the present study was focused on the isolation of potent keratinase producers, followed by optimization of keratinase production, characterization of the keratinase enzyme with subsequent application of keratinase on feather. As a result, the following sections of this chapter highlight the study's numerous significant findings:

- A total of 43 bacterial isolates were obtained from several samples (inside poultry house, poultry waste, sludge and waste feathers) through an enrichment process using feather meal media under submerged conditions. However, out of the 43 isolates, only 21 isolates were found to show proteolytic activity on a skimmed milk agar plates. The qualitative screening of the 21 isolates on chicken feather meal agar further revealed their keratinolytic activity. The highest keratin-degrading bacteria was further identified by quantitative screening using chicken feather as the sole source of carbon and nitrogen. The isolate was later identified by 16S rDNA sequencing as *Pseudomonas aeruginosa* S-04. The nucleotide sequence was deposited to the National Biotechnology Information Center (NCBI) Genebank with accession number, MT626658.
- Studies on optimization of various physical and chemical parameters were performed using a basal salt medium with feather to optimise keratinase production. Keratinolytic activity was determined by the standard Sigma method using keratin azure as substrate. The optimal incubation period for *P. aeruginosa* S-04 keratinase production was found to be 72 h, and this incubation period was used in subsequent experiments. Although keratinase production was significant in the pH range of 7-10, optimum keratinase production was noted at pH 9.5. Studies on the effect of temperature (30-40°C) on keratinolytic enzyme production revealed 35°C as the optimum growth temperature. Optimization of feather concentration (0.5 - 5%) revealed that feather concentration at 1% level was ideal for optimum keratinase production. Further, experiments conducted with variable inoculum size (1 to 5%, v/v) revealed that an inoculum size of 1% gave optimum keratinase activity. Inclusion of additional carbon source (molasses, starch, maltose, glucose, sucrose and lactose) on keratinase production and extent of keratin degradation showed a decrease in keratinase production, upon the addition of starch, maltose, glucose, sucrose and lactose. This indicates that the presence of readily available carbon sources reduces keratinase production. The addition of molasses, however, increased keratinase production and was used in further experiments. The addition of nitrogen sources revealed a decrease in keratinase production for urea, peptone, and ammonium chloride. A marginal increase in keratinase production was observed during the addition of tryptone whilst the addition of casein recorded a 1.1 U/ml increase in keratinase production. Overall, the enhancement in keratinase

production was 1.97-fold under optimized conditions when compared to pre-optimized conditions.

- Keratinase purification was performed by ammonium sulphate precipitation followed by ion-exchange chromatography using DEAE column, concluded in 29% yield and a 2.4-fold purification. The molecular weight of keratinase of *P. aeruginosa* S-04 was estimated by SDS-PAGE analysis to be at ~35.5 kDa. Zymogram and SDS-PAGE analysis further revealed that the keratinase under study was monomeric in nature.
- Characterisation of the purified keratinase exhibited its maximum activity at pH 9.5 and 60°C temperatures. Keratinase from *P. aeruginosa* S-04 can thus be classified as alkalophilic and thermotolerant. The pH stability testing of keratinase showed more than 95% stability for pH 7 to 9.5 after 2 h of incubation. The keratinase also showed relatively good thermal stability from temperatures 4 to 40°C, retaining almost 80% of its activity at 40°C after 2 h of incubation. Metal ions like Fe^{3+} and Mn^{2+} stimulated enzyme activity whereas Na^{+}, $Mg^{2+}$and Zn^{2+} ions inhibited the enzyme activity. The least enzyme activity was found in the presence of EDTA whereas PMSF did not inhibit the activity, suggesting that this enzyme belongs to the metalloprotease family. Only reducing agent, dithiothreitol showed a marginal increase in enzyme activity and β-mercaptoethanol inhibited enzyme activity. Kinetic parameters of keratinase resulted in K_m of 7.62 mg/ml and V_{max} of 200 U/mg using the Lineweaver-Burk plot.
- Gravimetric analysis of feather degradation using keratinase of *P. aeruginosa* S-04 revealed efficient degradation of the feather within 24 h using 500 U of keratinase. The HPLC analysis of the feather hydrolysate showed the presence of 17 amino acids. In this regard, the amino acids with the highest concentration were glycine (36.4%), proline (8.5%), serine (8.1%), glutamic acid (8%), valine (5.9%) and leucine (5.54%). Amino acids, alanine (3.2%), arginine (4.9%), aspartic acid (4.5%), isoleucine (3.4%), phenylalanine (4.0%), threonine (3.1%) and tyrosine (2.7%) were also observed in appreciable amounts. However, lower levels of histidine (0.07%), methionine (0.45%), lysine (0.94%) and hydroxyproline (0.24%) were noted.

  This study has revealed a novel *P. aeruginosa* S-04 strain, with remarkable keratinolytic activity and significant potential in keratin degradation, thus

providing a new biocatalyst for biotechnological processes revolving around keratin hydrolysis. Further research could be focused on the expression of the *P. aeruginosa* keratinase gene on prokaryotic and eukaryotic hosts to develop a more robust strain with improved keratinase production to satisfy increased industrial demands. Moreover, the use of statistical optimisation using Plackett-Burman Design or Response Surface Methodology for improved keratinase production should also be another potential research area to be explored. Additionally, only a few keratinase-based formulations prepared predominantly from *B. licheniformis*, beneficial to increasing the nutritional value of livestock feeds, have been commercialised so far. The commercialisation of more products with similar nutritional benefits would offer the agricultural industry more choices to enhance the feed quality. Furthermore, due to the availability of more keratinase-based formulations will ultimately lead to competition among suppliers resulting in a reduced price of the product which ultimately results in the cheaper formulation of livestock feeds. Further investigation into the structure of the keratinase as well as the mechanism of keratinolysis needs to be elucidated to fully understand the function of the enzyme and the process of keratinolysis.

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