Isolation & Characterization of Keratin Degrading Bacterial Isolate

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MICROBIOLOGY

By

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Candidate's Declaration

I hereby declare that the work reported in the M.Sc. dissertation thesis entitled **"Isolation & Characterization of Keratin Degrading Bacterial Isolate", in partial fulfilment of the requirements for the award of the degree of Master in Science in Microbiology submitted in the department of Biotechnology & Bioinformatics,** Jaypee University of Information Technology, Waknaghat, Himachal Pradesh, India, is an authentic record of my work, carried out under the supervision of **Dr. Ashok Kumar Nadda (Assistant Professor)** at Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat, Himachal Pradesh-173234, India.

I also authenticate that I have carried out the above mentioned project work under the proficiency stream.

The matter embodied in this dissertation thesis has not been submitted this work elsewhere for the award of any other degree or diploma.

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CERTIFICATE BY SUPERVISOR

This is to certify that the work reported in the M.Sc. dissertation thesis entitled **"Isolation & Characterization of Keratin Degrading Bacterial Isolate",** submitted by Pramita Ghosh (217857), at Jaypee University of Information Technology, Waknaghat, Himachal Pradesh, India, is a bonafide record of her original work and has not been submitted elsewhere for any other degree or diploma programme.

Pramita Ghosh (217857)

The above statement made is correct to the best of my knowledge.

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ABSTRACT

Chicken feather wastes are generated as one of the abundant by product, by the poultry meat processing units. These contains 90% of keratin in their composition. So, undoubtedly these are the promising source of amino acids and the bioactive peptides of the proteins. But unfortunately, most of these wastes are disposed by landfilling and incineration, instead of being their conversion into value-added products. Keratin is one of the structural and insoluble protein, that provides the structural toughness to the animal feathers. It also serves its fundamental protective capabilities, in the outermost dead layer of epithelium of the epidermis. It has recalcitrant properties because of the strongly stabilized and tightly packed protein chains. It is resistant against classical hydrolysis techniques (by proteolytic enzymes, like papain, trypsin and pepsin) as well. So, before enzymatic treatment of feathers, they are pre-treated by the alkaline agents, in microwave or autoclave. On the other hand, keratinase is the member of proteolytic enzyme family, that endowed with the degradation of the keratin into amino acids and peptides. In the space of green industries, their potential multifaced implementations are gaining traction.

CHAPTER 1

INTRODUCTION

Nowadays, modern science is favoring the use of waste biological materials to form value-added products, biotechnologically. Those can efficiently replace currently available synthetic products. In today's globe, most countries are implementing those biotechnological approaches, to improve their economic and environmental conditions [1]. So, the need for the continuous research for the formation of biologically modified new products, through efficient approaches, is developing rapidly. In this context, microbial enzymes are predominantly occupying a vast space, in bio-economics. However, a broad range of microbial resources and their viable metabolites, are still remaining under-exploited. Due to the lack of proper studies and research evidences. Within these, microbial proteases hold 60% of enzyme market. And keratinase is one of them [6,2]. In biotechnology, keratinase hold its position as one of the most promising candidate. Because of its catalytic and robustic properties [20]. The necessity of feeding the globally arising population, is directly resulting on the incrementation of biological waste materials. Those are affecting the environment by generating an inevitable amount of pollutants, having varying pollution degree. The law of environmental protection, promotes the efficient management of recalcitrant wastes, by transforming them into value-added essential products, through recycling technologies. Protein hydrolysates of high-quality can be obtained from the keratinous biomass, by using microbial valorization mechanism. However, the amount of microbial keratinases, varies from cultivation media to media, even under same optimized conditions. The precedence is taken by the diversified range of keratinolytic microbes (especially allochthonous and autochthonous microbial strains, belonging from various ecological niches), as they are considered to be the most viable agent, used for the bioconversion of keratinous wastes. And they possess as such no virulence traits (like dermatophytes have). On the other hand, these keratinolytic wastes materials are heat treated and then mixed with other some animal waste materials, then milled into power like consistency, to make them usable as the protein supplement. And then by mixing it with the food substance of animals. This is known as the "animal flour". However, recent studies have demonstrated that this can cause some severe diseases (like Creutzfeldt Jakob disease, swine fever, mad cow disease, spongiform bovine encephalopathy) in animals, being one of the best enigmatic (prion) carriers. Nevertheless,

this problem can be resolved through the incineration process, where prions are broken down [25]. In current green economy, the potential biotechnological applications are bestowed by microbial keratinases, because of its unique properties. Significantly, the bio-economical landscape can be revolutionized through the continuous exploration of new keratinases, having unique functions and novel properties. For example, thermostable protease, isolated from Bacillus licheniformis (a typically thermophilic bacteria), can be mentioned. This is widely used in detergent industries [21,24]. Inspite of owing high amount of disposed poultry wastes each year, only a very small amount of these materials is used for developing insulation materials and other products. They contain nearly 70% of high-valued amino acids, vitamins and growth factors. And approximately 85% of its content is in the form of crude protein. As these wastes are highly resistant against hydrolysis, so before applying degradation techniques, they undergo some pre-treatment methods, that prepare them to be easily degradable. Among these pre-treatment methods, chemical methods (like application of alkaline and acidic solution) and physical methods (like puffing and pressure) are mostly applied. All these techniques, have certain drawbacks, such as they damage the products sustainability and consume high amount of energy [23].

As dehairing agents, huge number of chem-based products are used, in the tannery industries. Fatty emulsified matter, soda-lime, sulphide, salts, chromate, lime waste liquor and solvent flashy, are used as the main component of all these dehairing agents. These are not only environmental pollutant but also hazardous for both animal and human health. Some of these are corrosive as well. Recently, government have forced some of the tanneries plant to close them down, to prohibit their adverse impacts on human health and protect the environment [24]. On this context, the application of microbial keratinase, has been observed. They also retain the native structure and properties of products. Microbial degradation, highlights on the identification and cultivation of suitable microbial strain. They are screened in appropriate culture media, to obtain high yield in low time period. Furthermore, the enzymatic characteristics, strategies applied for enzyme purification, and the keratinases' heterologous expression, are been reported currently. But this biodegradation process of feather, faces some challenges, during the time of scaling up, in the fermenter.

Till date, in industries to achieve the degradation of chicken feathers at about 2% (w/v), in a fermenter of 14L, the culture of DB 100 (p5.2), a recombinant strain of *Bacillus subtilis*, has been developed. Mainly soluble peptides and some amino acids are produced, as the result

of feather hydrolysis by keratinases. Those products, are observed to show antioxidant properties and play vital role to protect human and animal from the free radicals. Being the potential source of antioxidant and protein, they are used commercially. And are efficiently replacing currently available chemically derived antioxidants [3].

CHAPTER 2

2. REVIEW OF LITERATURE

2.1 Keratin

Keratin is an important ubiquitous, fibrous, water insoluble protein, present in nature. From the body parts of humans and animals, keratin can be isolated, readily. Also, can be obtained from the sources, like feathers of birds, hair, scales, nails, hooves, wools and stratum corneum of mammals, intrinsic part of epidermis and other skin appendages [5,1,20]. In the epithelial cells of the digestive organs (intestines, gallbladder, pancreas and liver), and in the silk obtained from silkworms and the webs of spiders, the presence of keratin has also been reported. The presence of keratin, in goblet cells, hepatobiliary ductal cells, acinar cells, small intestine's enterocytes, hepatocytes, colon, oval cells, has also been observed. In broad spectrum, keratin is present starting from the daily house hold dust to the very common contaminants of laboratory [12,20]. In several fields, chicken feathers are significantly used as the biological source of keratin. In feather, the amount of available keratin possesses 20kDa molecular weight. It is comprised of cysteine, serine, proline and glutamine like vital amino acids and also an assortment of 17 types of amino acids. These amino acids are-histidine, lysine, glycine, arginine, valine, alanine, phenylalanine, tyrosine, aspartic acid, glutamic acid, proline, serine, threonine, methionine, leucine, isoleucine and cysteine [7,20]. The degradation of polypeptides and the dis-integration of the keratin's insoluble macromolecules, are largely relies on the extracellularly secreted microbial keratinases [20]. The proteolytic degradation of cysteine-rich pig bristles, hooves and animal feathers are opening up the path for the formation of glutathione, by the production of cysteine-rich oligopeptides.

This glutathione is further hydrolyzed in cysteine, glycine and glutaminate, by the gamma-glutamyl (take part in balancing the intracellular cysteine level). This gamma-glutamyl belongs to transpeptidase family and are isolated from *Bacillus* sp. Then microbial cells uptake that resulted glycine, glutaminate and cysteine. The enhancement of keratin degradation can be done by the upward regulation of transcriptase gamma-glutamyl in the medium [19]. In the primary sequence of keratin, the high amount of cysteine, differentiates it from another fibrous proteins, like elastin and collagen [9]. In vertebrates, there are 54

conserved genes, those encodes for keratin only. During the mitotic cell division, the synthesis of keratin takes place [1]. Keratin is well-known for its withstand degradative nature, by some common types of proteases, like pepsin, papain and trypsin [20]. In terms of abundancy, in nature keratin is holding third position, just after chitin and cellulose. Though it is unable to transport within the cells but it plays a very vital role as the protective barrier between the environment and animal cells. It also provides toughness against both biotic and abiotic stresses, to the animal cells and organs. Also, keratin is involved in a series of functions in epithelial tissues, which includes both the structural and non-structural functioning of cells, along with cell signaling [1,18].

The high number of hydrogen bonds make the structure of Keratin very stable (because of tight packaging of α helix and β sheet of the polymer chains, that further form the supercoiled structure), highly recalcitrant to degradation (because of orientation and inherent interactions within the bonds) and resistant against chemicals as well [1]. This structural stability is also provided by the packaging of polypeptide chains, forming intra or inter cellular cross-linked disulfide bonds, through hydrophobic interactions, hydrogen bonds and cysteine residues. Based on the secondary structure of polypeptide chains and the composition of amino acids, keratin can be classified into two categories. They are -

(1) α -Keratin - Contain less than 10% cysteine. Observed majorly in epithelia of mammals. Particularly remarkable for its flexibility, adaptability, strength, toughness, versatility, insolubility, stability, and elasticity, even during the exposure to mechanical stress. Polypeptide chains of cysteine, leucine, arginine and hydrophobic amino acids, like- valine, alanine, methionine, isoleucine and phenylalanine are there in the right-handed structure of α-helix of α -keratin. α-keratin, is molecularly composed of two curled loops alpha helical peptides of keratin, both consisting the structure of a directional head (has a typical secondary structure, because of glycosylation and phosphorylation) and a tail (plays a vital role in the organization of intermediate filament), forming a dimeric coil structure. The coiled amino acids, then form the structural fibrous protein, i.e. similar to the α-helix of an ordinary protein structure. Through disulfide bonds, cysteines are bonded together and form 45 nm long dimers. Those dimers are then coiled by the curling of two polypeptide chains. A protofilament structure is formed, by the arrangement of bonds within one dimer and the other dimer's termini. Then a protofibril is formed by the clusters of two protofilaments. And further an intermediate filamentous structure is formed by associating these four protofibrils. These intermediate filaments act as the essential subunit in the structure of α-

keratin and impacts on its quality (specifically on the keratin's hydrogen bonds) and its water content. Compromised firmness and the quality of the keratin, is the result of high water content, present within the intermediate filaments. A super coiled structure of approximately 7 nm diameter, is formed by the shrinked intermediate filaments. That can be further classified as, Type I, i.e., acidic and Type II, i.e., basic. Within the high residual concentration of phenylalanine, glycine and tyrosine, these are submerged. In mammals, the structure of α-keratin is differed by the variations of intermediate filaments. Through the transcription and translation when the synthesis of protein occurs, the α-keratins are formed. But they die just after their maturation and form the keratinized tissue, i.e., the non-vascular unit [20].

(2) β-Keratin - Posses 10-14% of cysteine content. Present in reptilian and avian animal tissues (like claws, scales and feathers). Provides enhanced protection, rigidity and prevent the structure from outer attack as well [5,12,20]. In the skin appendages, within the epidermis's pre-corneous layer β-keratin is found. In cells, β- keratin is able to mask and replace α-keratin, by forming a hard layer of corneous. β-keratin is little less elastic but more easily accessible to keratinase enzyme than α-keratin, because of less number of disulfide bonds within the fibril molecules (3-4 nm in length). Those molecules are supramolecularly formed by the pleated β-sheets with the help of filamentous framework. And are associated by the C and N terminal domains, crosslinked through disulfide bonds [20].

Because of high content of sulphur, nitrogen and carbon, keratin-containing wastes (mainly animal feathers and hairs from poultry industries) are considered as one of the major resources of some economical products. In both these structures, the fibrils of keratin are parallelly twisted to form macro and micro fibrils. And confer stability to the fibers of keratin [9,19,20].

Keratin can be further classified, based on their Sulphur content. They are - (i) Hard keratin, containing high amount of disulphide bonds, that form the tough structure of keratin, found in nails and hooves. and (ii) Soft keratin, the low content of disulphide bonds results in the pliable conformation of the keratin, found in skin. The stiffness and viscoelastic property of a particular keratinous substance is defined by the degree of hydration of keratin molecules [6,8]. Keratins can be further classified based on the pI (Isoelectric Point). They are- type I and Type II (basic and acidic). This classification can be affected by the post-translational modifications. In case of transformed modification site, the intermediate filaments will be

prompted as failed, because of lacking the post-translational alterations. Subsequently, the status of altered post-translational modifications will results in the adaptation of keratin [20].

2.2 Keratinase

Keratinase is a monomeric enzyme and a member of protease family (also known as endopeptidases), that effectively degrade keratin into non-nutritive amino acids, like- lysine, alanine and lanthionine. This degradation occurs by identifying hydrophobic substrates and also by cleaving the non-terminal peptide bonds of polypeptide chains. Depending on the catalytic activity, keratinases can be classified in the following subfamily of chymotrypsin like and subtilism-like proteases, of S8 family (serine protease) [4,3,7].

Inspite of having very complicated structure, the disulfide bonds of keratin-rich poultry wastes are easily breakable by keratinase enzyme. And thus, make it accessible for manufacturing diverse range of products, those are usable in various field [1,7]. Especially the crude keratinase significantly promotes the digestibility of amino acids, present in animal feathers. At present, commercially available keratinases are classified under the family of S8. But not all the proteases those belongs to S8 family, are able to degrade keratinous wastes. [13,19]. From the M12 metalloproteases family, *Myroides profundi* (a deep-sea bacterial strains), has been reported to have synergistic role and elastinolytic activity, against collagen (collagen hydrolysis). Later on, through an optimized scale up process, the specific properties of collagen structure were confirmed [19]. For the complete conversion of keratin into amino acids and peptides (used as the supplementation of proteins in the animal feed formulations), several keratinases are needed. Because of preferences of different cleavage site. This keratin degradation includes two steps. These are- (1) release of keratin peptide and (2) the degradation of that peptide. Some reducing agents or disulfide reductase, act as the catalysts in that reduction reaction [1,7].

However, the entire degradation mechanism of the keratinous substrates, are not completely elucidated. But it can be stated that, the reduction of the cysteine bridges significantly impacts on the whole degradation process. It has been conceived that the cleavage of peptide bonds and the reduction of disulfide bridges, make keratinous materials enzymatically dismembered. Biochemical assay of keratinase has proved its activity (hydrolyze) against the both soluble (such as hemoglobin, bovine serum, casein, albumin and gelatin) and insoluble (like collagens, nails, azokeratin, elastin, horns, hairs, silks, feathers and wools) proteinaceous substrates. And also, its specificities against these substrates [2,4,1,7,14].

Because of this specific nature, the keratinase emerges itself as a potential substitution of proteases. And are widely used in detergent and leather industries and generate contamination free wastes [18]. It has been observed that an array of microbes from diverse ecosystem, have been associated with keratinolytic proteases activity. Where they use the provided media as the major source of carbon and nitrogen, required for their growth and metabolism. Till date, there of 14 types of microbial keratinase has been reported, those belongs to proteases family [2,4,1,7,14]. Identified keratinases are genetically characterized by sequencing KerA obtained from *B. licheniformis* PWD-1 [7]. The microorganisms secret their keratinase enzyme into the extracellular matrix, once they are cultured in keratin-containing media. There are some organisms, those are able to secret keratinase both intracellularly and extracellularly, simultaneously [1].

2.3 Microbial Keratinase

The biological degradation of keratinolytic wastes, involving microorganisms includes a sequence of certain mechanisms. Those are- Adhesion, Colonization and then the Amplification of the enzyme by the deprivation and break down of the substrate. Primarily, the microorganisms degrade the keratin by sulfitolysis (the breaking down of disulfide bonds) and proteolysis (proficient proteolytic attack on substrate, by utilizing the extracellularly secreted keratinase enzyme) followed by the consumption of the non-keratinous lipid elements. In case of fungal application, this degradation process starts with the mycelial growth of fungi over the surface of keratinolytic wastes, followed by sulfitolysis and then proteolysis [12,20].

Being protein rich substances, the decomposition of keratin includes costly techniques, as it is highly resistant towards any physical and chemical agents. To design efficient (in terms of environmental and economic friendly decomposition methods, while reducing the amount of released carbon dioxide) several others methods has been invented. They are- by using UV radiations, acids, bases, high temperature and pressure, enzymes etc. [7].

In Hydrothermal Method, in the presence of any alkali or acid, high temperature of about 80-140°C and high steam pressure of 10-15 psi, is applied. Free amino acids, oligopeptides and even water-soluble polypeptides, are the yields of this process. The only limitation of this technique is the formation of some non-nutritive amino acids, such as from lysine and cystine the formation of lanthionine (the content of lanthionine is inversely proportional to the amount of amino acids, present in the feather) and lysinoalanine, respectively. It also

causes the loss of some essential amino acids, like- tryptophan, lysine and methionine, during the partial or complete hydrolysis of keratinous wastes [8]. Another satisfactory qualitative conversion method is the alkali hydrolysis method. It employs the hydrolysis of keratin fibers. The application of 0.1N NaOH results in the rapid dissolution of wools. But this method is less effective than acid hydrolysis method. Also destruct some of the amino acids, like- cystine, threonine, arginine and serine. Another method is Stem Explosion (SE), a subtype of keratin hydrolysis by hydrothermal method. This method includes, rapid release of the pressure after it has been shortly exposed to high temperature steam. And as a result, the constitutive components released out. The drawbacks of this method are the low yield (of only about 18.66%) and the destruction of crosslinks and crystals of the macromolecular chains of fibers. This destruction results in the alterations of the fiber's entities. And further that results in the reduction of moisture regain, strength and its solubility in caustic solution [8].

An excellent amount of yield (63.5-87.7%) has been observed from the degradation of chicken feather, in the ionic liquid solution of 1-Butyl-3-Methylimidazolium Chloride ([BMIM]Cl). Another convenient and rapid method employed for the extraction of protein from human hair (for its further examination of biochemical properties) is oxidation and reduction method. In this method the conversion of keratin containing wastes into hydrolysates takes place. This is done by removing the proteins, present in the human hair's cortex part, by using mixture of urea and thiourea, as reductant. The resulting fraction of human hair majorly consists of proteins associated with keratin (molecular weight 6-30kDa) and α-keratin i.e., hard (molecular weight 40-60kDa). Further, keratin can be achieved by the selective purification of KAPs (Keratin Associated Proteins), by ethanol [8].

The nature has been blessed by the planet earth with the abundancy of keratinophilic soil microflora, that can be readily isolated. They are present in variable niches, thriving under diversified environmental and ecological conditions. To screen the keratin degradation by microbial keratinase, monitorization of some factors are mostly required. They are- temperature, weather, composition of keratin, the water content, the culture medium and the culture time, concentration of feather etc. The variations in the growth nutrients, present in the culture medium, plays an important role in the expression of the particular metabolites encoding gene (gene of interest) [1,7,8,12]. The presence of keratinous substrates induced the expression of these genes and also the activates of the redox reaction. The level of substrate's degradation and the degradative product's concentration shares an intimate

correlation [3,1,7]. Disulfide reductase and keratinase, initiates the keratin degradation, by unstabling the resistance and the structural stiffness of keratin against proteolysis. This is occurs through the disruption of disulfide bonds [20]. On the other hand, the alkalization of the fermentation medium is also important, for the enhancement of sulfitolysis (decomposition of keratin). Cysteine residues are produced as thiol, by the break down disulphide linkages and proteolysis [3,1,7]. Thermally stable keratinase is obtained from few actinobacteria, those having potential industrial applications. These keratinases can be further diversified based on the following parameters. They are - optimal temperature (majorly between 37℃ - 65℃), optimal pH (generally between pH 7.0 - 9.0), and molecular weights (ranging between 17 - 240 kDa), metal ions (Pb^{2+}, Hg^{2+}, Cu^{2+}, Fe^{3+}, Ni^{2+}, and Cd^{2+}) [3,1]. Also, the presence of metal chelating agents, like ethylenediaminetetraacetic acid (EDTA), organic ligands (like 1,10- phenanthroline), reducing agents, solvents and surfactants, in the medium impacts on the stabilization, inhibition and enhancement of the keratinase activity. Through the implementation of certain amino acids like threonine, valine and methionine, the enzymatic activity of keratinase, can be determined [17]. Its activity has also been studied to be inhibited by diiodo propyl fluorophosphates (DFP), phenylmethanesulfonylfluoride fluoride (PMSF) [7].

On the other hand, the presence of some surfactants like Tween 80, Tween 20, Triton X-100, some organic solvents, like isopropanol, DMSO and methanol, and some reducing agents, like β-marcaptoethanol, cysteine, sodium sulfate and DTT (dithiothreitol), promote the stabilization and also keratinase activity [7]. The mechanism of keratin degradation can be predicted through bioinformatics, by the accumulation of the genome sequences of the microorganisms. But even today, no keratinase is there that is able to completely degrade and solubilize native keratin. Only a small amount of keratinous substances can be fully decomposed [8,10]. That further results in the significant reduction of the environmental and health hazards of animals and human [1]. The easiest degradation method is the enzymatic lysis (by using proteases). However, the most popular and robust technique, reported till date is the thermal degradation (pyrolysis). The only limitation of this method is its high energy demand and costly technology [7].

Among all the keratin-degrading strains, *Bacillus* is the predominant one in respect of producing high yield (highest enzymatic activity was observed, 156 U/ml). Overexpression of keratinase (compared to other species of feather degrading organisms) and also the reduction the cost and time, has been observed in *Bacillus* [5,9,17]. Serine (from

family S8) was the first described protease, was isolated from *B. licheniformis*. Also, *Bacillus*, possess the potential to prove itself as the best candidate, that can be used in different sector of industries [18,19]. But till today, in GenBank only two keratinase genes have been successfully deposited after sequencing of *Bacillus cereus* [13]. *Bacillus subtilis*, *Bacillus cereus*, *Bacillus lichenifomis*, *Bacillus pumilus* are mostly found. Apart from these - *Vibrio*, *Actinobacteria*, *S. albus*, *Streptomyces pactum*, *Brevibacillus*, *Pseudomonas*, *Serratia*, *Fervidobacterium*, *Rummeliibacillus*, *Achromobacter*, *Keratinibaculum*, *Rhodococcus*, *Microbacterium*, *Aeromonas*, *Sporosarcina*, *Burkholderia*, *Exiguobacterium*, *Stenotrophomonas*, *Meiothermus*, *Amycolatopsis*, *Geobacillus*, *Hoolocks*, *Paenibacillus* are there as well. Examples of some non-sporogenic keratin-degrading microorganisms are - *Lysobacter*, *Kocuria*, *Fervidobacterium*, *Nesterenkonia sp*. Among the gram-negative bacteria *Chryseobacterium*, *Xanthomonas*, *Stenotrophomonas*, and *Thermoanaerobacter*, needs to be highlighted [1,5,15,16,20]. It has been reported that *Actinobacteria* can produce thermally stable keratinase, and can readily be used in the industries. Particularly *Arthrobacter*, *Bevibacterium*, *Nocardiopsis*, and *Streptomyces* has been observed to exhibit significant keratinolytic activity [1,5].

In hooves and feathers, there are certain types of saprotrophs fungi (for example- *Onygena corvina*). Those have been shown to possess wide spectrum of keratin-degrading enzymes.

Keratinase from some strains of fungi, has also been isolated from human and animal tissues, to degrade keratin. For example- *Aspergillus*, *Rhizomucor*, *Paecilomyces*, *Doratomyces*, *Trichoderma*, *Trichophyton* mentagrophytes, *Fusarium*, *Acremonium*, *Onygena*, *Cladosporium*, *Microsporum canis*, *Microsporum gypseum*, *Lichtheimia*, *Aphanoascus*, *Chrysosporium*, and *Scopulariopsis* [1,5,15,16,19,22].

Among fungi dermatophytes are been reported for having extensive keratinase activity. And the secreted keratinase, can be obtained easily. However, all these keratinolytic fungi, appears to be slow in the degradation process. But for waste conversion under industrial setting they are preferred over bacterial keratinases, because of low cost and easy accessibility. With the association of keratinase, these keratinolytic fungi produces sulfide, that is further utilized in the sulfitolysis process. By this process, the path of keratin's easy degradation is paved out. This is occurs by the breakdown of cysteine's disulfide bonds and also by producing proteolytic enzymes [22, 20].

Table 1and Table 2 Name of the Microorganisms and Fungi, Their source and Name of their Substrates

Table 1

Name of Microorganism	**Source**	**Activity of enzyme**	**Reference**
Bacillus cytotoxicus	Hot springs	Azocasein as substrate	25
Bacillus Subtilis	Poultry feather wastes	Casein agar as substrate	26
Chryseobacterium aquifrigidense	Soil of poultry wastes disposal site	Skim milk agar as substrate	27
Pseudomonas aeruginosa	Soil	Skim milk agar as substrate	28
Streptomyces Pactum	Wastewater of poultry processing unit	Keratin azure as substrate	29

Table 2

Name of Fungi	**Source**	**Activity of enzyme**	**Reference**
Chrysosporium pannicola	Sewage sludge	Sabouraud dextrose agar as substrate	30
Microsporum gypseum	Sewage sludge	Sabouraud dextrose agar as substrate	30
Chrysosporium tropicum	Chicken feathers	Sabouraud dextrose agar as substrate	31
Aspergillus clavatus	Soil	Hammerstein casein solution as substrate	32

Trichophyton rubrum	Clinical isolates	Keratin azure as substrate	33
Aspergillus niger	Soil	Keratin azure as substrate	33

Production Condition for the microbial keratinases

No specific culture method has still been established. Because of physiological diversity in within the keratin-degrading microbial strains [15,19]. After the isolation of microbial strain, from different environmental resources, on particular type of artificial growth culture they are cultivated. During their growth, to obtain high yield of keratinases optimized conditions are maintained sincerely. For the growth of keratinophilic microorganisms, commonly used media is skim milk agar. Whereas, for the cultivation of fungi, sabouraud's dextrose media is used [22]. As per recent studies, high degradation potential of feathers has been found in mesophilic microbial strain (for example, microorganisms belong to the group of *Cytophaga-Flavobacterium*). They consume less amount of energy than that of generally used thermophilic strains [13,14]. Fungal strains demand more time than bacterial strains for keratin degradation. Approximately, 4-8 weeks are needed, for the fungal growth. After the 8^{th} weeks of cultivation, fungus can degrade 40% of keratin, under optimized conditions [22]. The production of microbial metabolites, are influenced by the following studied factors, availability of growth nutrients in the culture medium, culture conditions and the inherent cell precursors. The variations in the physico-chemical conditions of the fermentation, depends on the genetic diversity of the keratinolyitc microorganisms [5,7]. The yield and the sustainability of microbial keratinase can be affected by the prolonged period of fermentation. According to the report, there is slight difference between fungal and microbial mechanism of keratin degradation, because of the different sequence of amino acids [1,6,20]. To enhance the activity of microbial keratinase, slight genetic modification is required. The best and cheapest way to obtain that, is the removal of inhibitory conditions. Here, non-reusable and immobilized keratinase can be opted. These immobilized enzymes are well known for their high stability than the free enzymes. Recent advances in the field of enzyme technology, have proved that it is absolutely possible to trigger several entities of an enzyme, simultaneously. Implementation of different techniques of genetic engineering, make it possible. These approaches are- truncation, DNA shuffling, site-directed

mutagenesis, directed evolution and saturation mutagenesis [9]. It would be highly beneficial to discover easily accessible microbial keratinases, by standardization and well-characterization. And this can even create a benchmark, in the field of modern enzyme technology [10]. Nowadays, the extraction of the bacterial keratinases in in-vitro conditions, have been implemented in the multiple sectors of sewage treatment, for the bioremediation [17].

Biotechnological approaches for the degradation of feather meals, by improving its quality and quantity, few steps are been employed. These are - the denaturation of disulphide bonds by cleaving it and then substrate's proteolytic hydrolysis. This hydrolysis can be performed using the microbes (example- Actinomycetes). Those are able to degrade the poultry wastes, through fermentation process and enzymatic reaction [7]. The following two steps, i.e., the synthesis of the enzyme and then the hydrolysis, are drastically favored by certain conditions, hence staging is essentially required. A step, involving a membrane of cross-flow ultrafiltration, physically differentiates previously mentioned two steps. Cyclically, these two steps are repeated to obtain the semi- continuous operation [11]. In its infancy, the keratin degradation by enzymatic hydrolysis, is an industrial process. Hence, it is desirable to lead the degradation process to be performed at loadings of high amount solids. And that will be results in minimizing the energy consumption, size of the bioreactor, amount of process water. While simultaneously maximizing the amount of product tier [11]. Apparently, during the processing of poultry products, several types of by-products are also produced. Those are inedible and unfit for any other applications of human welfare. All these wastes materials, plays a very prominent nutritious home for the microorganisms and helps in resulting their toxicity. Hence, they are potential danger point for the human and animals. Considering all the drawbacks of mentioned techniques, microbial approaches appears to be the most viable option [12].

Apart from these, effective production of keratinase and also the degradation of feathers, are been studied using various techniques. To ensure no modification of the native structure and conformation of the enzyme, the fermentation process should be optimized [7]. On this context, several different approaches (both statistical and classical) have already been adopted. The statistical approaches are somehow dependent on the classical approaches. On the other hand, only one variable is considered at a particular time (OVAT, One Variable At a Time). However, there are certain dis-advantages of this technique too. This method is unable to provide the interaction patterns within the considered parameters. Therefore, it has

been shown that the fusion of both these methods is able to affect bacterial productivity, significantly [13].

Based on the solubility, fractionation of the protein is done. And several electrophoretic and chromatographic methods are opted for their purification. They produce ammonium salts and free amino acids, like cysteine and methionine, by cutting the bonds of keratin. Under microscope, this hydrolysis method has been studied. It includes the adhesion of microbes on the feather as the very first step, followed by penetration. And then the separation of its barb into power like gelatinous mass. After that the degradation and rupture of the feather into needle-like structure, takes place. Later on, the production of keratinase can be obtained through the solid state or submerged fermentation method. By these fermentation methods, the conversion of non-soluble keratin into polypeptide and the production of keratinase by the microorganisms, can be determined. Having the diversified range of enzymes, microbes in the fermentation media, secrete several bioactive compounds, after using free-flowing liquid substrates. Hence, in the form of feedstock formulations, the crude hydrolysates can be used. Commercially, to improve the degree of hydrolysis upto several folds, cocktail of microbes, can be used [7,20]. Therein, a very strong incentive lies to optimize and stimulate high-fidelity dynamic processes. This takes place by the successful parameterization of a model of keratin hydrolysis in lab-scale. This model designing is necessary in terms of valorization of keratinolytic wastes management. So that, it can further aim towards the exploration of future advances, regarding the precise explanation of effects of apparent inhibitors at higher level of substrate concentration [11].

Being rich in amino acids, keratinous wastes can adversely affect environment, atmosphere, soil and even water resources, if they are not treated properly, after disposal. According to the statistical report every year throughout the world the amount of chicken is slaughtered for meat purpose is almost around 58×10^9 [8]. And worldwide the annual amount of feather wastes is almost about $8\text{-}9 \times 10^5$ Tons. The frontline countries, in terms of producing highest amount of keratinolytic wastes are- Brazil, United State of America, India and China [12]. On the other hand, this rapidly increasing amount of unwanted and concomitant waste have urged its utilization as the great protein (posses ±90% crude protein, 66.2% more than fish and 42.5% more than soybean) and amino acids rich, low cost resource [5]. Technical approaches are been implemented for the production of diverse range of value-added products. That also enables a sustainable waste management system. Examples of such products are pharmaceuticals, chemicals, cosmetics and skin care products having anti-aging

properties, supplements of animal feeds, bio-degradable foils, glues, films, biofertilizers, cleaners (dusters made up of feathers), bedding and decorative items (in very minute quantities) and even to produce some of rare amino acids (like proline, serine and cysteine) [7,8]. In crop production as an important source of nutrients, animal-based wastes can be used (though till date, this implementation as the supplementations of dietary protein, is not so widely distributed). Promotion of green economy, through the proper utilization of robust functionality of keratinases', are highly in interest [14,20,24].

According to recent reports, keratinase can be effectively used to remove dag (recalcitrant of the deposits, consists of organic matter. Such as, straw, feces, urine, feed, soil and hair) from sheep, cattle and goats. It basically weakens the interactions between the dag and the animal hair. Hence favor the easy removal of dag by washing [9,10]. In modern medicinal science, the implementation of keratinases, have proven to have potential efficacy in the treatment of human health issues. The inactivation and break down of prion proteins those are misfolded, has been observed. Researchers are contributed to understand the possible connection between the pathogenicity of prion with specific types of dementia [10].

These keratinous wastes are not only derived from poultry sources but also from human activities, like barber shops, parlors, tannery industries and municipal drainage. These cause stream pollution and environmental imbalances as well [7].

In nature it can lead to further decrease in environmental diversity, eutrophication and soil acidification, due to the over nitrogen deposition. Considering these issues, there are following methods, accepted as the keratinolytic wastes management methods. Those are- (1.1) Incineration, the combustion and further conversion of keratinolytic wastes mostly into water and CO_2, above 850℃, to destroy potential source of contamination. This method results in the emission of bad odors and some of toxic gases such as sulfur dioxide, phenols, nitriles, pyrroles, pyridines, carbonyl sulfides, hydrogen sulfides and ammonia. (1.2) Landfilling, the economic investments, associated with the disposal and management of keratinous wastes are extremely high and the accessibility of landfilling space is decreasing as well [7,8]. The leachate of landfilled wastes material can contribute to the increasing of nitrogen content in the surrounding soil. And it will result in harming of ecosystem by leading the algal blooms. (1.3) Mechanically grinding of wastes, after hydrolyzing it under pressure, heat and drying. Though this grounded powder can be applied as the source of nitrogen in soil enhancer organically and in animal feed, but it has certain limitations. Those are loss of several amino acids, because of extreme high heat treatment. And lastly (1.4)

Composting of wastes, an aerobic biological approach, for degradation of wastes and using the ultimate composted product as biofertilizer [12].

Recently, the novel bioeconomic initiatives are focusing on the upgradation of decomposition mechanisms. This minimize and recycle poultry abattoirs. This is extensively developing as the last alternative. Also attracting the scientists for further research and developments in this field [7,8,12].

2.4 Production of keratinase

As per the compilations of many literature, the production of keratinase has been observed in the shake-flask. Once, the keratinolytic microorganisms are cultivated in the keratinous substrate containing medium, they secret extracellular keratinases predominantly. In medium keratin acts as an inducer, during the reduction of disulfide bonds by peptidases. The amount of produced keratinase in the medium is affected by physical factors, such as - agitation speed, temperature, aeration, pH and incubation time. Only after employing the entire living cells, the sulfitolysis process can be considered as feasible [20].

2.4.1 Purification of produced keratinase

To enable further characterization of the interested protein, the purification step is opted. To obtain the high level of purity of keratinase, chromatographic methods (like, ion-exchange chromatography and gel filtration chromatography), dialysis and precipitation of ammonium sulfate is used. Basically, these techniques select particularly a few numbers of proteins from the mixture. However, there are certain advantages and dis-advantages of these techniques [20].

Advantages

- Ion-exchange chromatography - Easy analysis of even very small quantity of sample. High resolution can be obtained.
- Gel filtration chromatography - Obtain high resolution.
- Precipitation of ammonium sulfate - Enables scale-up.

Dis-advantages

- Ion-exchange chromatography - Expensive and time taking.

- Gel filtration chromatography - Expensive and time taking.
- Precipitation of ammonium sulfate - Denaturation of some organic solvents can happen. After the precipitation of salt, dialysis needs to be done. Sometimes enzyme get inactivated [20].

2.5 Commercial Implementations of Keratin

- **Fertilizers -** The recycled keratinolytic biomass, are used as highly nitrogenous bio-fertilizers. To get improved crop yield of flowering plants (belonging from herbaceous perennial family) hydrolyzed form of feathers are used. Thermophilic actinomycetes treated feathers are also been used as the bio-fertilizer, in ryegrass cultivation**.**
- **For the dressing of wounds -** Non-antigenic material (for the dressing and healing of wounds) can be derived from the keratinolytic biomass. To speed up the healing process of wounds and the stimulation of cellular migration, keratin have been used in some in-vitro studies. Somehow, the bioactive content of keratin elevates wound healing. Keratinocytes activation and the secretion of peptides, initiates this healing process. It maintains a moist and healthy healing environment to the wounds. This is takes place through the absorption of exudates, secreted from the wounds. This absorption is possible because of having sponge like permeability in its network structure. Particularly, for the treatment of dry wounds there are different forms of ointment or gels, obtained from keratin. But, keratinocytes remain inactive in case of chronic and non-healing wounds.
- **In cosmetic industries -** The wide range of hair and skin care product, relies on the implementation of keratin. This keratin is acquired from various bio-resources like hooves, horns, feathers and wools. For the formation of beauty blends, shampoo, conditioner, hair creams and masks, serum and styling sprays, gels and powders, keratin alongwith others polymers are used. The reason behind these much influence of keratin on the cosmetic market, is the ability of keratin to provide heathier, supplemented treatments to skin and hair. Being auxiliary building blocks of hair, keratin is well known to reinforce the integrity of hair. And also in curly hair it simultaneously reduces the fizziness, and prepare it for easy to style.

In Pharmaceutical Industries

- **In microneedles -** For the safe and efficient delivery of pharmaceutical components **(**such as calcein, bovine albumin**,** serum albumin and drugs) keratin mediated microneedles are used.
- **Hydrogels of keratin -** For the sustainable release of antibiotics and reinforcement of various therapeutic agents, the hydrogels of keratin are used. In advanced medical science and tissue engineering these hydrogels are used widely.

- **In nanoparticles -** Silver nanoparticles coated with keratin, in aqueous environment shows higher level of stability. Remarkably, it is denoted for having anti-cancer properties. And are considered as one of the most effective carriers of anti-cancer drugs.
  The pH sensitivity of keratin, can be credited undoubtedly for providing the ability of targeting specifically tumors.
  Similarly, the gold nanoparticles of keratin layered, demonstrates antioxidant and antimicrobial activities. These nanoparticles build compatibility with the living cells, against *Staphylococcus aureus*.
- **Films of keratin -** In pharmaceuticals, for the expansion of corneal epithelial tissues and also, for the reconstruction of ocular surface, these films are used. They have strong mechanical properties. And are able to replace commercially available chem-derived thermoplastics.
- **For the formation of rubber and bio-sorbent -** To obtain purified water and also to remove heavy metals contamination, from the waste water, the prospectus applications of keratinous materials can be observed. For the absorption of some metals like- aluminium, mercury, chromium, copper, cadmium, silver and lead, keratin is isolated from wools. Mimicking the properties of synthetic rubber (used as fillers) keratin of avian feathers can be applied. This can be the best inexpensive, easily accessible and eco-friendly substitute of conventional rubber.

In the formation of hygiene products

- **Feminine hygiene products -** To make sanitary towels and disposable sanitary pads, the hydrophobic, easy to compress and flexible chicken feathers are considered as the major raw material. The semi-permeable nature of feathers is provided by the tiny pores, present between the barbules and barbs. It basically enhances its wettability and help to hold the moisture content within it.
- **Diapers production -** To form disposable diapers, applications of animal wools are increasing rapidly. It is water-resistant, breathable, highly flexible, bacteriostatic and pocket friendly to maintain. They contain 30% of moisture and also able to stretch itself up to 70%, in its dry conditions. But interestingly, still retain in its native size and shape.
  So, it can efficiently replace currently available synthetic products.

2.6 Applications of keratinase in industries

Extensive applications of keratinase in the field of nanotechnology, biomedical, pharmaceutical, biotechnology, cosmetics, leather processing units, waste management plants, production of feed items and in the formulation of detergents, have been successfully implemented.

Keratinases are proved to biologically degrade gelatin. Hence used in the silver recovery from gelatin and photographic X-ray plates. Wasted X-ray plates have protein in their content. This protein is insoluble and recalcitrant against any regular (by proteolytic enzymes) degradation mechanisms. The recovery of silver, includes few steps. First of all, by incineration of the films, from gelatin the bounded silver is removed. Then by applying keratinolytic enzymes, from the layer of protein that silver is isolated.

For treating keratinous wastes in environment friendly and economic way, bacterial keratinases are used widely. In textile industries the properties of keratinase, modify the quality of wool and silk. It has been reported, that the shrink proof wools with improved tensile strength, can be obtained by treating the fabrics (blended with polyester) with the strain of *Pseudomonas*. In the production unit of animal feed, the animal feathers are valorized into peptides and then into amino acids, by microbial keratinases. Industrially, horn meals are produced from bovine horn and hoof, by the process of de-keratinization. For the commercial production of some cosmetic items (like skin brightening and bleaching creams, masks) the formulations of keratinases (as depilatory agents) are used. In parlors and salon, keratinases are applied as a potential alternative of commonly used salicylic acid, to remove black spots from fingers and toes. There keratinase degrade the thick layer of dead skin, known as hyperkeratosis.

CHAPTER 3

3.1 MATERIALS

3.1.1 For isolation, screening and characterization of bacterial strain

- Soil sample from feather dumpsite.
- Autoclaved petri-dishes, test tubes, glass spreader.
- Sieve.
- Distilled water.
- Nutrient agar and nutrient broth.
- Skim milk agar and skim milk broth.
- Gram staining and endospore staining kit.
- Test kit for- Catalase test, Methyl Red Test, Simmon Citrate Test, Urease Test and Indole Test.

➢ **For characterization of the keratinase enzyme, isolated from the white colony strain**

- Basal media.
- 0.05M of pH 8.0, Tris HCl.
- 0.4 M TCA.
- 0.1 N HCl and 0.1 N NaOH.
- Keratin, Casein, Peptone and Skim milk powder.

3.2 METHODS

➢ **For isolation, screening and characterization of isolated microbial strain**

Soil sample was collected from the chicken feather dumpsite, situated near the dhaba site of JUIT. Collected sample was sieved carefully to remove all the hard, insoluble and unwanted particles. Then Preserved that sample properly. In autoclaved distilled water, dissolved that soil sample. Later, that solution was serially diluted up to 10^6 times. In autoclaved nutrient agar plate, spreading those serially diluted solutions, was done. After spreading, incubated those plates, in incubator at 37°C. Periodically checked bacterial growth on those plates. After 72-75 hrs. of incubation, a few bacterial colonies were observed. After 4 days of incubation, prominent colonies were observed.

Three types of colonies were observed

White colonies, Pink colonies and Yellow colonies

Then those three colonies were streaked separately on individual nutrient agar and skim milk agar plates, for further screening. And after incubation characterization of colony morphology, was performed. By - Gram staining and Endospore staining.

Gram staining

Thin smear was prepared on clean, oil-free glass slides, by dropping loop full of suspended bacterial culture. Air dried the slide or with the help of very gentle heat, to prevent the culture loss (during washing). As primary stain, crystal violet was dropped on the smear. After 30 secs, excess stain was washed off with water. Then Gram's iodine was added. And after 60 secs it was washed off with ethyl alcohol and then with distilled water. After that added a few drops of safranin on the smear (counter-stain). Washed the excess stain and air dried the slides. Slides were microscopically observed under 100X.

Endospore staining

On clean, oil-free glass slides, smear of bacterial suspended culture was prepared. Fixed the smear by air drying and mild heat. Then added malachite green on the smear and heated it on water steam for 5 mins. Gently washed the slides under running tap water. Safranin was added (as counterstain). Then washed the slides with distilled water, to remove excess amount of stain from the smear. Dried the slides and microscopically observed under 100X. According to the observation, white colony forming microbial strain was selected for further screening, as it was more efficiently growing on both skim milk agar plate and skim milk broth. Later, the biochemical characteristics of the white colony forming microbial strain, was determined. By using - Catalase test, Methyl Red Test, Simmon Citrate Test, Urease Test and Indole Test.

Catalase test

On a clean and oil-free glass slide, smear of bacterial suspension was prepared. Air dried the smear. And then dropwise added hydrogen peroxide on that smear. Observed the bubbles formation, as a result of released O_2.

Methyl Red test

In two clean test tubes, MR broth was prepared and autoclaved. One test tube was inoculated with the microbial strain and another was kept uninoculated, used as control. Incubated both the test tubes

at 37℃. Then added few drops of methyl red to each test tube, as indicator. Color change was observed.

Simmon Citrate Test

Simmon citrate agar plates were prepared under aseptic conditions. Then inoculated one plate and keep another as uninoculated control plate. Incubated both the plates at 37℃. And observe the microbial growth and change in color of the plates.

Urease Test

Urea agar plates were prepared. Inoculated and kept control plate. Plates were incubated at 37℃. Coloration was observed.

Indole Test

Tryptone broth was prepared in two test tubes and autoclaved. Inoculated and uninoculated both the test tubes were incubated at 37℃. Dropwise Kovac's reagent was added to both the test tubes and mixed gently. Change in color was observed.

Furthermore, the optimization of the growth factors of that strain was performed, at different temperature (28, 33, 37, 42, 47 and 52℃) and varying pH (3, 4, 5, 6, 7, 8, 9 and 10). Optical density was measured at 590 nm, using microtiter plates, in spectrophotometer. Graph was plotted to observe the optimum growth conditions of the strain.

- **For characterization of the keratinase enzyme, isolated from the white colony strain**

Basal media was prepared, maintaining 7.5 pH and autoclaved. Inoculated the medium and incubated at 37℃, 150rpm. After incubation, inoculated media was centrifuged at 5000rpm, at 4℃ for 20 mins. Supernatant was obtained. The reaction mixture was prepared, by mixing that obtained supernatant with the substrate, i.e., the mix solution of Tris HCl and keratin. Then incubated at 40℃ for 10 mins and reaction mixture was prepared. Added TCA in the reaction mixture. Centrifuged the mixture at 3830 rpm at 4℃ for 30 mins. Optical Density was measured at 280 nm.

For optimization of enzymatic activity of keratinase at different temperature

The reaction mixture of substrate and supernatant was incubated at different temperature of 28, 33, 37, 42, 47 and 52℃. Added TCA in the reaction mixture. Centrifuged the mixture at

3830 rpm at 4℃ for 30 mins. Optical Density was measured at 280 nm and graph was plotted.

For optimization of enzymatic activity of keratinase at different pH

pH of the reaction mixture was adjusted at 3, 4, 5, 6, 7, 8, 9 and 10. By adding 0.1 N NaOH and 0.1 N HCl. Then incubated at 40℃ for 10 mins. Added TCA in the reaction mixture. Centrifuged the mixture at 3830 rpm at 4℃ for 30 mins. OD was measured at 280 nm and graph was plotted.

For optimization of enzymatic activity of keratinase at different substrates

In the reaction mixture different substrates (keratin, peptone, peptone and skim milk powder) were added. Incubated for 10 mins at 40℃. Then TCA was added. Centrifuged the mixture at 3830 rpm at 4℃ for 30 mins. After that OD was measured at 280 nm and graph was plotted.

CHAPTER 4

4. Results

For satisfying the first objective of the following project work, the isolation of the keratinolytic strain from soil sample, was done. Observing proper collection protocols, the sample was collected from the dumpsite of chicken feather wastes near JUIT DHABA [13] (shown in Fig 1). Both the collection site and the sample are shown in Fig 1 below.

Fig 1 Collected soil sample

After proper refining of the soil, samples were serially diluted [13]. And prepared spread plates. After incubation, plates with mixed culture were obtained. From these plates the colonies were isolated. And streaked them separately. These plates were then incubated for 3–4 days. And the following growth was observed (Shown in Fig 2).

First was white, isolated colony, second was pink, isolated colony and third was yellow, sticky colony (Shown in Fig 2).

Fig 2 White Colonies Pink Colonies Yellow Colonies

After observing the growth on the culture plates gram staining was performed, and under a 100X microscope, the following (shown in Fig 3) images were observed.

The white colony forming organism was observed as gram negative rod-shaped organism. Pink colony forming organism was gram negative cocci shaped and the yellow colony forming was gram negative short rod- shaped organism (shown in Fig 3).

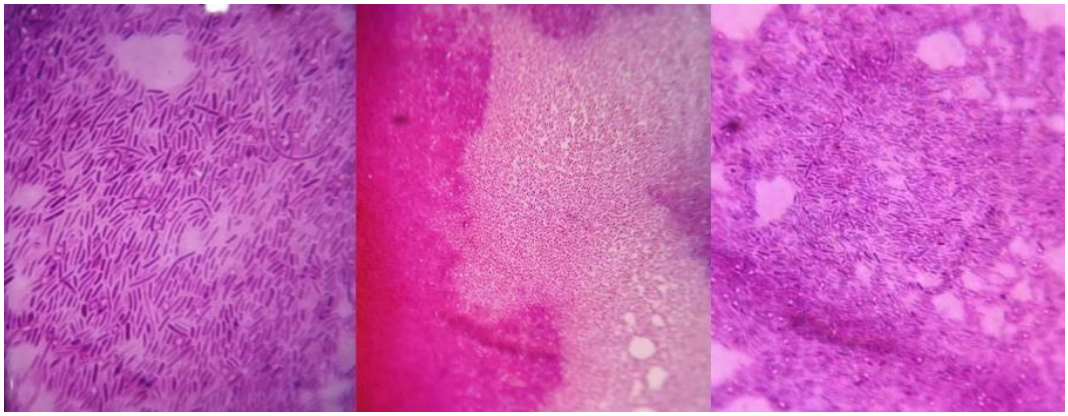


Fig 3 Gram negative Rods (White Colonies) Gram negative Cocci (Pink Colonies) Gram negative Short Rods (Yellow Colonies)

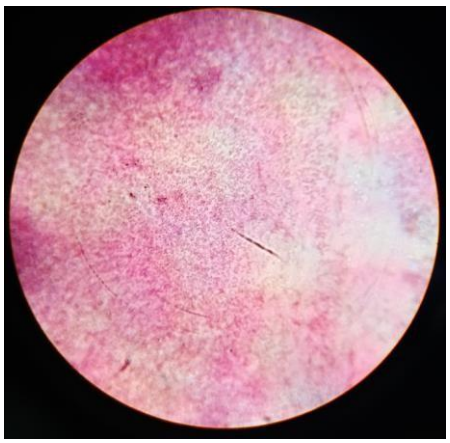


Fig 4 Endospore staining of white colony forming microbial strain (non-spore forming)

After the pure culture was procured, the strain of white colony forming organism was selected for further screening and optimization. After performing endospore staining of the white colonies, the following image was observed. White colony forming microbe was identified as non-spore forming organism (As shown in Fig 4).

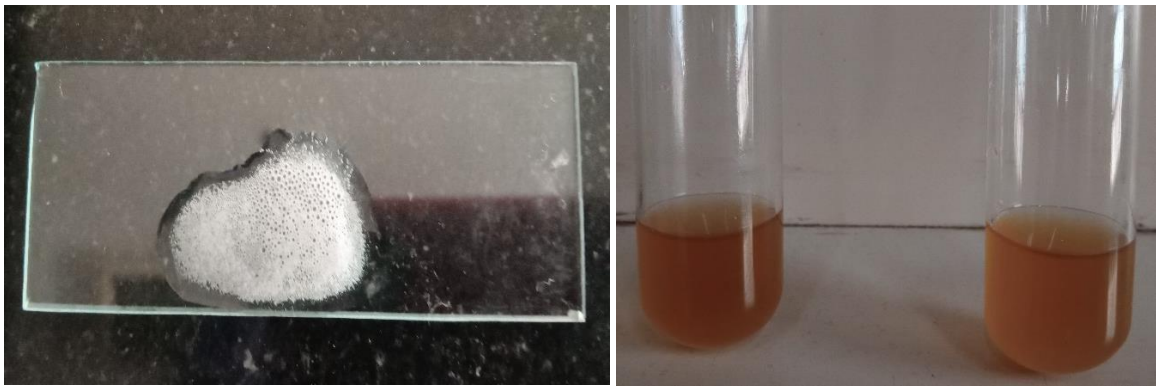


Fig 5 (a) Catalase test (b) Methyl Red Test

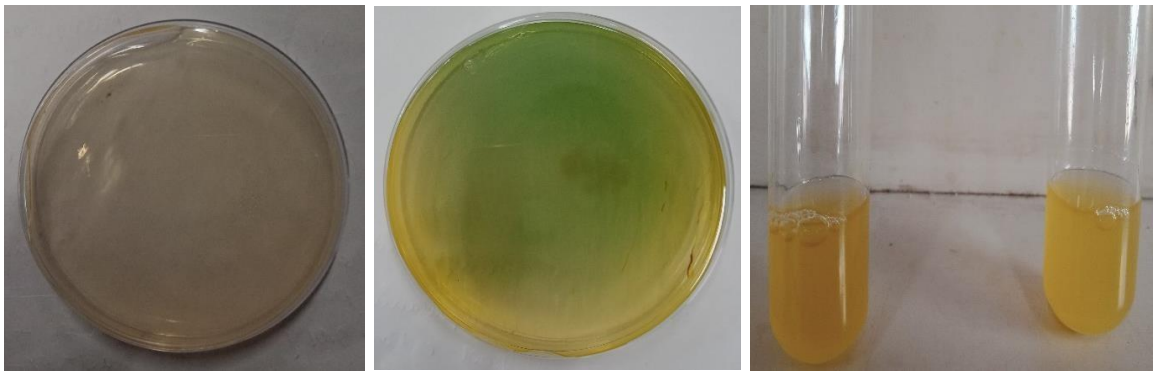


Fig 6 (a) Urease Test (b) Simmon Citrate Test (c) Indole Test

Furthermore, for the biochemical characterization of the white colony-forming microorganism, the above-mentioned biochemical tests were performed [13]. And the results are summarized in Table 3.

Positive catalase test [Fig 5 (a)], indicates that the organism possesses catalase enzyme. That results in the hydrolysis of hydrogen peroxide. Oxygen gas is produced in the form of bubbles.

Negative methyl red test indicates [Fig 5 (b)], that the organism does not produce any acidic product. This organism decarboxylated the fermented products, and formed acetoin. That results in the inclination of the pH of the medium to the neutral by declining the acidity. So, no red color formation was observed.

Negative urease test [shown in Fig 6(a)], means that the organism does not produce urease, that produce CO_2 and ammonia, by fermenting urea.

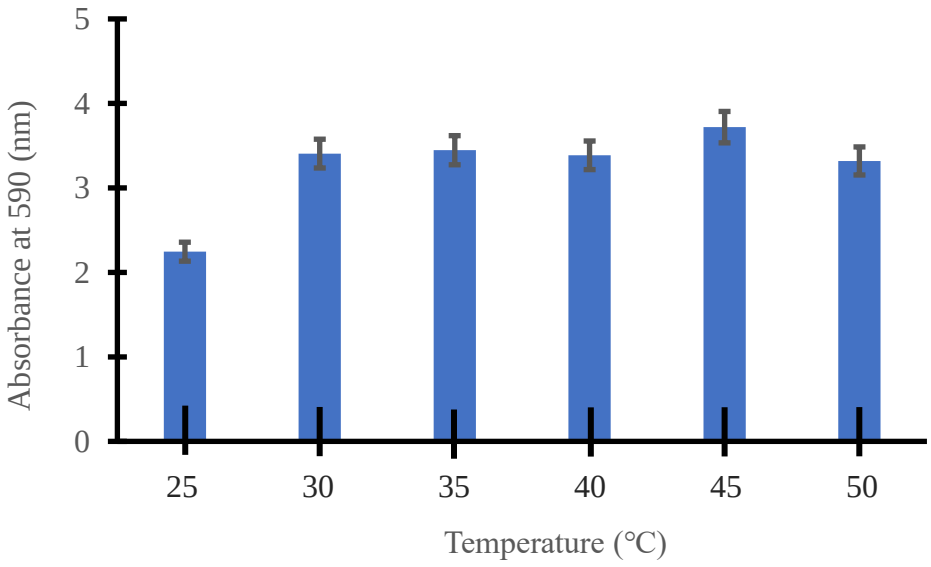
Negative result of simmon citrate test [mentioned in Fig 6(b)], indicates the inability of the organism to utilize citrate as the major source of carbon.

Negative indole test [shown in Fig 6(c)] means, the organism is unable to utilize tryptophanase, from tryptophan to produce indole.

Name of Biochemical Test	Result
Catalase Test	Positive
Methyl Red Test	Negative
Urease Test	Negative
Simmon Citrate Test	Negative
Indole Test	Negative

Table 3 Biochemical Tests

To determine the optimum growth of white colony-forming organisms, different growth parameters like temperature and pH, were considered. The bacterial culture was grown at different temperature (25, 30, 35, 40, 45, and 50°C) and pH (3, 4, 5, 6, 7, 8, 9 and 10) for 3–4 days to optimize. Considering varying temperatures and pH, the following growth curve was observed at 590nm using a UV spectrophotometer (shown in Fig 7) [13].



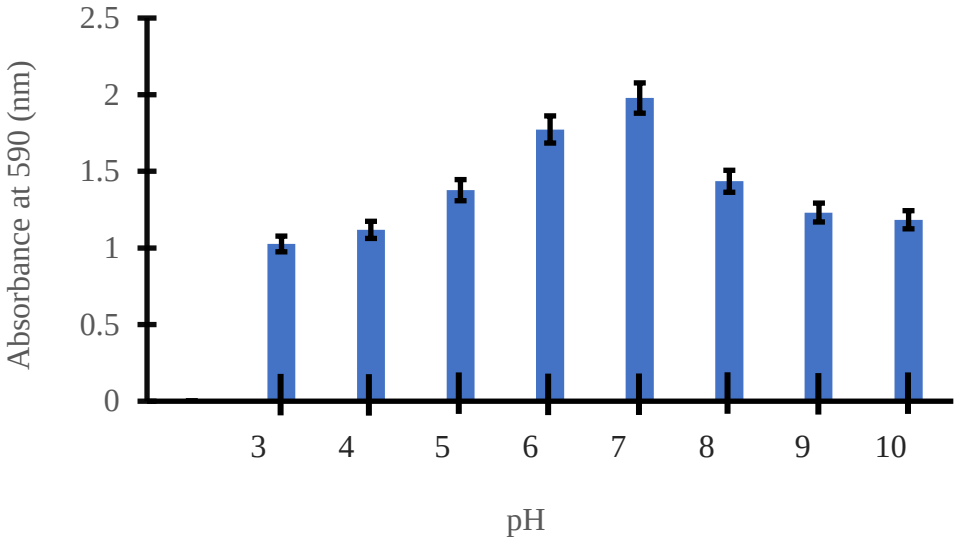


Fig 7 Determination of optimum growth pH and temperature of bacterial growth

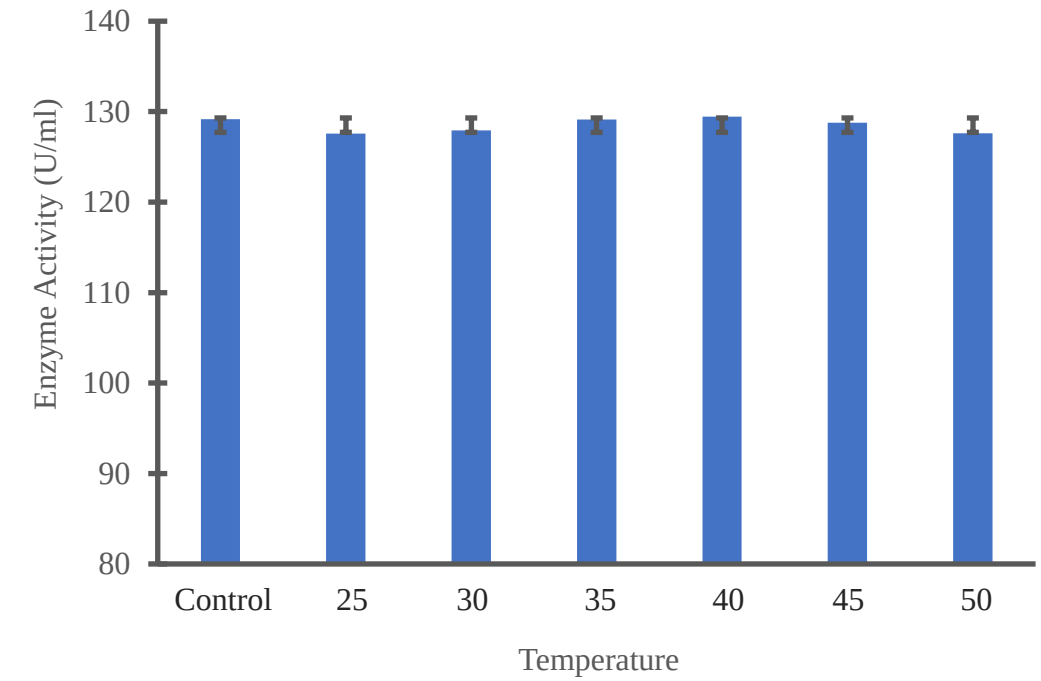
According to the graph, it was observed that the highest bacterial growth was at 45℃ and lowest was at 25℃**.** While in case of pH optimized growth was at pH 7 and very least growth was observed at pH 3 (mentioned in Fig 7). Indicating that the organism is able to grow only in neutral pH. And unable to grow neither in acidic nor in basic culture medium.

To optimize the enzymatic activity, under different temperature and pH, following graphs were plotted (Fig 8). In both these graphs (mentioned in Fig 8), the X-axis represents the growth parameters (temperature, pH, and different substrates), and the Y-axis represents the enzymatic activity of the keratinase enzyme obtained from the previously cultured white colony-forming organism. To determine the specific substrate, for keratinase activity, was performed after incubating the reaction solutions under operated condition of pH 7, at 42℃ at 150 rpm [3]. One unit of enzymatic activity (mentioned in the Y axis of Fig 8 and Fig 9) was determined by

$$U=4 \text{ X } n \text{ X } A280 / (0.01 \text{ X } 10)$$

Where, n denotes the factors of dilution, here n=1 and Incubation period =10 min

Total volume of the reaction solution =4 ml [3]



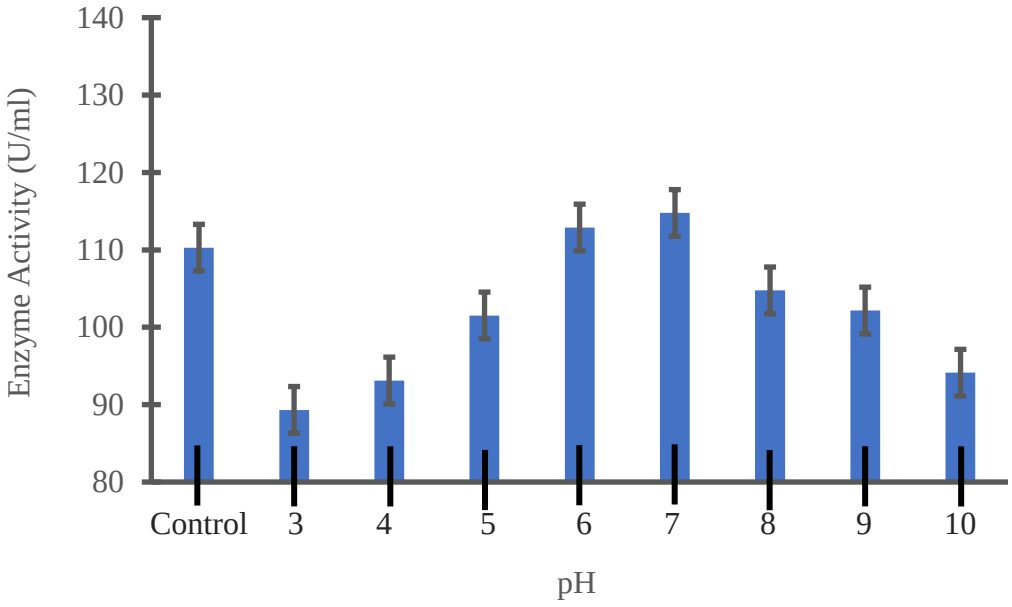


Fig 8 Assay the effect of different temperature and pH on the enzymatic activity of keratinase. Bacteria were grown on the basal media and the effects of different growth-impacting factors, like temperature (25, 30, 35, 40, 45 and 50°C), pH (3, 4, 5, 6, 7, 8, 9 and 10). After cultivation the highest enzymatic activity was observed at 40°C (according Fig 8). That indicates that the enzyme is not highly thermostable and remains active at moderate temperature [3].

The best enzymatic activity of the keratinase enzyme was observed (shown in Fig 8) maximally at pH 6-7 and minimally at pH 3 and pH 10. Means the organism is able to grow in almost neutral pH and unable to grow at highly acidic or highly alkaline conditions.

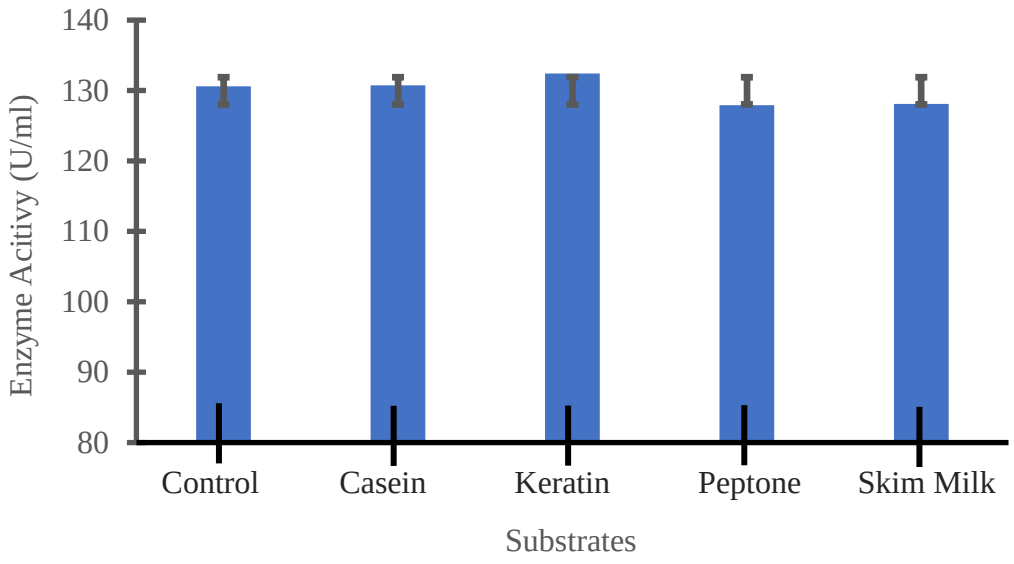


Fig 9 Assay the effect of different substrates on the enzymatic activity of keratinase

Another vital factor (for the assessment of enzymatic activity) is the presence of substrates (casein, keratin, peptone and skim milk), in the culture medium (shown in Fig 9). These were observed at 280 nm by a UV spectrophotometer [3,13]. Using keratin as its substrate [3]. Comparatively low activity was observed using casein and lowest activity was observed using peptone and then skim milk. The pH was adjusted by adding 0.1 N NaOH and 0.1 N HCl solution [3]. (mentioned in Fig 9).

Discussion

The main goal of this project work, was isolation and characterization of keratin degrading microorganism, that has been isolated from the dumpsite of feather wastes, located near JUIT. Isolated three colonies were gram negative, but shapes were different. They were grown both on nutrient agar plates and skim milk agar plates, under the optimized conditions. Skim milk agar was considered here as metabolizable protein source [36]. Further a particular strain was selected (white colony forming), based on the highest keratinolytic activity, among others. This strain was non-endospore forming and also the fastest growing, within these three strains. Preliminary biochemical tests were performed, to understand the characteristics of this strains [36]. This microorganism was able to degrade keratin under its optimum growth temperature of 45 °C and pH 7. With the increasing and decreasing pH, growth declination was observed. Hence, it can be state that the organism is thermophilic and is unable to growth neither in acidic nor in basic conditions [36].

Next the enzymatic activity of keratinase was also studied. And detected the optimum temperature and pH, to obtain its highest activity. Also display its activity on different substrates (used in the culture medium). The enzymatic activity of this microbial keratinase was observed at 40 °C, pH 6-7, using keratin as its substrate.

This keratinase enzyme shown its efficacy to the digestion of keratin, hence can be utilized to degrade keratinolytic wastes. And also, in the keratin processing [36].

CHAPTER 5

5. Conclusion

In recent biotechnological approaches, bacterial keratinases are used as an important biocomponent. They manage the keratinolytic wastes and health concerns of the animals as well. The expression and the suppression of the protease synthesizing genes are significantly influenced by the environmental and nutritional factors. Because of the diversified class of keratinolytic microbial strains, different optimal growth parameters and conditions are there. In this context, temperature is largely impacting factor on the rate of secondary metabolite's synthetization, biochemically. Here, mesophiles are preferred over others. Because of low energy requirement for their sustainable growth. pH is another influential factor, among others. The molecular transfer through the microbial cell membrane, according to its porosity, is being maintained by pH. Hence, the pH of the medium should be preserved necessarily, to obtain the maximum production of the microbial keratinase.

The disulfide bond's degradation in fermentation culture medium is signified by generating sulfhydryl groups, during the hydrolysis of feather. Commercially, to obtain high enzymatic yield, content analysis of amino acids along with its variants and hydrolysates (of keratinolytic biomass) should be performed. Keratinases, shows itself as a wonderful compatible substitute of chemically synthesized additives, used in the detergents of laundry.

In this project, the studied microbial strain has showed its best activity in neutral to slightly acidic pH at thermophilic temperature.

References

1. N. E. Nnolim, C. C. Udenigwe, A. I. Okoh, and U. U. Nwodo, “Microbial Keratinase: Next generation green catalyst and prospective applications”, Front. Microbiol., vol. 11, p. 580164, 2020.

2. A. Razzaq *et al.*, “Microbial proteases applications,” Front. Bioeng. Biotechnol., vol. 7, p. 110, 2019.

3. N. C. Barman et al., “Production, partial optimization and characterization of keratinase enzyme by Arthrobacter sp. NFH5 isolated from soil samples, ” AMB Express, vol. 7, no. 1, p. 181, 2017.

4. X. Zhang, Y. Shuai, H. Tao, C. Li, and L. He, “Novel method for the quantitative analysis of protease activity: The casein plate method and its applications,” ACS Omega, vol. 6, no. 5, pp. 3675–3680, 2021.

5. Q. Li, “Structure, Application, and Biochemistry of Microbial Keratinases,” Front. Microbiol., vol. 12, p. 674345.

6. A. Jana et al., “Keratinase biosynthesis from waste poultry feathers for proteinaceous stain removal'', ACS Sustain. Chem. Eng. vol. 8, no. 48, pp. 17651–17663, 2020.

7. M. P. Koentjoro and E. N. Prasetyo, “Advances in use of keratinase from feather wastes for feedstock modification,” Appll. food Biotechnol., vol. 8, no. 1, pp. 19–30, 2021.

8. D. S. Ningthoujam, K. Tamreihao, S. Mukherjee, R. Khunjamayum, L. J. Devi, and R. S. Asem, “Keratinaceous wastes and their valorization through keratinolytic microorganisms*”*. IntechOpen, pp. 2018.

9. C. L. A. de Menezes *et al.*, “Industrial sustainability of microbial keratinases: production and potential applications,” World J. of Microbiol and Biotechnol, vol. 37, no. 5, pp. 2021.

10. L. Lange, Y. Huang, and P. Kumar Busk, “Microbial decomposition of keratin in nature—a new hypothesis of industrial relevance,” Appll. Microbiol. Biotechnol., vol. 100, pp. 2083–2096, 2016.

11. A. Rodman, F. Falco, D. I. Gerogiorgis, and K. V. Gernaey, “Enzymatic keratin hydrolysis: Dynamic modelling, parameter estimation and validation,” in Computer Aided Chemical Engineering, Elsevier, pp. 1553–1558.

12. T. Kumar Kumawat, A. Sharma, and V. Sharma and S. Chandra, “Keratin waste: the biodegradable polymers”, in Keratin, M. Blumenberg, Ed. London, England: IntechOpen, 2018.

13. N. Nnolim, L. Mpaka, A. Okoh, and U. Nwodo, “Biochemical and Molecular Characterization of a Thermostable Alkaline Metallo-Keratinase from Bacillus sp. Nnolim-K1,” Microorg., 2020.

14. A. Riffel, F. Lucas, P. Heeb, and A. Brandelli, “Characterization of a new keratinolytic bacterium that completely degrades native feather keratin,” Arch. Microbiol., vol. 179, no. 4, pp. 258–265, 2003.

15. S. Pandian, J. Sundaram, and P. Panchatcharam, “Isolation, identification and characterization of feather degrading bacteria.” Accessed: Apr. 24, 2023.

16. H. Prasad V, G. Kumar, K. L, and B. R. Kv, “Screening of Extracellular Keratinase Producing Bacteria from Feather Processing Areas in Vellore, Tamil Nadu, India,” J. Sci. Res., vol. 2, no. 3, p. 559, 2010.

17. S. Balakumar, N. Mahesh, M. Arunkumar, R. Sivakumar, and V. Hemambujavalli, “Optimization of Keratinase Production by Keratinolytic Organisms under Submerged Fermentation,” Internat. J. Pharm. Tech. Res. CODEN, vol. 5, no. 3, pp. 1294–1300, 2013.

18. D. Balakrishnan and N. Padmanabhan, “Identification and Phylogenetic Analysis of Keratinase Producing Bacteria SNP1 from Poultry Field,” Internat. J. Biotechnol. Biochem., vol. 15, no. 1, pp. 39–51, 2019.

19. Y. Huang, M. Lezyk, F. Herbst, P. Kumar Busk, and L. Lange, “Novel keartinolytic enzymes, discovered from a talented and efficient bacterial keratin degrader,” Sci. Rep., vol. 10, no. 1, pp. 2020.

20. L. Matches, “Valorization of chicken feather through dekeratinization by keratinolytic Bacillus species to amino acid,” vital.seals.ac.za.

21. N. E. Nnolim and U. U. Nwodo, “Microbial keratinase and the bio-economy: a three-decade meta-analysis of research exploit,” AMB Express, vol. 11, no. 1, pp. 2021.

22. S. C. B. Gopinath et al., “Biotechnological Aspects and Perspective of Microbial Keratinase Production,” BioMed. Res. Interna., pp. 2009.

23. Z. Peng, X. Mao, J. Zhang, G. Du, and J. Chen, “Effective biodegradation of chicken feather waste by co-cultivation of keratinase producing strains,” Micro. Cell Fact., vol. 18, no. 1, pp. 2019.

24. S. Sangali and A. Brandelli, “Isolation and Characterization of a Novel Feather-Degrading Bacterial Strain,” Appll. Biochem. Biotechnol, vol. 87, no. 1, pp. 17–24, 2002.

25. I. Cavello, B. Bezus, and S. Cavalitto, “The keratinolytic bacteria Bacillus cytotoxicus as a source of novel proteases and feather protein hydrolysates with antioxidant activities,” J. Gen. Eng. Biotechnol, vol. 19, no. 1, pp. 2021.

26. V. Sekar, M. Kannan, R. Ganesan, B. Dheeba, N. Sivakumar, and K. Kannan,

"Isolation and Screening of Keratinolytic Bacteria from Feather Dumping Soil in and Around Cuddalore and Villupuram, Tamil Nadu," Proc. Nat. Acad. Sci, Biol. Sci., vol. 86, no. 3, pp. 567-575, 2015.

27. A. Bokveld, N. E. Nnolim, T. O. Digban, A. I. Okoh, and U. U. Nwodo, "*Chryseobacterium aquifrigidense* keratinase liberated essential and nonessential amino acids from chicken feather degradation," Environ. Technol., vol. 44, no. 3, pp. 293-303, 2023.

28. Y. A. Moonnee, M. J. Foysal, A. Hashem, and M. F. Miah, "keratinolytic protease from Pseudomonas aeruginosa for leather skin processing." J. Gene. Eng. Biotechnol., vol. 19, no. 1, pp. 2021.

29. D. M. T. Tapia and M. L. G. Simões, "Production and partial characterization of keratinase produced by a microorganism isolated from poultry processing plant wastewater," Afr. J. Biotechnol., vol. 7, no. 3, pp. 2008.

30. T. M. Muhsin and R. B. Hadi, Mycopathologia, vol. 154, no. 4, pp. 185-189, 2002.

31. M. M. Koutb, E. A. Hassan, F. M. Morsy, and M. M. K. Bagy, "Optimization of keratinase production by keratinolytic fungus Chrysosporium tropicum and its potentiality in bidegradation of chicken feathers," J. Umm Al-Qura Univ. Appll. Sci., vol. 9, no. 1, pp. 50–56, 2023.

32. S. Timorshina, E. Popova, V. Kreyer, N. Baranova, and A. Osmolovskiy, "Keratinolytic Properties of Aspergillus clavatus Promising for Biodegradation," Int. J. of Environ. Res. Public Health, vol. 19, no. 21, p. 13939, 2022.

33. J. Qiu, C. Wilkens, K. Barrett, and A. S. Meyer, "Microbial enzymes catalyzing keratin degradation: Classification, structure, function," Biotechnol. Adv., vol. 44, no. 107607, p. 107607, 2020.

34. A. Blanco and G. Blanco, ‘Proteins”, in Medical Biochemistry, Elsevier, 2017, pp. 21-71.

35. A. Riffel and A. Brandelli, “Keratinolytic bacteria isolated from feather waste”, Braz. J. Microbiol, vol. 37, no. 3, pp. 395-399, 2006.

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