Valorization of chicken feather through dekeratinization by keratinolytic Bacillus species to amino acid

University of Fort Hare

Together in Excellence

Masters dissertation

BY

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DECLARATION

I, Lupho Matches, hereby declare that this dissertation contains my very own original work with exception of citations. The work is being submitted for a Master of Science degree in the Department of Biochemistry and Microbiology, Faculty of Science and Agriculture, University of Fort Hare, Alice. This work has never been submitted for any degree before at any University.

Name: Lupho Matches

Signature:…….

Date:……

DEDICATION

This work is dedicated to my parents, Mr T. Kokwe and Mrs Z.G Kokwe, my daughter Othalive, my grandmother and my sister. I would not be here without their prayers, love, support and encouragement. I am forever grateful for their comfort and prayers during hard times.

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I am deeply indebted to my parents, Thokozani and Zoleka Kokwe, I live to make them proud each day. To my sister Simamkele, your love and support are very much appreciated.

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Contents

List of abbreviations

PMSF	Phenylmethylsulphonyl fluoride
EDTA	Ethylenediaminetetraacetic acid
DTT	Eithiothreitol
DMSO	Dimethyl sulfoxide
SDS	Sodium dodecyl sulfate
AEMREG	Applied and Environmental Microbiology Research Group
SAMRC	South African Medical Research Council
DSI	Department of Science and Innovation
BSA	Bovine serum albumin
OD	Optical density

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GENERAL ABSTRACT

The poultry meat processing sector generates chicken feathers as by-products, and they are 90% keratin in composition. Keratin is an insoluble and structural protein that shows recalcitrance to hydrolysis by classical proteolytic enzymes, including trypsin, pepsin, and papain. Keratinases are a group of proteolytic enzymes endowed with keratin degradation into peptides and amino acids. They are recently gaining traction for their multifaceted potential application in the green industrial space. Hence, keratinolytic bacteria previously isolated from dumpsite were identified using 16S rDNA sequencing. The optimal fermentation conditions were determined for enhanced extracellular keratinase production and chicken feather degradation. Also, the amino acid analysis of the chicken feather hydrolysates was carried out. The biochemical properties of the keratinases were also determined. Based on 16S rDNA sequencing and phylogenetic analysis, the isolates coded as SSN-02 and HSN-03 showed a high percentage of sequence homology with *Bacillus* spp.; hence, they were identified as *Bacillus* sp. NFH5 and *Bacillus* sp. FHNM, respectively. *Bacillus* sp. NFH5 showed optimal keratinase production of 1149.99 ± 80.99 U/mL after 96 h of incubation time, in optimized fermentation conditions that included pH (4.0), chicken feather (1.5%, w/v), inoculum size (3%, v/v) and temperature (30 ^{o}C). Similarly, *Bacillus* sp. FHNM demonstrated the maximum keratinase production of 480 ± 41.14 U/mL 144 h post cultivation, in optimized fermentation conditions with pH (7.0), chicken feather (2.0%, w/v), inoculum size (3%, v/v) and temperature (30 ^{o}C). For *Bacillus* sp. NFH5 chicken feather hydrolysate, the amino acids in relatively higher concentration (>1.0g/100g sample) include arginine (1.8), serine (1.16), aspartic acid (1.95), glutamic acid (2.47), proline (1.16) and glycine (1.45). *Bacillus* sp. FHNM feather hydrolysates, contained (g/100g of sample): arginine (1.9), serine (1.4), aspartic acid (2.5), glutamic acid (2.51), glycine (1.51), proline (1.13), leucine (1.030, histidine (1.25), and lysine (1.06) (g/100g of sample) in high

concentration. The keratinases were optimally active at pH 8.0. *Bacillus* sp. FHNM showed an optimal temperature of 100 ^{o}C; while *Bacillus* sp. NFH5 keratinase displayed optimal activity at 90 ^{o}C. EDTA and 1,10-phenanthroline inhibited the keratinases, and the inhibition pattern indicated that they belong to metalloprotease. Keratinase from *Bacillus* sp. FHNM showed considerable residual activity in the presence of Co^{2+} (93%), Fe^{3+} (99%), and K^{+} (94%). *Bacillus sp*. NFH5 keratinase retained 92%, 92%, 93% of the original activity against Ba^{2+}, Na^{+} and Fe^{3+} treatment. *Bacillus* sp. FHNM keratinase was remarkably stable after 60 min of detergents treatment with residual activity of 89%, 96%, 81%, 73%, 96%, 88%, 88% and 98% for Omo, Surf, Ariel, Sunlight, Prowash, Freshwave, Sky, and Evaklin, respectively. Maq impacted the enzyme stability negatively, with residual activity of 48% after 60 min of incubation. Additionally, keratinase *Bacillus* sp. NFH5 retained 68%, 78%, 80%, 84%, 57%, 80%, 98%, 106% and 106% of the original activity against Omo, Surf, Ariel, Sunlight, Maq, Prowash, Freshwave, Sky and Evaklin, respectively. Therefore, these results suggest that *Bacillus* spp. could be ideal candidates for sustainable production of active keratinases and valorization of the abundantly generated keratinous biomass. The stability displayed by keratinases from *Bacillus* sp. FHNM and *Bacillus sp.* NFH5 suggests their promising candidacy for detergent formulation.

CHAPTER ONE

GENERAL INTRODUCTION

1.1. BACKGROUND STUDY

Feathers are generated from poultry slaughterhouses in sizeable amounts as by-products of poultry processing. The increase in the stream of wastes emanating from the sector results from the globally growing demand for poultry meat and its products. Chicken feathers are about 5 – 10% of the grown chicken mass, making them the poultry industry's primary waste products (Tesfaye et al., 2018). Chicken feathers are produced in a million tons throughout the year, with the USA, Brazil and China being the largest producers worldwide (Tesfaye et al., 2017; Tesfaye et al., 2018). South Africa, Egypt and Nigeria were significant producers of chicken feathers in Africa (Tesfaye et al., 2018). Feathers constitute over 90% keratin protein (Li, 2019), making them beneficial bio-resource to convert into valuable proteins (Khosa and Ulla, 2013). Regrettably, instead of being converted into valuable products, most of these feathers are discarded by burning and landfilling (Gurav and Jadhav 2013). Although feathers are without a doubt promising protein resources for bioactive peptides and amino acids, their usefulness is limited due to their immense resistance to proteolysis caused by their rigid structure (Onifade et al., 1998; Gupta and Ramnani, 2006; Li et al., 2020).

Feathers have stable structures that resulted from the presence of the tough protein called keratin. Keratin is a structural and fibrous protein found in feathers and is known for its recalcitrance (Li, 2019). The recalcitrance results from the tightly packed protein chains strongly stabilized by disulfide cross-linkages, hydrogen bonds and hydrophilic interactions. Keratin is grouped into alpha (α) keratin – found in nails, horns, hair and hooves, and beta (β) keratins found only in scales and feathers (Fraser and Parry, 2017). Previous researches have demonstrated the pre-treatment of feathers by autoclave and microwave or alkaline reagent before enzymatic treatment using savinase (Cheong et al., 2018). A few fundamental methods used to manage keratin wastes included incineration and landfill. However, these techniques

were harmful to the environment as they produced numerous harmful gases and decimated the protein contents of feather keratin. Chemical (acid and alkali) and physical (cooking or burning) methods currently used for the treatment of feathers are expensive, hazardous and also lead to the pollution of air, water, and soil (Gupta and Ramnani 2006). They also utilize immense energy and destroy certain vital amino acids such as methionine, lysine, and tryptophan, reducing the protein contents of the produced feather meal (Moritz and Latshaw, 2001; Acda, 2010). Therefore, an environmentally friendly method has been developed to manage keratinous wastes and replace the existing harmful methods. Microorganism has been found to degrade keratinous waste permitting their transformation into feedstuffs, organic fertilizers, and films (Brandelli et al., 2015).

Microbes harbour a battery of diverse enzymes that can be utilized for various industrial applications (Mazotto et al., 2013). Fungi and bacteria are reported to have produced keratinases in submerged and solid-state fermentation. The breakdown of keratinous wastes, including feathers, hair, hooves and horns, and the recycling of nutrients (carbon, nitrogen and sulphur) are carried out naturally by bacteria, fungi and actinomyces. The degradation of keratin is initiated by keratinases and disulfide reductases or redox potentials that disrupt disulfide bonds responsible for keratin's structural stiffness and its resistance to proteolysis (Gupta and Ramnani, 2006). Keratin degradation is described frequently in Gram-positive bacteria than in Gram-negative bacteria (Gupta and Ramnani, 2006). Keratin biodegradation has been described in Bacillus strains; non-sporogenic bacteria including Fervidobacterium, Lysobacter, Nesterenkonia, and Kocuria species (Vidmar and Vodovnik, 2018). Keratin degradation in Gram-negative bacteria was reported in Xanthomonas, Thermoanaerobacter, Stenotrophomonas, and Chryseobacterium (Vidmar and Vodovnik, 2018). Exploring the diversity of keratinolytic microorganisms is therefore crucial in the study of keratinases for

biotechnological uses. Even though keratinases produced by different microbes have been widely reported, keratinases' robust functionality and suitable for green technology are highly in demand (Vidmar and Vodovnik, 2018).

1.2. PROBLEM STATEMENT

Keratinous biomass such as feathers is generated in incredible amounts by the poultry industry each year due to increased demand for poultry meat and its products. The discarded feathers pollute the environment and lead to protein wastage (Mazotto et al., 2011; Sharma and Gupta, 2016). The polluted environment is, therefore, hazardous to humans and animals. Conventional approaches for feather degradation like steam pressure cooking and alkali hydrolysis utilise vast amounts of energy and destroy valuable amino acids (Lateef et al., 2015). Microbial degradation of feathers provides a feasible alternative with immense benefits. Some minute quantities of feathers are used as bedding, cleaning (feather dusters), and decorative materials. However, because of the enormous quantities of feathers generated daily, the new biotechnological approach based on microbial degradation represents a sustainable method of converting these residues into functional peptides and amino acids for various applications potentials (Brandelli et al., 2015; Sharma and Gupta, 2016). Keratinases provide practical uses in bioengineering applications involving keratin biomass from poultry and leather processing plants for non-polluting procedures. The transformation of recalcitrant feather keratin into feedstuffs, fertilizers, glues, and films occurs after enzymatic hydrolysis (Friedrich and Antranikian, 1996; Gupta and Ramnani 2006; Brandelli, 2008).

1.3. HYPOTHESIS

The research is based on the null hypothesis that Bacillus species isolated from a municipal dumpsite do not possess chicken feather dekeratinization potentials.

1.4. RESEARCH AIM

The study aimed to assess the amino acids profile of the chicken feather dekeratinization by keratinolytic *Bacillus* species.

1.5. RESEARCH OBJECTIVES

To identify the feather degrading isolates from AEMREG culture repository

To optimize the culture conditions for enhanced keratinase activity

To determine the biochemical characteristics of the produced keratinases

To profile the amino acids content of the chicken feather hydrolysates

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CHAPTER TWO

LITERATURE REVIEW

2. LITERATURE REVIEW

2.1. KERATIN

Keratin is a fibrous and insoluble material found in the external layer of skin in humans and animals. Keratin serves fundamental and protective capabilities, particularly in the epithelium and is found on the dead outermost layer of the epidermis, hair and wool, horns, hooks, hooves, feathers, and scales of reptiles, birds, amphibians, and warm-blooded animals (McKittrick, et al., 2012; Sharma and Gupta, 2016; Holkar, et al., 2018;.Feroz et al., 2020; Qiu et al., 2020). Keratin is also present in the digestive organs' epithelial cells such as the liver, pancreas, intestines, and gallbladder. It is also present in hepatocytes, hepatobiliary ductal cells, oval cells, acinar cells, and enterocytes of the small intestine, colon, and goblet cells (Sun et al., 1979; Zatloukal et al., 2004; Zhou et al., 2006; Sharma and Gupta, 2016).

Keratin may be grouped as alpha and beta keratin; based on their secondary structures. Alpha keratin is formed in mammalian skin, hair and wool, whereas beta keratin is formed in bird feathers and scales of reptiles (McKittrick, et al., 2012 Greenworld et al., 2014; Qiu et al., 2020). Keratin is known for its nature to withstand degradation by common proteases, including papain, trypsin and pepsin (Fang et al., 2013; Li, 2019). The bio-disintegration of insoluble keratin macromolecules relies upon the microbial secretion of extracellular keratinolytic enzymes that can degrade these polypeptides (Riffel and Brandelli. 2006). Their insolubility is from the presence of sulfur compounds bonded by the disulfide bridges. Keratin is comprised of an assortment of amino acids cysteine, alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine, with cysteine being the most abundant (Korniłłowicz-Kowalska and Bohacz, 2011; Sharma and Gupta, 2016; Kumawat et al., 2018).

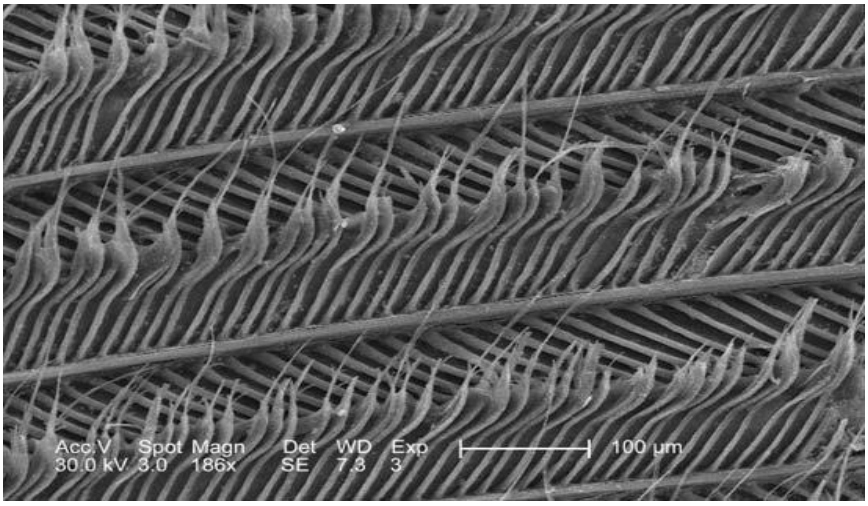

Figure 2.1: an image from a scanning electron microscope showing strands of feather keratin at 186x magnification (*https://www.britannica.com/science/keratin/media/1/315321/153502*).

2.2. STRUCTURE OF KERATIN

The alpha keratin has cysteine content fewer than ten percent and beta keratin with cysteine content between ten to fourteen per cent (Jin et al., 2017). Alpha keratin forms in mammal skin as a protective layer, and beta keratin in scales, feathers, beaks and claws (Alibardi, 2016). Alpha keratin comprises alpha-helical curled loops coordinated into moderate fibres (Meyers et al., 2008). Beta keratin comprises beta-pleated sheets that form supramolecular fibril groups (Bodde et al., 2011). The robustness and toughness of keratin are accomplished from the exceptionally packed alpha-helical and beta-sheet arrangements. Their recalcitrance results from the heightened levels of cross-linkages formed by the disulfide and hydrogen bonds, as well as hydrophobic interactions (Meyers et al., 2008). Post-translational modifications, phosphorylation, and glycosylation are significant for keratins' structural stability (Li, 2019). In some keratins, phosphorylation sites are the non-curved first structures containing threonine and serine.

Keratins may also be characterized as type I and type II (acidic and basic) depending on the

isoelectric point (pI) (Bragulla and Homberger, 2009). Post-translational modifications can influence the pI of keratin. Subsequently, only after the alteration of post-translational status will keratin adaptations occur. Lack of post-translational alterations will prompt failed intermediate filaments if the modification site is transformed (Bragulla and Homberger, 2009).

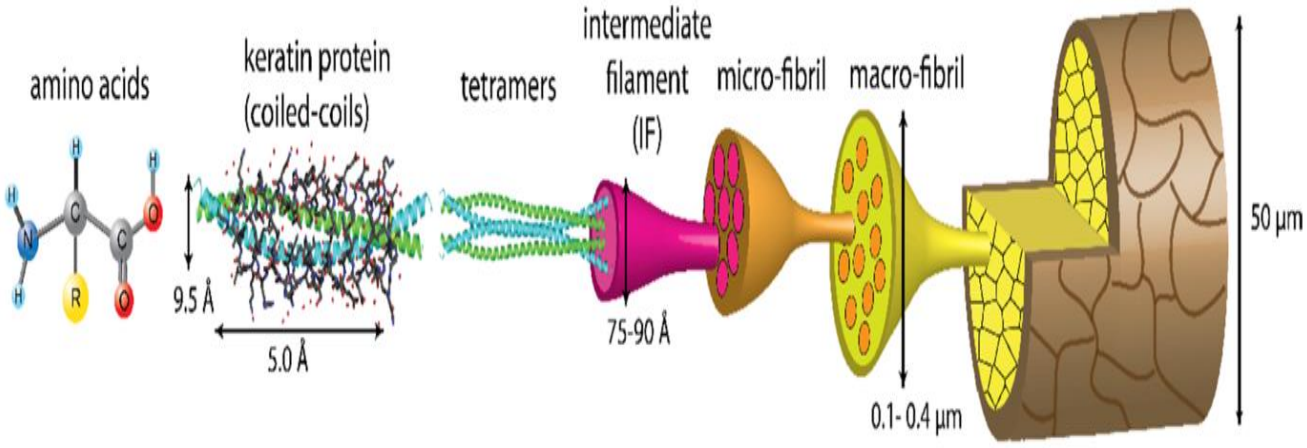

Figure 2.2: Structural components of keratin (De Oliveira et al., 2020).

2.2.1. Alpha keratin (α-keratin)

Alpha keratin is an essential constituent in hair, horns, mammalian claws, nails and the epidermis layer of mammalian skin that protect against mechanical stress. They are expressed in the epithelial cells of vertebrates (Kumawat et al., 2018). Alpha keratins are remarkable for their stability, versatility, rigidity, insolubility and adaptability. Alpha keratins have various amounts of hydrophobic amino acids such as methionine, phenylalanine, valine, isoleucine and alanine (Kumawat et al., 2018; Lange et al., 2016). The amino acids in alpha keratins form a repeating secondary structure making them a fibrous structural protein. The coiled secondary structure is similar to that of an ordinary protein α-helix (Lange et al., 2016). Their tight stable structures protect mammals. Alpha keratins are formed during protein synthesis through transcription and translation, but as they mature, they die and create a non-vascular

unit of keratinized tissue (Wang et al., 2016).

2.2.1.1. Structure of alpha-keratin

Alpha keratin is a polypeptide chain abundant in alanine, leucine, arginine, and cysteine, forming a right-handed α-helix (Pace and Scholtz, 1998; Burkhard et al., 2001). Two of the polypeptide chains curl together to form approximately 45 nm long coiled-coil dimers bonded together by disulfide bonds contributed by cysteine (Wang et al., 2016). These dimers form a protofilament by arranging their bonding termini with the termini of other dimers (Steinnert et al., 1985). A cluster of two protofilaments form a protofibril and four protofibrils form an intermediate filament that serves as an essential subunit of alpha keratins. The intermediate filaments shrink, forming a super-coil of about 7 nm in diameter, classified as type 1 (acidic) and type 2 (basic), and submerge in a high cysteine matrix glycine, tyrosine, and phenylalanine residues concentration. Therefore variations of these intermediate filaments account for α-keratin structures found in mammals (McKittrick et al., 2012).

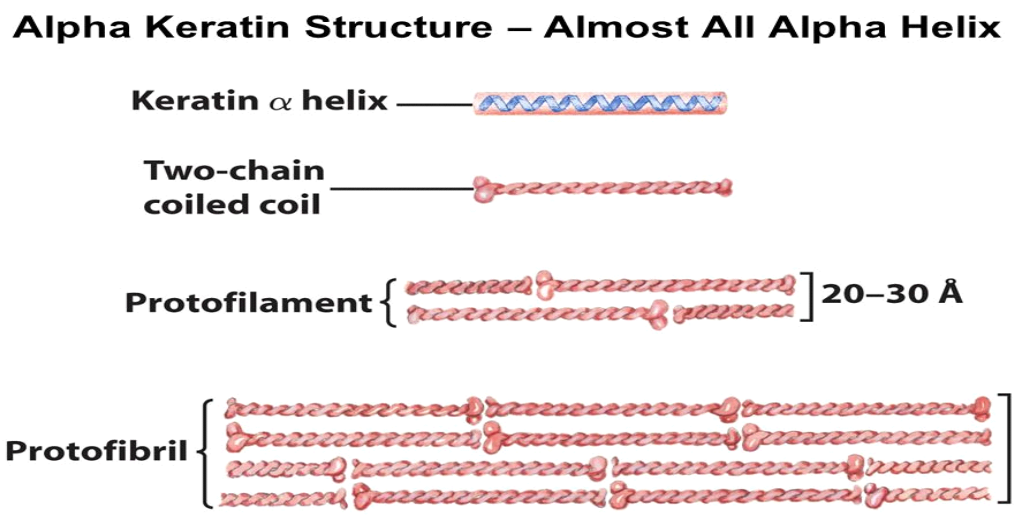

Figure 2.3: A 3-dimensional primary structure of α-keratin. Two molecules of α-keratin formed a coil; the two helices are stabilized by disulfide bonds and subsequently pairs up

with other coils to form protofilaments and protofibrils. Four protofibrils form the α-keratin intermediate filament (Muhammed, 2011).

2.2.1.2. Properties of alpha keratin

Structural strength is the most significant property of alpha keratin. Alpha keratin is known to retain its structure even when exposed to mechanical stress (Pan et al., 2013). The water content within the intermediate filament can affect the quality of alpha keratins; high water content results in compromised quality and firmness of keratins due to their impact on the hydrogen bonds within alpha keratin structures (Wang et al., 2016). The alpha keratin gene loci are comprised of a cluster of genes coding for diverse keratin types (Coulombe and Omary, 2002; Langbein and Schweizer, 2005; Alibardi, 2016).

2.2.2. Beta keratin (β-keratin)

Beta keratin is present in reptiles and avian feathers (Greenwold et al., 2013). Beta keratins are rich in cysteine residues; therefore, a higher content of disulfide bonds is available, allowing rigidity and enhanced protection from attack (Qiu et al., 2020). Beta keratin differs from alpha keratin as they are specialized to make rigid structures like scales, feathers and claws. About 80–90% of beta keratins are available in a mature feather (Tesfaye et al., 2017). Beta keratins are formed in the pre-corneous layer of the epidermis in skin appendages. Beta keratins can replace and mask alpha keratins in cells and form a hard corneous layer. Beta keratins are slightly less elastic than alpha keratins. The filaments of beta keratins are about 3-4 nm long (Toni et al., 2007). Beta keratin comprises a centred domain abundant in residues that favour building beta-sheet structures linked with the filament framework, N and C-terminal domains associated with the network and structure cross-linked by disulfide bonds (Fraser and Parry, 2011). The β-pleated sheets of β-keratins

arrange into a supramolecular fibril bundle (Li, 2019).

2.3. SIGNIFICANT SOURCES OF KERATIN

Keratin biomass is recoverable from animals and humans body parts. The skin and its appendages are abundant in keratin proteins. Nails, hair, feathers, wool, hooves, scales, and stratum corneum are the most significant keratin sources (Gupta and Sharma, 2006). Keratin has also been reported in spider webs and silk from silkworms (Saravanan, 2006).

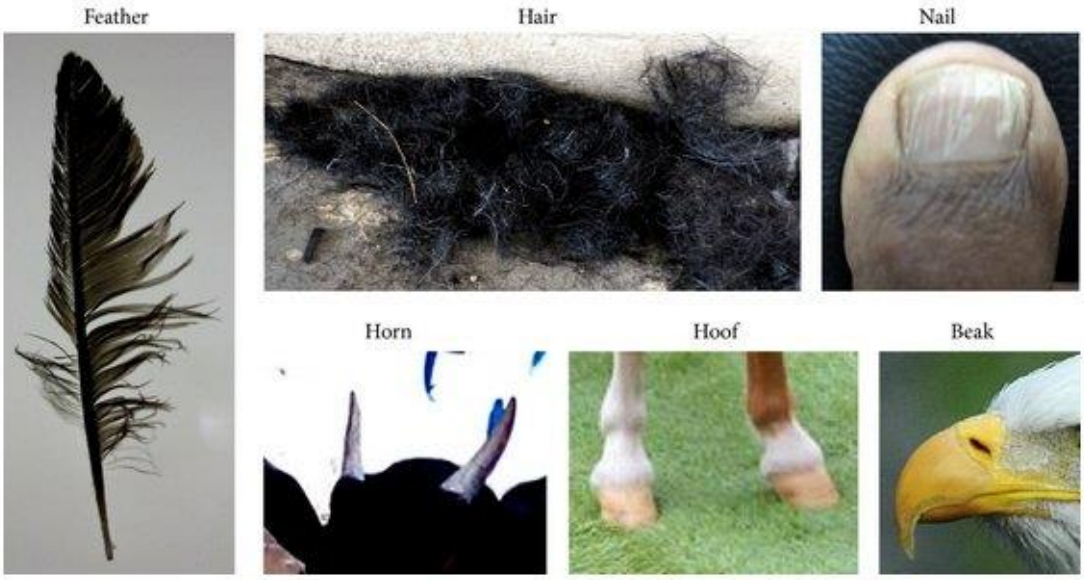

Figure 2.4: Various sources of keratin protein (Gopinath et al., 2015).

2.3.1. Feathers – a significant source of keratin protein

Keratin, recognized for recalcitrance, is the major structural protein of feathers. Feathers contain over 90% crude protein in the form of keratin (Habbeche et al., 2014). Poultry feathers are the most significant keratin sources and can be utilized in several fields (Sharma and Gupta, 2016). The keratin sequence in feathers is comprised of vital contents of cysteine, glutamine, proline, and serine, thus making feathers significant sources of vital amino acids

(Tesfaye et al., 2017).

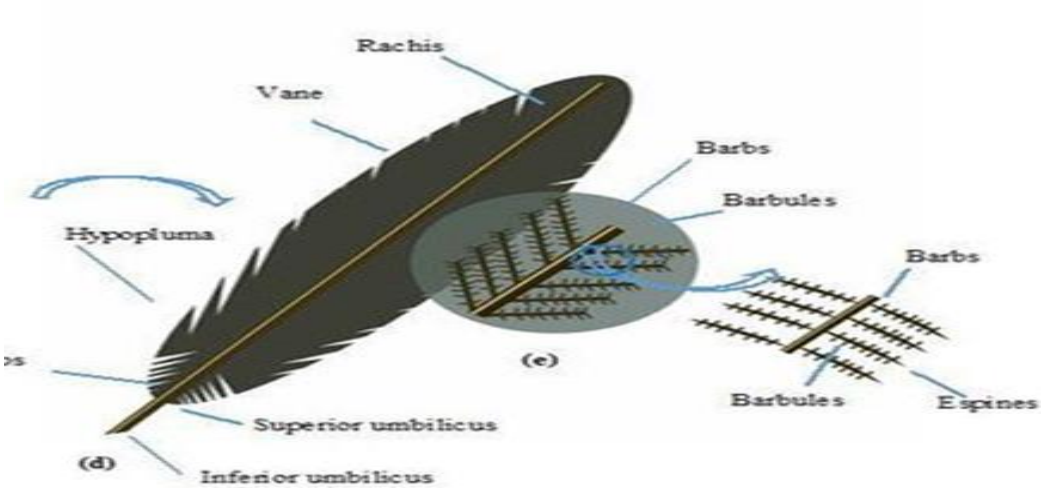

Figure 2.5: Feather structure (Pahua-Ramos et al., 2017).

2.4. COMMERCIAL USES OF KERATIN

2.4.1. Cosmetics

Keratin has been applied in skin and hair treatment in the cosmetics industry (Vermelho et al., 2010). Keratin and other characteristic polymers are utilized to make beauty blends for cosmetic applications (Lewandowska et al., 2015). Keratin acquired from feathers, horns, wool, and hooves can be utilized as an ingredient in hair products. Because keratin is the auxiliary building block of hair; hence, its products, supplements and treatments are thought to reinforce hair integrity and make it healthier. Hair cuticles absorb keratin proteins, resulting in smooth and glossy hair. Keratin reduces the fizziness in curly hair, making it easier to style.

2.4.2. Fertilizers

Keratinous wastes are used in the production of bio-fertilizers as a way of recycling biomass. The high nitrogen content in feathers makes them an excellent material to be used as

fertilizers. Hydrolyzed feathers were applied to improve herbaceous perennial flowering plants and other cultivation crops (Sharma and Gupta 2016). Feathers treated with thermophilic actinomycetes strains were used as fertilizers to cultivate ryegrass (Sharma and Gupta, 2016).

2.4.3. Wound dressing

In wound dressing, the material made from keratin biomass act as a non-antigenic wound healing material. The permeable sponge networks in keratin play a role in absorbing exudates from wounds and preserving a healthy and moist environment for the healing wound (Kelly et al., 2010). In vitro studies have described keratin to speed up the wound healing process by stimulating cellular migration into the wound (Pechter et al., 2012). Keratin is comprised of bioactive properties that elevate wound healing. The wound healing process is initiated by the release of keratin peptides from wound dressings, activating keratinocytes (skin cells) in the wound bed, thus stimulating them to proliferate, leading to wound healing (Than et al., 2012). In non-healing chronic wounds, keratinocytes have been reported to be inactive (Than et al., 2012). There are different forms of wound dressings for different types of wounds. Keratin wound dressings in the form of gel/ointment are suitable for dry wounds (Kelly et al., 2010; Wang et al., 2017).

2.4.4. Pharmaceuticals

2.4.4.1. Nanoparticles

Keratin based nanoparticles have demonstrated high effectiveness in anticancer drug-carriers, and their ability to target tumours is credited to pH sensitivity (Xu et al., 2014; Li et al., 2017; Varma et al., 2018). Keratin covered gold nanoparticles displayed antibacterial activity against *Staphylococcus aureus* through demonstrative compatibility with living cells; also,

they displayed antioxidant activities (Saravanan et al., 2011; Varma et al., 2018; Tran et al., 2018). Keratin covered silver nanoparticles, acting as anti-cancer agents, display higher stability in aqueous conditions (Reichl, 2009).

2.4.4.2. Keratin films

Keratin films have strong mechanical properties similar to those of commercially available thermoplastics. In the pharmaceutical industry, keratin films are utilized for ocular surface reconstruction and are also suitable for corneal epithelial cell expansion (Reichl, 2009; Varma et al., 2018).

2.4.4.3. Microneedles

Keratin microneedle has demonstrated safe potential in delivering drugs and other components such as serum albumin, bovine albumin and calcein (Varma et al., 2018).

2.4.4.4. Keratin hydrogels

Hydrogels have been reported to reinforce therapeutic agents' sustained-release, making them highly effective polymeric systems for tissue engineering and medical regeneration implementations. They have also been reported in the sustained release of antibiotics (Tachibana et al., 2005; Saul et al., 2011).

2.4.5. Bio-sorbent and Rubber

Keratin-based material has the prospect for application in water purification, particularly wastewater contaminated with heavy metals (Sharma and Gupta, 2016). Keratin from wool was reported to adsorb mercury, copper, silver, cadmium, lead, chromium, and aluminium (Khosa and Ullah, 2013).

Keratin derived from avian feathers, or feather meal can be used as filler in rubber compositions. The environmentally friendly components of the filler are inexpensive and easy to process.

2.4.6. Diapers and feminine hygiene products

2.4.6.1. Wool

Animal wool is in demand to manufacture diapers and feminine products because they are highly flexible, breathable, naturally bacteriostatic, water-resistant, and inexpensive to maintain (Kakonke et al., 2019). Wool fibres are considered alternatives to cellulose fibres because they can stretch to up to 70% in moist conditions and 30% in dry conditions and still retain their original shape, thus their current use in diapers and feminine production towels.

2.4.6.2. Feathers

Feathers have high moisture content and large numbers of tiny pores between barbs and barbules they have used to manufacture feminine products. The presence of tiny pores makes chicken feathers semi-permeable with increased wettability (Mahall, 2003; Afrin et al., 2009; Kakonke et al., 2019). Chicken feathers are highly flexible and hydrophobic with good compressibility and resilience, making them the right raw material for the production of disposable diapers and sanitary towels (Sharma and Gupta 2016; Tesfaye et al., 2017; Kakonke et al., 2019).

2.5. MICROBIAL DEGRADATION OF FEATHER KERATIN

2.5.1. Degradation by bacteria

Bacteria recovered from the soil in poultry farms have been reported as good feather degraders (Lucas et al., 2003; Brandelli, 2008; Bach et al., 2011; Sahoo et al., 2012; Nnolim

et al., 2020). Keratin degradation is commonly seen in Gram-positive bacteria, but a few Gram-negative bacteria strains were reported to degrade keratinous materials. Generally, bacteria of the genera; Bacillus, Stenotrophomonas, Pseudomonas, Brevibacillus, Fusarium, Geobacillus, Chryseobacterium, Xanthomonas, Nesterenkonia, Serratia, Keratinibaculum, Paenibacillus, Meiothermus, Rhodococcus, Achromobacter, Exiguobacterium, Rummeliibacillus, Sporosarcina, Brevibacillus, and Aeromonas were found to produce keratinases (Brandelli, 2008; Adelere and Lateef, 2016; Saarela et al. 2017; Li, 2019). Several studies have reported remarkable keratinolytic activities from strains of *B. licheniformis*, *B. pumilus*, *B. cereus*, and *B. subtilis* (Kim et al. 2001; Riffel et al., 2003; Lateef et al. 2010; Li, 2019). Bacterial keratinases display a wide range of optimal temperatures and pH, making them better candidates against fungal counterparts (Tamreihao et al., 2019). Some keratinolytic bacteria and the properties of their respective keratinase are presented in Table 2.2.

Table 2.1: Some bacterial keratinases and their properties

Bacterial species	**Catalytic type**	**Molecular weight (kDa)**	**Optimum pH**	**Optimum temperature (°C)**	**Reference**
Bacillus spp.	Metallo	134	7.0	40	Lee et al., 2002
Bacillus sp. JB 99	Serine	29	11	70	Shrinivas and Naik, 2011
Bacillus sp. SH-517	Metallo	51	7.5	40	Jeong et al., 2010
Bacillus altitudinis RBDV1	Serine	43	8.0	85	Pawar et al., 2018
Bacillus amyloliquefaciens S13	Serine	28	6.5	50	Hamiche et al., 2019
Bacillus cereus DCUW	Serine	80	8.5	50	Ghosh et al., 2008
Bacillus circulans	Serine	32	12.5	85	Benkiar et al., 2013
Bacillus licheniformis FK14	Serine	35	8.5	60	Suntornsuk et al., 2005
Bacillus licheniformis K-	Thiol	42	8.5	52	Rozs et al., 2001

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Bacillus licheniformis MSK103	Serine	26	9.0-10	60-70	Yoshioka et al., 2008
Bacillus licheniformis PWD-1	Serine	33	7.5	7.5	Hoq et al., 2005
bacillus pumilus NRC21	Serine-metallo	30	7.5, 8.5	50, 45	Tork et al., 2016
Bacillus subtilis MTCC (9102)	Metallo	69	6.0	40	Balaji et al., 2007
Bacillus tequilensis hsTKB2	Serine	59.89	10.5	70	Paul et al., 2014
Bacillus thuringiensis MT1	Metallo	80	9.0	50	Hassan et al., 2020
Bacillus thuringiensis serover israelensis H14 (IPS-82)	Serine	40	7.0	30	Poopathi et al., 2014
Chryseobacterium sp. Kr6	Metallo	64	8.5	50	Riffel et al., 2007
Chryseobacterium gleum	Metallo	36	8.0	30	Chaudhari et al., 2013
Nesternkonia sp. AL-20	Serine	23	10	70	Gessesse et al., 2003

2.5.2. Degradation by fungi and actinomycetes

The frequent keratinolytic fungi include Aspergillus, Penicillium, Fusarium, Microsporum, Trichoderma and Chrysosporium genera (Cao et al., 2008; Anithamand Palanivelu, 2013; Călin et al., 2017Hassan et al., 2020). Actinomycetes belonging to the genus Streptomyces and Arthrobacter were found to produce keratinases (Brandelli, 2008; Syed et al., 2009; Barman et al., 2017). The keratinolytic fungi produce sulfide together with keratinase, which is utilized in the process called sulfitolysis. In this process, cysteine's disulfide bonds are broken down, paving the path for easy degradation of keratin by the proteolytic enzyme produced. A few non-pathogenic fungi demonstrated the capabilities to degrade feathers into bioavailable proteins that may be suitable for animal feed supplementation or bio-fertilizer (Bhange et al., 2016). The sequence of amino acids in keratinases produced by fungi and actinomyces was different from keratinases produced by bacteria (Li, 2019). Some

keratinolytic fungi and actinomycetes with their respective keratinase properties are presented in Table 2.3 and 2.4, respectively.

Table 2.2: Some fungal keratinases and their properties

Fungal species	Catalytic type	Molecular weight (kDa)	Optimum pH	Optimum temperature (°C)	Reference
Aspergillus oryzae	metallo	60	8.0	50	Farag and Hassan, 2004
Doratomyces microsporum	serine	30-33	8.0-9.0	50	Gradišar et al., 2005
Myrothecium verrucaria	Serine	22	8.3	37	Moreira-Gasparin et al., 2009
Paecilomyces marquandii	serine	33	8.0	60-65	Gradišar et al., 2005
Scopulariopsis brevicaulis	Serine	36-39	8.0	40	Anbu et al., 2004
Trichoderma atrvoviride F6	serine	21	8.0-9.0	50-60	Cao et al., 2008

Table 2.3: Some actinomycetes keratinases and their properties

Actinomyces species	Catalytic type	Molecular weight (kDa)	Optimum pH	Optimum temperature (°C)	Reference
Streptomyces sp. *Strain* AB1	Serine	29.85	11.5	75	Jaouadi et al., 2010
Streptomyces sp. S7	Serine-metallo	44	11	45	Tatineni et al., 2008
Streptomyces albidoflavus	Serine	18	6.0-9.5	40-70	Bressolier et al., 1999
Streptomyces brevicaulis	serine	40-50	7.8	40	Malviya et al., 1992
Thermoanaerobacter kerationophilis	serine	135	8.0	85	Riessen and Antranikian, 2001

2.6. KERATIN DEGRADATION MECHANISM

The mechanism of keratin degradation by microorganisms is an intricate bioprocess. However, it is an economical approach to avoid the vast utilization of energy and the destruction of valuable amino acids (Gupta and Ramani, 2006; Korniłłowicz-Kowalska and Bohacz, 2011; Hassan et al., 2020). Keratin degradation mechanisms are varied depending on the organism (bacteria, fungi and actinomycetes) (Hassan et al., 2020). The crosslinking of keratins by disulfide bridges blocks putrefaction by common proteases (papain, pepsin and trypsin); keratinase is therefore required for keratin degradation new generation protease. The mechanism of keratin degradation by keratinase involves the splitting of disulfide bonds in the polypeptide chains of keratin. According to Kaul and Sumbali (1997), keratin degradation is assumed to comprise deamination and proteolysis. Kunert (1976) reported sulfitolysis during the study of wool degradation by *Microsporum gypseum*. During the process of sulfitolysis, the fragmentation of disulfide bonds that link the polypeptide chains in keratin by inorganic sulfite from fungus could result in thiol group formation. Studies by Kunert (1976) and Gradisar et al. (2005) documented the formation of cysteine and S-sulfocystein due to protein denaturation during the cleavage of disulfide bonds in keratin. The process of sulfitolysis results in the denaturation of keratin structure and gives rise to different vulnerable sites for a keratinase attack, thus further facilitating the release of peptides and amino acids.

In filamentous fungi and actinomycetes, the process of degradation begins with the growth of mycelia over keratin's surface, followed by sulfitolysis and proteolysis. The mechanism that relied on disulfide reductase action where the disulfide bonds could easily be reduced using *Vibrio* sp. Kr2 strain was proposed (Sangali and Brandelli, 2000). Yamamura et al. (2002) demonstrated techniques for the degradation of keratin by extracellular keratinases from

bacteria. This technique clarified keratin hydrolysis through the activity of disulfide reductase-like proteins and keratinases detected in the culture of *Stenotrophomonas* sp. D-1 during the decomposition of keratin. Throughout this process, the disulfide bonds were reduced by disulfide reductase, which resulted in moderately denatured proteins and thus providing keratinases with the ability to degrade keratin substrate and liberate vital products, including peptides and amino acids (Hassan et al., 2020).

Furthermore, Qu et al. (2018) conducted a study on the degradation techniques of feathers by *Stenotrophomonas maltophilia* CA-1 purified from the gut of spider *Chilobrachys guangxiensis*, which demonstrated remarkable results for the degradation of feathers by bacteria. From this study, three enzymes from the culture supernatant were identified as an alkaline serine protease, ABC transporter permease, and alkaline phosphatase (Qu et al., 2018). The study reported a total feather degradation within 20 h, which has been considered the most rapid process. Therefore, the three enzymes' degradation techniques were studied to uncover a novel mechanism for bacterial feather degradation.

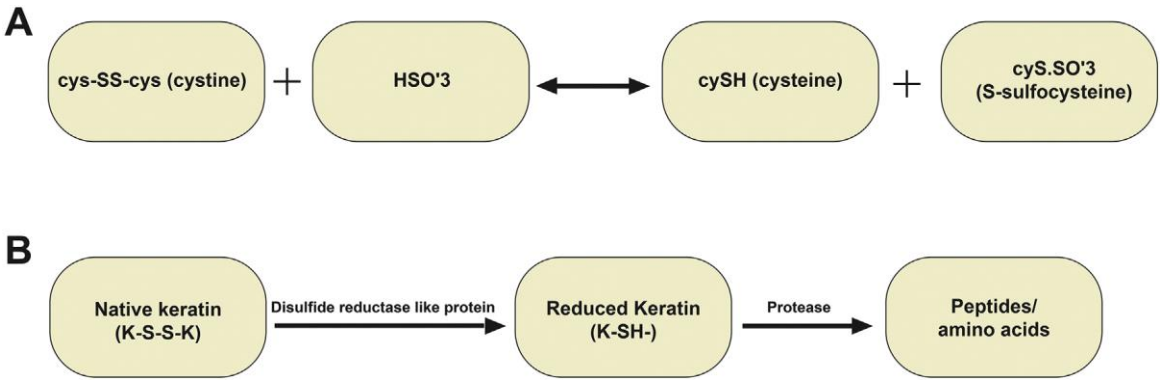

Figure 2.6: A- The process of sulfitolysis during keratin degradation, B- Mechanism of keratin degradation (Hassan et al., 2020).

2.7. KERATINASE – AN OVERVIEW

Keratinases are a distinct class of serine- or metallo-type of proteolytic enzymes recognized for degrading recalcitrant keratin protein (Brandelli and Reffel, 2005; Gradisar et al., 2005; Gupta and Ramani 2006; Mukherjee et al., 2008). The keratinases produced by different microorganisms have distinctive characteristics such as a distinct amino acid sequence, molecular weight, optimal pH and temperature (Brandelli, 2008). They present immense potentials for industrial applications because of their ability to cleave insoluble and recalcitrant keratins derived from feathers, hair and wool.

Keratinases are extracellular enzymes produced when cultivated in the presence of keratinous substrates. Although the enzyme is produced when keratin substrates are present, it can be produced in a medium containing non-keratin substrate such as soya beans, widely known to induce enzyme production (Pakshir et al., 2013). The reductase-like protein synergistically works with keratinases, facilitating complete keratin degradation (Yamamura et al., 2002).

2.7.1. Optimization of culture conditions for keratinase production

A specific growth medium containing salts, keratinous substrates, nitrogen and carbon sources is required for a successful keratinase production. Also, essential cultivation conditions, including temperature and pH, are required (Brandelli et al., 2010). The optimization of culture conditions involves changing one independent variable while keeping others at fixed levels (Brandelli et al., 2010). Most researchers use the statistical approach – response surface methodology to optimise the media components (Brandelli and Riffel, 2005; Brandelli et al., 2010).

2.7.2. Production of keratinases

Many literature compilations concerning keratinase production have been on shake-flask production (Gupta and Ramnani, 2006; Brandelli et al., 2010; Brandelli et al., 2015; Aderele and Lateef, 2016; Sharma and Gupta, 2016); but there are not many reports on the production of keratinase in solid-state fermentation (Williams et al., 1990). Extracellular keratinases are predominantly secreted when microorganisms are grown in keratin-containing substrates. Peptidases reduce disulfide bonds, while keratin serves as an inducer in a production medium (Adelere and Lateef, 2016). The process of sulfitolysis is considered feasible when complete living cells are employed (Aderele and Lateef, 2016). Several physical factors affect the amount of keratinase produced in a culture medium, including incubation time, temperature, pH, agitation speed and aeration.

2.7.3. Purification of keratinases

The purification enables further characterization of the protein of interest. The purification process selects a few proteins from the crude (Fang et al., 2013; Brandelli et al., 2015). For keratinase to be maintained in high purity, purification techniques in use include ammonium sulfate precipitation, dialysis, chromatographic techniques such as gel filtration and ion-exchange chromatography (Brandelli et al., 2015). However, these techniques may have advantages and disadvantages when applied to enzyme purification.

Table 2.4: The advantages and disadvantages of the purification process

Technique	Pros	Cons	Reference
Ammonium sulphate precipitation	Ability to scale-up	Requires dialysis after salt precipitation, and some organic solvent may denature or inactivate the enzyme	(Brandelli et al., 2015)

Gel filtration chromatography	Produce high resolution	Time-consuming and costly	(Gradisar and Friedrich, 2000)
Ion-exchange chromatography	Produce high resolution, analyses of small sample	Time-consuming and costly	(Fang et al., 2013)

2.7.4. Keratinase substrate specificity

Keratinases display broad substrate specificity, as they have sufficient activity on both soluble and insoluble protein molecules. For soluble proteins, keratinases are tested for their ability to hydrolyze casein, bovine serum albumin (BSA), gelatin and haemoglobin (Gupta and Ramnani, 2006). For insoluble proteins, keratinases are capable of feathers, collagen, horn, hair and azokeratin (Gupta and Ramnani, 2006; Brandelli et al., 2015).

2.7.5. Keratinase stability

Keratinase activity stimulated was previously reported in the presence of $CaCl_2$, $MgCl_2$, whereas $CuSO_4$ $CoCl_2$, $MnSO_4$ and $ZnSO_4$ inhibited the keratinase activity (Sivakumar et al., 2012). Reducing agents such as β-mercaptoethanol, dithiothreitol, and sodium sulphite have been reported to enhance keratinolytic activity, which indicates that keratin dismemberment by purified keratinases is efficient in the presence of reducing agents as they facilitate the hydrolysis of highly packed cysteine bridges (Yamamura et al., 2002; Riffel et al., 2003; Gupta and Ramnani, 2006). Furthermore, keratinases are generally stimulated or inhibited in non-ionic surfactants and organic solvents (Gupta and Ramnani, 2005).

2.8. INDUSTRIAL APPLICATIONS OF KERATINASES

Keratinolytic enzymes have been successfully applied in feed production, leather processing, detergent formulation, waste treatment, biomedical fields, pharmaceutical, nanotechnology,

cosmetics, among other fields of biotechnology (Gupta and Ramnani, 2006; Li et al., 2013; Adelere and Lateef, 2016). In the waste treatment industry, keratinolytic microbes are essential for feather waste management (Adelere and Lateef, 2016). The meat industry generates tons of keratin wastes due to the high demand for poultry meat; therefore, keratinolytic microbes play a significant role in managing the keratinous wastes.

There are reports of keratinolytic enzyme being used in the recovery of silver from gelatin. Silver could be recovered from the waste X-ray film or photographic film by a keratinase application. The waste X-ray film has been reported to contain an insoluble protein that is recalcitrant to degradation by common proteolytic enzymes (Mohammed, 2011). However, keratinolytic enzymes have shown to biodegrade gelatin, a constituent of waste X-ray film (Mohammed, 2011). Silver recovery requires removing bound silver from gelatin by burning the films. Then silver is isolated from the protein layer using keratinolytic enzymes. Thus, keratinases play a vital role in the recovery of silver (Mohammed, 2011). There are reports regarding the valorization of feathers into amino acids and peptides for animal feedstuff (Coward-Kelly et al., 2006). Industrial sectors also use the horn and bovine hoof to produce horn meals through dekeratinization (Brandelli et al., 2015). Keratinases can modify silk and wool; therefore, they are potential candidates for textile processing industries. Keratinases from a Pseudomonas strain used to treat polyester-blended fabrics and wool were shrink resistance and high tensile strength (Cai et al., 2011). In cosmetic industries, keratinase was used to formulate depilatory agents for shaving hair and skin lightening creams (Yang, 2012). Keratinases have also demonstrated the potential to degrade the thickened layer of dead skin (hyperkeratosis) found in toes and fingers; therefore, they are a viable alternative to the common method of using salicylic acid (Gupta and Ramnani, 2006). Some keratinase have been used to formulate different products at a commercial scale, and they are presented in Table 2.5.

Table 2.5: Selected keratinase-based products in commercial use (Hassan et al., 2020)

Product name	**Microbial source**	**Manufacturer**
Versazyme	*B. licheniformis*	BioResourse international Inc
Valkerase	*B. licheniformis*	BioResourse international Inc
Prionzyme (TM)	*B. licheniformis*	Genencor international Inc
Protease P4860	*B. licheniformis*	Alcalase, Novozymes Crop
Protease P5860	*Bacillus* sp.	Esperase, norvozymes crop
Protease P3111	*Bacillus* sp.	Savinase, norvozymes crop
Proteinase k	*Tritirachium album*	New England biolabs
Keratoclean®Hydra PB	*B. licheniformis*	Proteos biotech
Keratoclean sensitive PB	*B. licheniformis*	Proteos biotech

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CHAPTER THREE

KERATINASE PRODUCTION AND CHICKEN FEATHER DEGRADATION BY KERATINOLYITC *Bacillus* spp

ABSTRACT

Chicken feathers are generated as by-products from poultry processing farms, and they are 90% keratin in composition. Keratin is an insoluble and structural protein that shows recalcitrance to hydrolysis by classical proteolytic enzymes, including trypsin, pepsin, and papain. Keratinases have opened a new avenue for the management of keratin-rich agro residues, and this approach of wastes handling is attractive from an ecological perspective. Hence, keratinolytic bacteria previously isolated from the dump site were identified using 16S rDNA sequencing. The optimal fermentation conditions were constructed for enhanced extracellular keratinase production and chicken feather degradation. Also, the amino acid analysis of the chicken feather hydrolysates was carried out. Based on 16S rDNA sequencing and phylogenetic analysis, the isolates coded as SSN-02 and HSN-03 showed a high percentage of sequence homology with *Bacillus* spp.; hence, they were identified as *Bacillus* sp. NFH5 and *Bacillus* sp. FHNM respectively. *Bacillus* sp. NFH5 showed optimal keratinase production of 1149.99 ± 80.99 U/mL after 96 h of incubation time, in optimized fermentation conditions that included pH (4.0), chicken feather (1.5%, w/v), inoculum size (3%, v/v) and temperature (30 ^{o}C). Similarly, *Bacillus* sp. FHNM demonstrated the maximum keratinase production of 480 ± 41.14 U/mL 144 h post cultivation, in optimized fermentation conditions with pH (7.0), chicken feather (2.0%, w/v), inoculum size (3%, v/v) and temperature (30 ^{o}C). The chicken feather hydrolysate by *Bacillus* sp. NFH5; the amino acid content was in high concentration (>1.0g/100g sample) include arginine (1.8), serine (1.16), aspartic acid (1.95), glutamic acid (2.47), proline (1.16) and glycine (1.45). *Bacillus* sp. FHNM feather hydrolysates, contained (g/100g of sample): arginine (1.9), serine (1.4), aspartic acid (2.5), glutamic acid (2.51), glycine (1.51), proline (1.13), leucine (1.030, histidine (1.25), and lysine (1.06) (g/100g of sample) in high concentration. Therefore, these results suggest that *Bacillus*

spp. could be ideal for sustainable production of active keratinases and use in the valorization of keratinous biomass.

3. INTRODUCTION

Keratin is an insoluble and structural protein that shows recalcitrance to hydrolysis by classical proteolytic enzymes, including trypsin, pepsin, and papain (Altun et al., 2006). Keratinous biomass is generated as by-products from meat and poultry processing farms. Methods that have been previously used for the treatment and recovery of keratinous waste's protein values have disadvantages that include environmental pollution and vast energy investment, which ultimately affect the quality of the products (Korniłłowicz-Kowalska and Bohacz, 2011). An eco-friendly method is therefore essential for recycling these keratinous wastes into valuable products. At present the frequently used enzymes that metabolize several natural substrates belong to the hydrolase group and mostly of microbial origin (Mitidieri et al., 2006). Various species of fungi, actinomycetes and bacteria have been implicated in keratin biodegradation. Among the bacterial species, prolific keratinase production and efficient keratin degradation have been demonstrated by the genus Bacillus (Gradišar et al., 2005; Jaouadi et al., 2010; Cedrola et al., 2012). Keratinases have opened new avenue for the management of keratin-rich agro residues and this approach of wastes handling is attractive from ecological perspective.

Keratinases are promising candidates in numerous areas of biotechnology due to their robustness and catalytic tendencies (Verma et al., 2017). They display catalytic efficiency over a broad range of pH and temperature. The optimal pH conditions for most keratinases are from the neutral to alkaline pH range (Bouacem et al., 2016; Su et al. 2017). Keratinase-assisted generation of high quality keratin hydrolysates has revolutionized the application of keratin protein in the present day bio-based industry. Analysis of keratin hydrolysates has

shown the presence of various amino acids of proteins and bioactive peptides (Sharma and Gupta, 2016). These bioavailable and assimilable protein sources from cheap and readily available keratinous residues have shown immense potentials for application in livestock production. In this study bacteria previously isolated from agro-wastes dump sites that showed remarkable chicken feather degradation were used. The fermentation conditions were optimized for improved keratinase production and efficient chicken feather degradation. Also, the protein hdrolysates from chicken feather degradation were analyzed for various amino acids of protein.

3.1. MATERIALS AND METHODS

3.1.1. Preparation of keratin substrate

Keratin substrate used in the study was prepared from chicken feathers collected from poultry farm. The chicken feathers were washed with tap water to remove the blood stains, and subsequently dried in an oven at 60 ^{o}C for 48 h. After that, they were pulverized into fine powders and stored in an air-tight container at room temperature.

3.1.2. Bacterial isolates

Chicken feather degrading bacteria coded as SSN-01 and HSN-01 which were used for the study were previously isolated from municipal dump site and preserved in 20% glycerol at -80 ^{o}C.

3.1.3. Fresh inoculum preparation

The bacterial isolates were resuscitated by passaging in basal salt media (BSM) that contained 0.3 g/L K_2HPO_4; 0.4 g/L KH_2PO_4; 0.2 g/L $MgCl_2$; 0.22 g/L $CaCl_2$ and 10 g/L chicken feather powder (CFP) in 100 mL Erlenmeyer flasks. The flasks were inoculated with 10 µL of the stock cultures and incubated in rotary shaker at 30 ^{o}C for 96 h. After incubation,

a loopful of each culture was streaked on chicken feather powder agar (CFPA) plates and incubated at 30 °C for 24 h. The CFPA plates were prepared with similar constituents of BSM and CFP with addition of bacteriological agar (15 g/L). Colonies from the plates were harvested into microtube with normal saline and homogenized by vortexing. After that, the optical density of the bacterial suspension was adjusted 0.1 at 600 nm with the aid of spectrophotometer. The standardized bacterial suspension therefore served as the fresh inoculum for subsequent experiment.

3.1.4. Keratinase production

The production medium was composed with the following; 0.3 g/L K_2HPO_4; 0.4 g/L KH_2PO_4; 0.2 g/L $MgCl_2$; 0.22 g/L $CaCl_2$ and 10 g/L CFP in 250 mL Erlenmeyer flasks. The flasks were sterilized by autoclaving and the medium pH was aseptically adjusted to 6.0 prior to inoculation with 2% (v/v) inoculum. The flasks were incubated at 30 ^oC under constant agitation (150 rpm) for 96 h. After the incubation, the fermentation medium was centrifuged at 15,000 rpm for 10 min, and the supernatant served as crude enzyme for the subsequent analysis.

3.1.5. Assay for keratinase activity

Keratinase activity assay was done following an established method (Nnolim et al., 2020). Briefly, 0.5 mL of 10 g/L of keratin azure in tris-HCl (pH 8.0; 0.1 M) was mixed with 0.5 mL of crude keratinase solution and incubated in a water bath at 50°C for 1 h. After incubation, the reaction mixture was placed on ice cold water to stop the reaction. Subsequently, the mixture was centrifuged at 12,500 rpm for 10 min to remove undegraded keratin azure. An aliquot of the supernatant was used to determine the azo dye released from the substrate by reading the absorbance at 595 nm using the SYNERGYMx 96 wells microplate reader (BioTek, USA). The reaction mixture containing only crude enzyme and buffer without the

keratin azure served as control. One Unit (U) of keratinase was defined as the amount of enzyme causing 0.01 absorbance increases under the standard assay protocol described.

3.1.6. Total protein determination

Bovine serum albumin (BSA) was used as a standard protein to construct a standard curve for extrapolating the protein concentration in the test sample as described by Bradford (1976).

3.1.7. Thiol concentration determination

Thiol groups emanating from the chicken feather keratinolysis was determined using the method of Ellman (1959). Ellman's reagent (5,5-dithio-bis-(2-nitrobenzoic acid); DTNB) (Sigma-Aldrich, St. Louis, MO, USA), 4 mg was dissolved in I mL of phosphate buffer (pH 8, 0.1 M). Thereafter, 500 µL of distilled water was mixed with 250 µL crude extract and 50 µL of DTNB solution. The mixture was allowed to stand at room temperature for 5 min. The yellow colour development that showed the reduction of DTNB by free sulfhydryl groups to give 2-nitro-5-thiobenzoic acid (TNB) was monitored at 412 nm.

3.1.8. Molecular identification of the chicken feather degrading bacteria

The genomic DNA of the chicken feather degrading bacteria coded as SSN-01 and HSN-01 was extracted using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research). The 16S target region was amplified using the OneTaq® Quicklod® 2X Master mix with universal oligonucleotides 27F: 5'- AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTTACGACTT-3' as the forward and reverse primers, respectively. The PCR products were electrophoresed on an ethidium bromide-stained agarose gel and subsequently, gel extracted with Zymoclean™ Gel DNA Recovery Kit (Zymo Research). The extracted fragments were sequenced in the forward and reverse direction (Nimagen,

BrilliantDye™ Terminator Cycle Sequencing Kit v3.1, BRD3-100/1000) and purified (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™). The purified fragments were evaluated on the ABI 3500xl Genetic Analyzer (Applied Biosystems, Thermofisher Scientific), and further analyzed using CLC Bio Main Workbench v7.6. BLAST search in NCBI was used to retrieve related sequences and the phylogenetic tree was constructed in MEGA X.

3.1.9. Optimization of physiochemical conditions for keratinase production

Optimization of the fermentation conditions followed one variable at a time approach. The effect of initial medium pH on keratinase production was studied by varying the initial medium pH from 3.0 to 11.0 at an interval of 1 unit. Similarly, the effect of incubation temperature on keratinase production was evaluated by incubation the production flasks at different temperatures (25℃, 30°C, 35℃, 40℃ and 45℃). Also, the inoculum size influence on extracellular keratinase production by the isolates under investigation was assessed by using different concentrations of the standardized bacterial suspension that ranges from 1-6% (v/v), at an interval of 1%. Effect of chicken feather concentrations on keratinase production was investigated by using different feather concentrations that ranged from 0.5-3% (w/v) at an interval of 0.5%.

3.1.10. Time course study

The kinetics of keratinase production by the study isolates was investigated at an optimized fermentation conditions. The production was carried out in 250 mL Erlenmeyer flasks containing improved fermentation media and aliquots were withdrawn in an aseptic condition at an interval of 24 h and were used to determine the keratinase production, pH change, thiol concentration and total protein content.

3.1.11. Profiling of amino acid contents of the chicken feather hydrolysates

The amino acid analysis (except cysteine and tryptophan) was carried out by acid hydrolysis, pre-column derivatisation, separation by HPLC and detection using a fluorescence detector (Einarsson et al., 1983). Cysteine determination involved oxidization of cystine/cysteine to cysteic acid, acid hydrolysis, pre column derivatisation, separation by HPLC and detection using a fluorescence detector (Gehrke et al., 1985, Cunico et al and Williams, 1984). Additionally, Tryptophan was assessed through enzymatic hydrolysis, separation by HPLC and detection using a fluorescence detector (De Vries et al., 1980).

3.2. RESULTS AND DISCUSSION

3.2.1. Kertinolytic Bacterial identification

Following the 16S rDNA sequencing and phylogenetic analysis, the isolates coded as SSN-01 and HSN-01 showed higher percentage of sequence homology with other *Bacillus* spp. including *Bacillus* sp. UFSC-31S3 (accession number MT269033) and Bacillus sp. 44 (accession number MW092680), therefore, they were identified as *Bacillus* sp. NFH5 and *Bacillus* sp. FHNM respectively. The nucleotide sequences of *Bacillus* sp. NFH5 and *Bacillus* sp. FHNM were assigned accession numbers MW165830 and MW165831, respectively, during their submission to the NCBI GenBank. The phylogenetic tree showing the study isolates and other *Bacillus* spp. from the database is presented in Figure 3.1. The keratinolytic potentials of *Bacillus* species cannot be over emphasized. The group has been extensively reported to demonstrate extraordinary keratinolytic activity towards keratinous biomass degradation (Nnolim et al., 2020).

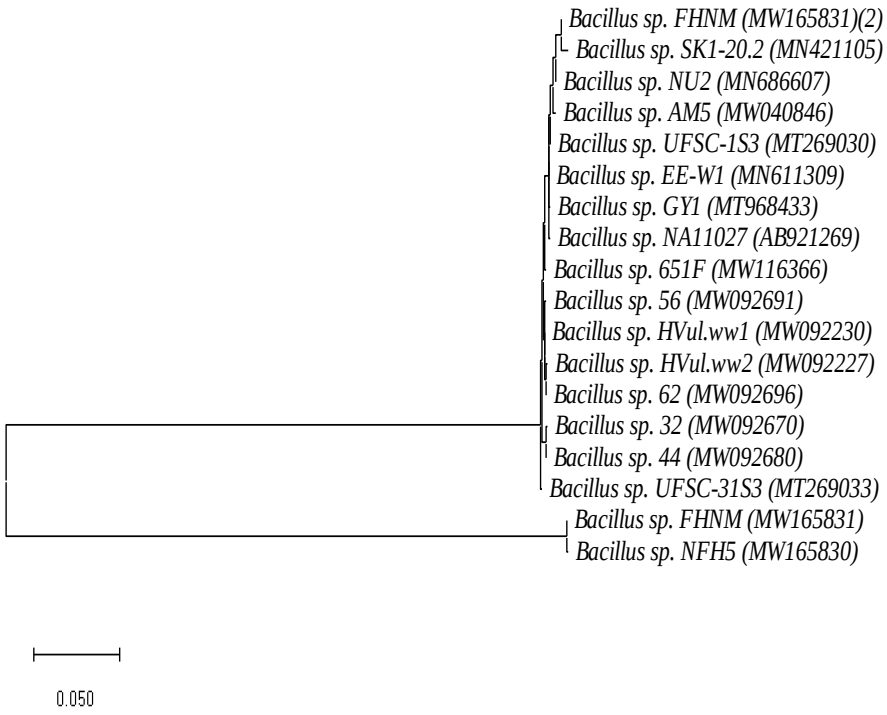


Figure 3.1: The phylogenetic tree based on 16S rDNA sequence analysis of the isolates *Bacillus* sp. FHNM and *Bacillus* sp. NFH5, and selected closely related strains from the database. The accession numbers are displayed in parenthesis.

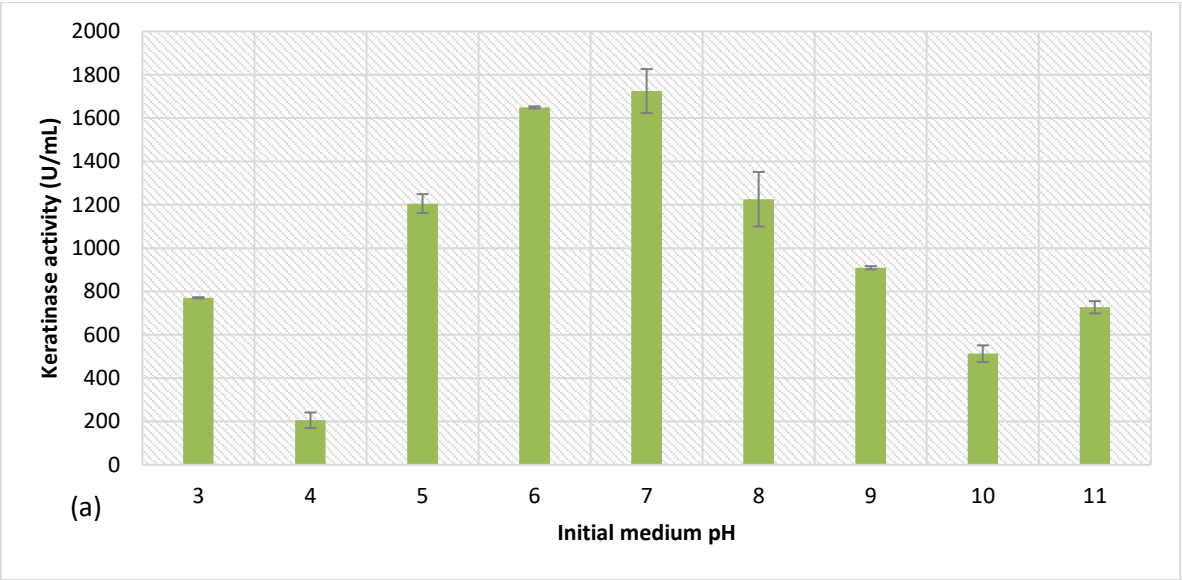
3.2.2. Fermentation conditions optimization

Keratinase production by the *Bacillus* spp. was assessed from pH 3-11, and the results showed that the isolated had extracellular keratinase activity at a broad spectrum of pH conditions (Fig. 3.2). *Bacillus* sp. FHNM recorded considerable keratinase production at pH 3 with enzyme activity of 770.71 U/mL (Fig. 3.2a). The keratinase activity decreased at pH 4, and subsequently increased from pH 5, with maximum extracellular keratinase production of

1724.55 U/mL at pH 7. Beyond the optimal pH, keratinase activity decreased with more alkalinisation of the initial medium pH (Fig. 3.2a).

Bacillus sp. NFH5 showed remarkable keratinase activity from pH 3 to pH 8, with the maximum keratinase production of 1198.18 U/mL at pH 4 (Fig. 3.2b). The enzyme production drastically decreased after pH 8, with the least activity of 331.82 U/mL obtained at pH 10.

The pH of a medium is an important factor that influences the entire microbial metabolism by regulating the function of genes, permeability of cell membrane and transportation of nutrients and metabolites across the microbial membrane. Hence, maximum secretion of metabolite of interest occurs when the prevailing pH is satisfactory for the synthesis and secretion of a particular metabolite. Maximum keratinase production at a neutral pH by other *Bacillus* spp. has been previously reported (Jain and Sharma, 2012; Alahyaribeik et al., 2020). *Bacillus* sp. NFH5 optimal keratinase production was similar to another study in this field (Nnolim et al., 2020). Also, Kim et al. (2001) documented remarkable keratinase activity of some *Bacillus* spp. in weak acidic conditions. Many reports have indicated that keratinase production by bacterial isolates was optimally achieved from neutral to alkaline condition (Sutornsuk and Sutornsuk, 2003; Sivakumar et al., 2012). The significant enzyme production of the study isolates at a broad range of pH suggests their numerous biotechnological application potentials.



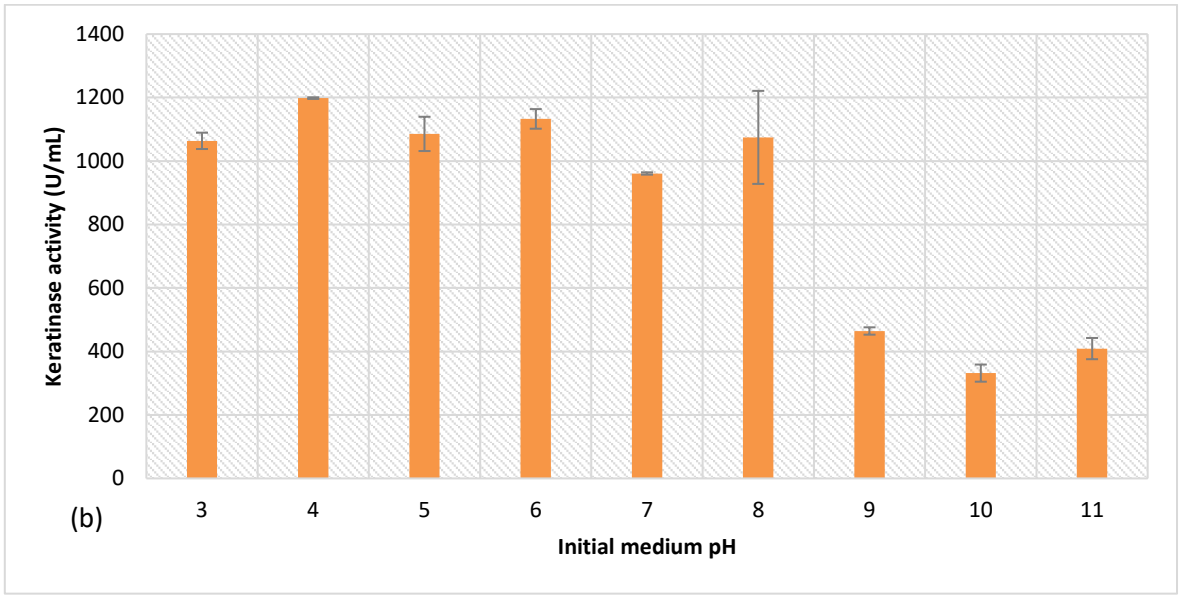
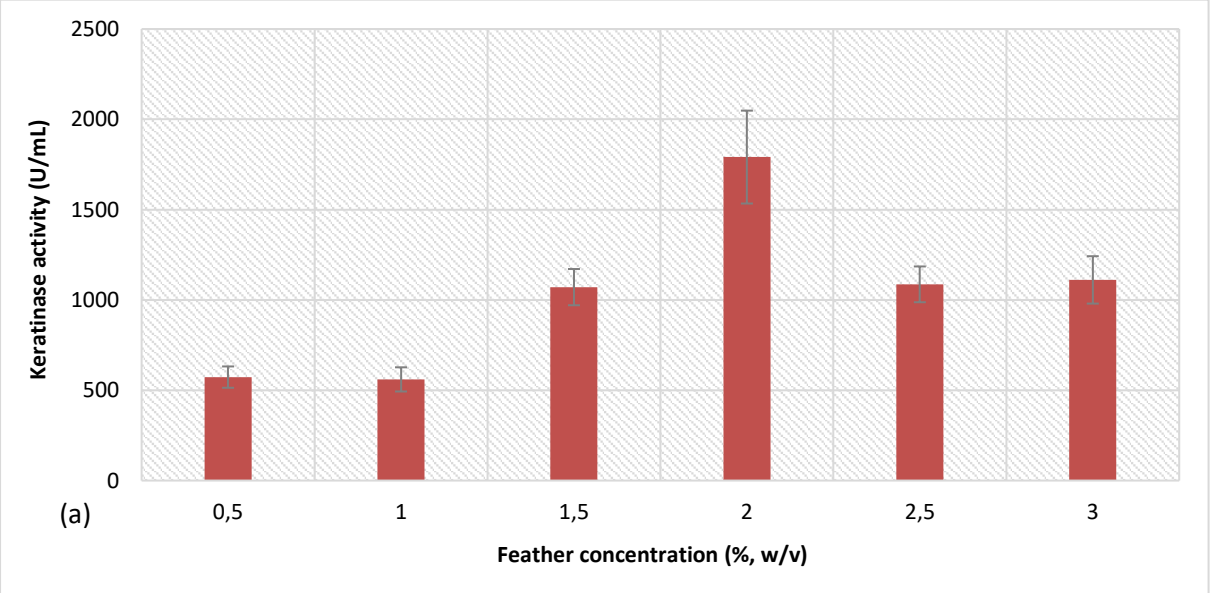


Figure 3.2: Effect of initial medium pH on keratinase production by (a) *Bacillus* sp. FHNM (b) *Bacillus* sp. NFH5.

The effect of feather concentration on keratinase production was studied, and the findings indicated that extracellular keratinase secretion by the *Bacillus* spp. increased with increasing medium feather concentration (Fig. 3.3). Consequently, *Bacillus* sp. FHNM achieved maximum keratinase production of 1790.9 U/mL at 2% (w/v) chicken feathers, and the

enzyme activity decreased afterwards (Fig. 3.3a). For *Bacillus* sp. NFH5, the optimal extracellular keratinase activity of 1819.99 U/mL was obtained in a medium with 1.5% (w/v) chicken feathers (Fig. 3.3b). Above 1.5% chicken feathers, the enzyme production gradually decreased and finally dropped significantly at 3% chicken feathers with enzyme activity of 1070.91 U/mL.

Keratinases are generally produced in media that contained keratin substrates as the principal source of carbon and nitrogen (Nnolim et al., 2020). The pattern of feather requirements for optimal extracellular secretion of keratinases by the study *Bacillus* spp. differed significantly, hence, showing the strains diversity. Different bacterial species show variable chicken feather optima for the keratinase coding gene expression and extracellular enzyme secretion (Chen et al., 1995; Cai et al., 2008; Alahyaribeik et al., 2020). Keratinase production by *Bacillus weihenstephanensis* PKD5 was remarkably inhibited by 1.5 to 2% (w/v) feather (Sahoo et al., 2012). High feather concentrations were reported elicit substrate inhibition by increasing the medium viscosity and decreasing the medium concentration of dissolved oxygen; hence affect microbial growth and metabolism (Suntornsuk and Suntornsuk, 2003). The ability of *Bacillus* sp. NFH5 to show improved keratinase production at broad range of feather concentration indicate its relevance in sustainable biotechnology.



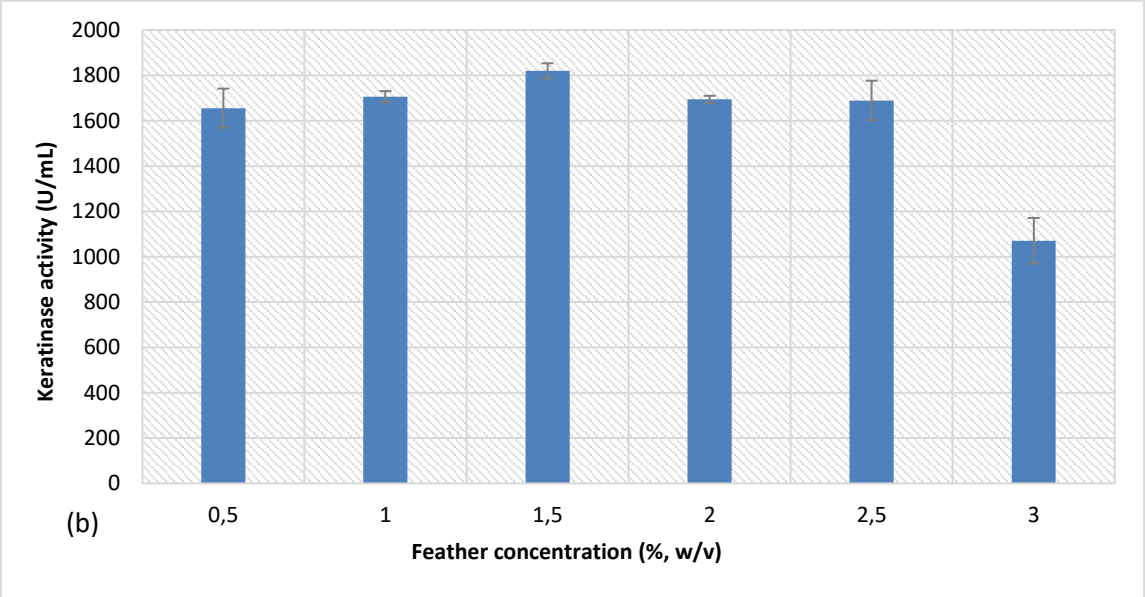


Figure 3.3: Effect of feather concentration on keratinase production by (a) *Bacillus* sp. FHNM (b) *Bacillus* sp. NFH5.

Inoculum sizes were varied from 1 – 6%, and the results showed that *Bacillus* sp. FHNM optimally produced keratinase at 6% (v/v) inoculum. However, there was no significant difference in keratinase activity among inoculum sizes 3%, 5% and 6% (Fig. 3.4a).

For *Bacillus* sp. NFH5, optimal keratinase production was obtained at 3% (v/v) inoculum. Although, there was no statistical difference in enzyme activity at 1%, 3% and 6% inoculum sizes (Fig. 3.4b).

Inoculum size has been reported to influence the enzyme production (Hameed et al., 1999). Similar findings were reported by Sivakumar et al. (2013) where 3% (v/v) was found to be optimum for keratinase production from *B. cereus* and *Pseudomonas* sp. Also, inoculum side that ranged from 2-5% was ideal for the maximum keratinase production similar study (Mabrouk et al., 1999; Kanekar et al., 2002).

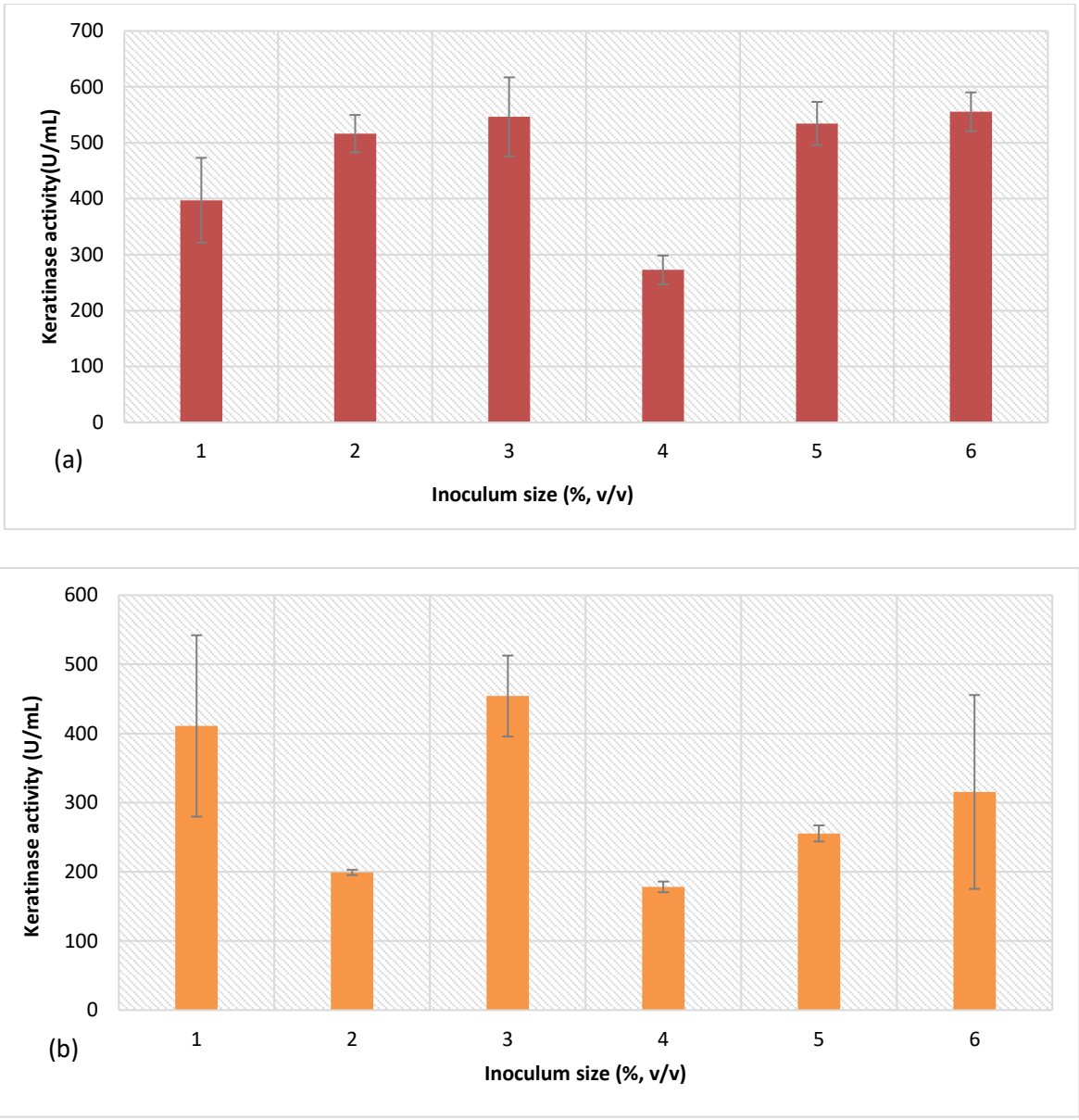
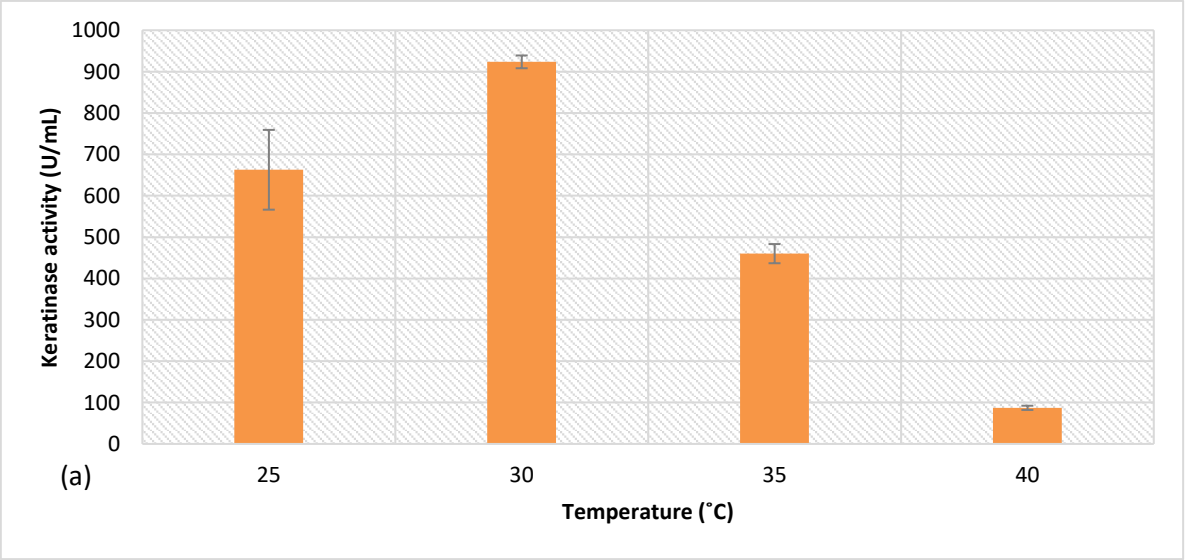


Figure 3.4: Effect of inoculum size on keratinase production by (a) *Bacillus* sp. FHNM (b) *Bacillus* sp. NFH5.

The effect of incubation temperature on keratinase production by *Bacillus* spp. was investigated. The results indicated that the isolates remarkably produced keratinases at optimal temperature of 30 °C (Fig. 3.5a and b). Above 30 °C, the keratinase activity consistently decreased with further increase in incubation temperature.

The observed results indicated that both *Bacillus* sp. FHNM and *Bacillus* sp. NFH5 optimally display the keratinolytic potentials at mesophilic condition, and might be a reflection of the temperature condition of the environmental source where these bacteria were isolated. Previous studies have documented that keratinase producing bacteria function exceptionally well at temperatures ranging from 25 ºC – 40 ºC (Suntornsuk and Suntornsuk, 2003; Jeong et al., 2010; Pereira et al., 2014; Nnolim et al., 2020). However, there have been reports on optimal keratinase production by bacteria at elevated temperature condition (Sivakumar et al., 2012; Akhter et al., 2020). The optimal demonstration of keratinase activity by the isolates under investigation at mesophilic condition underpins their significance in the biotechnological development.



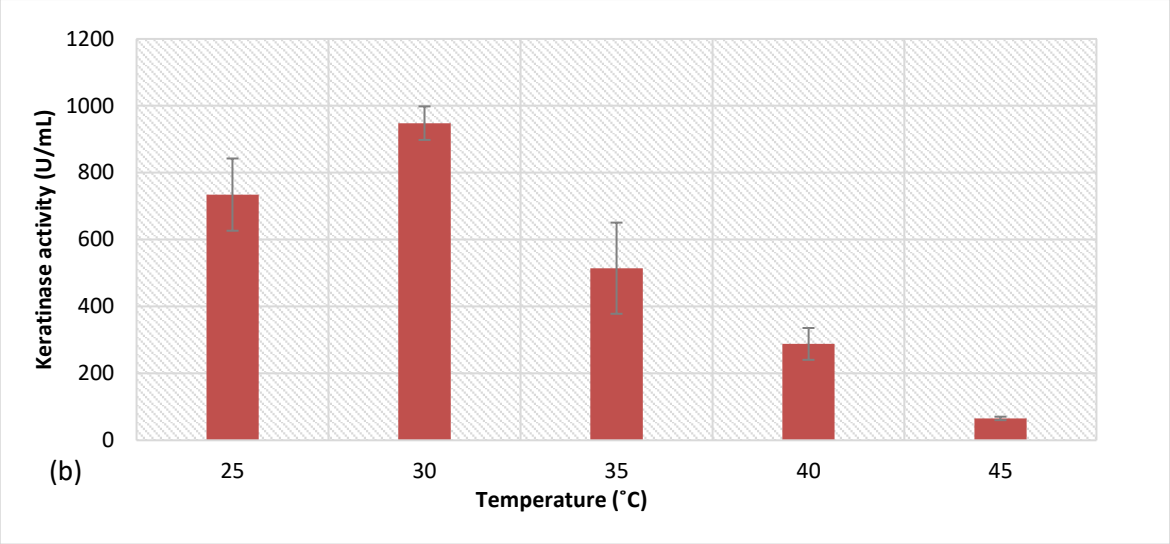


Figure 3.5: Effect of incubation temperature on keratinase production by (a) *Bacillus* sp. FHNM (b) *Bacillus* sp. NFH5.

3.2.3. Time course study

The effect of incubation period on keratinase activity of the *Bacillus* spp. was investigated.

For *Bacillus* sp. FHNM, the results showed that keratinase activity and total protein increased with prolonged incubation, with respective optimum at 144 h (480±41.14 U/mL) and 168 h (0.55±0.06 mg/mL) as shown in Figure 3.6a. However, keratinase production decreased at 168 h. Likewise, 1ncrease in incubation time results in generation of high concentration of thiol groups in the fermentation medium. The maximum thiol group concentration of 3.84±0.09 mM was obtained at 96 h of incubation period (Fig. 3.6b). Beyond 96 h, the thiol concentration decreased with further increase in incubation time. The pH of the medium was relative stable during the fermentation process.

For *Bacillus* sp. NFH5, the keratinase production and protein concentration peaked after 96 h and 120 h of incubation with enzyme activity and protein content of 1149.99±80.99 U/mL and 0.38±0.05 mg/mL respectively (Fig. 3.7a). The enzyme activity and protein concentration decreased drastically at 144 h and 168 h of incubation respectively. The thiol groups

accumulated in the medium as the incubation time increased. The maximum concentration of 9.48±0.79 mM was then recorded at 120 h of incubation period (Fig. 3.7b); and subsequently decreased at 144 h. Furthermore, the initial medium pH changed considerably from the initial value of 4.0 at the start of fermentation to a final value of 7.41 after 168 h (Fig 3.7b).

The optimal keratinase production from *Bacillus* sp. NFH5 was in accordance with the report by Akhter et al. (2020) where *B. cereus* had highest yield of keratinases after 96 h of incubation. Similar to one of our findings, a study by Sahoo et al. (2012) recorded maximum keratinase production by *B. weihenstephanensis* PKD5 at 144 h of incubation period, which decline thereafter. The high concentrations of total protein quantitated in the fermentation medium indicated the presence of high amount of soluble proteins. The remarkable concentration of thiol groups indicated efficient chicken feather degradation by the produced keratinolytic enzymes. Similar thiol concentrations were reported in other study (Nnolim et al., 2020), and this finding indicates the relevance of this isolates in bioconversion of the recalcitrant keratinous residues into quality protein hydrolysates for other biotechnological applications. The change in medium pH has been attributed to increasing ammonia concentration originating from the deamination of soluble degradation products (Cai et al., 2008; Patinvoh et al., 2016).

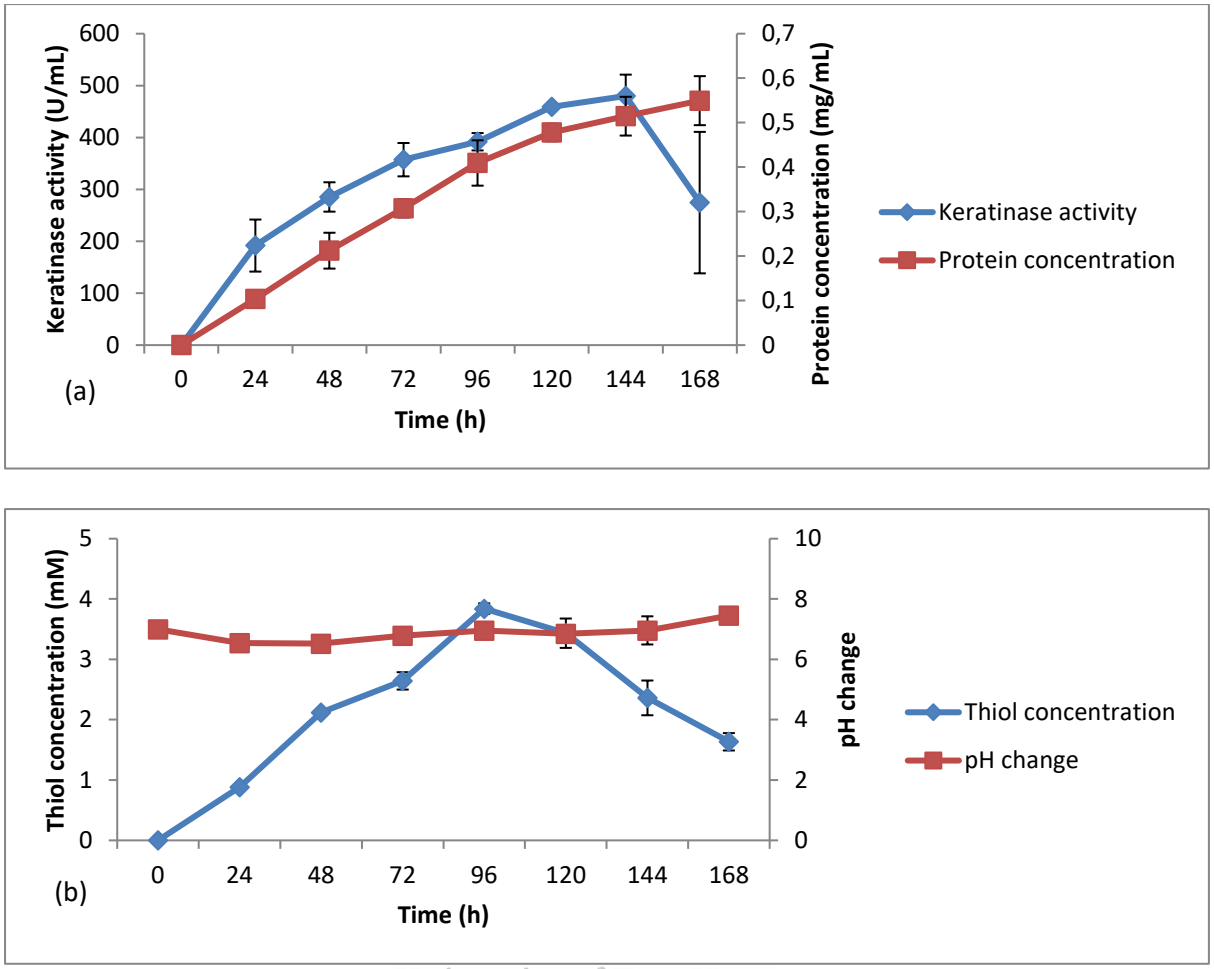
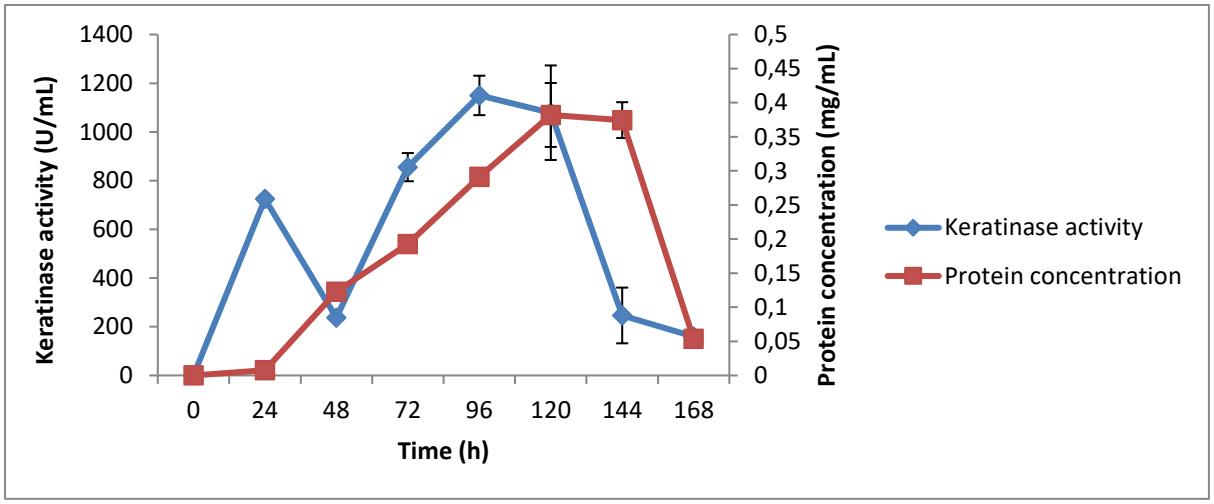


Figure 3.6a and b: Time course study of keratinase production by *Bacillus* sp. FHNM under optimized fermentation conditions.



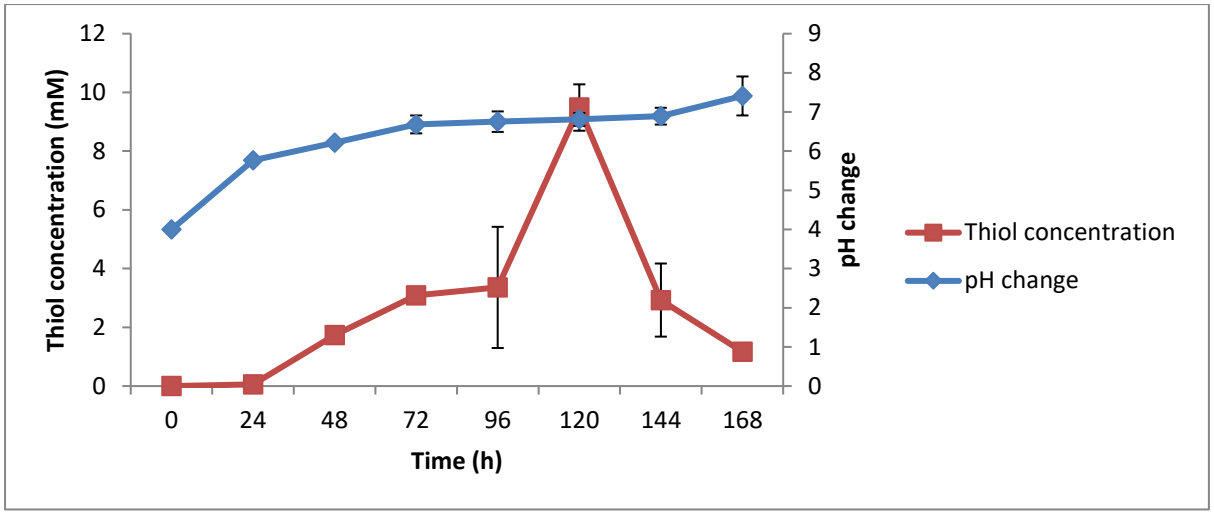


Figure 3.7a and b: Time course study of keratinase production by *Bacillus* sp. NFH5 under optimized fermentation conditions.

3.2.4. Determination of amino acid contents of the feather hydrolysates

The chicken feather hydrolysates that emanated from fermentation process were analyzed for various amino acids of protein. For the hydrolysates from *Bacillus* sp. FHNM, the amino acids with the most abundance (>1.0g/100g of sample) include arginine (1.9), serine (1.4), aspartic acid (2.5), glutamic acid (2.51), glycine (1.51), proline (1.13), leucine (1.030, histidine (1.25), and lysine (1.06) as presented in Table 3.1. However, these amino acids HO-proline, methionine, tyrosine, tryptophan and cysteine showed the respective lowest concentration of 0.01, 0.25, 0.27, 0.26 and 0.38 (g/100g samples).

For *Bacillus* sp. NFH5 chicken feather hydrolysate, the amino acids in relatively higher concentration (>1.0g/100g sample) include arginine (1.8), serine (1.16), aspartic acid (1.95), glutamic acid (2.47), proline (1.16) and glycine (1.45) (Table 3.2). On the other hand, HO-proline, methionine, tyrosine, tryptophan and cysteine recorded the lowest abundance of 0.1, 0.31, 0.38, 0.37 and 0.34 (g/100g sample).

The ability of the keratinolytic bacteria to mediate the liberation the various amino acids from chicken feathers promote their significance for sustainable recycling of keratinous agro-wastes into valuable products with enormous application potentials. The low abundance of some amino acids may be attributed to the site of cleavage of feather keratin by the keratinolytic enzymes or low concentration of these amino acids in the feather biomass. Likewise, studies have documented low contents of methionine, lysine, histidine and tryptophan in feather meal (Baker et al., 1981; Moritz and Latshaw, 2001; Adejumo et al., 2016).

Table 3.1: Amino acid profile of *Bacillus* sp. FHNM chicken feather hydrolysate

Amino acid	Concentration (g/100g sample)
Cysteine	0.38
Tryptophan	0.26
Arginine	**1.90**
Serine	**1.40**
Aspartic acid	**2.50**
Glutamic acid	**2.51**
Glycine	**1.51**
Threonine	0.89
Alanine	0.99
Tyrosine	0.27
Proline	**1.13**
HO-Proline	0.01
Methionine	0.25
Valine	0.93
Phenylalanine	0.73
Isoleucine	0.58
Leucine	**1.03**
Histidine	**1.25**
Lysine	**1.06**

Table 3.2: Amino acid profile of *Bacillus* sp. NFH5 chicken feather hydrolysate

Amino acid	Concentration (g/100g sample)
Cysteine	0.34
Tryptophan	0.37
Arginine	**1.80**
Serine	**1.16**
Aspartic acid	**1.95**
Glutamic acid	**2.47**
Glycine	**1.45**
Threonine	0.83
Alanine	0.89
Tyrosine	0.38
Proline	**1.16**
HO-Proline	0.01
Methionine	0.31
Valine	0.99
Phenylalanine	0.78
Isoleucine	0.59
Leucine	0.99
Histidine	0.99
Lysine	0.93

3.3. CONCLUSION

In conclusion, the keratinolytic bacteria previously isolated from dump site was identified as *Bacillus* sp. FHNM and *Bacillus* sp. NFH5 based on 16S rDNA sequencing and phylogenetic analysis. The isolates produced keratinases at mesophilic conditions and at a broad spectrum of pH. The optimal extracellular keratinase activity was obtained at 144 h and 96 h for *Bacillus* sp. FHNM and *Bacillus* sp. NFH5 respectively. The degradation of chicken feathers resulted in accumulation of high concentration of thiol groups in the fermentation medium, and this signified the keratinolytic efficiency of the study isolates. The quantitative analysis of the fermentation broth showed presence of different amino acids of protein in variable concentrations. Therefore, these results suggest that the study isolates - *Bacillus* spp. could be

ideal candidates for the sustainable production of active keratinases as well as valorization of the abundantly generated keratinous biomass.

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CHAPTER FOUR

CHARACTERIZATION OF KERATINOLYTIC PROTEASES FROM *Bacillus* SPP

ABSTRACT

Keratinases are a group of proteolytic enzymes that are endowed with degradation of keratin into peptides and amino acids. They are recently gaining attractions from biotechnologists because of their multifaceted application potentials. In the present study, *Bacillus* sp. FHNM and *Bacillus* sp. NFH5 were used to produce keratinases in relatively cheap media. The biochemical properties of the keratinases were also determined. The keratinases were optimally active at pH 8.0. *Bacillus* sp. FHNM showed an optimal temperature of 100 ^{o}C; while *Bacillus* sp. NFH5 keratinase displayed optimal activity at 90 ^{o}C. The keratinases were inhibited by EDTA and 1,10-phenanthroline; and the inhibition pattern indicated that they belong to metalloprotease. Keratinase from *Bacillus* sp. FHNM showed considerable residual activity in the presence of Co^{2+} (93%), Fe^{3+} (99%), and K^{+} (94%). *Bacillus sp*. NFH5 keratinase retained 92%, 92%, 93% of the original activity against Ba^{2+}, Na^{+} and Fe^{3+} treatment respectively. *Bacillus* sp. FHNM keratinase was remarkably stable after 60 min of detergents treatment with residual activity of 89%, 96%, 81%, 73%, 96%, 88%, 88% and 98% for Omo, Surf, Ariel, Sunlight, Prowash, Freshwave, Sky, and Evaklin, respectively. Maq impacted negatively on the enzyme stability, with residual activity of 48% after 60 min of incubation. Additionally, keratinase *Bacillus* sp. NFH5 retained 68%, 78%, 80%, 84%, 57%, 80%, 98%, 106% and 106% of the original activity against Omo, Surf, Ariel, Sunlight, Maq, Prowash, Freshwave, Sky and Evaklin, respectively. The stability displayed by keratinases from *Bacillus sp.* FHNM and *Bacillus sp.* NFH5 suggests their promising candidacy for detergent formulation.

4. INTRODUCTION

Keratinases are a group of proteolytic enzymes that are endowed with degradation of keratin into peptides and amino acids. They are recently gaining attractions from biotechnologists because of their multifaceted application potentials. Keratinases are produced by microorganisms within the confines of fungi and bacteria (Li, 2019). Keratin protein is the predominant part of feathers, hair, horns, hooves and wool (Brandelli et al., 2010). Keratin shows high degree of resistance to degradation to proteolytic enzymes because of the existence of structural stability prompted by the inner crosslinking of disulfide bonds, hydrogen bonds and hydrophobic interactions (Hill et al., 2010), which also enable the protein to withstand harsh environmental condition (Manczinger et al., 2003; Kreplak et al., 2004).

A number of bioengineering techniques has been employed for the study of keratin biodegradation mediated by extracellular keratinases produced by microorganisms to generate quality protein hydrolysates that serve as substrates in hair care products formulation, animal feeds upgrade, and laundry detergents production (Gupta and Ramani, 2006; Gupta et al., 2013). The capability of keratinases to show robustness in wide range of temperature and pH conditions has underscored their relevance as better replacements of some proteolytic ensyme currently utilized in numerous biotechnological processes (Xie et al., 2010; Rai et al., 2011). Versazyme is a keratinase-based feed additive formulated to improve the nutritional values of livestock feedstuffs (Wang et al., 2008). This bio-innovative development makes it imperative for the continuum in isolation and characterization of microbial keratinases with novel properties. In the present study, *Bacillus* sp. FHNM and *Bacillus* sp. NFH5 with efficient chicken feather degradation activity were used to produce keratinases in low-cost media. The biochemical properties of the produced keratinases were determined.

4.1. MATERIALS AND METHODS

4.1.1. Keratinase production and assay for keratinase activity

Keratinase production was carried out as described in chapter 3, section 3.1.4. The assay for keratinase activity was done in accordance with the previous method (Nnolim et al., 2020), as described in chapter 3, section 3.2.5.

4.1.2. Effect of pH and temperature on keratinase activity and stability

The effect of different pH values on the activity of keratinases was studied from pH 5-11 by using various buffer solutions: sodium citrate (pH 5.0); potassium phosphate (pH 6.0–7.0); Tris-HCl (pH 8.0–9.0); and Glycine-NaOH (pH 10.0–12.0) at 0.1 M strength.

Effect of pH on the stability of keratinases was evaluated by pre-incubating the enzyme-buffer solutions for 240 min. Aliquots were withdrawn at a regular interval (30 min) to determine the residual keratinase activity under standard assay conditions. *Bacillus* sp. FHNM keratinase was evaluated at pH 8 and 9; while *Bacillus* sp. NFH5 keratinase was assessed at pH 6, 8 and 9.

For the temperature study, the effect of temperature on the activity of keratinases was carried out at temperatures that ranged from 30 °C–100°C. The thermal stability of the keratinases was investigated by preheating the enzyme solution at 60 °C, 70 °C, 80 and 90°C for 120 min. Aliquots were withdrawn at 30 min interval to determine the residual activity under the standard assay protocol. The enzyme activity determined without preheating served as the control and was taken as 100%.

4.1.3. Effect of metal ions on the stability of keratinases

For the effect of metal ions on the stability of keratinases, 5mM of Fe^{2+}, Co^{2+}, Fe^{3+}, K^{+}, Ca^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, Na^{+}, Ba^{2+} and Al^{3+} were pre-incubated with crude enzyme solutions at 40

°C for 1 h. After preincubation, the residual enzyme activity was determined under standard assay conditions. The enzyme solution pretreated with distilled water only served as control and was taken as 100%.

4.1.4. Effect of chemical agents on the stability of keratinases

Chemical agents including phenylmethylsulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), 1,10-phenanthroline, dithiothreitol (DTT), sodium dodecyl sulfate (SDS) were evaluated at 5 mM. Also, hydrogen peroxide (H_2O_2), dimethyl sulfoxide (DMSO), acetonitrile, triton X-100 and tween-80 were evaluated at a final concentration of 1% (v/v). The enzymes were preincubated with the respective chemical agent for 1 h at 40 ^{o}C. After that, the residual keratinase activity was determined under standard assay protocols. The enzyme solution pre-treated with water only served as the control and was set as 100%.

4.1.5. Effect of laundry detergents on the stability of keratinases

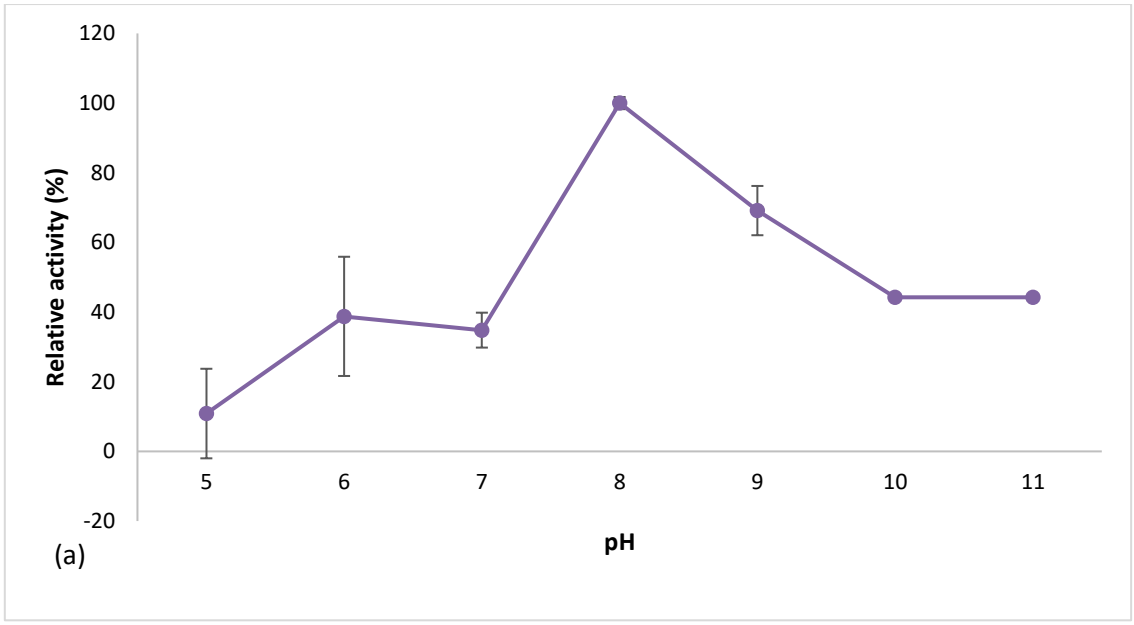
The effect of laundry detergent on the stability of keratinases from *Bacillus* sp. FHNM and *Bacillus* sp. NFH5 stability was evaluated using the method described by Paul et al. (2014). Solid laundry detergents including Sunlight, Omo, Surf (Unilever, South Africa), Ariel (Procter and Gamble, South Africa), Maq (Bliss brands (Pty) Ltd, South Africa), Fresh wave (Boxer superstores, SA), Evaklin (Oceanfield Group, SA), Sky (KT WASH (Pty) Ltd, SA) and Pro wash (Spar, SA) were each dissolved in tap water and diluted to a final concentration of 7 mg/mL. The endogenous enzymes were inactivated by heating at 100 °C for 30 min. Then, the crude enzyme was mixed with the detergent solution at a ratio of 4:1 and pre-incubated at 40 °C for 60 min. The residual keratinase activity was determined at 30 min interval under the standard assay conditions. Keratinase solution incubated with tap

water only served as the control and was taken as 100%.

4.2. RESULTS AND DISCUSSION

4.2.1. Effect of pH and temperature on keratinase activity

The effect of pH on the activity of keratinases from *Bacillus* sp. FHNM and *Bacillus* sp. NFH5 was investigated. The keratinase from *Bacillus* sp. FHNM was optimally active at pH 8.0 (Fig. 4.1a). It barely catalysed reaction at pH 5.0, and also showed 44% relative activity at pH 11.0. Similarly, *Bacillus* sp. NFH5 keratinase showed optimal catalytic efficiency at pH 8.0 (Fig. 4.1b). Similarly, KerBNK1 from *Bacillus* sp. displayed optimal biocatalysis at pH 8.0 (Nnolim et al., 2020). Additionally, Sahoo et al. (2012) and Lin et al. (2009) achieved similar results for keratinases from PKD5 and KS-1, respectively. In contrary, Sharma and Gupta (2010) documented pH 9.0 as the best condition for the best performance of the keratinase characterized in their study. Generally, keratinase showed optimal activity from neutral to alkaline condition (Brandelli et al., 2010), and this attributed has supported their various application potentials such as detergent formulation, feed production and leather processing.



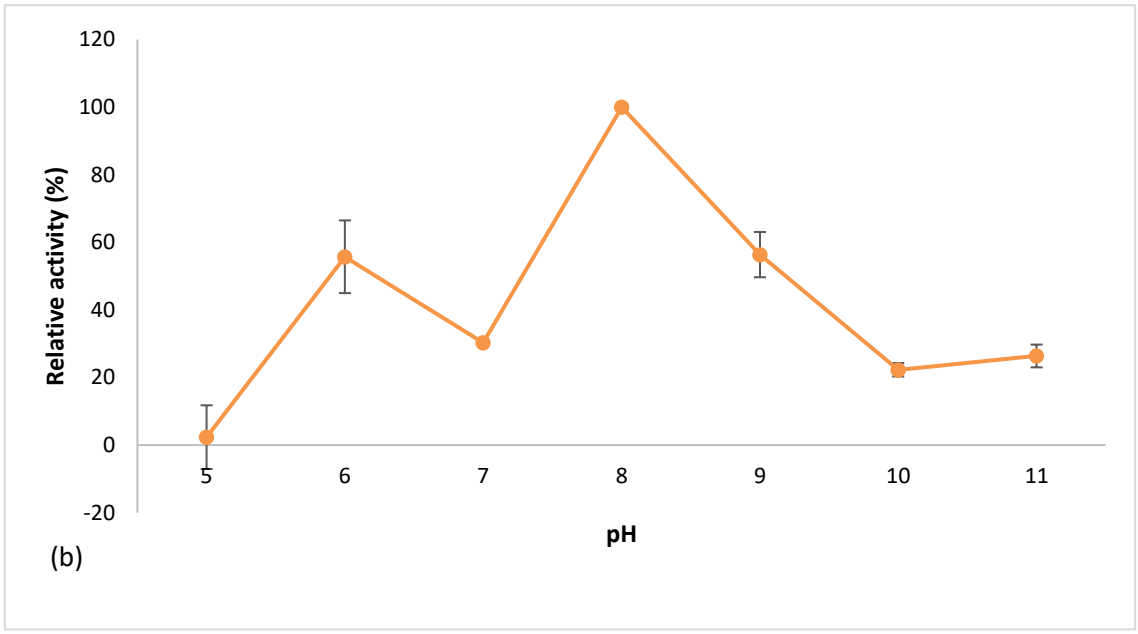
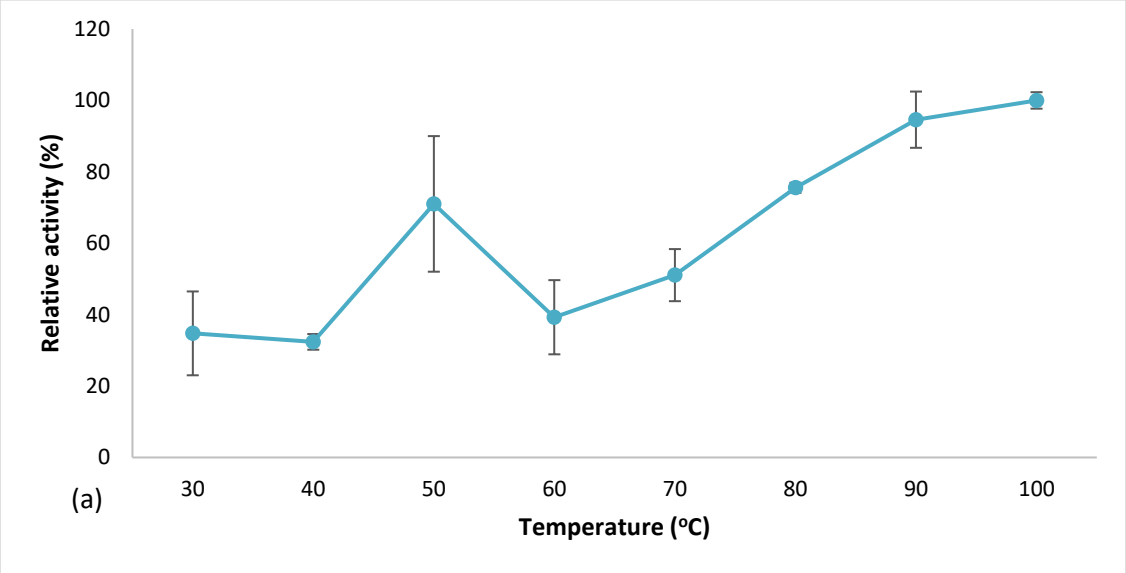


Figure 4.1: Effect of pH on the activity of keratinases from (a) *Bacillus* sp. FHNM (b) *Bacillus* sp. NFH5.

The effect of temperature on the activity of keratinases from *Bacillus* sp. FHNM and *Bacillus* sp. NFH5 was studied from 30 ºC to 100 ºC. The results showed that the catalytic efficiency of *Bacillus* sp. FHNM keratinase increased with temperature elevation. However, the enzyme activity decreased at 60 ºC, and then, increased with application of higher temperature, with optimal activity recorded at 100 ºC (Fig. 4.2a). Likewise, the activity of keratinase from

Bacillus sp. NFH5 increased as the assay temperature was raised, with maximum activity at 90 ^{o}C (Fig. 4.1b). The enzyme showed 95% relative activity at 100 ^{o}C. The thermoactivity of the study keratinases is similar to that reported for keratinase from Fervidobacterium islandicum that was optimally active at 100 ^{o}C (Nam et al., 2002). Most bacterial keratinases' optimal activity occurs between 40 and 70 ^{o}C (Sharma and Gupta, 2010; Brandelli and Daroit, 2014). However, keratinase activity at temperature up to 100 ^{o}C has been scarcely reported. The findings of this study suggest the application potentials of the study enzymes in processes that require high investment of temperature such as degradation of prion (Okoroma et al., 2013).



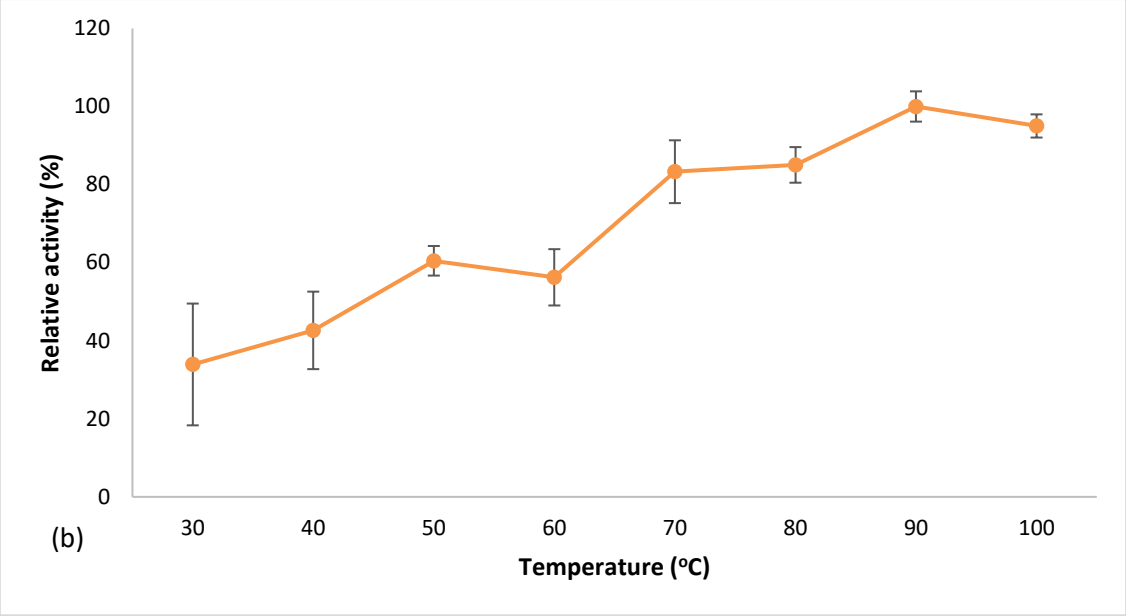
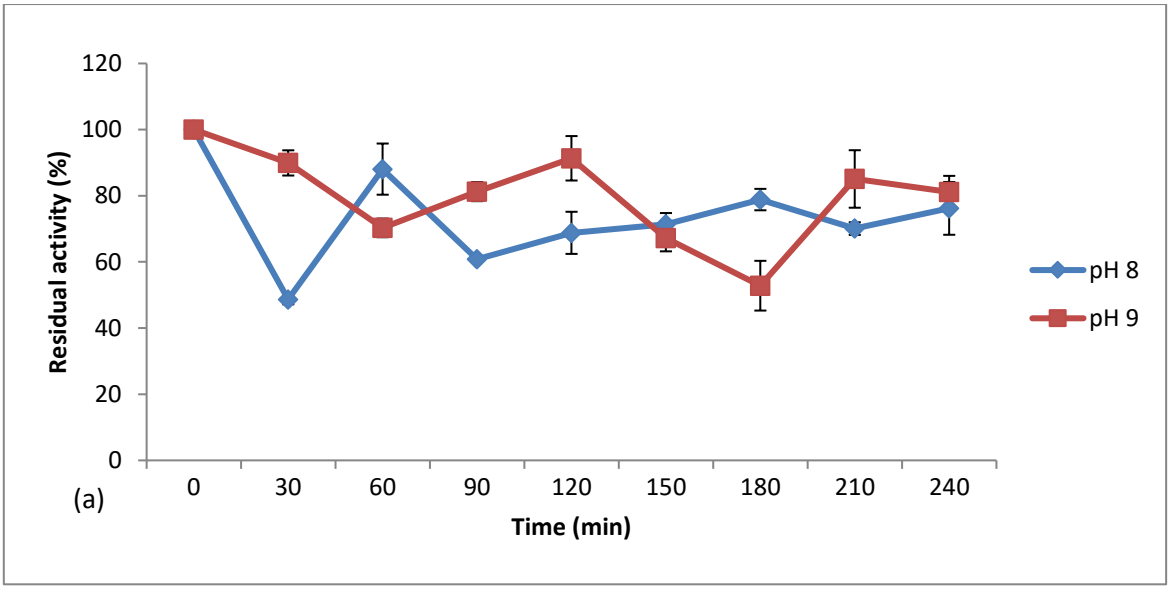


Figure 4.2: Effect of temperature on the activity of keratinases from (a) *Bacillus* sp. FHNM (b) *Bacillus* sp. NFH5.

4.2.2. Effect of pH and temperature on keratinase stability

Bacillus sp. FHNM keratinase stability at pH 8 and 9 was studied, and the results indicated that the enzyme was remarkably stability with residual activity of 76% and 81% at pH 8 and pH 9 respectively after 240 min of pre-incubation (Fig. 4.3a). Keratinase from *Bacillus* sp. NFH5 retained 45%, 83% and 69% of the original activity at pH 6, pH8 and pH 9 respectively, after 240 min (Fig. 4.3b). pH stability is imperative for industrial application of proteolytic enzymes that have demonstrated some unique properties. Sharma and Gupta (2010) reported that KP-1 keratinase showed remarkable stability from pH 5-9. The efficiency of the study enzymes at alkaline condition promotes their significance in green technology.



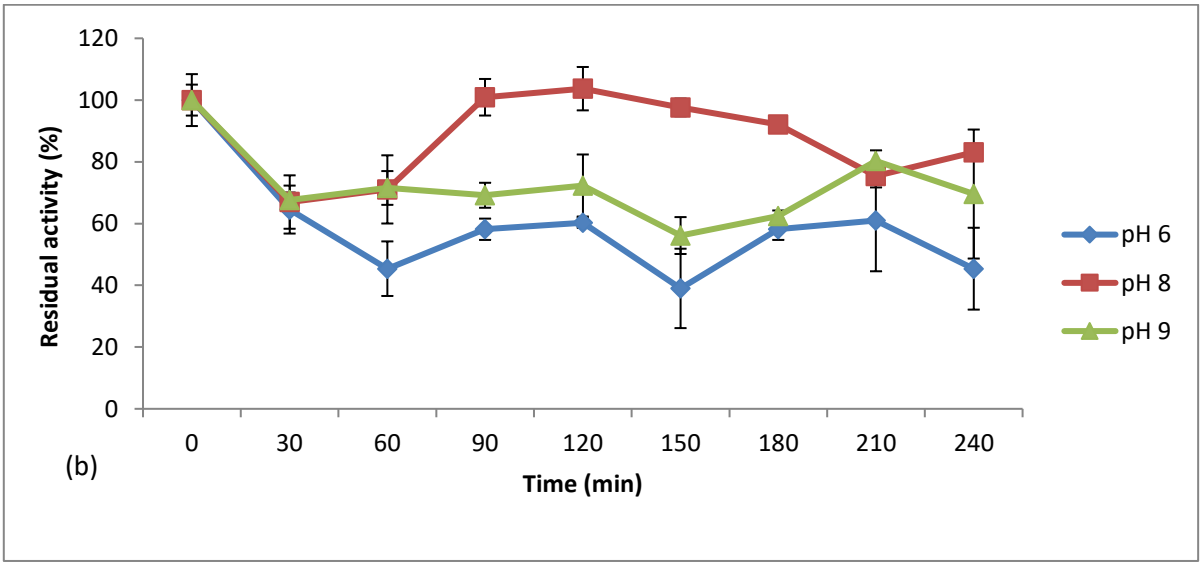


Figure 4.3: Effect of pH on the stability of keratinases from (a) *Bacillus* sp. FHNM (b) *Bacillus* sp. NFH5.

4.2.3. Effect of temperature of the stability of keratinase

Keratinases from *Bacillus* sp. FHNM and *Bacillus* sp. NFH5 generally showed low thermal stability. *Bacillus* sp. FHNM keratinase retained 65% and 38% of the original activity after 30 min and 60 min of heating at 70 ^{o}C, respectively (Fig. 4.4a). Similarly, *Bacillus* sp. NFH5 keratinase showed 48% and 76% residual activity after 30 min of heating at 60 ^{o}C and 70 ^{o}C,

respectively (Fig. 4.4b).

Keratinolytic peptidases from *Bacillus* spp. showed similar thermostability pattern even at lower temperatures (Gegeckas et al., 2018). However, other studies have reported remarkably thermostable keratinase of Bacillus origin (Gupta et al. 2015; Nnolim et al., 2020).

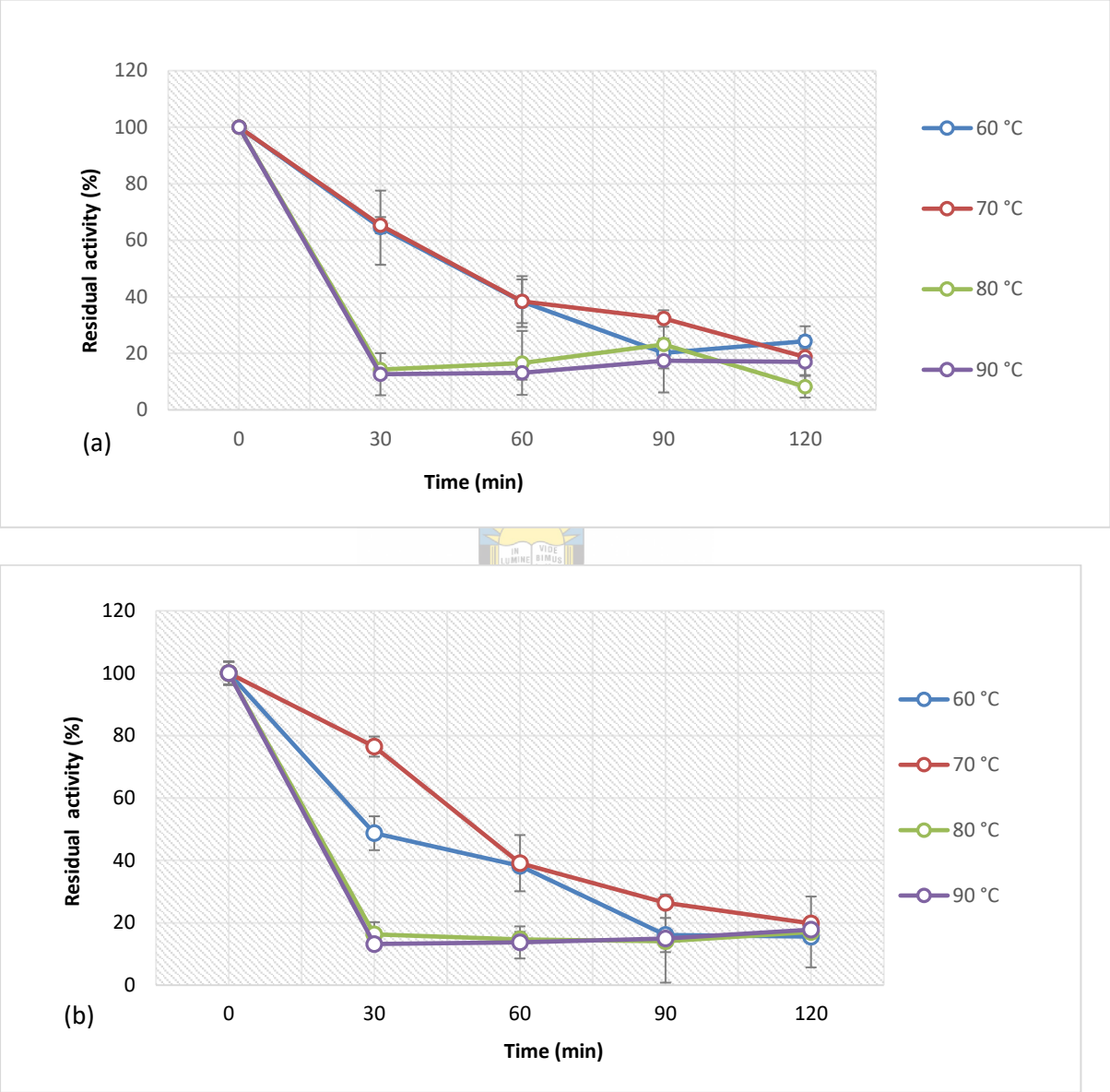


Figure 4.4: Effect of temperature on the stability of keratinases from (a) *Bacillus* sp. FHNM (b) *Bacillus* sp. NFH5.

4.2.4. Effect of metal ions and chemical agents on the stability of keratinase

The effect of metal ions on the stability of keratinases from *Bacillus* sp. FHNM and *Bacillus*

sp. NFH5 was evaluated, and the results showed that the metal ions generally elicited partial inhibition of the keratinase activity. Keratinase from *Bacillus* sp. FHNM showed considerable residual activity in the presence of Co^{2+} (93%), Fe^{3+} (99%), and K^{+} (94%) as shown in Table 4.1. *Bacillus sp*. NFH5 keratinase retained 92%, 92%, 93% of the original activity against Ba^{2+}, Na^{+} and Fe^{3+} treatment (Table 4.2). Most studies have reported that heavy metals such as Cu^{2+}, Zn^{2+}, and Co^{2+} completely inhibited keratinase activity (Riffel et al., 2003; Thys et al., 2004; Cai et al., 2008; Nnolim et al., 2020). The overall findings suggest that the study keratinases could moderately tolerate metal ions, and this moderate inhibition could be as a result of allosteric regulation of the enzyme structural conformation at such high concentration of the metal ions tested (Nnolimn et al., 2020).

The impact of chemical agents on the stability of keratinases from *Bacillus* sp. FHNM and *Bacillus* sp. NFH5 was investigated (Table 4.3 and Table 4.4). The results showed that EDTA and 1,10-Phenanthroline significantly inhibited the enzyme activity, while marginal drop in enzyme activity was obtained with PMSF. This pattern of inhibition suggests that the study keratinases belong to metallo-type of protease. Similar sensitivity of keratinases to protease inhibitors was previously reported (Thys et al., 2004; Tatineni et al., 2008; Nnolim et al., 2020). Partial inhibition of keratinase activity by PMSF was reported in similar studies (Cai et al., 2008; Gong et al., 2015). The keratinases showed variable loss of activity in the presence of other chemical agents tested. This may be attributed to the interaction of these chemical agents with either the essential residues in the catalytic cleft or side chain amino acids of the keratinases (Tatineni et al., 2008).

Table 4.1: Effect of metal ions on the stability of keratinase from *Bacillus sp*. FHNM

Metal ion	Concentration (mm)	Residual activity (%)
Control	0	100±2.72
Fe^{2+}	5	87±2.37
Co^{2+}	5	93±10.46
Fe^{3+}	5	99±2.26
K^{+}	5	94±5.63
Ca^{2+}	5	79±4.64
Mg^{2+}	5	83±0.19
Cu^{2+}	5	80±3.01
Zn^{2+}	5	88±4.71
Na^{+}	5	87±2.69
Ba^{2+}	5	86±10.05
Al^{3+}	5	82±1.56

Table 4.2: Effect of metal ions on the stability of keratinase from *Bacillus* sp. NFH5

Metal ion	Concentration (mM)	Residual Activity (%)
Control	0	100±4.36
Fe^{2+}	5	79±1.44
Co^{2+}	5	89±3.26
Fe^{3+}	5	93±0.70
K^{+}	5	89±4.54
Ca^{2+}	5	88±3.65
Mg^{2+}	5	88±1.47
Cu^{2+}	5	83±9.92
Zn^{2+}	5	84±2.31
Na^{+}	5	92±4.77

Ba^{2+}	5	92±0.53
Al^{3+}	5	82±5.91

Table 4.3: Effect of chemical agents on the stability of keratinase from *Bacillus sp*. FHNM

Chemical agent	**Concentration**	**Residual activity (%)**
Control	0	100±0.65
PMSF	5mM	89.70±1.08
EDTA	5mM	21.28±7.60
1,10-Phenanthroline	5mM	42.11±0.77
DTT	5mM	89.822±3.78
H_2O^2	1% (v/v)	88.90±1.27
DMSO	1% (v/v)	85.01±24.93
Acetonitrile	1% (v/v)	84.43±1.53
Triton X-100	1% (v/v)	94.97±0.68
Tween-80	1% (v/v)	80.66±2.61
SDS	0.5% (w/v)	84.67±18.73

Table 4.4: Effect of chemical agents on the stability of keratinase from *Bacillus sp.* NFH5

Chemical agent	**Concentration**	**Residual activity (%)**
Control	0	100±15.01
PMSF	5mM	83.21±5.82
EDTA	5mM	42.29±1.72
1,10-Phenanthroline	5mM	47.60±7.12
DTT	5mM	67.98±7.48
H_2O_2	1% (v/v)	69.35±1.05
DMSO	1% (v/v)	65.58±22.52
Acetonitrile	1% (v/v)	82.19±12.37

Triton X-100	1% (v/v)	81.68±3.85
Tween-80	1% (v/v)	54.11±8.06
SDS	0.5% (w/v)	52.39±11.09

4.2.5. Effect of laundry detergents on the stability and activity of keratinase

The effect of some selected laundry detergents, in commercial use, on the stability of keratinases from *Bacillus* sp. FHNM and *Bacillus* sp. NFH5 was examined. Generally, the keratinases showed significant loss of activity after 30 min of incubation in mixture with some laundry detergents (Fig. 4.5 and 4.6). The enzymes residual activity was promoted after 60 min of incubation. *Bacillus* sp. FHNM keratinase was remarkably stable after 60 min of detergents treatment with residual activity of 89%, 96%, 81%, 73%, 96%, 88%, 88% and 98% for Omo, Surf, Ariel, Sunlight, Prowash, Freshwave, Sky, and Evaklin, respectively (Fig. 4.5). Maq impacted negatively on the enzyme stability, with residual activity of 48% after 60 min of incubation. Keratinase *Bacillus* sp. NFH5 retained 68%, 78%, 80%, 84%, 57%, 80%, 98%, 106% and 106% of the original activity against Omo, Surf, Ariel, Sunlight, Maq, Prowash, Freshwave, Sky and Evaklin, respectively (Fig. 4.6). Likewise, Maq displayed the highest inhibitory potential against the *Bacillus* sp. NFH5 keratinase.

These findings of the present study were comparable to other previous reports (Jaouadi et al, 2009; Paul et al., 2014; Reddy et al., 2017; Nnolim et al., 2020). The level of impact of the different laundry detergents on the keratinase stability may be attributed to varieties in the respective detergent ingredients, and such varieties have been accounted for to influence the strength of detergent endogenous biocatalysts (Reddy et al., 2017). The exceptional stability displayed by keratinases from *Bacillus* sp. FHNM and *Bacillus* sp. NFH5 suggests their promising candidacy for detergent formulation.

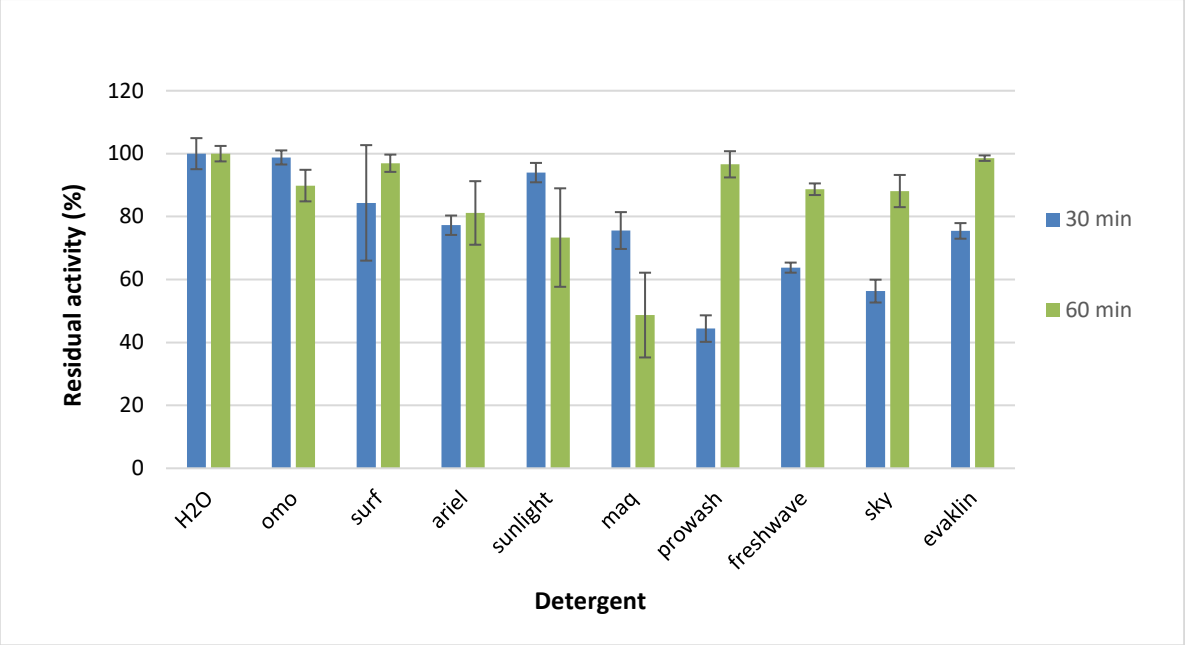


Figure 4.5: Effect of laundry detergents on the stability of keratinase from *Bacillus* sp. FHNM

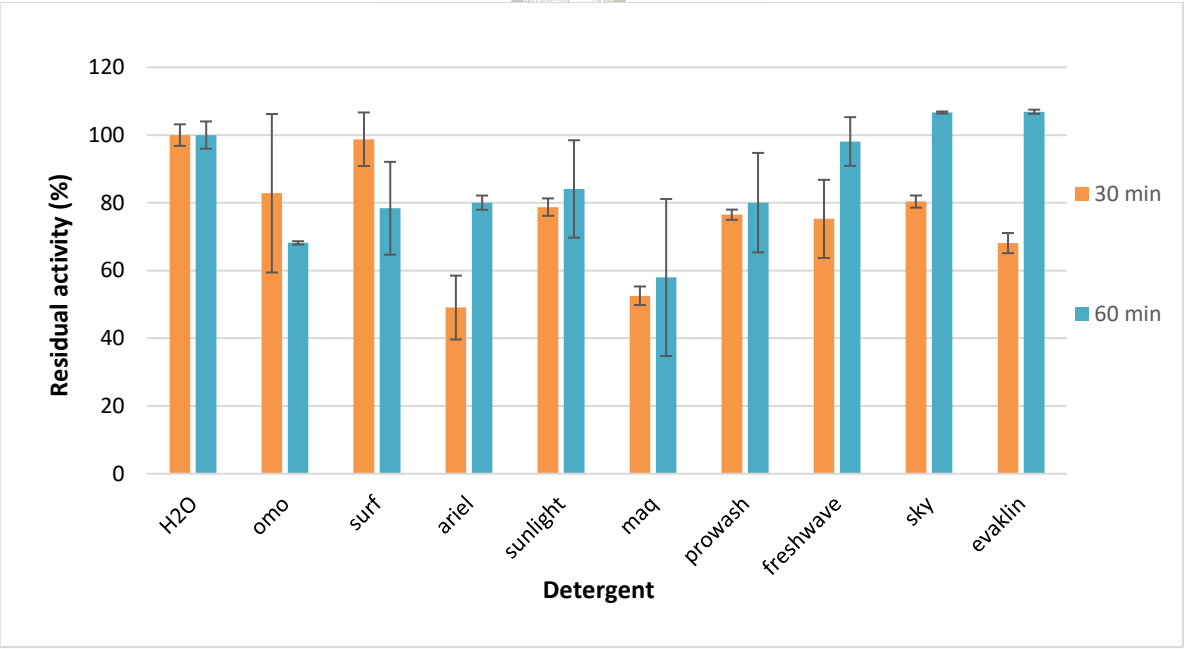


Figure 4.6: Effect of laundry detergents on the stability of keratinase from *Bacillus* sp. NFH5.

4.3. CONCLUSION

In conclusion, the keratinases *Bacillus* sp. FHNM and *Bacillus* sp. NFH5 showed optimal catalytic efficiency at pH 8.0 and temperature of 100 ^{o}C. The enzymes displayed considerable pH stability and low thermostability. They were showed high stability in the various metal ions tested. However, the enzymes were partially inhibited by some chemical agents used. The sensitivity of the keratinases to the protease inhibitors indicated that they belong to metallo-type of protease. The evaluation of the keratinases stability in the presence of some commercially available laundry detergents indicated that they are potential bio-additives in detergent formulation.

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CHAPTER FIVE

GENERAL DISCUSSION AND CONCLUSION

5.1. GENERAL DISCUSSION

The efficiency of keratinase production by microbial producers is dependent on significant fermentation process variables such as the concentration of keratinous substrates, medium pH, incubation temperature, and agitation speed. The influence of nutritional and environmental conditions on the synthesis of proteases plays a significant role in the expression or suppression (Srivastava et al., 2020). Due to strain variations, each bacterium possesses its own distinct optimal conditions for keratinase production (Nnolim et al., 2020). The pH of the culture medium is the most importance factor as it affects the membrane porosity and controls the transportation of molecules in and out of a bacterial cell membrane (Cunha et al., 2010). Therefore, maximum keratinase secretion occurs when a satisfactory culture medium pH is preserved. From the obtained results, *Bacillus* sp. FHNM maximally produced keratinase at neutral pH condition. This finding is in accordance with keratinase production by other bacterial species (Kim et al., 2001). In addition, *Bacillus* sp. NFH5 was very active at pH range within the acidic spectrum, and similar reported was document for a keratinolytic Bacillus sp. for efficient keratinase production (Nnolim et al., 2021).

Substrate concentration is significant for the efficiency of protease production. From the obtained results *Bacillus* sp. FHNM performed exceptionally well at 2% (w/v) chicken feather concentration even though 2% has limited keratinase production by keratinolytic bacterium (Sahoo et al., 2012). Similarly, *Bacillus* sp. NFH5 displayed optimal keratinase production at 1.5% (w/v) chicken feathers. Temperature is one of the most influential factors that affect the rate of biochemical synthesis of secondary metabolites. Therefore, the evaluation of temperature optima for both *Bacillus* sp. FHNM and *Bacillus* sp. NFH5 indicated their mesophilic condition preference towards extracellular secretion of keratinases. Mesophiles are importance in sustainable development as no high energy investment will be required. These findings are in agreement with previous studies that reported mesophilic

conditions as optimal temperature range for keratinase secretion (Jeong et al., 2010; Nnolim et al., 2020). Degradation of chicken feather resulted in the generation of sulfhydryl groups in the fermentation medium which signified significant hydrolysis of disulfide bonds. This attribute of the study isolates indicates their significance in the sustainable recycling of keratinous biomass. The analysis of amino acid content of the chicken feather hydrolysates showed presence of various amino acids of protein. This observation suggests that *Bacillus* sp. FHNM and *Bacillus* sp. NFH5 could be used for conversion of feathers into nutritionally rich protein hydrolysates with biotechnological application potentials.

Keratinases from *Bacillus* sp. FHNM and *Bacillus* sp. NFH5 were catalytically active at alkaline conditions which indicate their potential application in leather processing and detergent formulation. Keratinolytic proteases optimally activity has been generally recorded from neutral to alkaline pH (Brandelli et al., 2010). The keratinases were thermally active, with optimal temperature of 100 ^{o}C. Similar thermoactivity pattern was reported for keratinase from *F. islandicum* (Nam et al., 2002). The sensitivity of the enzymes to the protease inhibitors indicated that they are metallo-keratinases. Similar keratinase inhibition pattern in the presence of metal ion chelators was previously reported (Thys et al., 2004). The keratinases under study also showed considerable stability in the presence of metal ions and chemical agents, and this signifies their potential industrial applications. The evaluation of the enzyme compatibility with some selected commercially available laundry detergents indicated that the keratinases retained remarkable residual activity after 1 h of detergent treatment. The findings are comparable to other studies where bacterial keratinase stability was assessed in the presence of laundry detergents (Paul et al., 2014; Reddy et al., 2017).

5.2. CONCLUSION

In conclusion, *Bacillus* sp. FHNM and *Bacillus* sp. NFH5 optimally produced extracellular keratinases in a relative cheap medium that contained chicken feather as the only source of carbon and nitrogen. The isolates showed excellent keratinase production from acidic to neutral pH and under mesophilic conditions. The analysis of feather hydrolysates recovered from the fermentation broth indicated presence of various amino acids of protein in variable concentration, which suggested the isolates dexterity with bioconversion of recalcitrant keratinous wastes. The metallo-keratinases were efficiently active at alkaline conditions and showed high tolerance with chemical agents and metal ions. They also displayed excellent compatibility with laundry detergents, and such property has promoted them as potential laundry detergent bio-additives. Low enzyme yield has been one of the factors affect the commercialization of some keratinases with robust biochemical properties and biotechnological tendencies. Therefore, cloning and expression of these keratinases encoding genes in industrially suitable hosts might promote the enzymes yield.

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