ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

INSTITUTE OF BIOTECHNOLOGY



Production and Evaluation of the Biotechnological Potential of Alkaliphilic Protease from Tannery Waste Microbes

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A Thesis Submitted to the School of Graduate Studies of Addis Ababa University in Partial Fulfillment of the Requirement for the Degree of Masters of Science in Biotechnology

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February, 2025

Addis Ababa, Ethiopia

Declaration

I, Yoseph Sisay Tegegn, hereby declare that the thesis entitled: **"Production and Evaluation of the Biotechnological Potential of Alkaliphilic Protease from Tannery Waste Microbes"** is my original work and has not been submitted for any degree or diploma at any other University or Institution. I confirm that I have acknowledged all sources and references used in this work in accordance with academic standards. This thesis is submitted in partial fulfillment of the requirements for the degree of Master of Science in Biotechnology (Industrial Biotechnology).

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I certify that Yoseph Sisay Tegegn M.Sc. thesis entitled **"Production and Evaluation of the Biotechnological Potential of Alkaliphilic Protease from Tannery Waste Microbes"** is the final version. Therefore; I, as an advisor, approved it as the final document to be accepted as fulfilling the requirement for the degree of Master of Science in Biotechnology (Industrial Biotechnology).

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AKNOWLEDGEMENTS

First and foremost, I would like to express my deepest gratitude to the **Almighty God** for His countless blessings, guidance, and strength throughout this journey. I am forever grateful to the **Virgin Mary**, whose divine intercession and presence have been a constant source of comfort and inspiration. Without their grace, completing this work would not have been possible.

I owe a special debt of gratitude to my advisor, Addis Simachew (PhD.), for his exceptional guidance, encouragement, and patience throughout the course of my research. His expertise and insight not only helped shape the direction of my work but also inspired me to explore new ideas and challenge myself academically.

I extend my heartfelt thanks to my home institution, Bio and Emerging Technology Institute for the sponsorship. The support, formalized through the sponsorship letter, allowed me to pursue this academic endeavor with confidence and access to all necessary resources.

I am profoundly grateful to Addis Ababa University, particularly the Institute of Biotechnology, for accepting me into their esteemed M.Sc. program. The opportunity to study at such a prestigious institution has been a transformative experience, both academically and personally.

I would also like to extend my deepest thanks to Mr. Solomon Enquahone and Mrs. Tirunesh Shiferaw, whose friendship and professional expertise were indispensable to the success of this research. Their tireless efforts in maintaining laboratory equipment, preparing materials, and assisting with experimental procedures ensured that everything ran smoothly and efficiently.

I wish to express my deepest gratitude to my parents for their endless love, support, and encouragement. From the very beginning, their intimated belief in my abilities has been my greatest source of motivation. Their sacrifices, wisdom, and guidance have shaped the person I am today, and for that, I am forever thankful.

Lastly, to my beloved wife, Betelehem Fikade, I offer my deepest appreciation for her boundless love, patience, and support. Her resilient belief in me, even during the most challenging times, has been a source of strength and motivation. Her sacrifices, encouragement, and understanding have made this accomplishment possible, and for that, I am eternally grateful.

Table of Contents

AKNOWLEDGEMENTS i
LIST OF TABLES vi
LIST OF FIGURES vi
LIST OF APPENDICES vii
ABBREVATIONS AND ACRONYMS viii
ABSTRACT ix
1. INTRODUCTION 1
1.1. Background 1
1.2. Statement of the problems 3
1.3. Objectives 4
1.3.1. General objective 4
1.3.2. Specific objectives 4
2. LITERATURE REVIEW 5
2.1. Tannery wastes 5
2.2. Microbial enzymes 6
2.3. Types of proteases 7
2.3.1. Serine proteases 7
2.3.2. Cysteine proteases 8
2.3.3. Aspartic proteases 8
2.3.4. Metalloproteases 9
2.4. Nomenclature of proteases 10
2.4.1. Trivial names 10
2.4.2. Systematic names 10
2.5. Microbial sources of protease 11
2.6. Protease activity optimization 13
2.6.1. Effect of pH in protease activity 13
2.6.2. Effect of temperature in protease activity 13
2.6.3. Effect of salt concentration in protease activity 14
2.6.4. Effect of inoculum size in protease activity 14
2.7. Protease activity measurement 15

2.8. Locally available substrates for mass production 15
2.8.1. Carbon sources 16
2.8.2. Protein sources 17
2.9. State of fermentation 18
2.9.1. Solid state fermentation (SSF) 18
2.9.2. Submerged Fermentation (SmF) 18
2.9.3. Comparison between SSF and SmF 19
2.10. Applications of protease 19
2.10.1. Detergent industry 20
2.10.2. Leather industry 20
2.10.3. Silver recovery 20
2.10.4. Food industry 21
2.11. *Alkalihalophilus pseudofirmus* 22
2.12. Enzymes produced by *Alkalihalophilus pseudofirmus* 23
3. MATERIALS AND METHODS 24
3.1. Sample area description 24
3.2. Samples collection 25
3.3. Enrichment of the samples 25
3.4. Isolation and screening of alkaline protease producing bacteria 25
3.5. Production of alkaline protease 26
3.6. Characterization of *Alkalihalophilus pseudofirmus* YATW18 26
3.6.1. Morphological characterization 26
3.6.2. Biochemical characterization 27
3.6.3. Molecular characterization 27
3.7. Optimization of culturing conditions 29
3.7.1. Inoculum size optimization 30
3.7.2. Carbon source optimization 30
3.7.3. Nitrogen source optimization 30
3.7.4. Salt concentration optimization 30
3.7.5. Locally available substrate optimization 31
3.8. Physicochemical characterization of alkaline protease 31

3.8.1. Enzyme activity 31
3.8.2 Tyrosine standard curve preparation 32
3.8.3. pH profile of alkaliphilic protease 34
3.8.4. Temperature profile and stability of alkaliphilic protease 34
3.8.5. Salt profile of alkaliphilic protease 35
3.8.6. Incubation time profile of alkaliphilic protease 35
3.8.7. Metal ions profile of alkaliphilic protease 35
3.9. Partial purification of alkaliphilic protease 36
3.9.1. Ammonium sulphate precipitation 36
3.9.2. Centrifugation 36
3.9.3. Re-suspension and dialysis 36
3.9.4. Purity Assessment 37
3.10. Evaluation of alkaliphilic protease for its potential industrial applications 37
3.10.1. Stain removal for detergent application 37
3.10.2. Dehairing for tannery application 38
3.11. Data analysis 39
4. RESULTS 40
4.1. Characterization of tannery waste water 40
4.2. Screening of alkaline protease producing bacteria 40
4.2.1. Primary screening 40
4.2.2. Secondary screening 42
4.3. Characterization of *Alkalihalophilus pseudofirmus* strain YATW18 43
4.3.1. Morphological characterization 43
4.3.2. Biochemical characterization 44
4.3.3. Molecular characterization 45
4.4. Optimization of cultivation conditions 47
4.4.1. Effect of inoculum size 47
4.4.2. Effect of carbon and nitrogen sources 47
4.4.3. Effect of salt concentration 49
4.4.4. Effect of locally available substrates 49
4.5. Physicochemical characterization of alkaline protease 50

4.5.1. Effect of pH in alkaliphilic protease activity 50
4.5.2. Effect of temperature in alkaliphilic protease activity and stability 51
4.5.3. Effect of salt concentration in alkaliphilic protease activity 53
4.5.4. Effect of incubation time in alkaliphilic protease activity 54
4.5.5. Effect of metal ions in alkaliphilic protease activity 55
4.6. Partial purification of alkaliphilic protease 56
4.6.1. Ammonium sulphate precipitation 56
4.6.2. Purity assessment using SDS-PAGE 57
4.7. Evaluation of alkaliphilic protease for its potential industrial applications 58
4.7.1. Stain removal efficiency for detergent industry 58
4.7.2. Dehairing capacity for leather industry 59
5. DISCUSSION 62
5.1. Screening of alkaline protease producing bacteria 62
5.2. Optimization of cultivation conditions 63
5.3. Physicochemical characterization of alkaline protease 64
5.3.1. Effect of pH in alkaliphilic protease activity 64
5.3.2. Effect of temperature in alkaliphilic protease activity and stability 65
5.3.3. Effect of salt concentration in alkaliphilic protease activity 66
5.3.4. Effect of incubation time in alkaliphilic protease activity 66
5.3.5. Effect of metal ions in alkaliphilic protease activity 67
5.4. Partial purification of alkaliphilic protease 67
5.5. Evaluation of alkaliphilic protease for its potential applications 68
5.5.1. Stain removal efficiency for detergent industry 68
5.5.2. Dehairing capacity for leather industry 69
6. CONCLUSION AND RECOMMENDATIONS 70
6.1. Conclusion 70
6.2. Recommendations 70
7. REFERENCES 71
APPENDICES 92

LIST OF TABLES

Table 1: Chemicals used in various chemical processing steps and their impacts on life……6

Table 2: Different sources of isolation of alkaline protease producing microorganisms…….12

Table 3: Characterization of tannery wastewater samples concerning different parameters……40

Table 4: Clear zone comparison of bacterial isolates…….....41

Table 5: Morphological characterization of *Alkalihalophilus pseudofirmus* strain YATW18….44

Table 6: Biochemical characterization of *Alkalihalophilus pseudofirmus* strain YATW18……45

Table 7: Significant alignments produced from sequences of strain YATW18……...45

Table 8: Effect of carbon and nitrogen sources on alkaline protease production…….48

Table 9: Effect of salt concentration on alkaline protease production……..49

LIST OF FIGURES

Fig. 1: Protease enzyme classification……...9

Fig. 2: Several industrial applications of alkaline protease……...22

Fig. 3: Sample area description of Awash tannery with respect to sample points……24

F**ig. 4:** Tyrosine standard curve…….33

Fig. 5: Isolation and screening of alkaline protease producing bacteria……...42

Fig. 6: Enzyme activity of strain YATW18 at 12-hour intervals during the incubation period....43

Fig. 7: Neighbor-joining phylogenetic tree construction……..46

Fig. 8: Effect of inoculum size in alkaline protease production……47

Fig 9: Effect of locally available substrates in alkaline protease production ……50

Fig 10: Effect of pH in alkaline protease activity……..51

Fig. 11: Effect of temperature (A) in alkaline protease activity (B) in alkaline protease stability without calcium (C) in alkaline protease stability with 5mM calcium……53

Fig. 12: Effect of salt concentration in alkaline protease activity…….54

Fig. 13: Effect of incubation time in alkaline protease activity……55

Fig. 14: Effect of metal ions in alkaline protease activity…….56

Fig. 15: Partial purification of crude enzyme using ammonium sulphate…….57

Fig. 16: SDS-PAGE image and band pattern……57

Fig. 17: Egg yolk stains removal efficiency of different treatments…….58

Fig. 18: Partial and complete dehairing capacity of different treatments……..61

LIST OF APPENDICES

Appendix 1: Industrial visits in Leather Industry Development Institute……92

Appendix 2: Enzyme substrate complex at different ph level after dark room incubation……..92

Appendix 3: Partial purification of crude enzyme using ammonium sulfate……...92

Appendix 4: Preparation of egg yolk and staining on white cotton……..93

Appendix 5: Washing and drying of sheep and goat skins……...93

Appendix 6: Observation of physical appearance of bacterial colonies……...93

Appendix 7: Gel electrophoresis image and band pattern of the genomic DNA…….94

Appendix 8: Gel electrophoresis image and band pattern of the PCR products……...94

ABBREVATIONS AND ACRONYMS

APS	Ammonium per sulfate
ATCC	American Type Culture Collection
BOD	Biological oxygen demand
BRC	Biological Resource Center
CAGR	Compound annual growth rate
COD	Chemical oxygen demand
CTAB	Cetyl tri-methyl ammonium bromide
GRAS	Generally regarded as safe
LIDI	Leather Industry Development Institute
mS/cm	Milli Siemens per centimeter
OD	Optical density
OVAT	One variable at a time
PCR	Polymerase chain reaction
ppt	Parts per trillion
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SmF	Submerged fermentation
SSF	Solid state fermentation
TDS	Total dissolved solids
TE	Tris ethylene diamine tetra acetic acid
TEMED	Tetra methyl ethylene diamine
TSS	Total suspended solids
UNECA	United Nations Economic Commission for Africa
UNIDO	United Nations Industrial Development Organization

ABSTRACT

The global leather industry generates significant volumes of tannery waste, contributing to environmental degradation and public health concerns. The study investigated the production of alkaliphilic protease from tannery waste microbes, and its potential applications for detergent and leather industries were also evaluated. After the isolation of more than 100 bacterial strains and screening of 22 alkaline protease-producing bacteria, strain YATW18 has been identified as the most efficient producer of extracellular alkaline protease with 92.65 U/ml enzyme activity in 48 hours. Molecular characterization has assured the genus and species name of strain YATW18 to be Alkalihalophilus pseudofirmus. The partial sequence of Alkalihalophilus pseudofirmus strain YATW18 16S rRNA gene was submitted to the NCBI database and the accession number was obtained as PP942174. Fructose as a carbon source exhibited the highest enzyme activity with an average yield of 82 $\pm$ 2.98 U/ml, and peptone as a nitrogen source resulted in the highest enzyme activity of 96.86 $\pm$ 2.98 U/ml. From locally available substrates, water hyacinth has demonstrated the highest alkaline protease yield of 661.85 $\pm$ 10.98 U/g. The mean differences in optimizing parameters including carbon sources, nitrogen sources, and locally available substrates were statistically significant at $P \leq 0.05$. Physicochemical characterization of alkaline protease showed optimum activity at 55℃ and pH 10. The enzyme reached its saturation level within 20 minutes of incubation time and then linear activity was observed until 45 minutes. Alkaliphilic protease (77.92 U/ml and 99.6 U/ml) showed high protein (egg yolk) stain removal efficiency and dehairing (goat and sheep skins) capacity.

Keywords: *Alkalihalophilus pseudofirmus*, Enzyme activity, Industrial enzymes, Leather industry, Molecular characterization, Optimization, and Strain YATW18

1. INTRODUCTION

1.1. Background

Tannery waste is a potential source for the production of alkaline protease for various industrial applications. Liquid waste from the leather industry is rich in proteins and is also alkaline, making it a good alternative for isolating alkaliphilic, protein-degrading bacteria (Ahsan *et al*., 2023). The leather industry generally uses hides and skins as raw materials, which are by-products of the meat products industry (Azom *et al*., 2012). Tanning is a process that transforms animal hides into fine, smooth and flexible leather products. However, tanning produces various types of solid and liquid waste (Mohamed *et al*., 2021). According to the European Commission, the tanning process comprises four stages: beam house operation, wet finishing, dry finishing and wrapping (Dixit *et al*., 2015). Untreated synthetic chemicals and proteins generated throughout the tanning process would cause serious health and environmental problems. Therefore, there is an urgent need to develop various technologies to combat environmental pollutions and health risks originated from the leather industry.

One way to reduce the environmental impacts, caused by the excessive amount of wastes generated from tanneries, is their conversion into commercially valuable products. However, many chemical transformations using inorganic catalysts in various industries have inherent disadvantages from economic, energetic and environmental perspectives (Verma *et al*., 2021). Chemical hair removal in the leather industry uses more than 100 chemicals, including lime (CaO) and sulfides (Na_2S/NaSH) (Hashem *et al*., 2020). Pre-tanning process is necessary to remove about 90% of all impurities and produce semi-finished leather products. However, it also produces harmful gases and solid wastes, which pose a significant challenge to the environment (Chiampo *et al*., 2023). The major pollutants produced during pre-tanning, tanning and re-tanning processes include organic wastes, inorganic contaminants, and heavy metals, which account for almost 70% of all pollutants (Bhardwaj *et al*., 2023). Tannery wastes are not only pollutes the water, soil and air in the surrounding area, but also causes serious health problems such as asthma, hepatitis, dermatitis, various malignancies and neurological disorders among tannery workers and local residents (Alemu and Tegegne, 2022). In Ethiopia, the annual volume of liquid waste from the 27 tanneries was estimated to be 3,600,000 to 4,500,000 cubic meters based on their annual production capacities (Adem, 2019). Therefore, it has been classified as

one of the most polluting industries and there is concern that leather production could harm the environment (Verma and Sharma, 2023). Therefore, the leather industry needs to address these issues.

In contrast to chemical processes, biocatalysts offer ecological and clean solutions and are becoming an innovative approach to chemical technology. Due to their specificity, their activity under difficult conditions, their ability to produce natural products, their environmental friendliness and biodegradability, the use of enzymes is the preferred method for eliminating industrial pollutants generated in tannery wastewater (Khambhaty, 2020). To overcome the above problems caused by chemicals and waste in the leather industry, protease is promising due to its significant advantages such as shortened processing time, biodegradable effect, better product quality, low energy consumption, lower cost, non-toxic and environmentally friendly (Agyei *et al*., 2016). Extracellular protease, a versatile and commonly used enzyme, may provide an alternative method to chemical hair removal to minimize the toxic effects of chemicals during the pre-tanning, and tanning process in the leather industry.

Recently, bacterial alkaline protease has attracted attention and emerged as environmental friendly alternative to inorganic catalysts. Alkaline protease is active in the alkaline pH range. Among the different types of proteases, alkaline protease finds many applications in industries such as detergents, pharmaceuticals, food, leather processing, and protein waste bioremediation (Nadeem *et al*., 2013). Their use in raw hide processing as a replacement for hazardous chemicals has led to an improvement in the quality of leather products without harming the environment (Khambhaty, 2020). In particular, extracellular alkaline protease is important for the hydrolysis of residual proteins and allows bacteria to uptake and utilizes hydrolysis products by readily growing under extreme pH conditions (Khan *et al*, 2023). Therefore, the present study focused on the Production and Evaluation of the Biotechnological Potential of Alkaliphilic Protease from Tannery Waste Microbes.

1.2. Statement of the problems

In Ethiopia, the annual processing capacity of tanneries reaches approximately 48 million (32.4 million sheep and 15.5 million goat) skins and 2.9 million hides (Tadilo and Ambaw, 2024). The leather industry generates a significant amount of tannery wastes throughout the processing of hides and skins, which poses severe environmental and ecological challenges due to its high organic load, toxic chemicals, and significant contribution to the pollution (Vidaurre-*Arbizu et al*, 2023). Current waste management strategies remain insufficient, as they often fail to address the dual challenges of environmental sustainability and resource recovery (UNECA, 2023).

Meanwhile, the growing demand for industrial enzymes, particularly Alkaliphilic proteases, due to their stability and functionality in high-pH environments, makes them ideal for industrial applications. In Ethiopia, the biotechnological potential of alkaliphilic protease-producing microbes from tannery waste remains underexplored (Masi *et al*., 2021). Despite the abundance of organic material in tannery waste, which could serve as a substrate for microbial growth, there is a lack of comprehensive studies investigating its use for the cost-effective production of industrially valuable enzymes. The global market for industrial enzymes was valued at $9.70 billion in 2019 and $10.34 billion in 2020. This market is expected to reach $16.29 billion by 2027 with a compounded annual growth rate (CAGR) of 6.6% in 2027 (BCC, 2021). Therefore, this study aims to address environmental and industrial challenges, by investigating the biotechnological potential of alkaliphilic proteases isolated from tannery waste microbes.

1.3. Objectives

1.3.1. General objective

The main objective of the study is to produce and evaluate of the biotechnological potential of alkaliphilic protease from tannery waste microbes.

1.3.2. Specific objectives

The specific objectives of the study are to:

- ✓ Isolate, screen, and characterize alkaline protease producing bacteria from tannery waste.
- ✓ Optimize cultivation conditions of bacteria for maximum alkaliphilic protease production.
- ✓ Characterize alkaliphilic protease with various physicochemical parameters.
- ✓ Evaluate alkaliphilic protease for its potential biotechnological applications.

2. LITERATURE REVIEW

2.1. Tannery wastes

Tannery waste, also known as tannery by-products, refers to materials produced during the leather tanning process. This waste may include trimmings, fleshings, hair, and other organic materials generated from leather manufacturing processes (Patel *et al*., 2021). The tannery sector is thought to be one of the biggest sources of pollution in the world, producing 50,000 liters of wastewater and about 6 million tons of solid waste annually per ton of leather produced (Abate *et al*., 2021). It is a fact that tannery waste contains high concentrations of organic point source emissions, such as chromium and other heavy metals, which can be dangerous to the environment and human health if not properly managed (Singh *et al*., 2023). Numerous chemicals are used, and a large range of waste products are generated at each stage, resulting in ongoing environmental damage (Khambhaty, 2020).

Some approaches that can be used for tannery waste management include treatment processes to remove pollutants, recycling of byproduct wastes, and proper and efficient methods of disposal to avoid harming the environment (Rigueto, 2020). Improper disposal of these wastes has detrimental effects on the soil, human health, and water bodies (Table 1). Waste management should be a crucial component of tanneries' operational plans to reduce the potential hazards of tannery waste on the environment and people's health (Oruko *et al*, 2021). Tanneries are increasingly recognizing the need to process leather in more environmentally friendly ways. This involves improving waste treatment processes, using cleaner production methods, and embracing the principles of a circular economy to minimize waste (Chojnacka *et al*., 2021). Sustainable waste management within tanneries is crucial for reducing the environmental impact of chemicals, as well as for enhancing overall performance and reputation within the leather industry (Rigueto *et al*., 2020). Physical, chemical, and biological techniques can be applied independently or in tandem to achieve leather industry objectives (Patel *et al*., 2021). Biological approaches are the most environmentally benign methods for treating industrial effluents. (Sharma *et al*., 2021) shown that pH is a major factor influencing the removal effectiveness of organic material from tannery effluent. The leather industry faces significant issues in waste management, particularly wastewater treatment.

Table 1: Chemicals used in various chemical processing steps and their impacts on life (Khambhaty, 2020)

Steps	Chemical used	Reason	Impact
Soaking	Water, Sulphide	To make skin dirt free and soft	Deficiency of some micronutrients in soil, such as Zn, Cu, and Fe
Liming	Lime and Sodium Sulfide	Removal of hair and unwanted materials	Cause toxicity to living things
De-liming	Ammonium Sulphate	To decrease the pulping of skin and hide	Cause toxicity to living things
Tanning	Tannis, Cr tanning agent	To convert skin or hide to stable materials	Skin irritation, eardrum perforation, nasal irritation, Ulceration , and lung carcinoma in humans as well as animals
Finishing	Resins, Fillers, Dullers,	To ensure homogeneous, long lasting, attractive hides	Adverse effects on human eyes, lungs, liver, kidney, and thyroid

A huge volume of wastewater must be appropriately treated before discharge into the surrounding environment, necessitating the use of an efficient treatment facility (Khambhaty, 2020). Leather industries are among those that produce a lot of proteinase biomass. Leather biomass, notably trimming, fleshing, and shaving, contains protein (Patel *et al*., 2021). The discarded leather biomass comprises 30-35% collagen, keratin, and fat (Rigueto *et al*., 2020). The valorization of this waste leads to the creation of sustainable, biodegradable, and environmentally friendly high-value bio-products.

2.2. Microbial enzymes

Over time, the relationship between humans and enzymes has evolved. Even in ancient times, before the concept of enzymes was understood, the people of Egypt made beer and wine through

enzymatic fermentation. Nowadays, enzymatic studies have made significant progress thanks to increasing knowledge and improved techniques for protein extraction and purification. This has led to the development of many enzymes with high levels of purity for use in research and biotechnological applications (Chapman *et al*., 2018). Enzymes are involved in many traditional food processes, such as cheese making, beer brewing and the wine industry. Recent advances in biotechnology, particularly protein engineering, have enabled the development of enzymes with improved properties. This has enabled the development of novel, tailored enzymes for completely new applications in which enzymes have not previously been used (Rigoldi *et al*., 2018). Enzymes are essential for the maintenance of human life because they accelerate various biological reactions. They are found in almost all living organisms, `including microorganisms, plants and animals (Masi, 2020). However, enzymes derived from bacteria and fungi are more popular due to their catalytic activity, easy handling, lower production cost, availability, stability, specificity, environmental friendliness and non-toxicity (Gupta *et al*., 2015). Many microbial enzymes are used as biocatalysts for the production of various products from different substrates.

2.3. Types of proteases

Proteases are enzymes that break down proteins into smaller peptides or amino acids. They are a key for digestion, immune response, blood clotting, and cell signaling among other biological processes (Meloun and León, 2023). Proteases are categorized differently in their mechanism of action, substrate specificity, and optimum pH range (Chen *et al*., 2019). Types of these proteases include serine proteases, cysteine proteases, aspartic proteases, and metalloproteases (Fig. 1).

2.3.1. Serine proteases

Serine proteases are enzymes that play crucial roles in various important bodily functions. These enzymes have a serine residue at the active site, which is necessary for their catalytic activity. They control processes such as digestion, blood clotting, immunity, and more by cleaving peptide bonds in proteins, thus activating or deactivating them (Bansal *et al.,* 2023). The mechanism of action of serine proteases involves a nucleophilic attack, where the hydroxyl group from the serine residue attacks the carbonyl carbon of the peptide bond. This results in the formation of a covalent acyl-enzyme intermediate that then cleaves off protein fragments (Paschkowsky *et al*., 2019).

2.3.2. Cysteine proteases

Cysteine proteases are a type of protease that contains cysteine in their active sites. They play a crucial role in various biological processes, including protein degradation, immune responses, and cell signaling (Tummers and Green, 2017). The active site of a cysteine protease contains a cysteine group, which acts as a nucleophile when it attacks the carbonyl of peptide bonds (Elsässer and Goettig, 2021). This is followed by the displacement of a water molecule from the tetrahedral intermediate to regain its full negative charge (Narwal *et al*., 2018). Cysteine proteases regulate many physiological functions in animal and plant cells. They are selective in their choice of substrates and target specific protein sequences for cleavage (Guevara and Daleo, 2018). The activities of cysteine proteases are carefully controlled through various methods, such as post-translational modification of gene products and the presence of specific inhibitors (Zamaraev *et al*., 2017). Deregulation of cysteine proteases has been implicated in numerous diseases, including cancer, neurodegenerative disorders such as Alzheimer's disease, and inflammation-related diseases like arthritis (Turk, 2019). Cysteine protease inhibitors are being considered potential drugs for cancer therapy, autoimmune diseases, and infectious diseases because they reduce the activity responsible for unwanted degradation (Stoka *et al*., 2016). Understanding the structure and function it offers to cysteine protease is crucial for designing selective inhibitors against these enzymes without interfering with other proteases in the human system (Phan *et al*., 2021).

2.3.3. Aspartic proteases

Aspartic proteases have two aspartic acid residues in their active sites, which are crucial for their catalytic activity (Lehrbach and Ruvkun, 2016). Aspartic proteases are involved in important biological processes such as digestion, blood coagulation, and protein turnover (Herman *et al*., 2024). Pepsin, found in the stomach walls, is an example of an aspartic protease that aids in the pre-digestion of dietary proteins (Ashaolu *et al*., 2024). Renin is another significant aspartic protease that plays a role in regulating blood pressure by cleaving angiotensinogen to angiotensin I (Pagliaro *et al*., 2022). HIV protease is also an example of an aspartic protease and is targeted by antiretroviral drugs to inhibit virus replication (Voshavar, 2019). The structure of aspartic proteases typically consists of two lobes that create a cleft where the active site is located

(McGillewie *et al*., 2017). The two aspartate residues within the active site act as catalysts for cleaving peptide bonds within proteins (Mahanti *et al*., 2016).

2.3.4. Metalloproteases

Metalloproteases are a type of enzyme that is essential for biological functions. They work by breaking down peptide bonds in proteins and contain a metal ion, zinc, which is crucial for their chemical reactions. These enzymes are involved in important activities such as cell communication, protein breakdown, and tissue. They are also associated with diseases like cancer, inflammation, and neurodegenerative disorders (Giebeler and Zigrino, 2016). The process by which metalloproteases work involves the metal ion interacting with acids in the targeted protein to break down the peptide bond and disintegrate the protein into smaller pieces (Banerjee and Ray, 2017). Metalloproteases have a preference for specific amino acid sequences within proteins, allowing them to control how target proteins function and influence cellular activities (Espíndola *et al*., 2021). Understanding both the structure and function of metalloproteases is crucial for the development of inhibitors that can target these enzymes without interfering with important biological processes (Raeeszadeh-Sarmazdeh *et al*., 2020).

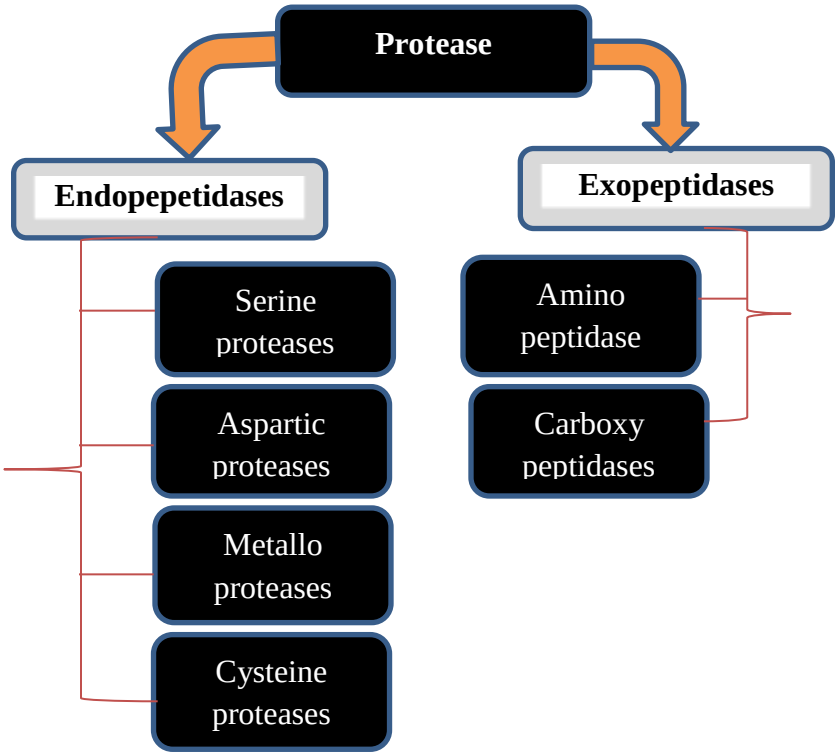


Fig. 1: Protease enzyme classification (Sharma *et al*., 2019 with some modifications)

2.4. Nomenclature of proteases

The nomenclature of proteases is based on their structural characteristics, catalytic mechanisms, and substrate specificities. These enzymes, which catalyze the hydrolysis of peptide bonds, are classified using trivial and systematic names.

2.4.1. Trivial names

Trivial names are derived from historical, functional, or source-based conventions and are widely recognized in common usage (Wang *et al*., 2020). For example, serine proteases include trypsin, which cleaves after lysine or arginine residues, and chymotrypsin, which targets aromatic amino acids like tyrosine. Similarly, papain, a cysteine protease from papaya, and pepsin, an aspartic protease active in acidic environments, are examples of proteases named for their sources or primary functions. Metalloproteases like thermolysin and threonine proteases like the proteasome further showcase the diversity of these enzymes. Trivial names often simplify communication but may lack precision in describing the enzymatic action or mechanism.

2.4.2. Systematic names

Systematic names, assigned by the Enzyme Commission (EC), offer a standardized approach, categorizing proteases based on their catalytic mechanisms and substrate specificity (Elsässer and Goettig, 2021). All proteases are hydrolases under EC 3.4, with subclasses distinguishing between exopeptidases (EC 3.4.11–19) and endopeptidases (EC 3.4.21–25). For instance, trypsin is systematically classified as a serine endopeptidases (EC 3.4.21.4), while pepsin is an aspartic endopeptidases (EC 3.4.23.1). This system highlights functional distinctions and enables consistency across diverse research disciplines.

Alkaline proteases belonging to the serine protease classes under the EC 3.4.21 subclass in the Enzyme Commission (EC) classification system (Bansal *et al.*, 2023). These proteases are characterized by their ability to function optimally in alkaline pH conditions (pH 8–11) and their reliance on a serine residue in the active site for catalytic activity.

2.5. Microbial sources of protease

Microbial proteases are a class of enzymes that facilitate the breakdown of proteins into polypeptides, oligopeptides, and lastly, amino acids through hydrolysis (Razzaq *et al*., 2019). Proteases alone represent 60% of the global market among all industrial enzymes, with *Bacillus species* being the most productive (Mordor Intelligence, 2021). The alkaline protease, which is characterized by its high activity and stability in the alkaline pH range, is the most widely-used enzyme in industrial settings (Sharma *et al*., 2019). This type of protease is active in the neutral to alkaline pH range and is of significant importance due to its frequent application. Alkaliphilic microorganisms represent a diverse group of organisms that thrive in highly alkaline environments.

There exist two primary categories of organisms that thrive in basic conditions: alkaliphiles and alkalitolerant. Alkaliphiles are organisms that grow optimally at pH 9 and cannot survive at pH 7 or below, but can endure pH levels above 10 (Joshi *et al*., 2021). On the other hand, alkali-tolerant organisms can survive at pH 10 but experience the best growth at pH 7 (Kanekar and Kanekar, 2022). Extreme alkalophiles are classified into two subgroups: facultative and obligate alkalophiles. Facultative alkalophiles can grow proficiently at neutral pH, but they experience the best growth at pH 10 and above (Zhu *et al*., 2020). Obligate alkalophiles, on the other hand, cannot survive at pH 7 (Poli *et al*., 2017). These organisms have been found in various materials such as tannery waste (Masi, 2015; Hanif, 2020), leather soil samples (Dheeran and Kumar, 2023), and fish stomach waste (Kumari and Reshma, 2021).

Alkaline proteases are derived from various microorganisms, including *Bacillus subtilis, Aspergillus oryzae, Streptomyces cellulasae,* and *Aeromonas hydrophila* (Taher *et al.,* 2024). Commercial proteases, comprising neutral and alkaline proteases, are predominantly sourced from *Bacillus* species (Contesini *et al*., 2018). *Bacillus* sp. are ideal for industrial applications due to their rapid growth rate, which leads to shorter fermentation times, their ability to produce large quantities of proteins, and their generally recognized as safe (GRAS) status with the food and drug administration, particularly for species such as B. *subtilis* and *Bacillus licheniformis* (Elshaghabee *et al*., 2017).

Microbial isolates such as *Bacillus licheniformis*, *Bacillus coagulans*, *Bacillus polymxa*, and *Bacillus amyloliquefaciens* have shown promising results in producing alkaline protease (Danilova and Sharipova, 2020). These biocatalysts are extensively used in industrial and laboratory settings and have several applications (Ullah *et al*., 2022). *Bacillus* sp. is considered one of the best protease producers due to their inherent properties such as substrate stability, high activity, easy purification, long incubation time, and low cost (Purohit *et al*., 2024). This makes bacterial proteases a valuable asset in various industrial applications.

As shown in Table 2: bacteria, fungi, yeast and actinomycetes are the potential producers of alkaline protease. Among the microorganisms, bacteria are the most dominant group of alkaline protease producers.

Table 2: Different sources of isolation of alkaline protease producing microorganisms (Taher *et al*., 2024 with some modification)

Organisms	**Source of Isolation**
Bacteria	
Listeria monocytogenes	Degraded meat of cow
Bcillus cereus 146	Soil of a wood factory
Bacillus amovivorus	Degrade pulse sample
Bacillus megaterium	Thai fish sauce
Bacillus circulans	Vegetable waste
Geomicrobium sp. EMB2	Salt lake
Fungi	
Penicillum sp.	Soil from soymeal manufacturing industry
Mucor sp.	Herbivorous dung
Aspergillus ustus	Sediment samples
Penicillum sp.	Soil
Aspergillus flavus & Aspergillus terreus	Soil around leather industry

Streptomyces sp.	Soil
Streptomyces roseiscleroticus	Sediment sample
Streptomyces ambofaciens	Soil

2.6. Protease activity optimization

Optimizing, in general, refers to improving the performance of a system, a process, or a product in order to obtain the maximum benefit from it. The term optimization has been commonly used in bioprocesses as a means to identify the conditions that produce the best possible response. The 'one-variable-at-a-time' approach allows optimization of nutritional or cultural factors, by changing one factor at a time, while keeping other variables constant (Jacyna *et al*., 2022). Effects of pH, temperature and culturing conditions on protease production have been optimized simultaneously using response to surface methodology (Chaud *et al*., 2016). Commercially viable production of protease enzymes have been obtained through optimization of growth media (Vázquez *et al*., 2017).

2.6.1. Effect of pH in protease activity

Protease activity and stability are tightly linked to the pH of the environment, as they impact the ionization of amino acid residues critical for catalytic function. Most alkaline proteases exhibit optimal activity in alkaline pH ranges (pH 8–11), where their catalytic triads maintain proper alignment and charge distribution (Li *et al*., 2023). Studies have shown that even minor deviations from the optimal pH can lead to enzyme denaturation or reduced activity, underscoring the importance of precise pH control during industrial processes (Atiroğlu *et al*., 2024). Moreover, the pH not only affects the enzyme's catalytic efficiency but also plays a role in enhancing substrate binding and overall stability, making it a key parameter in enzyme engineering and industrial application strategies (Albayati *et al*., 2024).

2.6.2. Effect of temperature in protease activity

The effect of temperature on alkaline protease optimization is a critical factor in maximizing enzyme activity, stability, and industrial applicability. Temperature influences the kinetic energy

of molecules, directly impacting the rate of enzyme-catalyzed reactions and the structural integrity of the protease (Copeland, 2023). Alkaline proteases typically exhibit optimal activity within a specific temperature range, often between 40–60°C, depending on the source organism and environmental conditions (Arya *et al*., 2020). Higher temperatures can enhance reaction rates up to a threshold but may also lead to protein denaturation, reducing enzyme efficiency and longevity. Conversely, lower temperatures can decrease catalytic activity due to insufficient molecular collisions (Peters *et al*., 2023). To address these challenges, researchers employ strategies such as enzyme immobilization, thermo-stabilization through genetic engineering, and the selection of thermophilic microorganisms to develop proteases capable of withstanding higher temperature ranges. These advancements not only enhance enzymatic performance but also expand the potential applications of alkaline proteases in diverse industrial processes.

2.6.3. Effect of salt concentration in protease activity

Salt concentration significantly impacts the optimization of alkaline protease activity and stability, particularly in industrial and biotechnological applications. Salts influence the ionic strength and osmotic balance of the medium, which can affect enzyme conformation, substrate binding, and catalytic efficiency (Silva *et al*., 2018). Low to moderate salt concentrations may stabilize protease structure by maintaining proper electrostatic interactions and hydration shells around the enzyme. However, excessive salt levels can disrupt these interactions, leading to denaturation, aggregation, or inhibition of enzyme activity (Rabbani and Choi, 2018). Additionally, salt ions may compete with substrates for active site access or alter the charge distribution on the enzyme surface, further affecting activity. Proteases derived from halophilic or halo-tolerant microorganisms often demonstrate higher tolerance to salt concentrations, making them suitable for processes requiring saline conditions (Mokashe *et al*., 2018). Optimizing salt concentration is, therefore, a critical step in tailoring protease performance for specific applications, including detergent formulations, food processing, and wastewater treatment in saline environments.

2.6.4. Effect of inoculum size in protease activity

Inoculum size plays a critical role in the optimization of alkaline protease production, as it directly affects microbial growth dynamics, enzyme yield, and overall process efficiency (Ullah

et al., 2023). An optimal inoculum size ensures a sufficient number of actively growing cells, facilitating rapid substrate utilization and protease synthesis. Too small inoculum size may result in delayed growth and extended lag phases, reducing productivity. Conversely, an excessively large inoculum can lead to nutrient depletion, competition among cells, and the accumulation of inhibitory by-products, ultimately lowering enzyme yields (Basak *et al*., 2020). Furthermore, the inoculum size can influence the pH, oxygen availability, and viscosity of the medium, all of which are crucial for maintaining favorable conditions for protease production (Moreno *et al*., 2024). Therefore, determining and standardizing the ideal inoculum size is essential for maximizing productivity and ensuring consistency in large-scale enzyme production processes.

2.7. Protease activity measurement

Protease activity can be measured, either by Qualitative or Quantitative methods. Both type of method involves measurement of product formation or substrate degradation. Qualitative assay depend on the formation of clear zone, and proteolytic index on casein agar plates, as a result of protein degradation; quantification of protease activity using spectrophotometry has been measured the extent of proteolytic potential of the enzyme (Jesmin *et al*., 2022).The reaction mixture has been assayed, towards its absorbance value, using spectrophotometry at 280 nm (Ravindran *et al*., 2012) at 660 nm (Rawski, 2020) at 640 nm (Kumari *et al*., 2021). One unit of protease activity is defined as the amount of enzyme that liberates one micromole of tyrosine per milliliter per minute under standard experimental conditions (Rawski, 2020). The tyrosine concentration in the filtrate can be determined from a previously prepared standard curve. Generally, different wave length ranges as described above; to measure protease activity, bases the concentration of tyrosine in mg/ml (Hamdani *et al*., 2019).

2.8. Locally available substrates for mass production

Studies have been conducted to develop alkaline protease enzymes using diverse substrates. The substrates tested include shrimp and lobster shell powder (Wang *et al*., 2005) and pigeon pea litter (Senthilraja and Saravanakumar, 2011); “Feather” meal (Lemes, *et al*., 2023); “Nug” meal (Gessesse, 1997); agro-industrial wastes (Prakasham *et al*., 2006); coffee wastes (Murthy and Naidu, 2015); Jatropha seed cake (Sadaf and Khare, 2014); Defatted soybean cake (Agrawal *et al*., 2005); wheat straw, Paddy straw and bagasse (Razzaq *et al*., 2019).

Among the substrates mentioned, "Nug" meal (*Guizotia abyssinica*, commonly known as Niger seed) is particularly abundant in Ethiopia. Ethiopia is one of the primary producers of Niger seed, which is widely cultivated in the country for its oil production. The residual Nug meal obtained after oil extraction is a readily available by-product that can be utilized as a substrate for alkaline protease production (Gessesse, 1997; Ramachandran *et al*., 2007). This makes it a cost-effective and sustainable choice for enzyme optimization studies in Ethiopia.

2.8.1. Carbon sources

Various carbon sources can be used to support the production of proteases. These sources provide energy and carbon for the growth of microorganisms in the metabolic process to produce proteases (Galluzzi *et al*., 2018). Common carbon sources include glucose, sucrose, fructose, and maltose. It is not difficult for microorganisms to metabolize these sugars and produce proteases (Zhang *et al*., 2018). Other carbon sources such as glycerol, starch, and cellulose can also be used to produce proteases (Wu *et al*., 2019). The effect of these sources can vary in the amount and properties of proteases; therefore, careful selection based on appropriate conditions is required.

The choice of carbon source for protease synthesis can have a significant impact on the entire process. For example, the concentration and availability of a carbon source affect microbial growth rate and production (Tan and Li, 2018). The type of carbon source is also a key determinant, as it influences the properties of the protease in terms of stability, specificity, and activity of the isolate in issue (Abdeshahian *et al*., 2020). Understanding how carbon sources affect protease production allows them to better optimize their production to obtain the necessary features. In that instance, selecting carbon sources must be carefully considered and evaluated to produce proteases successfully.

As a result, alternate and renewable sources, as well as traditional ones, are being used for protease production (Singhania *et al*., 2021). Molasses, whey, and lignocelluloses are some of the agricultural and industrial byproducts employed as substrates (Mohanty *et al*., 2021). Such alternative carbon sources have the twin advantage of decreasing the environmental impact while also lowering the economic cost of protease synthesis (Ja'afar *et al*., 2022). However, with these

carbon sources, more extensive optimization and customization of production conditions are required to achieve the maximal yield of proteases of the appropriate grade (Wu *et al*., 2019).

2.8.2. Protein sources

Low-cost SSF (Solid State Fermentation) substrate selection is critical for successful metabolite production. Cow dung is a viable substrate for alkaline protease production and contains 80 ± 12 mg protein/g solid substrate. Organism uses 40 ± 4.5% protein for growth and protease synthesis (Vijayaraghavan *et al*., 2012).

Feathers are known to be composed of more than 90% protein, with keratin being the primary component. This fibrous and insoluble protein is held together by strong bonds, including disulfide bonds. Due to their high protein content, feathers are a valuable source of protein and amino acids for animal feed and other applications. Additionally, feathers have potential use in the fermentation industry, particularly in the production of commercial enzymes. The purification and characterization of alkaline proteases produced by two alkaliphilic bacteria, which can grow on feathers as their sole source of carbon and nitrogen were reported (Gessesse *et al*., 2003).

Researchers have found that pigeon pea waste can be used as a substrate to produce thermostable alkaline protease by *thermoalkaliphilic Bacillus* sp. JB-99 (Senthilraja and Saravanakumar, 2011). By exploring these low-cost and conveniently available materials, we can identify new microorganisms that have the potential to be valuable in creating products.

The use of low-cost materials for industrial enzymes can lower production costs. This is especially important in developing countries where the cost of enzymes can be an obstacle to the industrial use of alkaline proteases. (Gessesse *et al*., 1997) reported a method for producing alkaline proteases from *Bacillus sp*. using nug meal.

The cuttlefish industry generates a significant amount of waste, both solid and liquid. The solid waste can account for up to 35% of the food waste and contains important nutrients. A study conducted to evaluate the suitability of powder obtained from cuttlefish by-products as a medium for protease production and implied that cuttlefish by-products can be a great source of proteins (Chua *et al*., 2023).

2.9. State of fermentation

It is important to highlight that the process of fermentation is crucial for the production of enzymes. During this process, bacteria, yeast, or fungi are used to synthesize enzymes by breaking down organic compounds (Soccol *et al*., 2017). The different stages of fermentation can impact the quantity and quality of the enzymes being produced. Factors such as temperature, pH, dissolved oxygen, and nutrients play a vital role in determining the most suitable fermentation stage for optimal enzyme production (Idris *et al*., 2017).

2.9.1. Solid state fermentation (SSF)

Solid-state fermentation (SSF) is a technique in which microorganisms are cultivated on a solid supporting medium, such as agricultural residues, wheat bran, rice bran, soybean meal, bagasse, and coffee husk with low water content are commonly used as substrates due to their abundance, low cost, and nutrient richness (Singh *et al*., 2016). This method is particularly suitable for producing enzymes like alkaline proteases, as it offers higher yields, better stability, and reduced contamination risks compared to submerged fermentation (Jiang *et al*., 2021). Typically, alkaline proteases produced through SSF demonstrate high activity and stability under alkaline pH conditions, making them ideal for applications in detergents, leather processing, and waste management (Chen, 2013).

Microbial strains, particularly bacteria like *Bacillus subtilis* and *Bacillus licheniformis*, and fungi such as *Aspergillus* and *Penicillium* species, are widely used for SSF-based alkaline protease production (Wang *et al*., 2020). The fermentation parameters, including pH, temperature, moisture content, aeration, and incubation time, are critical in optimizing enzyme yield (Ruan *et al*., 2020b). Recent advancements in SSF techniques, such as bioreactor designs and substrate pre-treatment, have further enhanced alkaline protease productivity and scalability (Jyoti *et al*., 2020).

2.9.2. Submerged Fermentation (SmF)

Alkaline protease production through submerged fermentation (SmF) is a widely employed method in industrial biotechnology due to its scalability, process control, and high enzyme yield. SmF involves cultivating microorganisms in a liquid medium with sufficient nutrients, allowing

for precise regulation of environmental conditions such as pH, temperature, dissolved oxygen, and nutrient levels (Wang *et al*., 2017; De Andrade Carvalho *et al*., 2018). This method is particularly suitable for large-scale production and ensures consistency in enzyme quality and activity (Jiang *et al*., 2021).

The conditions created by the liquid medium in submerged fermentation are favorable for the growth and observable metabolism of microorganisms. The liquid medium often contains carbon and nitrogen sources such as glucose, starch, peptone, or soybean meal, which promote microbial growth and enzyme synthesis (Ruan *et al*., 2020a). Agitation and aeration are critical in submerged systems, as they ensure uniform nutrient distribution and adequate oxygen transfer, both essential for microbial metabolism and protease secretion (Ramesh *et al*., 2022).

2.9.3. Comparison between SSF and SmF

For commercial-scale alkaline protease production, submerged fermentation is the preferred method due to its scalability, precise process control, and consistent enzyme quality (Liu *et al*., 2019). SmF enables efficient regulation of parameters like pH, temperature, and oxygen levels, making it suitable for automated bioreactor systems and large-scale operations (Kumar *et al*., 2021). The liquid medium simplifies downstream processing, enhancing productivity and cost-effectiveness for industrial applications (Palladino *et al*., 2024).

In contrast, solid-state fermentation is more cost-effective and environmental friendly, particularly when using agro-industrial residues. However, SSF faces challenges in scale-up due to difficulties in maintaining uniform conditions like moisture and aeration (de Mello *et al*., 2024). While SSF may be viable in resource-constrained settings, SmF remains the more reliable choice for consistent and high-yield enzyme production at a commercial scale (Rudakiya, 2019).

2.10. Applications of protease

Proteases are enzymes that play an important role in various industries such as detergents, leather, protein hydrolysis, tanning, medicine, and more (Solanki *et al*., 2021; Fig. 2). Alkaline proteases are a crucial group of enzymes used in cleaning, food and feed industries, waste treatment and even silver removal from photographic films (Naveed *et al*., 2021). Many

industries have developed various products based on alkaline protease enzymes (Sharma *et al*., 2019).

2.10.1. Detergent industry

Enzymes play a crucial role in the cleaning industry as they can effectively remove a wide range of stain. Detergents utilize a variety of hydrolytic enzymes that function within the alkaline pH range (Yadav *et al*., 2019). The reason for using different enzymes as additives is that proteases can break down proteins, amylases are effective against starch, and lipases work best for removing fat or oil stains (Gürkök *et al*., 2019).

The alkaline protease is used in laundry detergents, household dishwashing detergents, and the production of industrial detergents (Sharma *et al*., 2019). However, commercially available proteins may not be stable around bleach or oxygen. New technology, rDNA, has allowed for the creation of bioengineered proteases that have better stability and lifespan (Naveed *et al*., 2021).

2.10.2. Leather industry

In the leather industry, the use of alkaline protease has been on the rise due to its remarkable keratinolytic activity. This powerful enzyme has proven to be an asset in the tanning process, where it is employed to soak, bate, and dehair leather (Wanyonyi and Mulaa, 2019).The enzymatic breakdown of unwanted pigments by protease results in the production of white leather, which is highly valued in the market (Zhou *et al*., 2018). Previously, other enzymes were used to produce leather, but they were found to be ineffective. In contrast, protease enzymes from certain bacterial species have been shown to be effective in hair removal, which is essential in leather processing (Arora *et al*., 2020). Proteases play a vital role in selectively hydrolyzing non-collagenous proteins, as well as removing non-fibrillar proteins such as albumin and globulin, which are undesirable in the final product (Sharma *et al*., 2019).

2.10.3. Silver recovery

Silver is a crucial industrial metal and has applications in jewelry, photographic films, electronic items, silverwares, and X–ray films. Alkaline protease finds a potential application in the bio processing of used X-ray films for silver recovery (Pawar *et al*, 2019). Silver recovered from X-

ray films using an alkaline protease produced from a fungal strain *Aspergillus versicolor* PF/F/107. It was observed that the gelatin layer completely decomposed in 20 min at 50^{o}C and pH 9.0, and 0.135 g of silver was retrieved with 0.34% yield (Choudhary, 2013). Alkaline protease from *Conidobolus coronatus* removed the gelatin layer within 6 min at 40^{o}C and pH 9 (Arun, 2021). A *Bacillus* sp. produced thermostable mutant alkaline protease has been identified as a promising agent for the recovery of silver. In this regard, S8 has particularly demonstrated as a significant potential (Arya *et al*, 2020).

2.10.4. Food industry

By adding alkaline proteases, it is possible to obtain protein hydrolysates that have high nutritional value. This type of hydrolysate is particularly important when it comes to producing baby food and fortifying soft drinks and fruit juices, as it contributes essential nutrients that are beneficial to human health (Ray, 2012; Singh *et al*., 2016). It has been discovered that the optimal method for obtaining protein hydrolysates with the most desirable characteristics is through the formation of extended protein hydrolysates (Tang *et al*., 2020; Xu *et al*., 2020). This process involves the sequential action of exoproteases and endopeptidases, combined with the release and development of the post-hydrolysis process. This is the most effective approach to obtain protein hydrolysates with superior characteristics. Furthermore, bioactive peptides that are produced through the hydrolysis of various dietary proteins play an important role in cells as antioxidants (Sonawane and Arya, 2017).

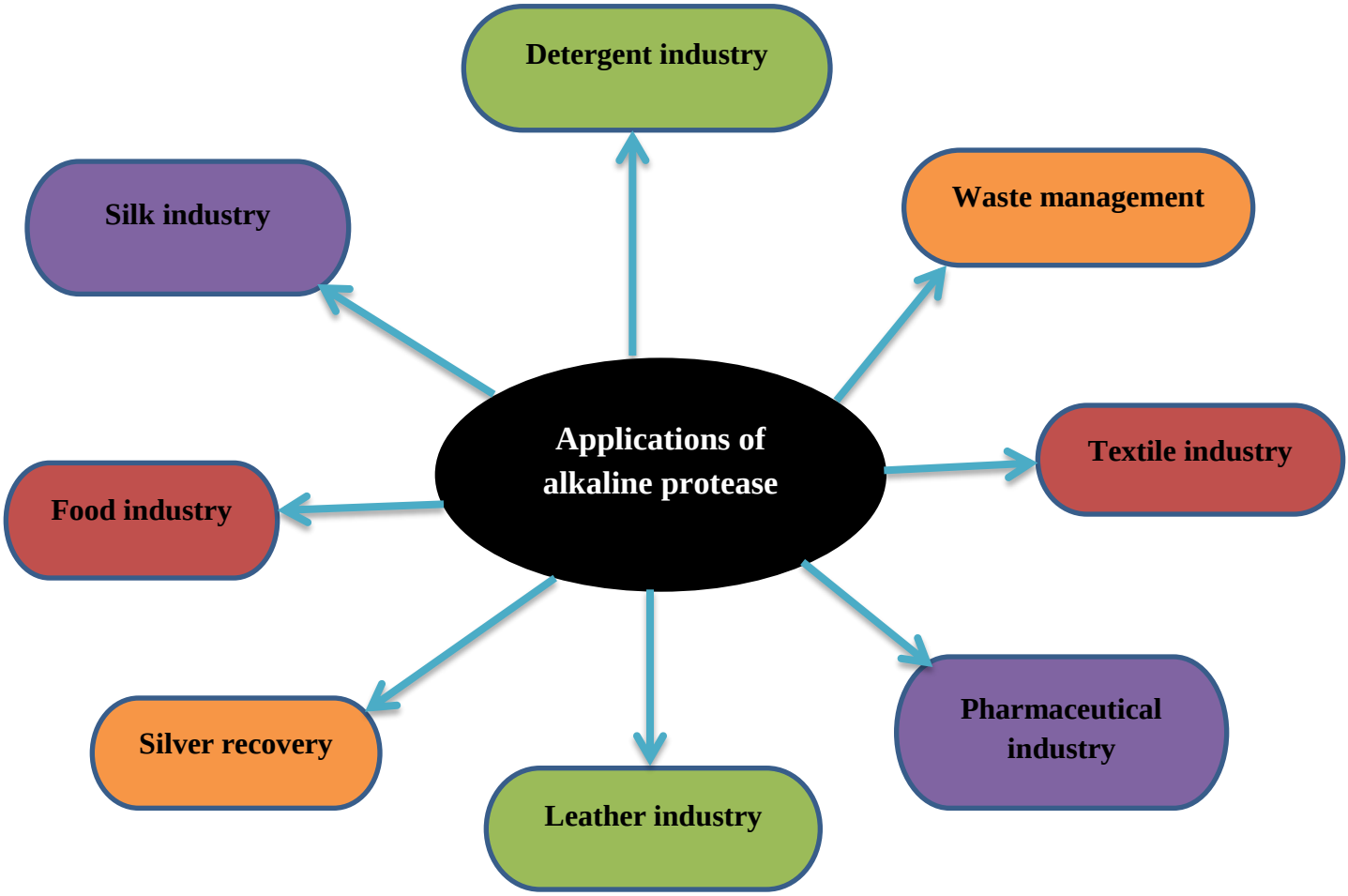


Fig. 2: Several industrial applications of alkaline protease (Ullah *et al*., 2023 with some modification)

2.11. *Alkalihalophilus pseudofirmus*

Alkalihalophilus pseudofirmus is a bacterium that can thrive in high pH (alkaline) and high salt concentrations. This species must have evolved in such environments and remains able to withstand these extremes. The extremophile has molecular mechanisms for maintaining cellular function and integrity under these conditions (Horikoshi, 2021). It could be the first organism discovered that likely uses different ion exchange mechanisms to maintain its pH gradient (Strange, 2020). It has developed a clever way to thrive in extremely challenging conditions (Durvasula and Rao, 2018). Its ability to survive in high pH and salinity environments makes it a compelling organism worthy of scientific attention (Joshi *et al*., 2021). The morphological traits

of *Alkalihalophilus pseudofirmus* are distinctive and unique. The bacterium has rod-shaped cells that typically range from 2-4 micrometers. These cells tend to form chain or cluster arrangements, creating colonies with varied sizes and shapes (Joshi *et al*., 2021). Furthermore, it is Gram-positive and has special structures like flagella for movement and pill for adherence, which help it, navigate and survive in harsh habitats (Arora *et al*., 2017). In addition to its rod-shaped cells and ability to tolerate high salt concentrations, the bacterium possesses a wide range of metabolic capabilities, allowing it to use different carbon sources for energy production (Yuan, 2018). It also produces specific enzymes to combat oxidative stress, a common issue in highly saline areas. These characteristics make *Alkalihalophilus pseudofirmus* a unique organism well-adapted to extreme conditions (De La Haba *et al*., 2024).

2.12. Enzymes produced by *Alkalihalophilus pseudofirmus*

Alkalihalophilus pseudofirmus is known to produce various enzymes that are essential for its survival in extreme conditions. Some of the enzymes produced by this microorganism include proteases, which break down proteins, amylases that aid in starch breakdown, and important lipases responsible for lipid hydrolysis (Prajapati *et al*., 2021; Harirchi *et al*., 2022; Lokre, 2017). These enzymes give *Alkalihalophilus pseudofirmus* the ability to thrive in high pH and high salt environments by facilitating nutrient digestion and cellular maintenance (Kuddus, 2018). Additionally, *Alkalihalophilus pseudofirmus* produces cellulases that help break down cellulose, a complex carbohydrate found in plant cell walls, and uses it as a carbon source, highlighting its metabolic versatility (Mevada *et al*., 2022). Moreover, it synthesizes oxido-reductases involved in redox reactions and catalases that aid in breaking down hydrogen peroxide (Fasman, 2018).

3. MATERIALS AND METHODS

Almost all the experiments were conducted in the laboratories of Microbial and Molecular Biotechnology at the Institute of Biotechnology (IoB), Addis Ababa University (AAU). Green fleshing of the raw skins was carried out at the Leather Industry Development Institute (LIDI), Ethiopia (Appendix 1).

All laboratory grade chemicals were purchased from local suppliers. Sheep and goat skins were purchased and collected from slaughter-houses, Addis Ababa. Ariel, OMO, largo and eggs were purchased from local shops, Addis Ababa.

3.1. Sample area description

Tannery waste samples were collected from, Awash leather industry. Awash tannery is located in Nifas Silk Lafto Subcity, Addis Ababa, Ethiopia. The geographical coordinates of the industry are 8°56′33″ N and 38°45′41″ E (Fig. 3). The factory is located on an area of 123,000 m². It is specialized in the manufacture of refined and quality leather products. The Awash tannery is capable of processing 3,600,000 sheep and goat skins as well as 396,000 hides per year. It generates 1000–5000 cubic meters of liquid waste daily (Amde, 2019).

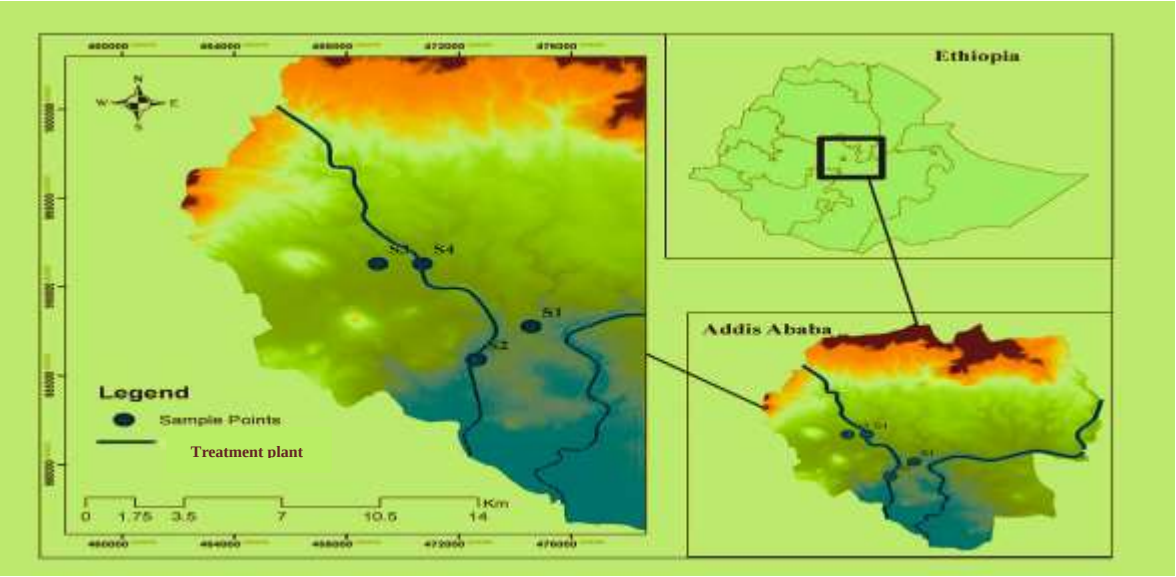


Fig. 3: Sample area description of Awash tannery with respect to sample points in treatment plant; thus, S1 (aerobic digestion), S2 (bulk), S3 (homogenization), and S4 (secondary treatment).

3.2. Samples collection

About 3 Liters of tannery waste-water was collected from Awash Leather Industry using 12 sterile bottles. Each four bottle samples were taken randomly from aerobic digestion, bulk, homogenization, and secondary treatment plants in triplicate to ensure reliability and then blended. Temperature, pH, and salinity of the samples were measured during sampling. TDS, TSS, COD, and BOD of the samples were measured offsite. The COD of the samples was determined by using dichromate method (Gnanavelu *et al*., 2021). The BOD was determined through iodometric or Winkler method (Bennett and MarkManuel, 2024). Finally, the samples were taken to the Microbial Biotechnology laboratory of the Institute of Biotechnology, Addis Ababa University, and stored at $4^{o}C$ for further studies.

3.3. Enrichment of the samples

One ml of the samples was enriched using a selective medium, containing 1% skim milk powder (as sole carbon and nitrogen source), 0.02% $MgSO_4.7H_2O$, 0.1% K_2HPO_4, 0.03% $CaCl_2.dH_2O$ and 1% $NaCl_2$ (Tiwari *et al*., 2015). The broth was maintained, at pH 10 using separately autoclaved 25% Na_2CO_3 (Gessesse *et al*., 2003). The inoculated medium was incubated in an orbital shaker incubator at $30^{o}C$ and 120 rpm for seven days.

3.4. Isolation and screening of alkaline protease producing bacteria

Alkaline protease producing bacteria were isolated and screened according to the method described by Gessesse *et al*. (2003) with some modifications. Skim milk agar medium was used, which contained skim milk (1%), NaCl (1%), yeast extract (0.05%), $MgSO_4.7H_2O$ (0.02%), $CaCl_2.2H_2O$ (0.03%), and K_2HPO_4 (0.1%). The media components were mixed and boiled on a hot plate until the crystals of the elements were dissolved. Then, the pH was adjusted to 7.5-8.0 using 1M NaOH and agar (2%) was added and allowed to boil. Next, all the media components were autoclaved at $121^{o}C$ for 15 minutes. A 25% Na_2CO_3 solution was prepared separately, autoclaved, and 1% added to the medium components to raise the pH to 10. Then, 0.1 ml from the selected dilutions of the enriched sample was spread on a plate containing skim milk agar medium, using a glass spreader and incubated in an incubator (Electrical Heat Thermostatic Incubator DH5000B) at $30^{o}C$ for 48 hours. More than 100 distinct fresh isolates were further

streaked on skim milk agar medium, using an inoculating loop, and then incubated at 30^{o}C for 48 hours. After each round of streaking, the cultures were uniformly re-streaked onto skim milk-agar medium. The plates were then incubated at 30°C for 48 hours. If a clear zone formed around the colonies due to skim milk hydrolysis, it was considered positive for proteolytic activity. The ratio of the diameter of the clear zone to the colony was calculated to assess extracellular protease production. Isolates with large clear zones were chosen for further studies.

3.5. Production of alkaline protease

To select a strain that produces the highest activity of extracellular alkaline protease in submerged fermentation, a loopful of refreshed culture was used to inoculate a 100 ml skim milk broth in 500 ml Erlenmeyer flask with the same medium as the isolation and screening, but without agar. This served as the starter culture. Subsequently, 3 ml of the 48-hours old starter culture was transferred to 100 ml of fresh medium and the flasks were placed in an orbital shaker incubator (121 rpm) at 30°C for 12-120 hours. Afterward, 1.5 ml of the fermented broth was aseptically removed at 12 hour intervals, centrifuged at 10,000 rpm for 5 minutes, and the resulting cell-free supernatant was used as a crude enzyme.

3.6. Characterization of *Alkalihalophilus pseudofirmus* YATW18

Characterizing *Alkalihalophilus pseudofirmus* involves a combination of morphological, biochemical and molecular techniques.

3.6.1. Morphological characterization

Colony characteristics such as form, color, opacity, texture, surface, edge, and elevation were examined microscopically and through direct observation of 48-hour-old colonies on skim milk agar plates (Hossain *et al*., 2021).

To determine the Gram reaction of bacterial isolates, a Gram test was performed using a 3% KOH solution. A small colony from an agar plate was picked using a loop and streaked onto a clean glass slide, leaving approximately a 3 mm distance from the edge. Subsequently, a 3% KOH solution was added, which was later diluted to 1%. The mixture was gently stirred for

about 60 seconds using the loop on the paddle. Observations were conducted to detect any changes in the consistency of the mixture in the glass slide.

3.6.2. Biochemical characterization

Carbohydrate fermentation tests (glucose, fructose, maltose, sucrose, and lactose) at a concentration of 1% were used to determine the ability of *A. pseudofirmus* YATW18 to ferment different carbohydrates to produce acid and/or gas (Hossain *et al*., 2021).

Enzyme activity tests (catalase, oxidase, amylase and lipase) were applied to determine the presence of product formation through specific substrate hydrolysis. Catalase test was performed by pouring 2-3 drops of 3% hydrogen peroxide solution onto the loopful of 48-hour-old colonies placed on a clean microscopic slide. Oxidase (Cytochrome Oxidase activity) of the *Alkalihalophilus pseudofirmus* YATW18 was conducted by taking 48 h old colony. A smear of cultures was made on Whatman filter paper and placed on a Petri dish. Then the compact smear was moistened with 2-3 drops of 1% solution of N, N, N, N′-tetramethyl-p-phenylenediamine di-hydro-chloride (TMPD). Amylase and lipase tests were performed by preparing starch agar plate and tributyrin agar plate, respectively. Then, the starch and tributyrin agar plate was inoculated with a streak of *A. pseudofirmus* YATW18 (Lotfy *et al*., 2017).

3.6.3. Molecular characterization

3.6.3.1. DNA extraction of the isolate

According to Surzycki, 2012, the DNA extraction from alkaliphilic protease-producing bacteria using the CTAB (Cetyl tri-methyl ammonium bromide) method involved several steps. First, 1.5 ml of the overnight bacterial culture was centrifuged at 10,000 rpm for 5 minutes, and the supernatant was discarded, leaving a cell pellet that was re-suspended in 567 µl of TE buffer. To lyse the cells, 30 µl of 10% SDS and 3 µl of Proteinase K (20 mg/ml) were added, and the mixture was incubated at 37°C for 1 hour. Following this, 100 µl of 5 M NaCl was mixed in, and 80 µl of CTAB/NaCl solution was added before incubating at 65°C for 10 minutes. The lysate then underwent phenol: chloroform-isoamylalcohol extraction by adding an equal volume of the mixture, vortexing for 30 seconds, and centrifuging at 10,000 rpm for 10 minutes. The upper aqueous layer was transferred to a new tube and subjected to further purification with an equal volume of chloroform-isoamylalcohol, followed by another round of vortexing and

centrifugation. The aqueous layer was then precipitated with 360 µl of isopropanol, and the DNA was pelleted by centrifuging at 12,000 rpm for 10 minutes. The resulting DNA pellet was washed with 1 ml of 70% ethanol, centrifuged again, and air-dried for 15 minutes before being re-suspended in 50 µl TE buffer. The extracted DNA was stored at 4°C for short-term use. Throughout the protocol, sterile techniques were employed, and toxic reagents like phenol were handled with caution.

3.6.3.2. Quality and quantity assessment of DNA

The yield of genomic DNA as well as its purity was determined qualitatively and quantitatively both by spectrophotometric method and by using the agarose gel electrophoresis (Cropper, 2019). First, a nano-drop spectrophotometer test was done by measuring the absorbance of the DNA sample at 260 nm and 280 nm to calculate the DNA concentration and its purity. The quantity of DNA was obtained from the absorbance values at 260 nm, whereas the 260/280, ratio of around 1.8 was generally considered to indicate pure DNA. Agarose gel electrophoresis was done to check the quantity of the extracted DNA. The genomic DNA (2 µl) was taken and mixed with a loading dye and then placed on an agarose gel and an electric current was applied to the gel. The genomic DNA was visualized under the UV light after staining the gel with Ethidium bromide. Based on the availability of the resource, SYBR Safe, GelRed, Thiazole orange, and Crystal violet can be used as a safer alternative to replace Ethidium bromide.

3.6.3.3. PCR amplification and sequencing

The PCR master mix was prepared with 19.5 µl PCR-dH_2O, 0.25 µl Taq DNA Polymerase, 0.25 µl $MgCl_2$, 0.25 µl DNTPs, 2.5 µl PCR-Buffer, and 0.125 µl forward primer-FD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and 0.125 µl reverse primer-RD1 (5'-AAGGAGGTGATCCAGCC-3'). The used primer pair was able to produce a PCR product of 1500 bp in size. The DNA template (2 µl) that was extracted was then mixed with the PCR master mix in the PCR tube, and the PCR was done in a thermo-cycler. The thermo-cycler was programmed to run a series of temperature cycles: 35 cycles of DNA amplification: the first cycle was initial-denaturation at 94°C for 5 min, after that, denaturation at 94°C for 30 sec, annealing at 55°C for 1 min and elongation at 72°C for 30 sec, the last step was final elongation at 72°C for 5 min (Bartlett and Stirling, 2008). The same primers were used to cycle-sequence

the PCR product on the ABI 3730XL genetic analyzer. The sequence work was outsourced and performed in Amsterdam, Netherlands by the company of MRC Holland.

3.6.3.4. BLASTn analysis

The use of BLASTn was to find sequences related to a reference sequence and define the degree of similarity to a particular nucleotide database (Ismail, 2022). First, using a spectrum from sequenced samples, the nucleotide sequence of interest was retrieved and prepared for submission. The sequence was entered into the BLASTn tool, accessible through the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The search was then performed and the program BLASTn aligned the query sequence with the sequences present in the database of the NCBI and ranked the sequences based on the score. The results were then summarized and presented regarding alignments, score values with the query and sequences of the database as well as E-values which aimed at indicating the statistical significance of the alignment and sequence descriptions.

3.6.3.5. Phylogenetic analysis

MEGA 11 software was used to perform phylogenetic analysis, and the molecular evolutionary relationship of the sequences was visualized. The nucleotide sequences of interest were collected and aligned in FASTA format. This sequence was then transferred to the MEGA 11 software. The software was started and the sequences were aligned with ClustalW for carrying out multiple sequence alignment properly. The sequence alignments underwent a process of phylogenetic tree and Neighbor-Joining tree construction. The bootstrap values that were based on 1000 replicates were chosen in this study. After that, the qualitative tree construction parameters were set to obtain the final phylogenetic tree.

3.7. Optimization of culturing conditions

To optimize the culture conditions for maximum alkaline protease production, various parameters were considered. All experiments were performed in triplicates to ensure data reliability. Statistical analysis, such as ANOVA, was used to determine the impact of culturing conditions on protease production and to identify statistically significant differences in enzyme activity.

3.7.1. Inoculum size optimization

Pre-culture was prepared in 1% skim milk broth medium by taking a loopful of bacterial colonies and incubating at 30°C and 120 rpm for 48 hours. Following sterilization, the production medium was inoculated aseptically with the pre-culture at varying inoculum sizes (1%, 2%, 3%, 4%, and 5%). Each flask was incubated at 30°C and 120 rpm for 48 hours. Enzyme activity was measured at 660 nm using casein hydrolysis method.

3.7.2. Carbon source optimization

To evaluate the effect of various carbon sources on alkaline protease production, a systematic study was performed using glucose, galactose, fructose, sucrose, maltose, starch, and dextrose as individual carbon sources. Skim milk broth medium (1%) was prepared, except for the carbon sources, which were replaced in each experiment with one of the seven tested carbohydrates at an equivalent concentration (1% w/v). Bacterial culture (3%) was inoculated into the production medium containing different carbon sources. The cultures were incubated at 30°C and 120 rpm for 48 hours. Enzyme activity was measured at 660 nm using casein hydrolysis method.

3.7.3. Nitrogen source optimization

To identify the optimal nitrogen source for alkaline protease production, a study was conducted using a range of organic and inorganic nitrogen sources, including peptone, yeast extract, ammonium nitrate (NH_4NO_3), ammonium sulfate ($(NH_4)_2SO_4$), ammonium chloride (NH_4Cl), and urea. Skim milk broth medium (1%) was prepared with a consistent carbon source, replacing the nitrogen sources in each experimental setup with one of the test compounds at an equivalent nitrogen concentration (0.5% w/v). Bacterial culture (3%) was inoculated into the production medium containing different nitrogen sources. The cultures were incubated at 30°C and 120 rpm for 48 hours. Enzyme activity was measured at 660 nm using casein hydrolysis method.

3.7.4. Salt concentration optimization

To optimize salt concentration for enhancing alkaline protease production, experiments were conducted by varying the total salt concentration in the production medium from 1% to 6% (w/v). Skim milk broth medium (1%) was prepared with salt concentrations adjusted incrementally (1%, 2%, 3%, 4%, 5%, and 6%). Bacterial culture (3%) was inoculated into the

production medium containing different salt concentrations. The cultures were incubated at 30°C and 120 rpm for 48 hours. Enzyme activity was measured at 660 nm using casein hydrolysis method.

3.7.5. Locally available substrate optimization

To optimize the use of locally available substrates for alkaline protease production, experiments were conducted using corn cob, coffee husk, wheat bran, water hyacinth, and feathers. All the substrates except "feather" were used as a carbon source, but feather was used as a sole protein source. Fresh water hyacinth plants were collected from local water bodies. The plants were thoroughly washed with tap water followed by distilled water to remove dirt, debris, and surface contaminants. The cleaned plants were air-dried to remove excess moisture and then oven-dried at 60°C until a constant weight was achieved. The dried water hyacinth was ground into a fine powder using a mechanical grinder. The powdered substrate was sieved. The water hyacinth was presented as a showcase, so the same preparation procedures were followed for all the substrates. The processed substrates were added to a minimal production medium containing salt and nutrient composition as usual in the synthetic media preparation. The production medium was adjusted in a 1:1.5 substrate-to-moisture ratio (w/v) with distilled water and was sterilized under standard autoclaving conditions. Bacterial culture (3%) was inoculated into the production medium containing different substrates. The cultures were incubated at 30°C for 3 days. Alkaline protease was extracted by soaking the fermented solid substrate with 5 ml of distilled water per gram of agricultural residue, totaling 50 ml. The mixture was shaken at 121 rpm for 30 minutes at room temperature. The solids were thoroughly mixed using a glass rod. Then, the suspension was hand-squeezed through a double-layered muslin cloth, and the particulate materials were clarified by centrifugation at 6000 rpm for 20 minutes. Finally, the harvested supernatant was used as a crude enzyme, and its activity was measured at 660 nm using the casein hydrolysis method.

3.8. Physicochemical characterization of alkaline protease

3.8.1. Enzyme activity

The protease activity was evaluated using casein as a substrate following the method outlined in the study by Gessesse *et al*. (2003). Firstly, a solution was prepared by mixing 2% casein with an

equal volume of 100 mM Glycine-NaOH buffer at pH 10.0, resulting in a final concentration of 1% casein and 50 mM Glycine-NaOH buffer at pH 10.0. Subsequently, 450 µl of this prepared substrate solution was dispensed into four clean Eppendorf tubes, labeled as triplicates for the enzyme and one for the enzyme blank. A reagent blank was also prepared, using 50 µl of distilled water instead of crude enzyme. Afterward 50 µl of crude enzyme was added to the enzyme-labeled tubes and incubated at 50°C for 30 minutes in the water bath.

Following this, 500 µl of 10% TCA (Trichloroacetic acid) was added to stop the reaction. The reaction mixture was left at room temperature for 10-15 minutes to allow undigested casein to precipitate. Afterward, the reaction mixtures were centrifuged at 10,000 rpm for 5 minutes. Following this, 500 µl of clear supernatant from each reaction mixture was transferred to the respective labeled clean test tubes, and 2.5 ml of 0.5 M sodium carbonate solution was added to each. Next, 500 µl of 1:5 diluted Folin-Ciocalteau's phenol reagent was added, thoroughly mixed by vortex, and then incubated in a dark room for 30 minutes (Appendix 2). Finally, optical density (OD) was measured using a spectrophotometer (JENWAY 6300 UV/Vis) at 660 nm. Enzyme activity was measured against enzyme blank and reagent blank. One unit of alkaline protease activity was defined as the amount of enzyme that liberates one micromole of tyrosine per ml per minute under standard experimental conditions (Rawski, 2020).

Relative activity was calculated as the percentage of enzyme activity in the sample compared to the sample with the highest activity.

$$\textbf{Relative Activity} = \frac{\textbf{Activity of sample (U)}}{\textbf{Maximum enzyme activity (U)}} \times \textbf{100}$$

Residual activity was calculated as the percentage enzyme activity in the sample compared to the activity of the control (untreated enzyme).

$$\textbf{Residual Activity} = \frac{\textbf{Activity of sample (U)}}{\textbf{Activity of control (U)}} \times \textbf{100}$$

3.8.2 Tyrosine standard curve preparation

To generate the standard curve, the following materials were used: 0.5M Na_2CO_3, Glycine-NaOH buffer (50mM, pH 10.0), 1:5 diluted 2N Folin Ciocalteau's phenol reagent, and the

tyrosine solution mixtures. Initially, a 200 µg/ml stock solution of tyrosine was prepared and appropriately diluted to achieve the desired tyrosine concentration. Subsequently, 2.5 ml of 0.5M Na_2CO_3 was added to each test tube, including the blank. Then, 500 µl of the above mixture was added to each test tube, followed by vortexing and incubation at room temperature for 10 minutes. After this, 500 µl of 2N Folin Ciocalteau's phenol reagent was added to each test tube. The solution was then vortex immediately and left at room temperature for 30 minutes. Finally, the absorbance (OD 660 nm) was measured using a spectrometer, and a standard curve was plotted using Origin Pro 2021 software, as shown in Figure 4.

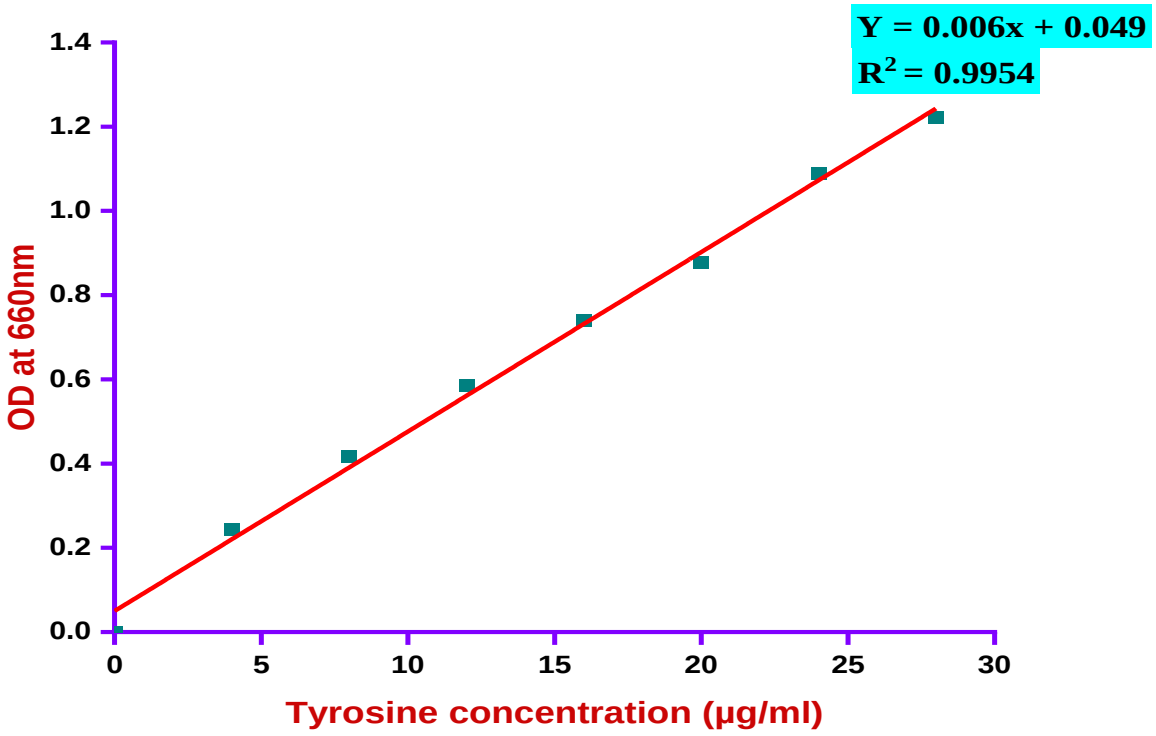


Fig. 4: Tyrosine standard curve

The $OD_{660\ nm}$ values obtained from the mentioned concentrations resulted in a regression equation of **Y = 0.006x + 0.049**. The high regression coefficient **(R^2= 0.9954)** indicates a strong positive correlation. Subsequently, by rearranging, solving, and substituting the absorbance values (not included here) into the previously provided equation, the corresponding concentrations can be determined.

$$\textbf{Enzyme Units (U/ml)} = \frac{(\textbf{166.83} \times \textbf{OD}_{\textbf{660 nm}} \times \textbf{Dilution factor}) - \textbf{8}}{\textbf{Incubation time}}$$

$$166.83 = \frac{\textbf{Maximum OD value throughout all reading}}{\textbf{Slope}}$$

$$8 = \frac{\textbf{Y-intercepts}}{\textbf{Slope}}$$

Incubation time = Optimized incubation time of the enzyme

3.8.3. pH profile of alkaliphilic protease

The research investigated how different pH levels affect the activity and stability of alkaline protease. Various buffers, such as KH_2PO_4 and K_2HPO_4 (pH = 6.0-7.5), Tris-HCl (pH = 7.5-9.0), Glycine-NaOH (pH = 9.5-11), Na_2PO_4 and NaOH (pH = 11-12), and KCl (12.5-13), all at a concentration of 50 mM and within 0.5 pH unit differences at 50°C, were used for the study. The enzyme assay utilized casein as a substrate and followed the outlined procedure in sub section 3.8.1 to calculate the relative activity.

3.8.4. Temperature profile and stability of alkaliphilic protease

The temperature profile of the alkaliphilic protease was determined by incubating the enzyme at various temperatures ranging from 30°C to 80°C in 5°C intervals in a water bath. The enzyme was tested using 1% casein in 50 mM Glycine-NaOH buffer as a substrate with a pH of 10.0. Activity was then determined using a standard protease assay procedure and relative activity was calculated to determine the optimum temperature for proteolysis.

The thermal stability of the enzyme was determined according to Gessesse *et al*. (2003). The enzyme was appropriately diluted with Glycine-NaOH buffer (pH 10.0, 0.1 M), with and without $CaCl_2$ (5 mM). The samples were then incubated at temperatures ranging from 45°C to 60°C for 1 hour in a water bath. After incubation, enzyme samples were withdrawn every 10 minutes and stored at 4°C. Residual activity was determined by using casein as a substrate with a standard protease assay procedure, taking the untreated enzyme as 100%.

3.8.5. Salt profile of alkaliphilic protease

The ionic strength of the alkaline protease was determined by adding NaCl to the reaction mixture in concentrations ranging from 0% to 20% (at 2.5% intervals, except for the first concentration). A standard protease assay was then conducted using 1% casein in 50 mM Glycine-NaOH buffer as a substrate at a pH of 10.0. Finally, the relative activity was calculated by setting the concentration with the highest activity as 100%.

3.8.6. Incubation time profile of alkaliphilic protease

The enzyme assays were conducted in a reaction consisting of 1% casein in 50 mM Glycine-NaOH buffer as a substrate, adjusted to a pH of 10.0 to match the optimal pH of the alkaliphilic protease. The experiments were carried out at 55°C, which is optimal for the alkaliphilic protease activity. All reactions were performed under controlled conditions to ensure reproducibility. The reaction mixtures were incubated for different time intervals: (5, 10, 15, 20, 25, 30, 35 and 40 minutes). Enzyme activity was measured after reaction termination, using the casein hydrolysis method as previously described in section 3.8.1. The incubation time profile was used to identify the optimal incubation time for maximum enzyme activity and to assess the stability of the enzyme over different time intervals.

3.8.7. Metal ions profile of alkaliphilic protease

Stock solutions of the metal ions (B^{3+}, Zn^{2+}, Mn^{2+}, Ni^{2+}, Cu^{2+}, Fe^{2+}, Co^{2+}, Mg^{2+} and Ca^{2+}) were prepared at higher concentration (50 mM) for ease of dilution. The stock solutions were diluted to obtain final concentration of 1 mM, 2.5 mM and 5 mM for the experiment. All reaction mixtures were kept under the same conditions of pH and temperature for consistency. All the reaction mixtures such as 1% casein, 50 mM Glycine-NaOH buffer and metal ions with different concentration were incubated at 55°C for 20 min. Enzyme activity was measured after reaction termination, using the casein hydrolysis method as previously described in section 3.8.1. The enzyme activity across different metal ion concentration was compared and the optimal metal ion concentration was determined.

3.9. Partial purification of alkaliphilic protease

Ammonium sulfate is commonly used for partial purification of alkaliphilic protease through ammonium sulfate precipitation followed by centrifugation, re-suspension in appropriate buffer, dialysis, and enzyme assay as well as determination of purity level.

3.9.1. Ammonium sulphate precipitation

The crude extract was centrifuged at 10,000 rpm for 5 min, and then 66.7g of solid ammonium sulfate was slowly added to 50 ml crude enzyme to reach the desired saturation level (Doonan, 2008). This operation was done with slow addition under continuous stirring to boost up even distribution and reduce localized super-saturation (Appendix 3). Finally, the sample was left to rest standing for 12 to 16 hours at 4°C, so that proteins would completely precipitate out (Scopes, 2013). Last of all, different saturation levels (20%-100%) within 10% intervals were adopted in a stepwise manner, for selective precipitation of proteins, including the target alkaliphilic protease.

$$\%\ \textbf{of Saturation} = \frac{\mathbf{533}\boldsymbol{g} \times \mathbf{S_1} - \mathbf{S_2}}{\mathbf{100} - \mathbf{0.3} \times \mathbf{S_2}}$$

S_1 = Initial saturation point, S_2 = Final saturation point, 0.3 = Specific volume of ammonium sulphate and 533 g = Ideal value of saturation level at 20°C

3.9.2. Centrifugation

After ammonium sulfate addition at every stage, the proteins were allowed to precipitate at 10,000 rpm for 15 minutes and the pellet was separated from the supernatant.

3.9.3. Re-suspension and dialysis

The protein precipitate was then re-suspended in a minimal volume 50 mM Glycine-NaOH buffer. To eliminate synthesizing osmotic pressure employed ammonium sulfate and other small molecules; the re-suspended protein solution was dialyzed in a beaker (5 liter) containing the same buffer (50 mM and 4 liter) employing a suitable 2 cm cut-off dialysis bag.

3.9.4. Purity Assessment

SDS-PAGE was used to determine the purity of the enzyme through molecular weight and protease band patterns on the baseline of the 250 kDa protein ladder. The partially purified protease sample was prepared, according to Kurien and Scofield (2012) by mixing it with an equal volume of SDS sample buffer. The mixture was then boiled for 5 minutes to denature the proteins. An appropriate percentage of polyacrylamide gel was prepared depending on the molecular weight of the proteins, which was 30%. The gel consisted of a stacking gel (with a lower acrylamide concentration) on top of a resolving gel (with a higher acrylamide concentration). The resolving gel preparation contained 3.3 ml of dH_2O, 4 ml of 30% acrylamide, 2.5 ml of Tris-HCl (1.5 M, pH 8), 50 µl of 10% SDS, 100 µl of 10% APS, and 20 µl of TEMED. Besides this, the stacking gel preparation contained 1.75 ml of dH_2O, 450 µl of 30% acrylamide, 630 µl of Tris-HCl (1.5 M, pH 6.8), 25 µl of 10% SDS, 50 µl of 10% APS and 15 µl of TEMED.

According to Kurien and Scofield (2012), the denatured protease sample (20 µl) and the 250 kDa protein ladder (5 µl) were loaded into separate wells of the gel. The gel was placed in an SDS-PAGE running apparatus filled with 300 ml of 10× running buffer. Electrophoresis was conducted at a constant voltage (85 V) until the dye front was reached at the bottom of the gel. After electrophoresis, the gel was removed and stained with Coomassie Brilliant Blue to visualize the proteins. Then, the gel was de-stained using 50% methanol and 10% acetic acid to remove excess stain and the proteins were visualized. Finally, the stained gel was analyzed by comparing the bands of the protease sample to the bands of the protein ladder (250 kDa). All experiments were conducted in triplicate to ensure the reliability of the results.

3.10. Evaluation of alkaliphilic protease for its potential industrial applications

3.10.1. Stain removal for detergent application

The performance of alkaline protease on removing protein stains and its compatibility with commercial detergents (Ariel, OMO, and Largo) were investigated using the method described by Zhang *et al.* (2021). In this experiment, white cotton cloth was used to test the cleaning abilities of commercial detergents (Ariel, OMO, and Largo), buffer, crude enzyme and partially purified enzyme. Ariel and OMO were diluted in tap water (7 mg/ml) according to the

manufacturer's specifications to simulate washing conditions. The endogenous enzymes contained in the Ariel were inactivated by heating the diluted solution for 1 hour at 100°C before the addition of the enzyme preparation. Crude alkaline protease of strain YATW18 (67.97 U/ml) was incubated in Ariel and OMO detergent solution for 30 min at 55°C, and then the remaining activity was determined following the standard protease assay conditions.

The study evaluated the washing performance of different detergent combinations and enzymes. First, egg stains were painted on to white cotton cloths (6 cm x 6 cm) and left to dry for 72 hours (Appendix 4). Then after, various methods were applied to remove the stain: distilled water (control) with buffer, Ariel with buffer, OMO with buffer, Largo with buffer, enzyme with buffer, enzyme and Ariel; with buffer, enzyme and OMO; with buffer, and enzyme, and Largo; with buffer. Finally, the cloth pieces were rinsed with tap water and air-dried. Finally, the dried pieces were visually inspected for cleanliness. All experiments were conducted in triplicate to ensure the reliability of the results.

3.10.2. Dehairing for tannery application

This experiment was conducted based on Wanyonyi and Mulaa (2019) with some modifications. The excess flesh and fatty tissue under the fresh goat and sheep skins were removed before they were treated with enzymes using a green fleshing technique. The skin pieces were washed with tap water (3 times) and then rinsed with distilled water to remove any chemicals that could hinder enzyme activity during dehairing (Appendix 5). The skin was then dried and cut into 12 cm x 12 cm pieces, each weighing 24.86 g. A cell-free supernatant with 99.6 U/ml of the crude enzyme was used as a source of crude enzyme for the experiment. The reaction mixture (50 ml) contained an equal volume of crude enzyme and 50 mM glycine-NaOH buffer at pH 10.00. A control was prepared under similar conditions, but it contained a crude enzyme that had been denatured by boiling at 100°C for 1 hour. Na_2S and CaO were used as positive controls. All the reaction mixtures; except a positive control were incubated at room temperature, until 20 hours; and 16 hours of its activity were also examined. The positive control was incubated at room temperature for 8 hours only. Dehairing activity of the enzyme was observed by pulling the hairs with fingers. Finally, the skin pieces were visually examined for complete dehairing activity and any changes.

3.11. Data analysis

The data from the optimization and application studies of alkaliphilic protease were analyzed using a one-way analysis of variance (ANOVA). All experiments were conducted in triplicate to ensure the reliability of the results. The mean values were compared using post-hoc multiple comparison analysis carried out with Duncan's homogenous test using SPSS version 29.0, at a significance level of $p<0.05$. The results of the characterization studies of alkaline protease, both in terms of activity and stability, were presented using three independent measurements and illustrated through graphs and columns using Origin Pro 2021 data analysis software.

4. RESULTS

4.1. Characterization of tannery waste water

The temperature of the waste-water taken was 28°C (Table 3). The pH level was determined to be 8.1. The salinity of the wastewater was identified to be 1%. The TDS of the samples were 9. 63 ppt. On the other hand, the total suspended solids (TSS) were determined to be 1044.7 mg/L. Besides, the COD level was established at 1276 mg/L, and the BOD at 375.4 mg/L (Table 3).

Table 3: Characterization of tannery wastewater samples concerning different parameters

No.	Parameters	Measured value	Place of measurement
1.	pH	8.1	On-site measurement
2.	Temperature	28°C	
3.	Salinity	1%	
4.	TDS	9.63 ppt	Off-site measurement
5.	TSS	1044.7 mg/L	
6.	COD	1276 mg/L	
7.	BOD	375.4 mg/L	

4.2. Screening of alkaline protease producing bacteria

4.2.1. Primary screening

There were visible distinct clear zones around the colonies which were evidence of skim milk hydrolysis (Fig. 5). The range of proteolytic index was from 2.5 mm (YATW5) to 7.9 mm (YATW9). The minimum proteolytic index of alkaline protease producing bacterial isolates were 2.5 mm (YATW5) followed by 2.9 mm (YATW14). The maximum proteolytic index out of 22 alkaline protease producing bacterial isolates was 7.9 mm (YATW9). Isolates with large clear zones (above 2.9 mm ratio) were chosen for secondary screening (Table 4).

Table 4: Clear zone comparison of bacterial isolates

No.	Bacterial isolates	Colony diameter (mm)	Clear zone diameter (mm)	Protolytic index (ratio)
1.	YATW1	3.7	18.7	5.1
2.	YATW2	3	19.7	6.6
3.	YATW3	3	17.3	5.8
4.	YATW4	3.3	21.3	6.5
5.	YATW5	3.7	9.3	2.5
6.	YATW6	3	22	7.3
7.	YATW7	2.7	20.7	7.7
8.	YATW8	3.3	20.7	6.3
9.	YATW9	2.7	21.3	7.9
10.	YATW10	2.7	17.3	6.4
11.	YATW11	3.3	22	6.7
12.	YATW12	3.7	22	5.9
13.	YATW13	2.7	21.3	7.1
14.	YATW14	3	8.7	2.9
15.	YATW15	3.3	21.3	6.5
16.	YATW16	2.7	8	3
17.	YATW17	3	9.3	3.1
18.	YATW18	3	20.7	6.9

19.	YATW19	3.7	22	5.9
20.	YATW20	4	20.7	5.2
21.	YATW21	2.7	20	7.4
22.	YATW22	3.3	17.3	5.2



Fig. 5: Isolation and screening of alkaline protease producing bacteria

4.2.2. Secondary screening

Among the 22 alkaline protease producing bacterial strains, the YATW18 strain was the most efficient in extracellular alkaline protease production (Fig. 6). The submerged fermentation process of the alkaline protease-producing bacterium yielded a fairly high enzyme level with a maximum amount between 36-60 hours of incubation period at 30°C and 120 rpm (Fig. 6). The enzyme production had reached the highest value of about 92.65 U/ml or 100% relative activity at its optimum incubation period of 48 hours.

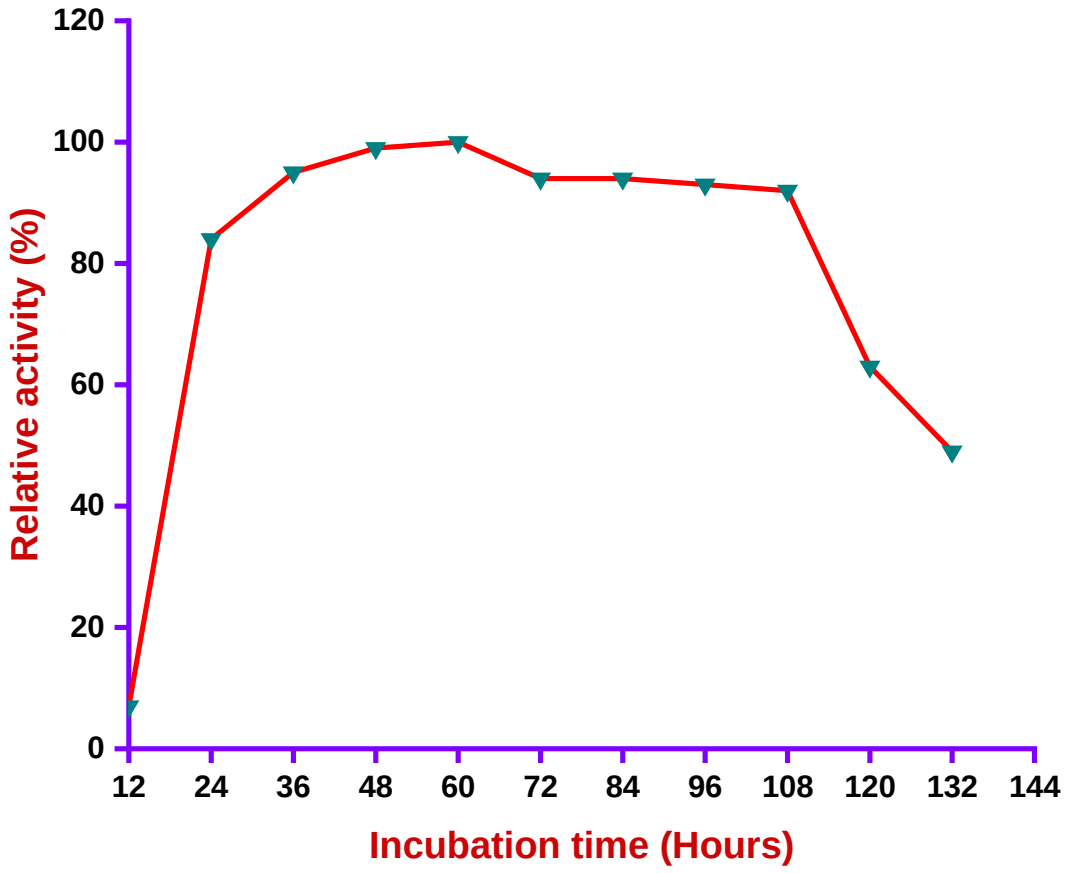


Fig. 6: Enzyme activity of strain YATW18 at 12-hour intervals during the incubation period

4.3. Characterization of *Alkalihalophilus pseudofirmus* strain YATW18

4.3.1. Morphological characterization

The colonies were circular and predominantly yellow (Appendix 6). They exhibited an opaque appearance and a smooth texture. The surfaces of the colonies were slightly glossy, with entire (smooth) edges and a mostly flat elevation with a slightly raised center (Table 5). The cells shape of this bacterium was typically rod-shaped and appeared in small clusters. It was observed that the bacterium exhibited motility, as indicated by its movement through the environment. *Alkalihalophilus pseudofirmus* strain YATW18 was determined to be Gram-positive (Table 5).

Table 5: Morphological characterization of *Alkalihalophilus pseudofirmus* strain YATW18

Test	Observation
Form	Circular
Color	Yellow
Opacity	Opaque
Texture	Smooth
Surface	Slightly glossy
Edge	Smooth
Elevation	Flat and slightly raised
Shape	Rod shaped and small cluster
Gram reaction	Gram positive
Motility	Positive

4.3.2. Biochemical characterization

The results indicated that the organism was able to ferment glucose, fructose, and maltose. Also, while using the Durham tubes, small bubbles were observed. But, when it came to sucrose and lactose, the color of the medium did not change and there was no gas production (Table 6).

In the catalase test, the bacterium produced a positive result, as indicated by the formation of air bubbles. The oxidase test revealed a positive reaction, characterized by the appearance of an intense deep blue color. In the amylase test, *Alkalihalophilus pseudofirmus* strain YATW18 exhibited negative amylase activity; there was no clear zone around the bacterial growth on the starch agar plate. However, the lipase test showed a positive result, with clear zones around the bacterial growth on the tributyrin agar plate (Table 6).

Table 6: Biochemical characterization of *Alkalihalophilus pseudofirmus* strain YATW18

Test	Observation
Glucose	Positive
Fructose	Positive
Maltose	Positive
Sucrose	Negative
Lactose	Negative
Catalase	Positive
Oxidase	Positive
Starch hydrolysis	Negative
Tributyrin hydrolysis	Positive

4.3.3. Molecular characterization

The genomic DNA (Appendix 7) was within the expected size range (5000 bp). The actual sizes of the PCR products (Appendix 8), was perfectly in agreement with the expected fragment sizes (1500bp). The overall identity, as well as coverage percentage in the query sequence, was high (Table 7). The partial sequence of strain YATW18 bacteria 16S rRNA gene was submitted to NCBI database and the accession number was obtained as PP942174 (Fig. 7).

Table 7: Significant alignments produced from sequences of strain YATW18

Description of the stain	Query Cover (%)	Identity (%)	Accession number	Sources of the strain
Alkalihalophilus pseudofirmus strain YATW18	100	100	PP942174	Tannery waste
Bacillus sp. NBRC 101252	100	99.65	AB681426	BRC
Bacillus pseudofirmus	100	99.65	AB201799	ATCC

Bacillus sp. (in: Bacteria) strain JSM	100	99.65	MG893133	Sandy soil
Bacillus pseudofirmus strain SA1	100	99.65	KM015448	Soil
Alkalihalophilus pseudofirmus strain SC171	100	99.65	OK563108	Mud
Bacillus pseudofirmus strain APT35	100	99.65	KC519407	Soil

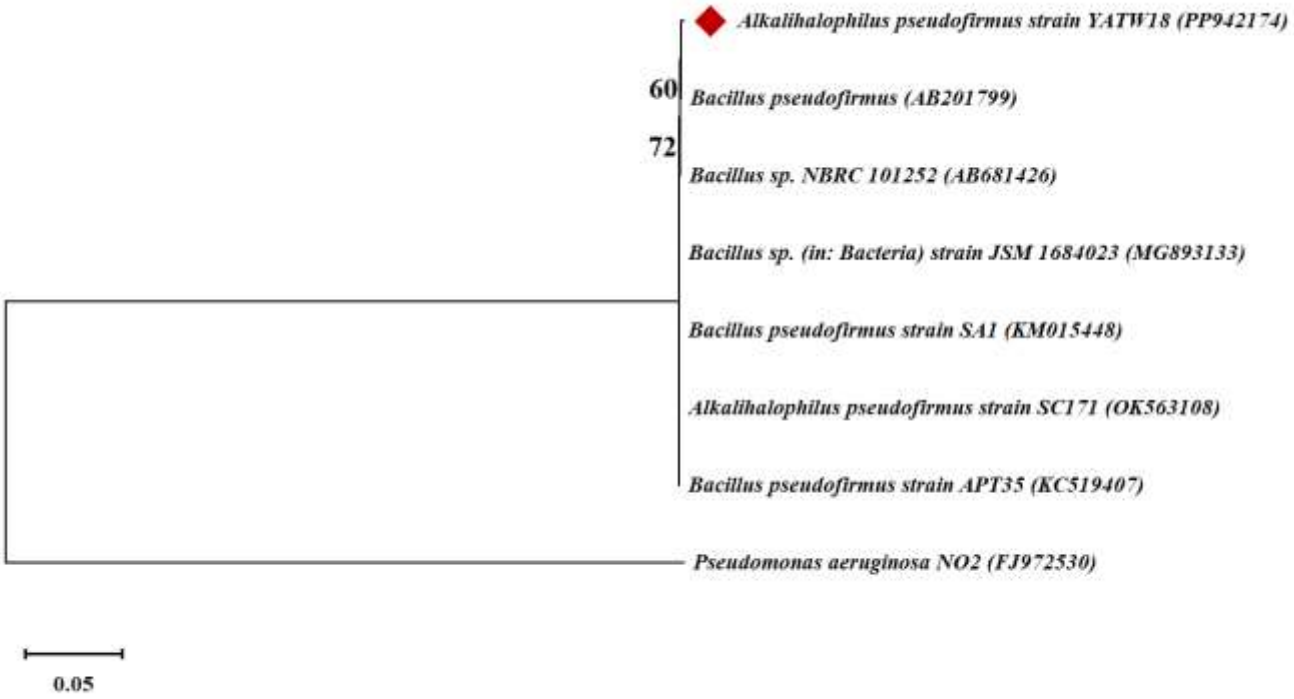


Fig. 7: Neighbor-joining phylogenetic tree construction showing the relationship among the alkaline protease producing bacterial strains studied and close matches; on the bases of 16S rRNA gene sequence. Numbers at nodes indicate percentages of occurrence in 1000 bootstrapped trees; only

values greater than 50% are shown. Scale bar 0.05 number of substitutions per nucleotide position. *Pseudomonas aeruginosa* NO2 (FJ972530) was used as an out-group. *Alkalihalophilus pseudofirmus* strain YATW18 was isolated in this study.

4.4. Optimization of cultivation conditions

4.4.1. Effect of inoculum size

It was observed that a 3% inoculum level was found to enhance the maximum enzyme production with relative activity of 100% (Fig. 8).

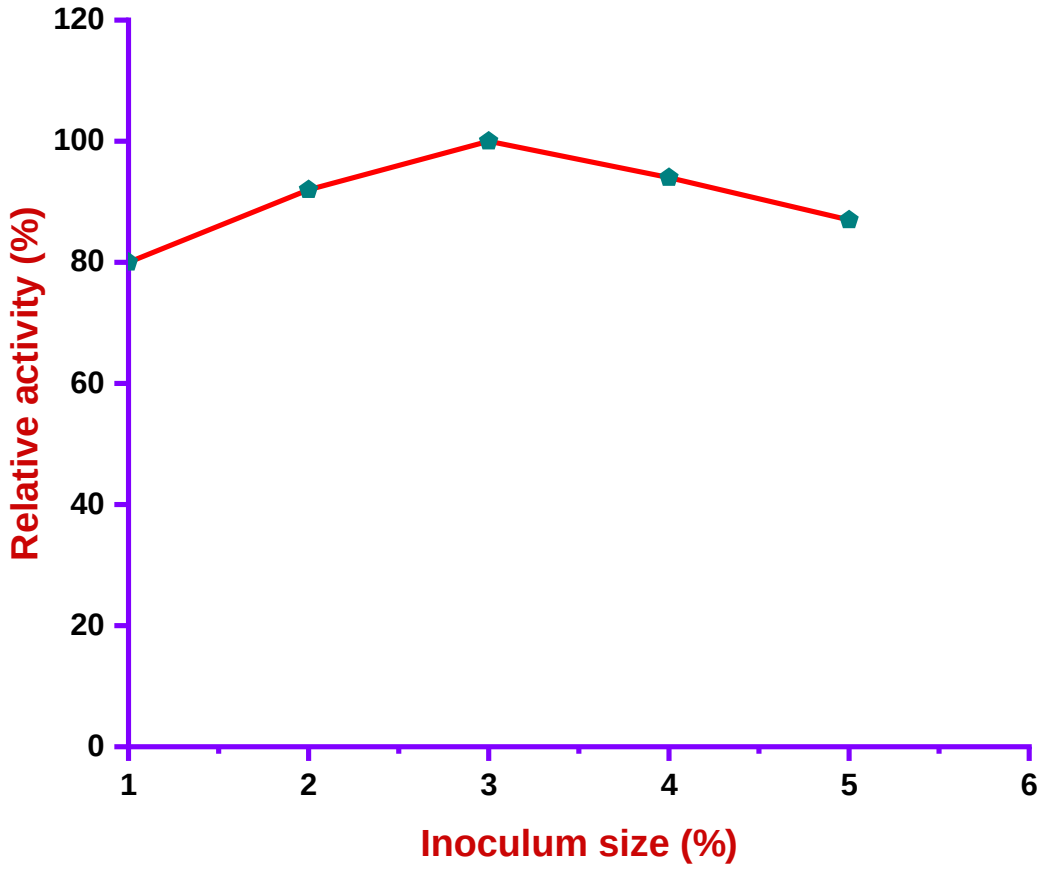


Fig. 8: Effect of inoculum size in alkaline protease production

4.4.2. Effect of carbon and nitrogen sources

Fructose exhibited the highest enzyme activity with an average yield of 82.10 ± 8.29 U/ml, while glucose yielded 63.47 ± 0.44 U/ml (Table 8). Carbon sources like galactose, sucrose, maltose,

and starch demonstrated lower enzyme activities, with yields ranging from 20.10 to 26.54 U/ml (Table 8). For nitrogen sources, peptone and yeast extract both resulted in the highest enzyme yields of 96.86 ± 2.98 U/ml and 95.59 ± 1.07 U/ml, respectively. In contrast, ammonium nitrate, ammonium sulfate, ammonium chloride, and urea produced significantly lower enzyme yields; with values ranging from 8.02 to 52.99 U/ml (Table 8). The mean difference was statistically significant at $P \leq 0.05$.

Table 8: Effect of carbon and nitrogen sources on alkaline protease production

Carbon sources	**Relative activity (%)**	**YATW18 alkaline protease yield (U/ml)** (**M ± SD**)
Glucose	77	63.47 ± 0.44^{b}
Galactose	14	20.10 ± 0.90^{d}
Fructose	100	82.10 ± 8.29^{a}
Sucrose	20	22.79 ± 0.23^{d}
Maltose	24	25.93 ± 0.94^{d}
Starch	20	26.54 ± 1.99^{d}
Dextrose	56	47.71 ± 2.76^{c}
Nitrogen sources		
Peptone	100	96.86 ± 2.98^{a}
Yeast extract	100	95.59 ± 1.07^{a}
Ammonium nitrate	7	12.89 ± 0.19^{c}
Ammonium sulfate	51	52.99 ± 2.67^{b}
Ammonium chloride	3	8.02 ± 0.98^{d}
Urea	1	8.97 ± 1.08^{d}

4.4.3. Effect of salt concentration

Initially, at 0% salt concentration, the enzyme yield was recorded 125.39 ± 4.01 U/ml, representing the highest yield and serving as the baseline with a relative activity of 100% (Table 9). As the salt concentration rose to 1%, the yield was slightly decreased to 86.27 ± 0.32 U/ml, indicating a relative activity reduction to 64%. Each increase in salt concentration was showed statistically significant reduction in the enzyme activity, with $P \leq 0.05$ confirming the significance of these changes (Table 9).

Table 9: Effect of salt concentration on alkaline protease production

Salt concentration (%)	**Alkaline protease yield (U/ml)** (M ± SD)	**Relative activity (%)**
0	125.39 ± 4.01	100[a]
1	86.27 ± 0.32	64[b]
2	48.98 ± 1.59	30[c]
3	40.47 ± 0.98	25[d]
4	30.44 ± 1.17	17[e]
5	28.49 ± 1.26	14[e]
6	21.81 ± 1.09	7[f]

4.4.4. Effect of locally available substrates

The yield and activity of alkaline protease from various locally available substrates, significant differences were observed (Fig. 9). Among the substrates tested, water hyacinth has demonstrated the highest alkaline protease yield of 661.85 ± 10.98 U/g, with a relative activity of 100%, the error bar was slightly larger than the others (Fig. 9). Corn cob gave a yield of 599 ± 6.51 U/g, and a relative activity of 97%, the error bar was minimal, similar to feather, indicating consistent results. Wheat bran yielded 532.7 ± 10.32 U/g with a relative activity of 91%, the

error bar was small. While feather had yields of 591.89 ± 4.31 U/g with relative activity of 96%, the error bar remains small. All substrates except Coffee husk exhibited moderate to high enzyme activity, with Water hyacinth were leading the group. Coffee husk not only had the lowest enzyme activity, but also showed minimal variability. The results were statistically significant with a level of significance set at $P \leq 0.05$.

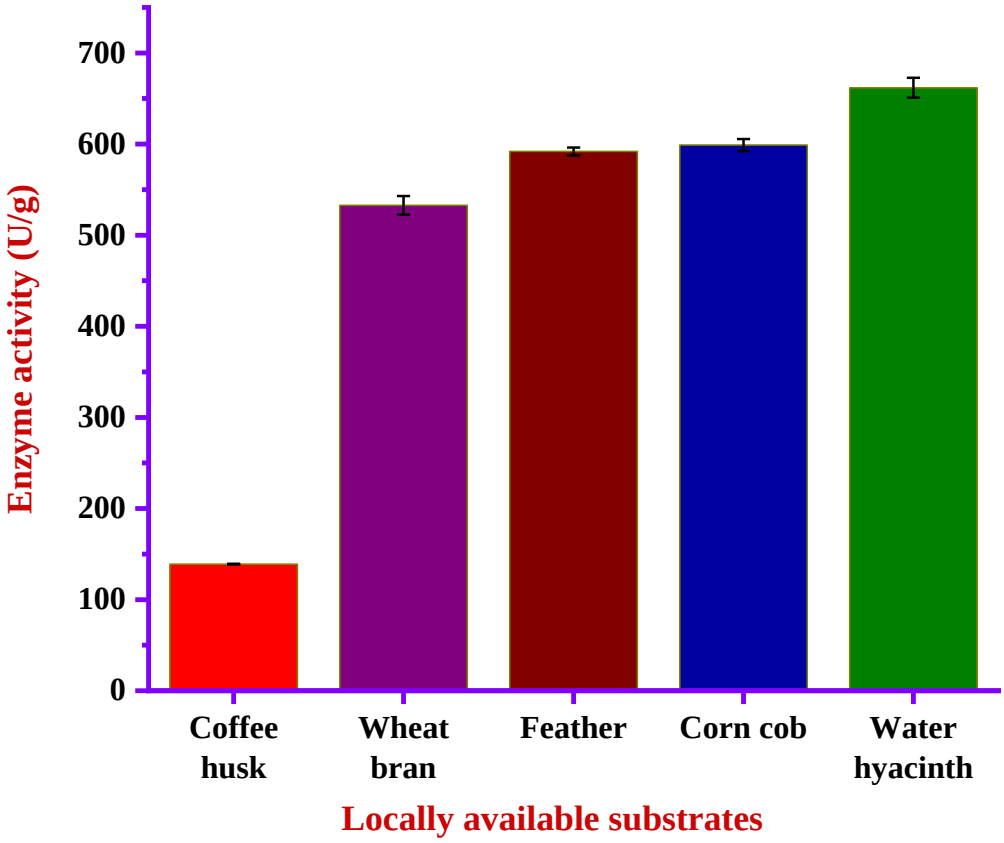


Fig 9: Effect of locally available substrates in alkaline protease production

4.5. Physicochemical characterization of alkaline protease

4.5.1. Effect of pH in alkaliphilic protease activity

At the pH ranging from 7.5 to 11, the activity of the enzyme was high with relative activity of 88% to 89% and reached its optimum at pH 10 with relative activity of 100% and then slightly declined, and totally declined after pH 11 (Fig. 10).

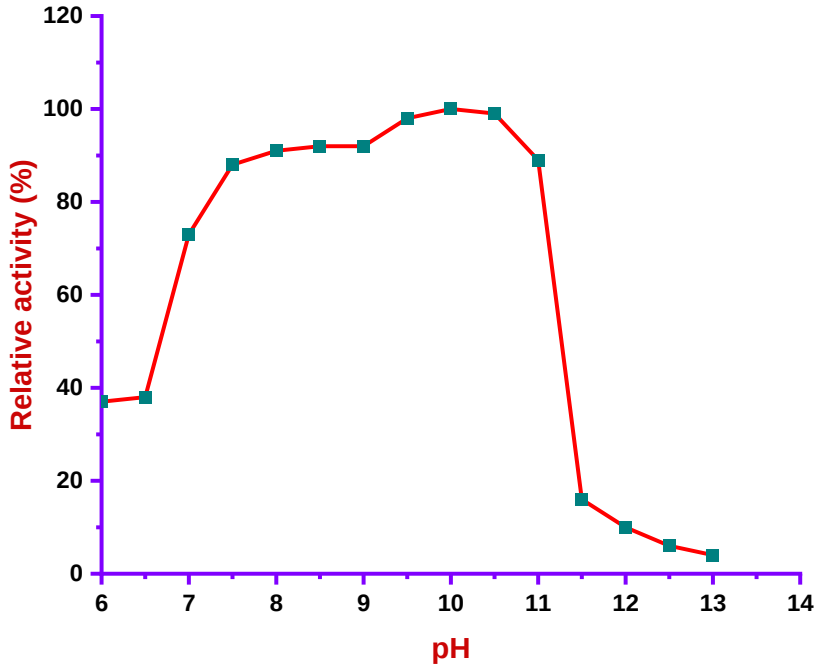


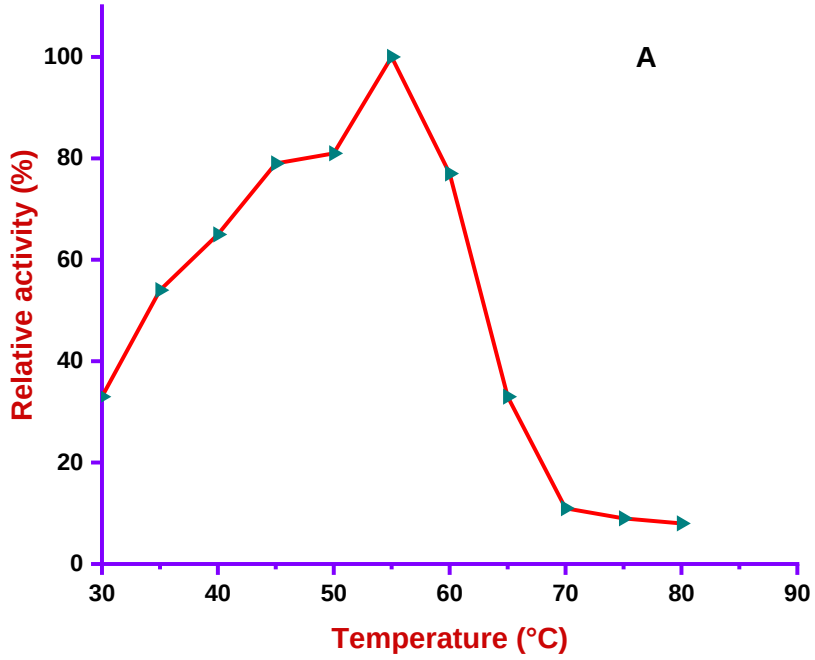
Fig 10: Effect of pH in alkaline protease activity

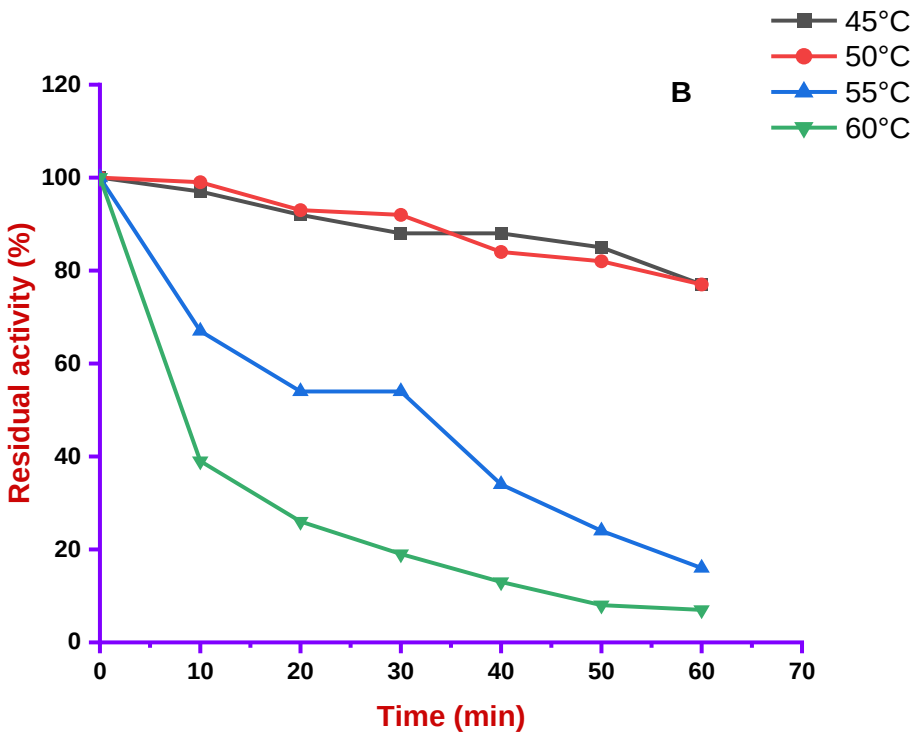
4.5.2. Effect of temperature in alkaliphilic protease activity and stability

As shown in Fig. 11A the analysis by the temperature profile showed that the enzyme had the least activity at 30°C with relative activity of about 30%. When the temperature raised up to 40°C the activity of the enzyme increased and reached about 60% and it was further increased and found to be 80% at 50°C. The protease activity reached its maximal value of about 100% at 55°C, the temperature identified as the optimal for proteolysis under the conditions of the study.

Fig. 11B shows the stability of the enzyme at four distinct temperatures of 45°C, 50°C, 55°C, and 60°C in time intervals of 60 min. The enzyme was stable and retaining more than 80% of its original activity throughout almost all temperature tested, except 60°C.

Fig. 11C demonstrates the thermo-stability of the enzyme at different temperatures in the presence of 5mM calcium at time intervals. The enzyme was found to be very stable at 45°C thereby retaining approximately 92% of its initial activity right through the incubation period of 60 minutes. At 50°C and 55°C the enzyme had a high activity level and a small decline after the 50 min.


A
Relative activity (%)
100
80
60
40
20
0
30
40
50
60
70
80
90
Temperature (°C)


B
45°C
50°C
55°C
60°C
Residual activity (%)
120
100
80
60
40
20
0
0
10
20
30
40
50
60
70
Time (min)

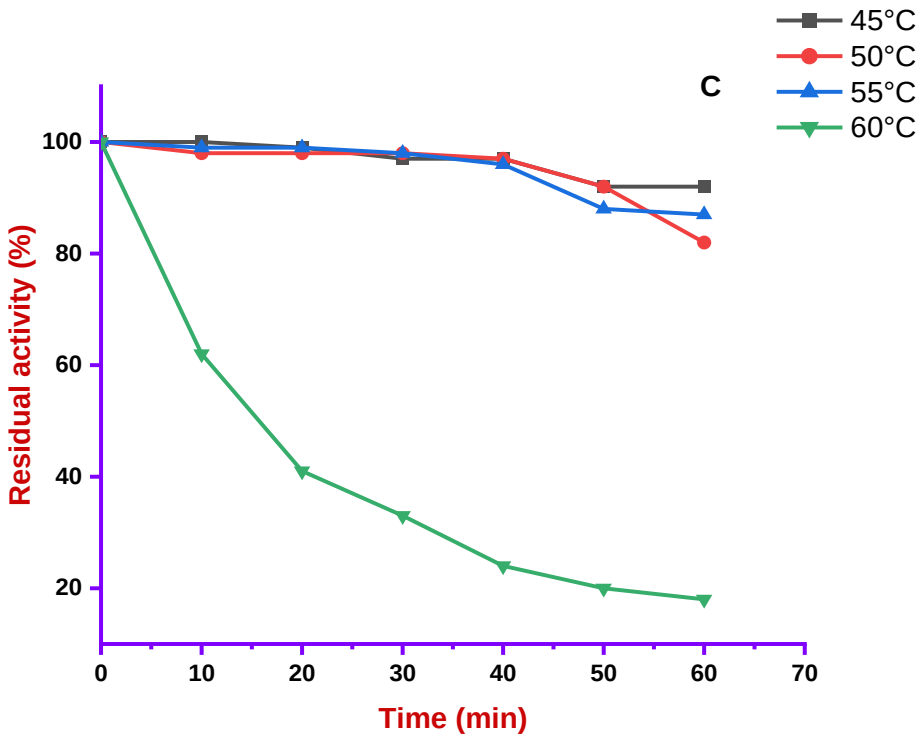


Fig. 11: Effect of temperature (A) in alkaline protease activity (B) in alkaline protease stability without calcium (C) in alkaline protease stability with 5mM calcium.

4.5.3. Effect of salt concentration in alkaliphilic protease activity

As shown in Fig. 12 the enzyme activity of alkaline protease from *Alkalihalophilus pseudofirmus* strain YATW18 demonstrated a notable tolerance to increasing salt concentrations. At 0% salt, the enzyme exhibits optimal activity. When the salt concentration is increased to 5%, the enzyme still retains 80% activity. At 10% and 15% salt, the enzyme maintains 60% and 40% activity, respectively, showing resilience to moderately high salt concentrations. Even at 20% salt, the enzyme retains 20% activity.

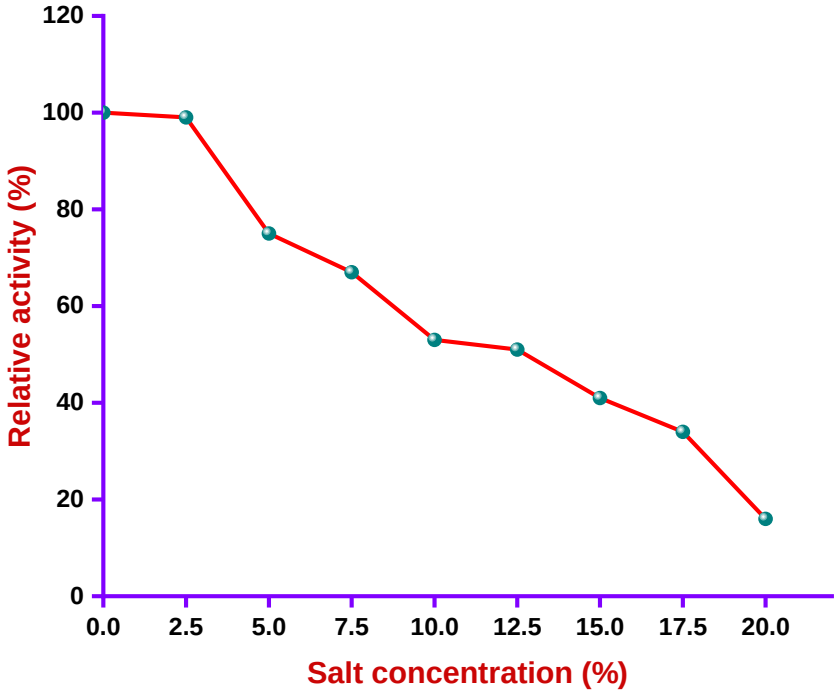


Fig. 12: Effect of salt concentration in alkaline protease activity

4.5.4. Effect of incubation time in alkaliphilic protease activity

The enzyme assays showed that the activity of the alkaliphilic protease in response to the incubation time was increased and reached an optimum level at 20 minutes of incubation (Fig. 13). At first, the relative activity increased rapidly, reaching out 60% at 5 min then increase to 85% at 10 min. This increment was sustained, and it was peaked 100% at 20 minutes. After this point, the relative activity remained relatively near this peak while oscillating slightly between 20 and 40 minutes into the process (Fig. 13).

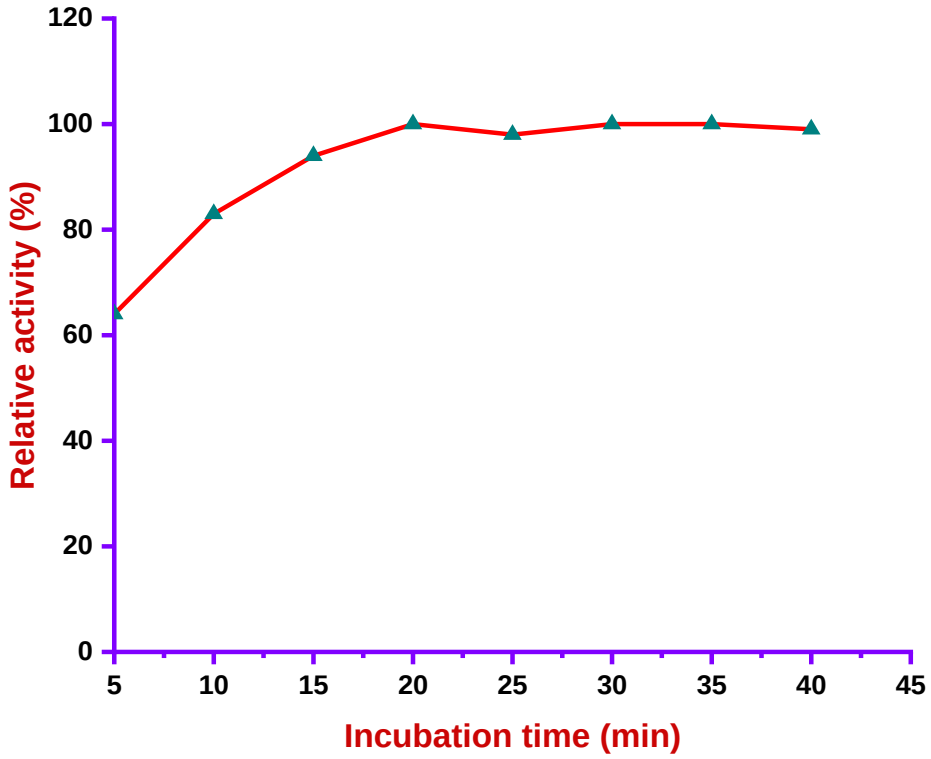


Fig. 13: Effect of incubation time in alkaline protease activity

4.5.5. Effect of metal ions in alkaliphilic protease activity

Most metal ions had minimal influence on activity across concentrations, with the relative activity close to or slightly below 100% (Fig. 14). Boron (B), Calcium (Ca), and Magnesium (Mg) showed very stable activity. Particularly at 5 mM, Nickel (Ni) and Zinc (Zn) showed a slight decline in activity as the ion concentration increases. Alkaliphilic protease containing Manganese (Mn) was observed with a noticeable drop in relative activity through increasing concentrations. For Mn, Ni, and Zn, some separation between bars at higher concentrations indicated a trend toward reduced activity (Fig. 14). Alkaliphilic protease stability had seen with some metal ions (B, Ca, and Mg). However, slight inhibitions were observed with increasing concentrations of Ni, Zn, and Mn.

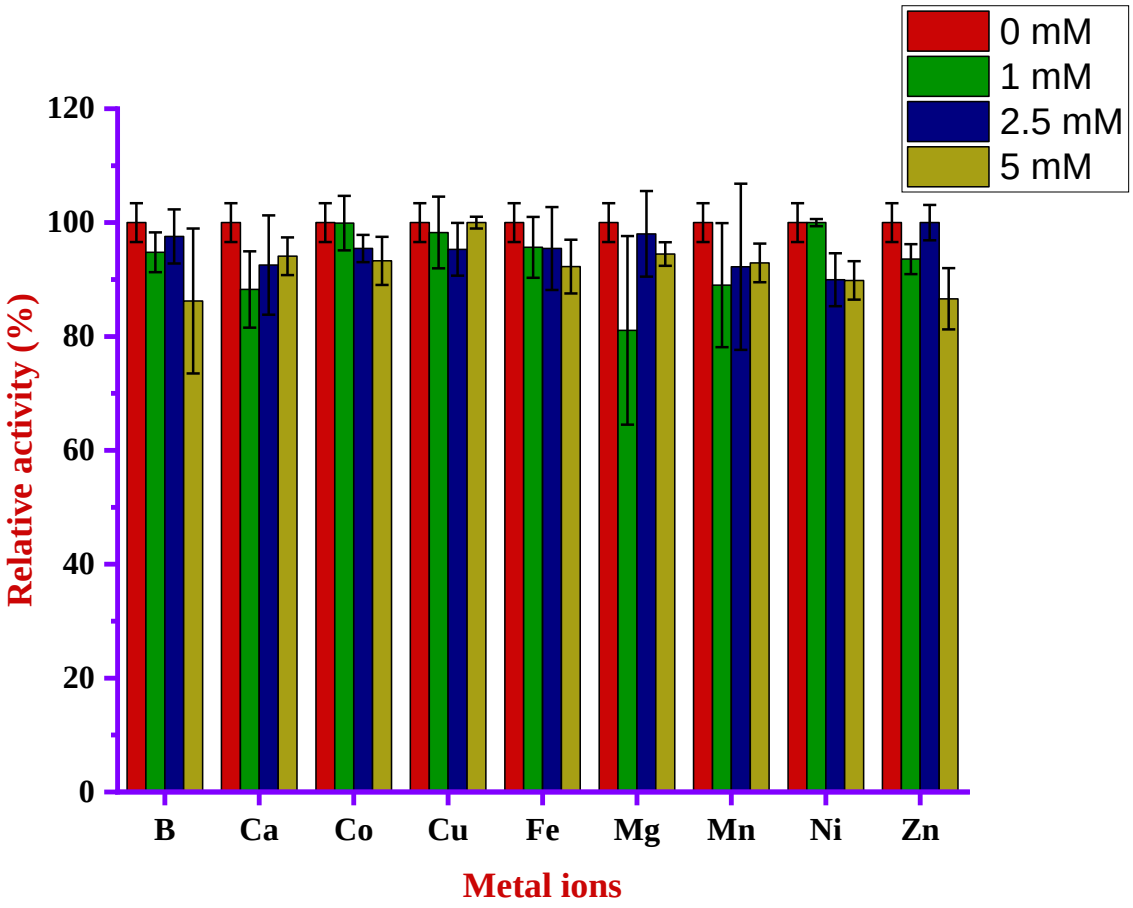


Fig. 14: Effect of metal ions in alkaline protease activity

4.6. Partial purification of alkaliphilic protease

4.6.1. Ammonium sulphate precipitation

The saturation level at which maximum relative enzyme activity occurred was in the 50% to 80%. Nevertheless, the relative activity was found to decrease steadily as the saturation level moved up above 80% and reduced sharply after 90% of the saturation level (Fig. 15).

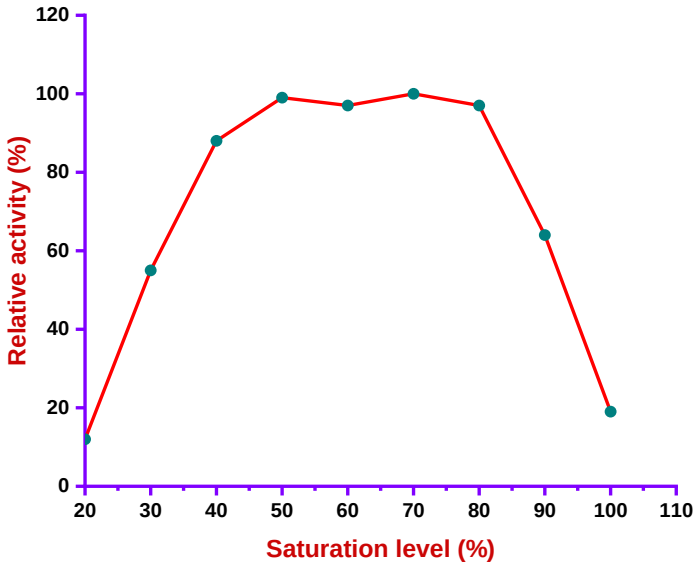


Fig. 15: Partial purification of crude enzyme using ammonium sulphate from (0-100) % saturation level

4.6.2. Purity assessment using SDS-PAGE

There was observed one faint band in S1 (crude extract) and two sharp and bright bands in S2 and S3 (partially purified) in SDS-PAGE (Fig. 16). Relative sizes of the proteins were 250 kDa and 125 kDa.

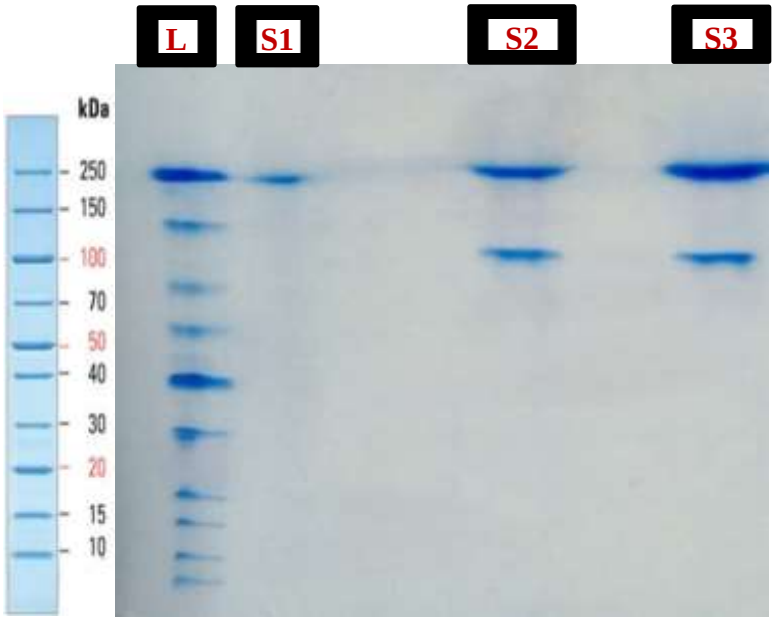


Fig. 16: SDS-PAGE image and band pattern of partially purified enzyme. L: protein ladder (250 kDa); S1: 0% saturation (positive control); S2: 50% saturation; S3: 70% saturation (125 kDa).

4.7. Evaluation of alkaliphilic protease for its potential industrial applications

4.7.1. Stain removal efficiency for detergent industry

Almost complete removal of egg yolk stains were observed on the pieces of cotton clothes washed with commercial detergents contained various amounts of the enzyme (Figs. 17A-C) compared to those clothes washed with the commercial detergents alone (Figs. 17D-F). As expected, no removal of the stains were recorded when the cloth pieces were washed with distilled water (control) (Fig. 17I). Interestingly, the stains on the cotton clothes were significantly removed when treated with crude and partially purified enzyme (Fig. 17G and H).

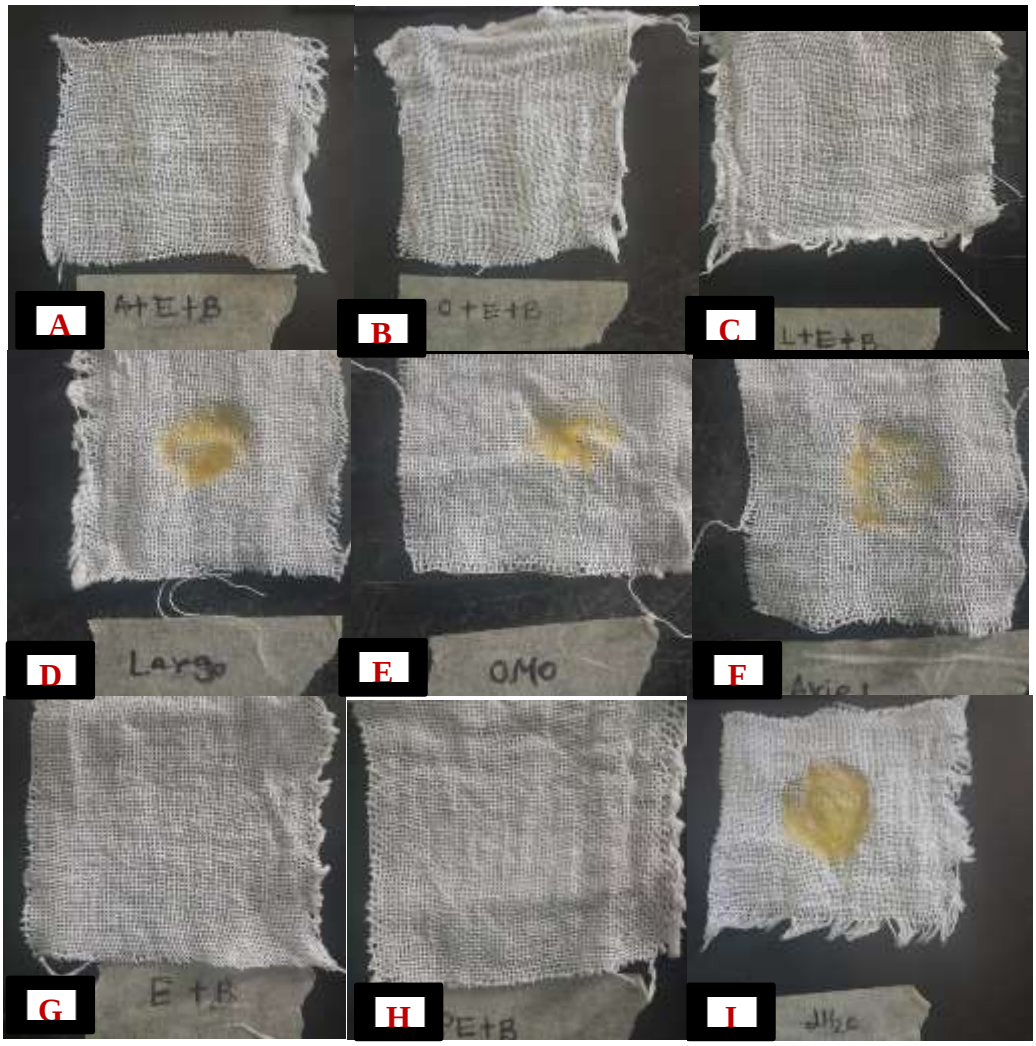


Fig. 17: Egg yolk stains removal efficiency of different treatments. (A) Enzyme + Ariel + 50mM Glycine-NaOH buffer, (B) Enzyme + OMO + 50mM Glycine-NaOH buffer, (C) Enzyme +

Largo + 50mM Glycine-NaOH buffer, (D) Largo + 50mM Glycine-NaOH buffer, (E) OMO + 50mM Glycine-NaOH buffer, (F) Ariel + 50mM Glycine-NaOH buffer, (G) Enzyme (77.92 U/ml) + 50mM Glycine-NaOH buffer, (H) Partially purified enzyme (44.36 U/ml) + 50mM Glycine-NaOH buffer, (I) dH2O + 50mM Glycine-NaOH buffer (negative control).

4.7.2. Dehairing capacity for leather industry

High dehairing properties were exhibited (Fig. 18). The skin pieces treated with the active enzyme were fully degraded, and it was easy to remove the hairs to get clean skin pieces (Figs. 18E and F). In contrast, the control sample did not exhibit any dehairing at all since the hair did not come off the skin easily (Fig. 18A and C). On the other hand, positive controls, Na_2S and CaO showed a good dehairing effect, but the skins looked more damaged compared to enzyme-treated skins (Fig. 18G and H).



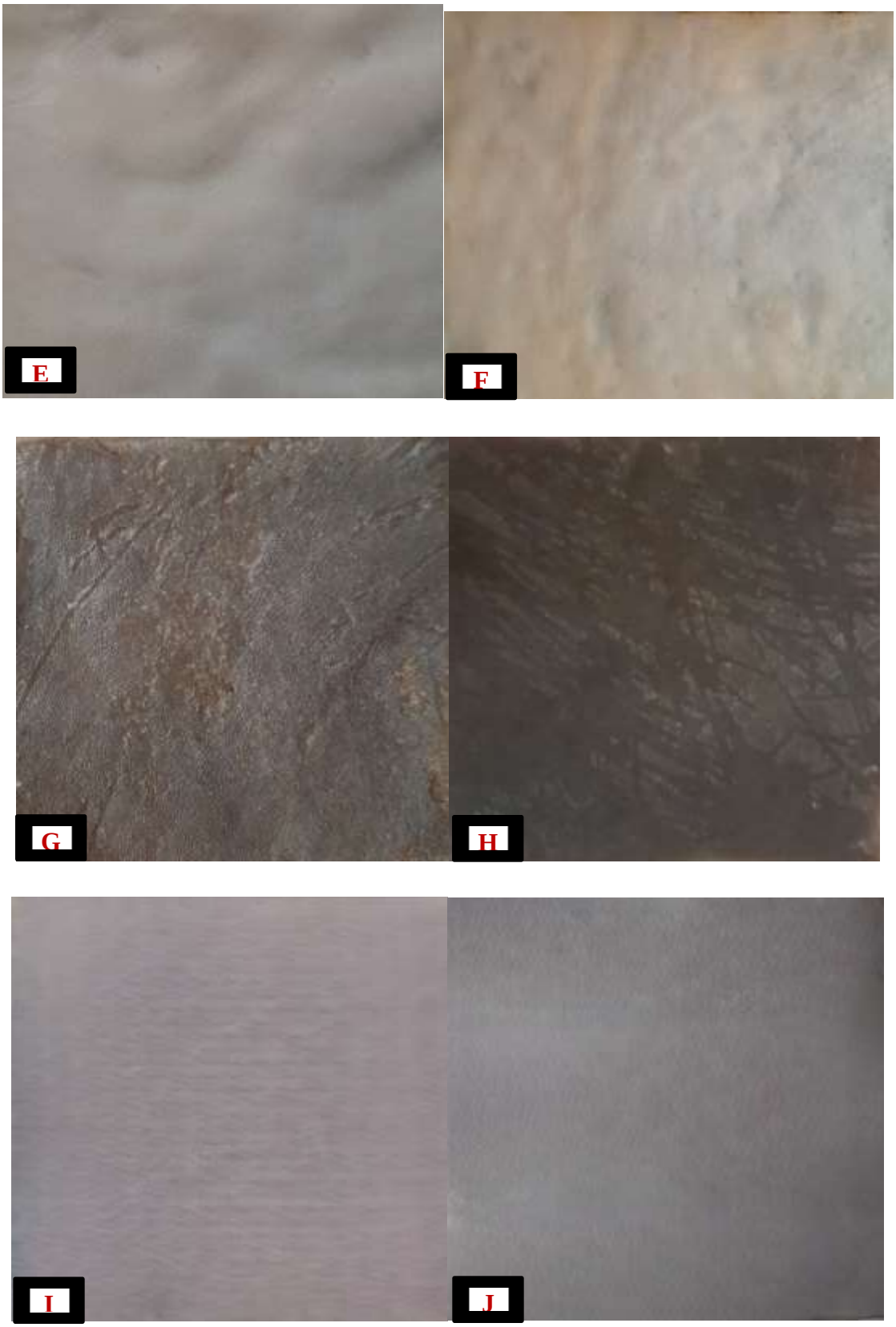

E
F
G
H
I
J

Fig. 18: Partial and complete dehairing capacity of different treatments. (A) Negative control (sheep skin treated with denatured enzyme) (B) Partial degradation of sheep skin treated with alkaline protease (After 16 hours; 79.68 U/ml) (C) Negative control (goat skin treated with denatured enzyme) (D) Partial degradation of goat skin treated with alkaline protease (After 16 hours; 79.68 U/ml) (E) Complete degradation of sheep skin treated with alkaline protease (After 20 hours; 99.6 U/ml) (F) Complete degradation of goat skin treated with alkaline protease (After 20 hours; 99.6 U/ml) (G) Complete degradation of sheep skin treated with Na_2S and CaO (After 8 hours) (H) Complete degradation of goat skin treated with Na_2S and CaO (After 8 hours) (I) Complete degradation of sheep skin treated with enzyme, and Na_2S and CaO (After 12 hours) (J) Complete degradation of goat skin treated with enzyme, and Na_2S and CaO (After 12 hours).

5. DISCUSSION

5.1. Screening of alkaline protease producing bacteria

The cut-off values for the proteolytic potential have been recorded as high among some of the microbial isolates which signifies a high potential for extracellular protease production, which has been confirmed by their ability to clear skim milk agar and form clear zones around their colonies (Ullah *et al.,* 2023). The existence of these clear zones as a measure of activity, thus said to be valid and affirms the capability of these isolates to digest casein; a protein present in skim milk agar medium (Fig. 5). The values of the proteolytic index obtained for the alkaline protease producing bacterial strains varied, this indicating differences in the secretory ability of enzymes among the tested strains (Table 4). The large variability in the proteolytic index brings differences in enzyme secretion, most of which might be gene-based, and aspects of metabolic rate. Additional studies on the molecular composition of these isolates may allow for identifying the specific proteases involved in casein hydrolysis and their application to increase the efficiency of the process in the industry (Kieliszek *et al*., 2021). In the same way, the isolates with larger clear zones were selected for investigation in the secondary screening, because the large hydrolysis zones are associated with high protease production, and therefore, the isolates are suitable for industrial application (Masi *et al*., 2021).

The results of the current work demonstrate that the *Alkalihalophilus pseudofirmus* strain YATW18 exhibits high efficiency, in the production of extracellular protease during submerged fermentation (Singh *et al*., 2023). The high enzyme levels attained in this strain within similar incubation period underline its ability for commercial protease production. These results are quite comparable to other studies conducted in this field (Jiang *et al*., 2021). However, certain differences can be observed regarding the time frames of enzyme production and the activity's maximum figures (Ramesh *et al*., 2022). As compared to the study of Kumar *et al*., 2012; which exhibited peak enzyme production after 48 hours, the present YATW18 strain had maximum production at 48 hours of incubation period. This efficiency might be an indication that this YATW18 strain possesses shorter production time, especially when used in industrial processes (Gupta *et al*., 2002). The reduced time with in the current study is very encouraging since it can further improve the cost efficiency of the process in industrial production. It also supposes that

YATW18 strain had a shorter generation time and an increased metabolic rate compared to other 22 alkaline protease producing bacterial strains during fermentation.

5.2. Optimization of cultivation conditions

When evaluating the efficiency of agricultural residues as substrates for alkaline protease production, water hyacinth, wheat bran, and corn cob turned out to be the most promising. The agro-industrial wastes are economical and easy to obtain as a low cost substrate for enzyme production (Kalaiarasi and Sunitha, 2009). Out of all the feed additives, corn cob, water hyacinth and wheat bran gave higher yield percentage than other feed additives like feather and coffee husk (Fig. 9). These findings are consistent with showed that wheat bran is better substrate for microbial enzyme production because of its nutritional content (Biswas *et al.*, 2019). The study also revealed that feathers available locally had a moderately positive effect on protease production, and showed that feather hydrolysates could be used successfully as a substrate for protease production in keratin-degrading microbes (Masi *et al.*, 2022). However, the lower yield obtained from feather and coffee husk indicates that all types of agricultural residues are not the same which is evident from previous findings where substrate type greatly influenced enzyme production (Wang *et al*., 2020). Surprisingly in this study, water hyacinth was used as a remarkable carbon source to produce highly active alkaline protease from *Alkalihalophilus pseudofirmus* strain YATW18.

The water hyacinth (*Eichhornia crassipes*) is an example of an aquatic plant that is regarded as the invasive one, and which creates certain ecological and economic troubles. Further, its application as a substrate for alkaline protease production holds potential environmental and economic benefits. When water hyacinth is utilized to produce enzymes for industrial use, the population of water hyacinth is regulated and therefore is useful to the environment (Ilo *et al*., 2021). The utilization of this invasive plant for industrial use turns it from a nuisance weed into a source of valued product, thus speaking positively to the enhancement of aquatic habitats (Guna *et al*., 2017). Water hyacinth is a natural product, renewable within a short period in many parts of the world including Ethiopia (Harun *et al*., 2021). Using it aligns with a circular economy, where waste is recycled; and drives sustainability in the biological processing companies. As it is an invasive weed, many regions actively seek to remove and manage its growth, sometimes

incurring costs for its eradication (Harun *et al*., 2021). Using an abundant substrate; lowers the cost of enzyme production, making it economically viable, especially for small and medium-scale enterprises. Therefore, the collection and processing of water hyacinths can help, in the provision of employment for the local people. This may allow income generation in rural and semi-urban centers, to improve standards of living as well as foster economic development of the area. In enzyme production, the substrates used such as the soybean meal or casein though common could be expensive since it might have to be imported. The use of water hyacinth for enzyme production eliminates worries about expensive imported raw materials and helps improve the economic viability of enzyme producers (Karouach *et al*., 2022). Consequently, integrating water hyacinth in enzyme production processes may thus be a strategic approach to improving sustainable biotechnology as well as circular bioeconomy, and turning an invasive species into economic resources (Cherwoo *et al*., 2024).

5.3. Physicochemical characterization of alkaline protease

5.3.1. Effect of pH in alkaliphilic protease activity

Alkaline protease from *Alkalihalophilus pseudofirmus* strain YATW18 was capable of operating under the most favorable pH of 8 to 11 (Fig. 10A), with observed optimum activity at pH 10. The detergent industry widely uses enzymes, especially proteases as additives to boost cleaning product performance. *Alkalihalophilus pseudofirmus* enzyme isolated from strain YATW18 operates at basic pH, which corresponds to the pH value in laundry detergents. Proteases that are used in laundry detergents have been designed to operate in a pH of range 8-11 (Razzaq *et al*., 2019). This positioning means the enzyme capable of attacking and removing proteinaceous materials, without being inactivated by the resultant high pH. The enzyme's high activity across a broad pH range makes it versatile for various detergent formulations, whether used in liquid, powder, or tablet form. The leather industry uses enzymes extensively in processing stages such as dehairing, bating, and soaking, where proteases help break down non-collagenous proteins (Wanyonyi and Mulaa, 2019). The protease from *Alkalihalophilus pseudofirmus* strain YATW18 revealed as very specific, efficient, and stable that can be regarded as advantageous for its usage in tannery applications. Dehairing is usually done by using chemicals or enzymes with an alkaline nature to removed hair plus epidermal materials. This protease thrives better at a pH range of 8-11 with optimum value at pH 10, which is in tandem with the conditions applied in

dehairing (Sharma *et al*., 2018). This protease is useful in the bating process where unwanted proteins are clear out from the hide resulting in softer skin. This characteristic of high activity in alkaline environment is useful in optimizing non-collagenous proteins removal, thereby improving the quality of leather.

5.3.2. Effect of temperature in alkaliphilic protease activity and stability

The temperature profile shows that the activity of the enzyme increases with the increase in temperature from 30°C to 55°C with optimum activity observed at 55°C and thus follows the kinetics of the enzyme (Fig. 11A). At 30°C, activity was considerably low most likely due to minimal intermolecular interactions (Taylor *et al*., 2020). The activity raised to 60 % when the temperature was 40°C and 80% at 50°C indicating that higher molecular activity and flexibility of the enzyme at relatively higher temperature (Govindasamy and Kumar, 2021). The activity was found the highest at 55°C; this is because proteolytic enzymes are known to be most efficient at this temperature. The observed increase in activity from 40°C to 55°C suggests that the enzyme is well-suited for warm and hot wash cycles, which typically range from 40°C to 60°C. This temperature stability ensures that the enzyme remains active during washing, enhancing the breakdown of protein-based stains. Many modern detergents aim to be effective at lower temperatures (30°C to 40°C) to reduce energy consumption (Cortez, *et al*., 2024). The enzyme's ability to retain 60% activity at 40°C indicates that it can still contribute significantly to stain removal in energy-efficient, cold-water detergents (Fig. 11B). The enzyme's optimal performance at 55°C aligns well with conditions used in industrial dishwashing and heavy-duty laundry applications (Stoner, 2004). Its high activity at elevated temperatures reduces the need for higher enzyme dosages, potentially lowering formulation costs. During dehairing, temperatures are typically maintained between 40°C and 55°C to facilitate the breakdown of keratin and other non-collagenous proteins. The enzyme's peak activity at 55°C suggests that it can efficiently accelerate these processes, reducing the time required for dehairing and bating, thereby enhancing the overall efficiency of leather processing. Leather processing often involves fluctuating temperatures, and the enzyme's broad range activity ensures stable performance across various stages of the process (Sivakumar, 2022). This temperature stability reduces the need for precise temperature control, simplifying operations and lowering energy costs.

5.3.3. Effect of salt concentration in alkaliphilic protease activity

The enzyme activity of alkaline protease from *Alkalihalophilus pseudofirmus* strain YATW18 demonstrates a notable tolerance to increasing salt concentrations, retaining significant activity even in the presence of salts (Fig. 12). Most household and industrial detergents are usually in one way or another compromised in areas where hard water and high mineral content are hindering the functioning of the enzyme (Galante and Formantici, 2003). An important advantage of this protease is that its activity remains rather high when the detergent contains 5-10% salts, which suggests good stability of the enzyme in hard water improving the efficiency of detergent preparations. Salt has been widely used in leather processing, especially in the soaking and curing of hides, for preservation and inhibition of microbial activity (Samidurai *et al*., 2022). It is also possible to work at higher concentrations of salt (up to 15%), thereby being active during these stages to facilitate the removal of non-collagenous proteins without being affected by the saline environment. The protease's salt tolerance also suggests its potential application, where high salt concentrations are used to preserve raw hides. The enzyme can operate effectively in this environment, helping to reduce unwanted proteins and impurities, which can enhance the quality of the final leather product.

5.3.4. Effect of incubation time in alkaliphilic protease activity

The enzyme activity indicates a slow increase in the rate of the reaction during the first few minutes of the incubation period and a striking elevation of the rate of the reaction between the 5-10 min intervals (Fig. 13). The enzyme needs a certain period for the optimum catalytic activity most probably because of substrate binding, enzyme activation, or completion of reaction equilibrium (Punekar, 2018). The optimum activity of the enzyme is achieved by the relative activity normalized to 100% in 20 minutes. All the active sites of the enzyme are nearly occupied, or fully occupied, and the reaction rate is not greatly affected by time (Romero *et al*., 2018). The observed alkaline protease activity in the early phase, followed by stabilization, aligns with typical enzyme kinetics, where the reaction progresses at an increasing rate until it achieves maximum velocity (Vmax). This pattern reflects the efficiency and stability of the enzyme over time, highlighting its ability to maintain consistent activity once optimal conditions are reached (Callender and Dyer, 2015). For industrial processes requiring precise timing or

sustained activity, the enzyme's ability to reach and maintain its peak performance within a short period of time is highly advantageous (Chapman *et al*., 2018).

5.3.5. Effect of metal ions in alkaliphilic protease activity

Testing the effect of the metal ions, boron, zinc, nickel, cobalt, magnesium, copper, and calcium in alkaline protease activity; showed comparable performance without significantly decreased activity of the enzyme throughout various concentrations, these results indicated most metal ions might not significantly hinder activity of the alkaline protease (Fig. 14). Lack of substantial inhibition at all the concentrations studied suggests, the system operates at a nearly baseline relative activity without much interference (Andreini *et al*., 2012). This characteristic of the enzyme's resistance to metal ion concentration variations is important and has marked implications in many industrial processes (Shahid *et al*., 2017). In detergent industry, metal ions are often present either as intentional additives, to enhance detergent functionality, or as byproducts of raw materials used in the manufacturing process (Baldiris *et al*., 2019). The enzyme's ability to maintain activity ensures its consistent performance in breaking down stains, even in chemically complex environments (Jakubowski *et al*., 2011). On the other hand, manganese depressed the enzyme activity in all the tested concentrations.

5.4. Partial purification of alkaliphilic protease

As expected, the larger proteins (250 kDa) migrated slower than the smaller one (125 kDa) when compared to those separated on SDS-PAGE. The lack of smearing or diffuse bands across the gel indicated that sample preparation, loading and separation through gel electrophoresis was done accurately (Sambrook, and Russell, 2001). The presence of two sharp, bright bands in both S2 and S3 shows that the purification process was highly effective. The two bands observed in S2 and S3 might represent different isoforms of the target protein or a protein complex that co-purifies with the target protein (Fig. 16). The faint band in S1, representing the crude extract, was expected when working with complex, unpurified samples. The lower intensity assured that the target protein was present in lower concentrations within the initial extract, but not absent. The faint band could also determine that the target protein was initially masked by the presence of numerous other proteins in the crude extract (Lek *et al*., 2016). During the purification, these

interfering proteins were effectively removed, allowing the target protein to appear more prominently (Alberti *et al*., 2018).

5.5. Evaluation of alkaliphilic protease for its potential applications

5.5.1. Stain removal efficiency for detergent industry

This study effectively presents how enzyme-enhanced commercial detergents remove stains better than commercial detergents that do not have enzyme boosters (Garcia *et al*., 2015). Very slight residues were noticed in all treated fabrics when enzyme-supplemented detergents were used (Figs. 17A-C) emphasizing that enzymes effectively eliminate protein such as egg yolk stains. The protein content of the egg depends on its size; the average egg contains about 6–7 grams of protein (Réhault-Godbert *et al*., 2019). Egg whites are especially renowned for their high levels of protein; however egg yolk contains more on a gram for gram basis, egg whites have 10.8g per 100g, and egg yolk contains 16.4g per 100g (Watkins, 2017). Thus, egg yolk stains were used as a potential substrate to the application of this experiment. The results provided evidence for the feasibility of adding enzymes into detergent mixes as a way of increasing their efficacy, especially in removing protein-based stains (Zhao and Russell, 2017). A strong qualitative difference could be observed between the enzyme-containing detergents (Figs. 17A-C) and the enzyme-free commercial detergents (Figs. 17D-F) where the egg yolk stain was not removed effectively. Interestingly, enzyme-only treatments (Figs. 17G and H) were also highly effective in removing egg yolk stains, despite the absence of additional detergent agents. In the case of enzyme-active laundry detergents, the observed efficiency indicates prospects for multi-enzymatic formulations of new-generation cleaning agents (Li and Ma, 2018). The research discovered that when proteolytic enzymes were added to the detergent formulation, the ability of the detergent to remove protein-based stains which were the most difficult to clean, is enhanced greatly. This could lead to improvement in the stain-removal mechanism, decreased use of harsh chemicals, and the minimum of use washing temperatures; thus, making detergents environment-friendly and energy-efficient (Nielson and Purnell, 2016).

5.5.2. Dehairing capacity for leather industry

The enzyme treatment demonstrated significant dehairing properties on both goat and sheep skin samples after incubation at room temperature for 16 and 20 hours, respectively (Figs. 18E and F). The ease of hair removal suggests that the enzyme effectively degraded the hair follicles, facilitating the detachment of hair without the need for harsh mechanical action (Sharma *et al*., 2018). This result highlights the enzyme's potential as an effective biological alternative to traditional dehairing chemicals in the leather industry. The use of enzymes could also allow for lower processing temperatures and milder conditions, reducing energy consumption and operational costs (Aravindan *et al*., 2017). In contrast, the untreated control samples (Figs. 18A and C) showed no visible dehairing effect, as the hair remained firmly attached to the skin. This outcome confirms that the enzyme activity is essential for the dehairing process. The enzyme-treated skins retained their integrity and appeared less damaged than those subjected to conventional dehairing agents like sodium sulfide (Na_2S) and calcium oxide (CaO) (Figs. 18G and H). While the positive controls did achieve dehairing, the skins showed visible signs of damage, likely due to the harsh chemical reactions involved. This finding suggests that enzyme-based dehairing is not only effective but also smooth on the skin, preserving its quality and making it more suitable for high-quality leather production (Kanth *et al*., 2019).

6. CONCLUSION AND RECOMMENDATIONS

6.1. Conclusion

The study proved that tannery waste has the potential to produce alkaliphilic protease through the isolation of efficient alkaline protease producing microbes, thus making waste disposal eco-friendly and also establishing an industry for extracellular enzyme production. This study was, therefore, able to develop the possibility of the production of alkaliphilic protease from *Alkalihalophilus pseudofirmus* strain YATW18. The protease was highly stable in a broad range of pH, which made it a suitable and reliable alternative for a sustainable industrial process. Crude enzyme enhanced its activity; by reducing the cost of purification process, and increased its viability for the commercial production at a large scale. The study also ensured that locally available substrates such as water hyacinth, corn cob, wheat bran, and feather are effective substrates for the enzyme production and hence have been indicated to be a cost-effective alternative of synthesizing industrially valuable enzymes.

6.2. Recommendations

- ✓ Further scale-up studies should be conducted to assess the economic viability of large-scale enzyme production for industrial applications.
- ✓ Based on the promising results, future research should focus on exploring additional biotechnological applications, such as in waste treatment, food formulation and bioremediation, to maximize their commercial potential.
- ✓ Exploration of genetic modification techniques to enhance the yield and stability of the protease should be pursued to improve its commercial feasibility.
- ✓ Additional purification methods beyond ammonium sulfate precipitation should be applied to achieve even higher enzyme activity levels for specialized applications.

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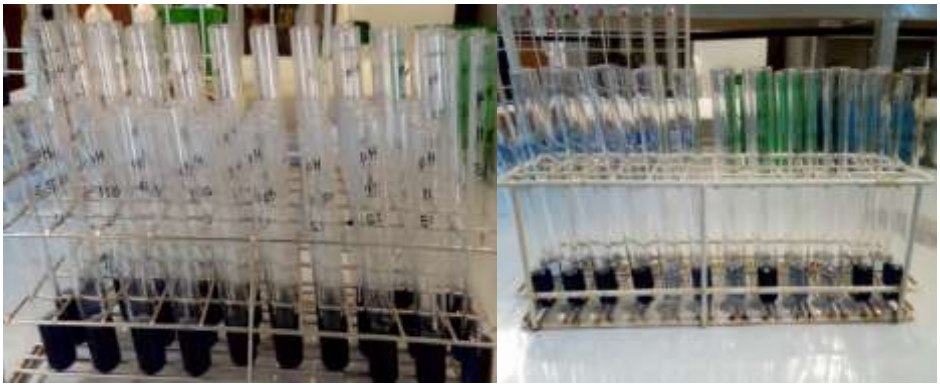
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APPENDICES

Appendix 1: Industrial visits in Leather Industry Development Institute, while green fleshing was carried out.



Appendix 2: Enzyme substrate complex at different pH level after dark room incubation



Appendix 3: Partial purification of crude enzyme using ammonium sulfate



Appendix 4: Preparation of egg yolk and staining on white cotton



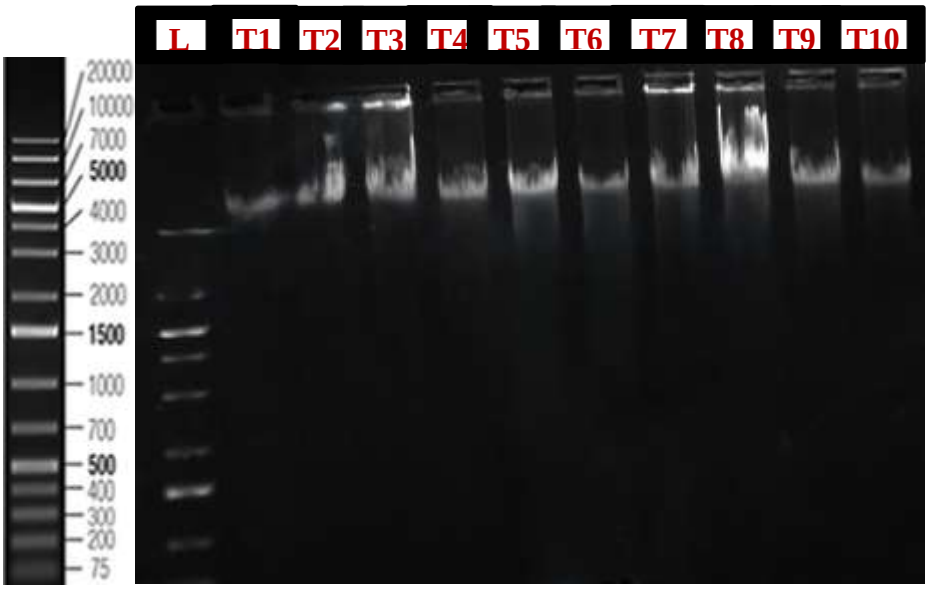
Appendix 5: Washing and drying of sheep and goat skins without green fleshing for a preliminary dehairing experiment



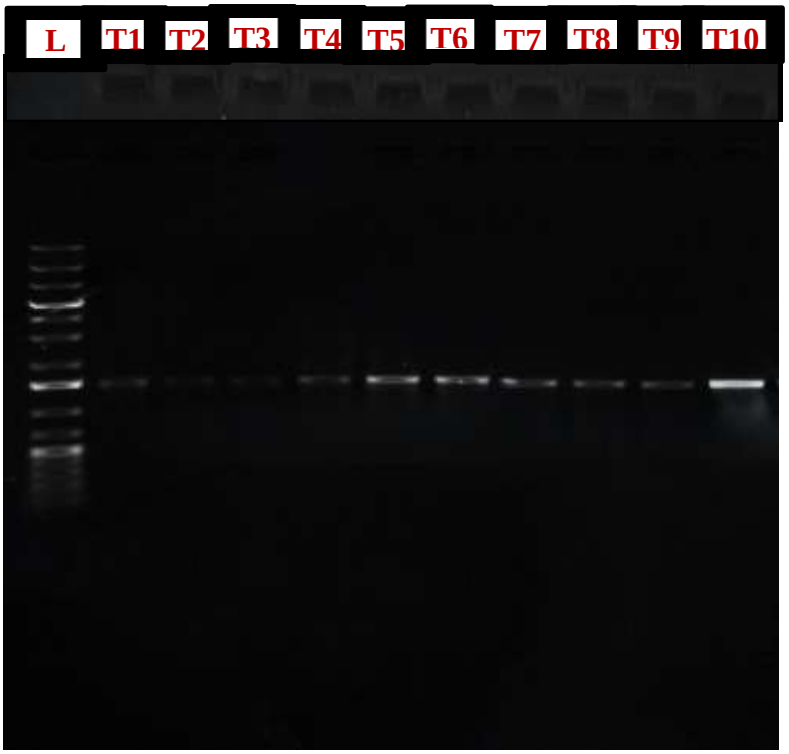
Appendix 6: Observation of physical appearance of bacterial colonies



Appendix 7: Gel electrophoresis image and band pattern of the genomic DNA



Appendix 8: Gel electrophoresis image and band pattern of the PCR products



L stands for DNA ladder (1Kb plus); T1=YATW19; T2=YATW12; T3=YATW10; T4=YATW6; T5=YATW8; T6=YATW15; T7=YATW4; T8=YATW7; T9=YATW1; T10=YATW18