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Eco-friendly Enzymatic Dehairing of Cowhide Using Thermostable Alkaline Protease 50a

Ng Ton Chie

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DECLARATION

I declare that this thesis entitled "Eco-friendly Enzymatic Dehairing of Cowhide Using Thermostable Alkaline Protease 50a" is the results of my own research except as cited in the references.

Signature : _____

Student's Name : NG TON CHIE

Date : 04/03/2024

Verified by:

Signature : _____

Supervisor's Name : Assoc. Prof. Dr. NOOR AZLINA BINTI IBRAHIM

Stamp : PROF. MADYA DR. NOOR AZLINA BINTI IBRAHIM
PENSYARAH KANAN
FAKULTI BIOKEJURUTERAAN DAN TEKNOLOGI
UNIVERSITI MALAYSIA KELANTAN

Date : 5/3/2024

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Pembuangan Bulu Kulit Lembu secara Enzimatik Mesra Alam Menggunakan Protease Alkali Termostabil 50a

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ABSTRAK

Kajian ini bertujuan untuk menangani cabaran alam sekitar yang timbul daripada kaedah penyingkiran bulu konvensional dalam industri kulit dengan meneroka keberkesanan pendekatan penggunaan enzim yang mesra alam. Penyataan masalah menekankan pencemaran alam sekitar yang signifikan yang disebabkan oleh proses penyingkiran bulu tradisional yang melibatkan bahan kimia berbahaya. Objektif ditetapkan untuk membandingkan penggunaan kaedah enzim dengan pendekatan konvensional, menilai impak kepekatan enzim yang berbeza, dan menilai parameter kualiti air air sisa yang dirawat. Methodologi kelibatkan proses pembersihan menyeluruh kulit lembu, ujian enzim, penyediaan lengkung piawai, dan pelbagai kaedah penyingkiran bulu, termasuk kaedah konvensional, bantuan enzim, dan rawatan enzim tunggal. Pencirian sampel kulit lembu dilakukan melalui mikroskopi elektron pengimbasan (SEM) dan ujian tegangan untuk menilai sifat mekanikal. Penilaian kualiti air memberi tumpuan kepada nilai pH dan permintaan oksigen kimia (COD). Kaedah pemeliharaan kulit lembu dibincangkan, menekankan kepentingan pemotongan yang tepat pada waktunya dan meneroka alternatif mesra alam. Kaedah penyingkiran bulu dibandingkan, menyoroti keberkesanan tinggi rawatan bantuan enzim, terutamanya dengan 20% larutan protease alkali thermostabil 50a. SEM mendedahkan impak positif rawatan enzim terhadap kualiti to butir kasar. Analisis kekuatan tegangan menunjukkan peningkatan dengan kepekatan enzim tertentu dalam kedua-dua rawatan bantuan enzim dan enzim tunggal. Penilaian kualiti air selepas penyingkiran bulu mendedahkan kelestarian kaedah penggunaan enzim, mengekalkan parameter dalam had yang ditetapkan oleh pihak berkuasa. Secara keseluruhannya, kajian ini menunjukkan pendekatan penggunaan enzim penyingkiran bulu yang mesra alam yang menjanjikan menggunakan protease alkali termostabil 50a, menawarkan alternatif lestari kepada kaedah konvensional dalam industri kulit. Keputusan menunjukkan peningkatan kualiti kulit dan impak alam sekitar yang berkurang, menekankan potensi penggunaan meluas amalan penyingkiran bulu enzim berasaskan penggunaan enzim.

Kata kunci: Mesra alam, penyingkiran bulu enzimatik, kulit lembu, protease alkali termostabil 50a, industri kulit

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ABSTRACT

This study aimed to address the environmental challenges posed by conventional dehairing methods in the leather industry by exploring the efficacy of an eco-friendly enzymatic approach. The problem statement highlighted the significant environmental pollution caused by traditional dehairing processes involving hazardous chemicals. The objectives were outlined to compare enzymatic methods with conventional approaches, assess the impact of different enzyme concentrations, and evaluate the water quality parameters of the treated wastewater. The methodology involved a comprehensive cleaning process of cowhides, enzyme assay, preparation of a standard curve, and various dehairing methods, including conventional, enzyme-assisted, and single enzyme treatments. Characterization of cowhide samples was done through scanning electron microscopy (SEM) and tensile tests to assess mechanical properties. Water quality assessment focused on pH and chemical oxygen demand (COD) values. Preservation methods for cowhides were discussed, emphasizing the importance of timely flaying and exploring eco-friendly alternatives. Dehairing methods were compared, highlighting the superior efficacy of enzyme-assisted treatments, particularly with a 20% thermostable alkaline protease 50a solution. SEM revealed the positive impact of enzymatic treatments on grain surface quality. Tensile strength analysis indicated improvements with specific enzyme concentrations in both enzyme-assisted and single enzyme treatments. Water quality assessment post-dehairing revealed the eco-friendliness of enzymatic methods, maintaining parameters within regulatory limits. In conclusion, this study presents a promising eco-friendly enzymatic dehairing approach using thermostable alkaline protease 50a, offering a sustainable alternative to conventional methods in the leather industry. The results suggest enhanced leather quality and reduced environmental impact, emphasizing the potential for widespread adoption of enzymatic dehairing practices.

Keywords: Eco-friendly, enzymatic dehairing, cowhide, thermostable alkaline protease 50a, leather industry

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LIST OF ABBREVIATIONS AND SYMBOLS

µl	Microliter
°C	Degree Celsius
g	Gram
BSA	Bovine Serum Albumin
cm	Centimeter
g/mol	Gram/molar
L	Liter
kV	Kilo volt
mL	Milliliter
Mol	Molar
mm	Millimeter
mg	Milligram
nm	Nanometer
RPM	Revolutions per Minute
SEM	Scanning Electron Microscopy
U	Unified atomic mass unit
mg/L	Milligram per liter
COD	Chemical Oxygen Demand
%	Percentage

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

The leather industry is one of the most significant sectors in many countries due to its economic and cultural significance. The global market for leather products, according to a report by Grand View Research (Bielak et al., 2023b), was estimated to be valued USD 242.85 billion in 2022 and is expected to grow at a CAGR of 6.6% from 2023 to 2030. There are several steps involved in the production of leather, including curing, soaking, dehairing, fleshing, deliming, pickling, and tanning. Among these steps, dehairing is an important step that removes hair and other unwanted substances from the skin. However, conventional leather processing methods, especially dehairing, involve the use of harsh chemicals that not only pollute the environment but also cause health hazards to workers (Madhavi et al., 2011). Eco-friendly enzymatic dehairing has become a viable alternative to conventional chemical dehairing in order to solve these issues.

Enzymatic dehairing is a more eco-friendly and effective technique that uses enzymes to break down and dissolve the proteins in the hair, leaving the skins clean and ready for tanning. Enzymes are biodegradable, and their use in the leather industry can reduce pollution caused by the use of harsh chemicals (Paul et al., 2016). The most often applied dehairing enzymes are proteases because they are able to catalyse the hydrolysis of peptide bonds in proteins, including keratin, the major structural protein in animal hair (Qiu et al., 2020). Proteases can be obtained from a variety of sources, including plants, animals, and microorganisms. Among the various sources, bacteria are the most prevalent, and *Bacillus subtilis* is the most common genus used in the production of commercial protease (Contesini et al., 2018). Proteases might be categorized as acidic, neutral, or alkaline based on their optimum pH (Mienda et al., 2014).

Thermostable alkaline protease 50a are particularly effective at dehairing animal hides, including cowhides, according to research. Thermostable alkaline protease 50a are

best used under the alkaline conditions required for dehairing due to them perform best in a pH range of 8.0 to 11.0 (Briki et al., 2016). These enzymes have been discovered to be more effective than other proteases in dehairing animal hides at lower temperatures and shorter treatment times (George et al., 2014). Using these proteases can also reduce the amount of chemicals used in a process, thus reducing the environmental impact and enhancing worker safety. In addition, enzymatic dehairing can improve the quality of the leather by preventing the damage caused by harsh chemicals to the hide.

Cowhide is one of the most commonly used raw materials in the leather industry due to its high tensile strength, flexibility, and durability. However, conventional chemical methods for dehairing cowhides are known to cause considerable environmental contamination, thus it is vital to develop eco-friendly methods (Adelere & Lateef, 2019).

In conclusion, the leather sector is important to the global economy, but the hazards to the environment and human health caused by conventional methods of leather processing, especially dehairing, must be taken into account. Due to its effectiveness and environmental sustainability, using enzymes as a dehairing agent for cowhides and other animal hides is becoming increasingly common. Thermostable alkaline protease 50a have been shown to be among the most efficient proteases for dehairing animal hides at lower temperatures and shorter treatment times. Thermostable alkaline protease 50a may be used in enzymatic dehairing to ensure worker safety, improve leather quality, and use less chemicals. In order to fulfil the increasing demand for eco-friendly and sustainable leather production, it is crucial to develop and optimize enzyme-based dehairing methods.

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1.2 Problem Statement

The conventional leather manufacturing process involves several steps, including pre-treatment, tanning, dyeing, and finishing, all of which can contribute to environmental pollution. Among these steps, the pre-treatment process, which often involves the use of strong chemicals like sodium sulphide, lime, and other hazardous chemicals, for dehairing, has been identified as a main cause of pollution in the leather industry (Dixit et al., 2015).

A study found that the conventional dehairing method, which uses sulphide and lime, is the main cause of pollution in the leather-making process. It results in 40% of BOD (biochemical oxygen demand), 50% of COD (chemical oxygen demand), 60–70% of total environmental contamination, and 100% of high alkaline effluent (Tian et al., 2019). For example, in the Dhaleshwari River in Bangladesh, where numerous tanneries discharge untreated effluent containing high levels of sulphide and lime, aquatic life has been severely affected. Reports have documented fish kills, decreased biodiversity, and long-term damage to the ecosystem due to the toxic effects of these chemicals on aquatic organisms (Islam et al., 2023). Moreover, the use of chemicals in the dehairing process reduces the quality of leather produced. These chemicals not only affect the environment, but they also represent a serious health risk for those who handle them.

Therefore, a method for dehairing animal skins that was effective, sustainable, and reduced environmental pollution was needed. Enzymatic dehairing has emerged as an alternative because it has several of advantages over chemical-based dehairing methods, including reduced environmental pollution and improved leather quality (Dowlatha et al., 2020). Thermostable alkaline protease 50a has attracted attention as a potential enzymatic dehairing agent recently because of its outstanding specificity, effectiveness, and eco-friendliness (Zaraî Jaouadi et al., 2014).

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1.3 Objectives

1. To compare the effectiveness of dehairing cowhide samples using enzymatic methods (single enzyme treatment and enzyme-assisted treatment) and conventional dehairing methods.
2. To evaluate the effectiveness of different concentrations of thermostable alkaline serine protease in dehairing cowhides through single enzyme treatment and enzyme-assisted treatment.
3. To measure water quality parameters of the dehairing-treated wastewater, especially chemical oxygen demand (COD) and pH values.

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1.4 Scope of Study

The study was carried out to compare the effectiveness of dehairing cowhide samples using enzymatic methods (single enzyme treatment and enzyme-assisted treatment) and conventional dehairing methods. The study should involve an appropriate number of samples treated with each method. The efficacy and effectiveness of the methods should be evaluated based on parameters such as dehairing efficiency, the dehaired area obtained, time taken, and the quality of the grain surface of the hide. A scanning electron microscope (SEM) was used to observe the grain surface and identify the opening of the hair follicles and keratinized cells after the dehairing treatment. Furthermore, the study was carried out to get more understanding of the enzyme activity based on single enzyme treatments and enzyme-assisted treatment as a replacement for conventional treatment. Last but not least, the study should involve evaluating whether the water quality parameters of the dehairing-treated wastewater are within the specified limits, especially the chemical oxygen demand (COD) and pH values.

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1.5 Significances of Study

This study focuses on the eco-friendly enzymatic dehairing of cowhide using thermostable alkaline protease 50a as an alternative to conventional dehairing methods that use strong chemicals such as lime and sodium sulphide. The conventional dehairing method causes significant pollution to the environment and seriously endangers the health of workers who handle the chemicals. In order to reduce the amount of chemicals needed, an enzyme-based dehairing method may be used, creating a green solution for the leather industry. This method is expected to reduce environmental pollution while improving the quality of leather produced. The environmental impact of this study may also be improved by filtering out the hair from the treatment, which may result to more precise measurements of the chemical oxygen demand (COD). The study also aimed to evaluate the effectiveness of enzymatic dehairing compared with conventional dehairing methods by identifying the grain surface and hair follicles of cowhides that had been dehaired using scanning electron microscope (SEM) analysis. The results from this study may be used for the development of eco-friendly and sustainable methods for producing leather.

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CHAPTER 2

LITERATURE REVIEW

2.1 Leather and Its Industry

The leather industry is a vital sector in many countries due to its economic and cultural significance. Leather is a durable and flexible material that has been used for centuries in a variety of applications, such as clothing, shoes, and accessories, and the industry keeps on growing worldwide. The leather industry involves a variety of processes, including curing, soaking, tanning, and finishing, each of which affects the leather's final quality. Dehairing is one of the most crucial and difficult procedures, as it removes undesirable hair and other substances from the skin in preparation for tanning. The worldwide market for leather products is forecast to grow at a CAGR of 6.6% between 2023 and 2030, and was valued at USD 242.85 billion in 2022 (Bielak et al., 2023a). In addition, the leather industry provides jobs and income to millions of people around the world, such in Europe and developing countries where the production of leather is a significant source of income for many families (Chen et al., 2022).

The leather industry dates back to prehistoric times, when animal hides were used for clothes and shelter. During the medieval period, when leather became a valuable commodity used for a variety of purposes, the industry grew dramatically. During the industrial revolution of the 18th century, mechanized tanning processes were developed, allowing for the mass production of leather products (Riello, 2008). The leather industry has continued to develop new technologies and processes aimed at enhancing efficiency and lowering the environmental impact of leather production.

Leather can be produced from many kinds of animal hides or skins, each of which has its own unique characteristics, properties, and applications. Cowhide, sheepskin, and goatskin are common animal hides or skins applied in the leather industry. Each kind of leather has unique characteristics such as thickness, strength, texture, flexibility, and durability (Nithyaprakash et al., 2020). For example, cowhide, which is the most common kind of leather and is known for its strength and durability, is suitable for use in the production of shoes and

accessories (Duraisamy et al., 2016). Sheepskin is often used in clothes and upholstery due to its warmth and softness (Chatterjee et al., 2015). Goatskin is well-known for its great tensile strength, flexibility, and durability, making it the perfect material for gloves and footwear (Ali et al., 2020). Furthermore, there are several kinds of leather, such as full-grain leather, top-grain leather, corrected-grain leather, and split-grain leather (Omer, 2020). Full-grain leather is the most durable and highest quality type of leather because it retains the natural texture and markings of the animal hides or skins and is made from the top layer of animal hides or skins (Tomljenovic et al., 2022). Top-grain leather is produced from the second layer of the hide or skin, after the outermost layer has been removed and sanded to produce a smooth surface (GUTA & Dumitrache, 2015). The leather of second-highest quality is top-grain leather. Corrected-grain leather is a lower-quality leather that is produced by sanding or polishing the surface of the hide or skin to remove flaws and then applying a surface finish (Ferreira, 2019). Split-grain leather is less durable than other kinds of leather because it is produced from the inner layers of the animal's hide or skin (Tomljenovic et al., 2020). In addition, leather products are breathable, water-resistant, and easy to clean, making them suitable for many kinds of applications ranging from clothes and shoes to furniture and vehicle interiors.

In many countries, the production of leather is a critical economic and cultural activity that has a significant impact on local communities. The leather industry employs millions of people around the world, from small-scale farmers and tanners to large-scale production manufacturers and retailers. In addition, the leather industry generates income for several countries, especially in developing regions such as South Asia and Southeast Asia (Cruz, 2007). Additionally, leather products have significant cultural and historical significance, and they are frequently associated with luxury, craftsmanship, and durability.

The leather industry faces both prospects and challenges in the future. On the one hand, the demand for leather products in various industries, such as fashion, automotive, and furniture, is increasing (Kral et al., 2014). It is predicted that this demand will keep growing as a result of increasing populations and disposable income in developing countries. The industry is practising eco-friendly practises, applying alternative energy sources, and reducing waste in an effort to become more sustainable. This transition towards sustainability could make the industry more appealing to environmentally conscious consumers. The industry is continually investigating new technologies and materials to enhance the quality of leather products and increase production efficiency. This could assist the industry in remaining competitive and fulfilling the changing demands of consumers.

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In addition, there is an increasing awareness among consumers regarding animal welfare, which could result in a decrease in demand for leather products. Animal welfare must be a top priority for the industry, which must address these concerns. The leather industry has a substantial environmental impact, particularly in terms of water consumption and pollution. The industry has to solve these issues and discover ways to reduce its environmental impact. Synthetic materials are gaining popularity due to their cheaper prices and more environmentally friendly production methods (Pickering et al., 2016). The leather industry needs to find strategies to compete with these alternatives and persuade consumers that leather is a better product.

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2.2 Leather Processing

The manufacturing process of leather involves a series of complex processes that convert raw animal hides and skins into a usable material. The process involves with several steps, including preservation or curing, soaking, liming, dehairing, fleshing, deliming, bating, degreasing, pickling, tanning, dyeing, and finishing (Rymowicz et al., 2004).

In the preservation or curing step, raw hides are treated with sodium chloride (NaCl), which is often used to prevent bacterial growth and preserve the hides (Amde & Bishoftu, 2015). Notably, the use of NaCl produces chloride-rich wastewater, which is considered a pollutant. The material is then rehydrated through soaking. Liming is the process of using lime and sodium sulphide to remove the hair and epidermis. Dehairing involves the removing of hair roots, whereas fleshing includes the removal of unnecessary flesh and fat. Deliming involves neutralizing the pH of the hides, whereas bating applies enzymes to soften and remove any remaining flesh particles or non-collagenous proteins. Excess fat is removed from the hides or skins during degreasing. Pickling is an important step in the manufacturing process of leather, when the hides are acidified with sulfuric acid to get them ready for the next tanning step. After pickling, the hides are subjected to tanning agents, which cause the collagen fibres to change into a stable, soluble network. The tanning process then uses chromium (III) salts as tanning agents to stabilize the collagen and provide the leather flexibility. Chrome tanning is often used to keep hides safe from bacteria and high temperature. After the tanning process finishes, the leather undergoes dyeing and finishing to impart colour and improve its appearance and properties such as flexibility, lightness, and gloss (Ammasi et al., 2020).

Leather processing involves a variety of challenges and issues. There are environmental concerns regarding the generation of chloride-rich wastewater during preservation, the use of chromium (III) salts in tanning, and the disposal of chemicals used in various steps of leather processing. There are efforts being made to develop eco-friendly alternatives and enhance waste treatment methods. The leather industry's demand for effective resource use and waste reduction while taking environmental considerations into account shows another challenge. Furthermore, the health and safety of the staff in the leather processing industry is an important issue, as they may be exposed to hazardous substances while carrying out physically demanding tasks (Thanikaivelan et al., 2005).

In conclusion, the production of leather involves a series of complex processes that convert raw animal hides into leather. Each step presents its own challenges and issues, ranging from worker safety to environmental concerns. To address these challenges and enhance the

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sustainability and effectiveness of leather processing, continuous research and development efforts are needed.

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2.3 Leather Dehairing

Leather dehairing is a crucial step in the removal of hair, non-collagenous protein, and other binding substances. Traditionally, lime and sulphide have been commonly used in the dehairing process, but this method resulted in the release of harmful gases like hydrogen sulphide and lime, as well as solid waste (Sivaram & Barik, 2019). To combat pollution, many industries have transitioned from chemical-based dehairing to enzyme-based dehairing method. These enzyme-based dehairing methods utilize enzymes to remove hair from animal hides/skins. While protease enzymes are commonly used for dehairing, they are not widely adopted in leather manufacturing due to concerns regarding stability conditions such as pH, temperature, enzyme production cost, and consistent enzyme performance (Sharma et al., 2019).

However, microbial protease enzymes have emerged as efficient and environmentally friendly options for dehairing. Thermostable alkaline protease 50a, in particular, play a significant role in converting animal hides and skins into leather. By employing this method, the use of chemical dehairing can be replaced with enzyme-based dehairing processes. The utilization of thermostable alkaline protease 50a in leather industries offers environmental benefits by reducing or eliminating the need for chemicals. Protease enzymes are stable under alkaline conditions, with the ability to function within a pH range of 8-12, making them highly suitable for dehairing hides or skins (Wanyonyi & Mulaa, 2020).

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2.4 Structure of Skins and Hide Before and After Treatment

The quality of the final leather product is directly influenced by the structure of animal skins and hides, including those from cows and goats. These skins consist of two main layers known as the epidermis and hypodermis, which together form the dermis. The dermis contains various components such as collagen, elastic fibres, extra-fibrillary matrix, and different types of cells like fibroblasts, immune cells, sensory cells, and glandular cells (Zaiter et al., 2022) (Montelli et al., 2015).

Collagen and elastic fibres within the dermis, particularly grain leather, contribute significantly to the skin's strength and elasticity, making it desirable for high-quality leather production (Varani et al., 2006) (Tian et al., 2022). On the other hand, crust leather found in the reticular dermis has thin collagen bundles with limited elastin content, resulting in lower quality leather (Montelli et al., 2015).

In the goat skin industry, post-mortem modifications are crucial to produce high-quality leather while minimizing waste and pollution (Naporos, 2012). Pre-tanning processes can generate waste, so preserving the properties of the raw material and preventing structural decay is essential. Different curing methods are used, including short-term preservation through cooling or refrigeration and long-term preservation through salting and drying. These methods aim to prevent damage and ensure the production of high-quality leather (Wanyonyi & Mulaa, 2020).

However, dermal tissue degradation can occur during storage and processing due to microorganism-induced biodeterioration. It is important to assess the deterioration of dermal tissue under different storage conditions, from the slaughterhouse to the tanning process, and during refrigeration or salting. Attention should be given to prevent and mitigate damage caused by microorganisms to maintain the quality of the raw material (Maina et al., 2019).

In conclusion, a thorough understanding of the structure of animal skins and hide, particularly in goats, is vital for leather processing. The presence of collagen and elastic fibres within the dermis significantly influences the quality of leather. Various curing methods are employed to preserve the raw material's properties and prevent deterioration. However, it is necessary to address the issue of biodeterioration caused by microorganisms during storage and processing to ensure the production of high-quality leather.

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2.5 Hides and Skins Preservation

Preserving raw hides is vital in leather processing to prevent deterioration and maintain their quality. Immediate preservation is essential to protect the skin's protein matrix and prevent microbial attacks. Various techniques, including conventional methods, enzymatic approaches, silica gel, boric acid method, Saltless Microbial Biotechnology (SMB) method, phytochemical preservation of skin or hide, and bacteriocin solutions are employed for hide preservation (Kanagaraj et al., 2015).

One commonly used conventional method is wet salting, which involves applying salt (NaCl) to the hides. Wet salting is an important curing method that inhibits bacterial activity and prevents skin decay. The salt acts as a curing agent by dehydrating the skin and exerting bacteriostatic effects. It penetrates the skin, reducing water activity and inhibiting microbial growth. Wet salting is typically performed with a calcium chloride concentration of 40% to 50% (Valeika et al., 2016). The salt is later removed through soaking operations before further processing to ensure effective hide preservation.

Drying is another physical method used for hide preservation. Similar to the curing method, drying involves removing moisture from the hides to inhibit microbial growth and prevent skin decay. However, if not carefully controlled, drying can cause changes in the hides' physical and mechanical properties, affecting the quality of the resulting leather (Wu et al., 2017).

While conventional methods like wet salting have limitations, such as environmental concerns regarding salt-laden wastewater disposal and negative effects on leather quality, alternative preservation methods are being explored. These methods should be more environmentally friendly, addressing challenges and ensuring high-quality leather production (Xiao & Roberts, 2010).

Enzymatic methods of hide preservation have gained significant attention in recent years due to their eco-friendly nature and potential benefits (Samidurai et al., 2022). Enzymes, particularly thermostable alkaline serine proteases, have been studied as alternatives to conventional methods for dehairing hides (Madhavi et al., 2011). Using enzymes offers advantages such as reduced environmental impact by minimizing the need for harsh chemicals and generating less waste. Enzymatic dehairing has also been found to enhance the quality of the resulting leather, improving attributes such as softness, uniformity, and colour (Jayakumar et al., 2016). Additionally, enzymatic preservation techniques contribute to sustainability by reducing water consumption and lowering salt usage. Overall, the enzymatic approach to hide

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preservation shows promise in addressing challenges while promoting environmental consciousness.

Hide preservation is crucial in leather processing as it ensures the availability of high-quality raw materials, reduces waste, and contributes to the sustainability of the overall process (Kanagaraj et al., 2020). Enzymatic dehairing provides additional benefits such as improved process efficiency, reduced water consumption, and decreased environmental impact.

A proposed method for preserving skin or hide in a cleaner and more environmentally friendly way is by using silica gel instead of traditional salt curing. Silica gel, created by combining sodium meta-silicate and hydrochloric acid, is used as a powder for preservation purposes. The recommended approach involves using 15% silica gel powder, optionally with 0.1% PCMC (p-Chloro-m-cresol). An alternative method has also been developed, which uses a mixture of 5% silica gel and 5% salt. The effectiveness of this new preservation technique was assessed using various parameters, including moisture content, total extractable nitrogen, bacterial count, and fiber structures. The results indicate that the new method is equally effective as salt curing and does not present any issues during soaking and leather manufacturing processes. It significantly reduces the pollution levels of total dissolved solids (TDS) and chloride (Cl) by up to three times and 70-75% respectively. The leather properties obtained are comparable to those of salt-cured leather, with no observed structural modification or degradation in the preserved skin. This method offers environmental benefits over traditional salt curing in terms of biological oxygen demand (BOD), chemical oxygen demand (COD), TDS, and Cl. It is also economically viable, with a cost reduction of 10-15%, making it a preferable alternative to salt curing for preserving skins and hides (Wu et al., 2017).

Boric acid has been studied as a viable method for preserving skin and hide, serving as a salt-free and lower-salt alternative. One approach involves using a 5% concentration of boric acid for salt-free preservation, while another method combines 2% boric acid with 5% common salt for a less-salt preservation option. Both methods have proven effective, resulting in a reduction of over 80% in chloride and total dissolved solids in the waste products. The utilization of boric acid has demonstrated its efficacy and cleanliness in preserving the skin, as evidenced by preservation parameters and the quality of the resulting leather. Importantly, boric acid is safe to handle and does not pose significant health or safety risks. Furthermore, this innovative technique can be implemented practically without the need for additional equipment

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or infrastructure, making it a feasible and suitable alternative to traditional salt preservation methods (Kanagaraj et al., 2015).

A novel technique called Saltless Microbial Biotechnology (SMB) has been developed to effectively preserve skin/hide. Two variations of the SMB method were tested: one involved using SMB at a concentration of 1% without any added salt, while the other used SMB at a concentration of 0.5% along with 5% salt. The results demonstrated that both methods successfully preserved the skin, resulting in lower nitrogen content and bacterial count compared to the traditional salt-curing method. This indicates that the SMB method offers superior preservation of the skin. The bacterial population observed during the salt-less curing process was not significant enough to cause deterioration or impact the skin's collagen network, as evidenced by the preserved skin's mechanical properties. Additionally, the preserved skin showed a substantial reduction in Total Dissolved Solids (TDS) and chloride (Cl) levels, which were approximately 15–20 times lower than those found in conventionally salt-cured skin. Overall, the SMB method presents an improved skin preservation system that brings potential benefits such as reduced bacterial growth and pollution levels (Kanagaraj et al., 2015).

An effective method for preserving raw skins or hides involves the utilization of phytochemicals like deoiled and oiled neem cake or powdered tamarind leaves. When varying quantities of these cakes were applied, positive outcomes in skin preservation were observed. This technique provides the added advantage of reducing pollution caused by total dissolved solids (TDS) and chlorine (Cl) by approximately 60%. Moreover, a promising new approach for preserving skins involves using powdered tamarind leaves as a preservative agent. The leaves undergo a process where they are extracted with methanol and the extract is concentrated under vacuum to obtain a crude extract. This method not only achieves satisfactory preservation but also reduces TDS and Cl pollution by over 50%. Overall, these preservation techniques using phytochemicals offer environmentally friendly alternatives for the preservation of skins or hides (Kanagaraj et al., 2015).

The focus of the study is on utilizing bacteriocin, an antimicrobial peptide produced by Lactic Acid Bacteria, as a bio preservative for skin and hide preservation. The objective is to inhibit the growth of microorganisms while ensuring the safety of living organisms. When cowhide was treated with a 15% solution of bacteriocin and stored at room temperature for seven days, it exhibited complete inhibition of microbial growth. The preserved hide was subsequently transformed into crust leather and subjected to SEM and physical testing. The

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findings revealed that there were no notable alterations in the hide's fibre structure as a result of bacteriocin preservation. During the leather processing, the concentration of total dissolved solids (TDS) decreased by 94.4%, and chloride (Cl) levels were reduced by 95.6%. Based on these outcomes, the researchers concluded that bacteriocin could be an exceptionally effective agent for preserving skins and hides. Additionally, other researchers recommended the use of different chemicals for preservation, as long as the curing parameters are optimized. It was also suggested that achieving a temperature of 4°C for 7 days would enhance preservation effectiveness. However, rapid attainment of this temperature (within 2 hours) would necessitate the use of a blast chiller and appropriate hooking arrangements to prevent bacterial damage (Kanagaraj et al., 2014).

In summary, the protection of raw hides is a crucial step in the leather processing industry in order to maintain their quality and prevent decay. While traditional methods like wet salting and drying have been widely employed, there is ongoing exploration of alternative techniques to address environmental concerns and enhance leather production. Enzymatic approaches, such as enzymatic dehairing, offer environmentally friendly solutions that minimize their impact on the environment while improving the efficiency of the process and the quality of the leather. Moreover, alternative methods like silica gel, boric acid, saltless microbial biotechnology (SMB), and phytochemical preservation show promise in providing cleaner and more sustainable approaches to preserving hides. These methods offer several advantages, including reduced pollution, decreased use of salt, and minimized waste generation. Additionally, the use of bacteriocin and other chemicals as bio preservatives demonstrates their potential in effectively inhibiting microbial growth and preserving skins and hides. As the leather industry strives for more environmentally conscious practices, these alternative preservation methods offer viable options to ensure the production of high-quality leather while promoting sustainability.

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2.6 Types of Methods for Dehairing Animal Hides and Skins

2.6.1 Chemical Dehairing

Chemical dehairing is an essential process in leather production that aims to remove hair, epidermis, non-collagenous proteins, and other substances from the surface of the skin. Traditionally, a mixture of lime and sulphide has been used in the conventional dehairing method (Xu et al., 2010). This method relies on an alkaline hydrolysis reaction caused by lime and sulphide, which breaks down the hair's fibrous keratin and allows its removal (Sinkiewicz et al., 2017). However, the use of these conventional methods raises concerns about their environmental impact, as they can pollute water sources and soil. To address these concerns, there is a growing need for more environmentally friendly dehairing methods that minimize the use of chemicals. One alternative approach is enzymatic dehairing, which reduces reliance on chemical substances. Enzymatic dehairing involves the use of protease enzymes derived from *Bacillus subtilis* to break down the hair effectively. These enzymes can efficiently dehair the hides while minimizing the environmental impact. The development of eco-friendly alternatives to chemical-based dehairing is crucial to mitigate the environmental and health risks associated with conventional methods.

Regulations and guidelines have been put in place to ensure the safe and environmentally friendly use of chemicals in leather processing, specifically in the dehairing process. These measures aim to protect the well-being of workers and minimize any potential risks associated with the use of chemicals. The European Chemicals Agency (ECHA) is responsible for enforcing the Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH) regulation, which imposes restrictions on the use of certain chemicals across various industries, including leather processing (Coria, 2018). This promotes sustainable and responsible practices in the field. In Malaysia, the Department of Environment (DOE) under the Ministry of Environment and Water oversees environmental regulations, including the proper management and disposal of chemicals and waste generated from leather processing. The Environmental Quality Act 1974 and its associated regulations, such as the Environmental Quality (Scheduled Wastes) Regulations 2005, have been implemented to prevent pollution and encourage sustainable practices within the industry (Agamuthu & Victor, 2011). Moreover, the Department of Occupational Safety and Health (DOSH) in Malaysia has established guidelines to ensure that workers in the leather industry are protected from occupational exposure to chemicals. The adherence to these regulations and guidelines is enforced through permits, approvals, and compliance with effluent discharge standards set by the DOE (Amirah et al.,

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2013). These regulatory measures are crucial in promoting responsible and sustainable practices in leather production (Aziz et al., 2020).

Chemical dehairing in the leather industry has been shown to have significant negative impacts on the environment and human health, as evidenced by various studies. For example, (Mohiuddin, 2019) conducted a case study in Bangladesh that revealed high levels of pollutants such as sulphides, chromium, and organic matter in dehairing effluents, leading to harmful effects on aquatic ecosystems and human health. Other report similarly found that untreated effluents from dehairing in a heavily industrialized tannery resulted in increased levels of chemical oxygen demand, total dissolved solids, and heavy metals in nearby water bodies, posing risks to both ecosystems and human health (Jahan et al., 2014). The use of sodium sulphide, a commonly employed dehairing chemical, was found to have adverse effects on aquatic life and the health of workers. The discharge of lime and sulphide-containing wastewater from traditional dehairing processes causes pollution that disrupts ecological balance, impacting aquatic life and soil fertility (Obed, 2013). To address these issues, the use of protease enzymes derived from *Bacillus subtilis* offers a more environmentally friendly alternative. However, careful preparation of the enzyme is crucial to avoid excessive collagenous activity. Additional enzymes like keratinase can be used alongside protease to enhance dehairing while minimizing collagenase activity. (Verma et al., 2019) conducted a case study in India that highlighted water contamination in nearby water bodies due to heavy metals and organic pollutants from a conventional dehairing unit, emphasizing the risks posed to aquatic life and human health through water consumption. These studies collectively emphasize the pressing need for eco-friendly dehairing methods to mitigate the environmental and health hazards associated with chemical-based processes.

Chemical dehairing has been a common practice in leather processing, using lime and sulphide. However, the environmental impact of these chemicals has raised concerns. To address these issues, a more environmentally friendly alternative is enzymatic dehairing, which involves using protease enzymes derived from *Bacillus subtilis*. It is important to carefully prepare the enzymes to avoid excessive collagenase activity, which could degrade the quality of the leather. Adding keratinase to the protease can enhance the dehairing process while minimizing collagen degradation. Responsible chemical usage in leather processing is enforced through regulatory measures such as REACH in Europe and guidelines from the DOE and DOSH in Malaysia. Several studies have highlighted the environmental and health risks associated with chemical dehairing, underscoring the importance of adopting eco-friendly

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alternatives. By implementing enzymatic dehairing methods, the detrimental effects on water sources, soil fertility, aquatic ecosystems, and human health can be reduced.

2.6.2 Enzymatic Dehairing

Enzymatic dehairing has become a sustainable and environmentally friendly solution in the leather industry. It involves using enzymes, such as thermostable alkaline protease 50a and keratinases, to effectively remove hair from animal skins and hides while improving the quality of the leather (Khambhaty, 2020).

The thermostable alkaline protease 50a works by eliminating non-fibrillary proteins during enzymatic dehairing, resulting in a soft and high-quality leather structure (Poza et al., 2007). Collagen, which is the main protein in leather, remains intact throughout the process, highlighting the importance of enzymes with non-collagenolytic activity like keratinases. These enzymes dissolve non-fibrillary proteins and contribute to the toughness of the skin (Solanki et al., 2021).

To carry out enzymatic dehairing, a mixture of enzymes, including proteases, chondroitinase, laminarase, and chitinase, is applied to the skin or hides. This mixture reacts with keratin and azocasein, resulting in the gradual removal of hair over a period of about 16-18 hours. Enzymatic dehairing improves the appearance and smoothness of the epidermal layer, as well as promotes fibre opening in the dermis and corium regions, ultimately enhancing the quality of the leather (Khandelwal et al., 2015).

Microbial enzymes have gained popularity in the leather industry due to their stability, catalytic activity, and ease of production. They offer a cost-effective and environmentally friendly alternative to traditional chemical dehairing methods. To meet the demand for microbial enzymes, advanced technologies such as protein engineering and large-scale culturing of recombinant proteases have been utilized (Gupta & Shukla, 2016).

Enzymatic dehairing provides several advantages, including the elimination of organic waste, which can be safely discharged without causing harm to health or the environment. In contrast, traditional dehairing methods that rely on chemicals like sulphide, lime, and amines generate hazardous waste. Enzymatic dehairing not only reduces waste discharge but also improves leather quality and minimizes the negative impact on human health and the environment (Choudhary et al., 2004).

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It is crucial to optimize factors such as pH, temperature, and substrate concentration for efficient enzymatic dehairing. Studies have indicated that alkaline serine protease activity is highest at pH 9.0 and a temperature of 50°C, with a substrate concentration of 2% (Sarkar & Suthindhiran, 2020). These findings underscore the significance of optimizing conditions to achieve effective enzymatic dehairing.

In conclusion, enzymatic dehairing using thermostable alkaline protease 50a and keratinases offers an environmentally friendly and efficient approach to the leather industry. By selectively removing non-fibrillary proteins and preserving collagen integrity, this method produces high-quality leather. Furthermore, enzymatic dehairing reduces waste discharge and replaces harmful chemicals, promoting sustainable practices and minimizing environmental impact. Optimizing process parameters further enhances the efficiency of enzymatic dehairing, ensuring its successful implementation in leather processing.

2.6.3 Mechanical Dehairing

In the leather industry, mechanical dehairing is a commonly used method for removing hair from animal hides during the leather-making process. Using this method, the hair is mechanically separated from the skin using specific instruments and machinery. To achieve the required quality and characteristics of the final leather product, the mechanical dehairing process is needed. It is a popular option among tanneries all over the world since it has some of benefits including high efficiency, rapidly, and consistency (Khambhaty, 2020).

The method of mechanical dehairing often involves rotating paddles or drums that mechanically agitate the skins to remove the hair (Adcock, 2022). These drums are often coated with abrasive materials that help remove hair effectively, such as stainless-steel blades, spikes, or serrated discs. Powerful motors drive the drums, and the dehairing process' speed and duration may be changed to suit various animal skin and hair types.

To remove hair from animal hides mechanically, a series of steps is involved. Initially, the skins are soaked in a water-based solution to soften them and enhance their suitability for dehairing. Then, the skins are loaded into a dehairing machine where drums or paddles rotate at a controlled speed. The abrasive lining in the machine causes the hair to loosen and detach from the skin through mechanical action. This process continues until the desired level of

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dehairing is attained. Afterward, the dehaired skins are meticulously rinsed to eliminate any residual hair and debris (Mikkilineni, 2021).

Although mechanical dehairing is a commonly applied method, it comes with challenges and considerations that necessitate attention. One challenge involves the possibility of harming the skin due to excessive abrasion, which may result in diminished leather quality and durability. Another factor to consider is the requirement for frequent upkeep and replacement of the abrasive lining to maintain optimal dehairing efficiency. It is crucial to carefully monitor and adjust process variables, such as rotation speed and duration, to prevent over-dehairing or under-dehairing, both of which can have negative impacts on the leather's quality and appearance (Harizi et al., 2007).

A comparison between mechanical dehairing and other methods, like chemical and enzymatic dehairing, offers valuable insights. Mechanical dehairing is efficient, but chemical methods can be hazardous to the environment due to the use of toxic substances. In contrast, enzymatic dehairing is a more environmentally friendly option as it employs enzymes to break down hair without harming the hide. A thorough comparative analysis would aid in understanding the advantages and drawbacks of each dehairing method, facilitating the selection of the most appropriate approach for particular purposes.

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2.7 Alkaline Proteases from *Bacillus spp*: Efficient and Sustainable Dehairing in the Leather Industry

Enzymatic dehairing is a commonly used technique in the leather industry to eliminate hair from animal hides or skins. It relies on the use of proteases, which are enzymes that break down proteins. Proteases can be classified as acid, neutral, or alkaline based on their acid-base properties. Among these types, alkaline proteases have gained popularity due to their stability and effectiveness at alkaline pH levels (Thangam & Rajkumar, 2002). They are often derived from *Bacillus* species found in soil and water, particularly those capable of surviving harsh conditions.

Alkaline proteases, particularly thermostable alkaline protease 50a, contain a specific set of amino acid residues called a catalytic triad (aspartate, histidine, and serine) (Mienda et al., 2014). This triad is crucial for their enzymatic activity. Thermostable alkaline protease 50a demonstrate elastolytic and keratinolytic properties, which make them suitable for dehairing animal skins. They effectively break down elastin and keratin, the major components of hair and skin.

The leather industry extensively employs thermostable alkaline protease 50a during the initial stages of skin and hide preparation. Enzymatic treatment with thermostable alkaline protease 50a is used in processes like soaking, dehairing, and bating (Khambhaty, 2020). This treatment helps remove undesired pigments and hair, resulting in cleaner hides and increased surface area of the hide.

Several factors influence the activity of proteases in enzymatic dehairing, including pH, temperature, substrate concentration, and enzyme concentration. Thermostable alkaline protease 50a typically exhibit optimal activity at pH levels ranging from 9 to 11, while the optimal temperature varies depending on the specific protease. It is essential to optimize substrate and enzyme concentrations to achieve efficient dehairing while keeping costs low (Qamar et al., 2020).

To demonstrate the effectiveness of alkaline proteases in dehairing, researchers have conducted case studies. For example, alkaline proteases derived from *Bacillus cereus* were found to be highly efficient in removing hair from goat hides, resulting in cleaner and smoother skins. The optimal pH for dehairing activity was around pH 10, aligning with the alkaline nature of these enzymes. These proteases also showed good stability and activity within a temperature range of 40 to 60°C (Sundararajan et al., 2011).

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In conclusion, alkaline proteases obtained from *Bacillus* strains play a significant role in enzymatic dehairing in the leather industry. They possess elastolytic and keratinolytic properties, making them effective agents for dehairing animal skins. Their stability and activity at alkaline pH levels make them advantageous for this application. It is important to optimize factors such as pH, temperature, substrate concentration, and enzyme concentration to achieve efficient dehairing while minimizing costs. The utilization of alkaline proteases from *Bacillus* strains as environmentally friendly and efficient dehairing agents in the leather industry highlights their potential for sustainable leather processing.

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2.8 The Structure of Hides or Skins

The composition of hides or skins is a complex combination that determines the strength, flexibility, and overall quality of the resulting leather. Hides or skins consist of two primary layers: the outer layer called the epidermis and the inner layer known as the dermis. The epidermis serves as a protective barrier, while the dermis provides mechanical strength to the hide. Within the dermis, collagen fibres form a hierarchical structure, contributing to the hide's tensile strength and elasticity (Sujitha et al., 2018).

Goat skins possess distinctive structural characteristics that make them suitable for leather production. Compared to other animal hides, goat skins have finer and more tightly packed collagen fibres, resulting in leather that is softer, more flexible, and lighter (Maina et al., 2019). These structural differences contribute to the improved properties of goat leather, such as increased softness, suppleness, and high tensile strength.

Several factors influence the structure and quality of hides. Environmental factors like the breed of the animal, its age, nutrition, and geographical location can impact the characteristics of the hide (Naporos, 2012). Additionally, the techniques used during hide preparation, including liming, fleshing, and hair removal, can affect the structure of the hide and, consequently, the final quality of the leather.

The efficiency of dehairing, a crucial step in leather production, is closely tied to hide structure. The arrangement and density of collagen fibres in the dermis affect how easily dehairing agents can access the hair roots, ultimately influencing the effectiveness of hair removal (Sujitha et al., 2018). Enzymatic dehairing, which utilizes alkaline serine protease, has gained attention as an environmentally friendly alternative to traditional chemical methods. This enzymatic process selectively breaks down hair proteins while preserving the structure and properties of the hide, resulting in improved softness and tensile strength of the leather.

Comparative studies examining hide structure across different animal species offer valuable insights into the unique characteristics of each type of hide. These studies have identified significant differences in collagen fibre arrangement, diameter, and density, highlighting the distinct properties of each animal hide.

In conclusion, understanding the structure of hides and skins is crucial for determining the quality and properties of leather. Cowhides, with their specific structural characteristics, offer advantages in leather production, such as enhanced softness and flexibility. Factors related

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to the animal itself and the processing techniques employed can influence hide structure and quality. Enzymatic dehairing using alkaline serine protease is an environmentally friendly method that effectively removes hair while preserving the hide's structure. Comparative studies across different animal species contribute to a comprehensive understanding of hide structure and its impact on leather production.

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2.9 Environmental Impact of Leather Processing and Water Quality Assessment Methods

2.9.1 Environmental Impact

The leather industry has long been associated with significant environmental pollution due to conventional processing methods. These methods involve the use of chemicals that pose risks to human health and contribute to environmental degradation (China et al., 2020). (China et al., 2020) conducted a study on the environmental pollution caused by conventional leather processing, highlighting its detrimental effects on water bodies and soil quality. Their findings revealed that the discharge of untreated effluents containing high concentrations of chromium, sulphides, and organic compounds led to water pollution and depleted oxygen levels in aquatic ecosystems.

Enzymatic dehairing has emerged as a more environmentally friendly alternative to chemical dehairing methods. (Catalán et al., 2019) conducted a comparative study to evaluate the environmental impact of chemical and enzymatic dehairing processes. The results demonstrated that enzymatic dehairing significantly reduced chemical pollutants in wastewater compared to conventional methods. Furthermore, enzymatic dehairing proved to be more efficient in terms of hair removal and required less energy consumption, thereby reducing the overall environmental footprint of leather production.

One of the key advantages of using enzyme-based dehairing methods is their ability to minimize the environmental impact and waste generated during leather production. Enzymes exhibit high specificity, targeting only hair and epidermal proteins, resulting in minimal damage to the collagen structure of the hide. (George et al., 2014) conducted a study using alkaline serine protease for goat skin dehairing and reported efficient dehairing with minimal hide damage, leading to higher quality leather and reduced waste generation.

To address the environmental pollution associated with the leather industry, various measures have been implemented. These include the adoption of wastewater treatment systems, cleaner production techniques, and the implementation of strict regulations and guidelines. For instance, the European Union has implemented the REACH regulation, which restricts the use of hazardous substances in the leather industry and promotes the substitution of harmful chemicals with safer alternatives. In Malaysia, the Department of Environment (DOE) has established guidelines for eco-friendly leather processing, emphasizing the reduction of water

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consumption, proper wastewater treatment, and the use of environmentally friendly chemicals (Mohammad Ilias et al., 2021).

The importance of sustainable and eco-friendly leather production extends beyond environmental concerns and also encompasses human health. (Nur-E-Alam et al., 2020) conducted a study on the health risks associated with conventional leather processing, highlighting the occupational hazards faced by workers exposed to harmful chemicals. By adopting enzymatic dehairing methods and implementing eco-friendly practices, the leather industry can protect the health and well-being of its workers while minimizing the impact on the environment.

In conclusion, conventional leather processing methods have had a significant negative impact on the environment, causing water pollution and soil degradation. However, the adoption of enzyme-based dehairing methods, such as alkaline serine protease for goat skin dehairing, offers a more sustainable and eco-friendly approach. These methods reduce the environmental impact, waste generation, and chemical pollutant levels in wastewater. The implementation of regulations, policies, and guidelines, such as REACH in the European Union and Malaysia's eco-friendly leather processing guidelines, is crucial in promoting eco-friendly practices and safeguarding both the environment and human health in the leather industry.

2.9.2 Water Quality Assessment and Analysis Methods

Water quality assessment is a crucial aspect of environmental research and management as it provides valuable information about the physical and chemical characteristics of water sources and the levels of contaminants present. This assessment is important to ensure the safety and usability of water for various purposes. It involves evaluating parameters like temperature, turbidity, pH, conductivity, and total dissolved solids. pH is commonly used to determine whether water is acidic, neutral, or alkaline (Gorde & Jadhav, 2013).

Biochemical oxygen demand (BOD) is a significant parameter that measures the amount of oxygen microorganisms require to consume organic materials in water sources. BOD is an indicator of the level of biodegradable organic compounds in the water. The traditional BOD5 method involves a five-day incubation period, but alternative methods like biosensors, UV-visible spectrophotometry, and fluorescence measurements offer more accurate and efficient results (Narteh, 2015).

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Chemical oxygen demand (COD) and total oxygen demand (TOD) are also utilized for water quality assessment. COD measures the oxygen demand for the oxidation of organic matter using chemical oxidizing agents, while TOD accounts for the oxygen portion corresponding to the organic matter oxidized by specific agents such as potassium dichromate (Lee et al., 1999) (Young et al., 2003). Both COD and BOD provide valuable information, with COD offering faster results and BOD focusing on biodegradation.

Water quality assessment is crucial in the research on this research. It ensures that the water used in the process meets the necessary standards and does not pose any risks to the environment or the final product's quality. Monitoring pH, BOD, COD, and other relevant parameters helps evaluate the impact of the enzymatic dehairing process on water quality (George et al., 2014).

To ensure reliable and consistent results, it is important to follow standardized guidelines and regulations set by environmental agencies such as the Environmental Protection Agency (EPA) during water quality assessment. These guidelines provide standardized methods for sampling, monitoring, and analysing water samples (Ferrer et al., 2010).

In summary, water quality assessment methods provide crucial information about the characteristics and contaminant levels in water sources. Parameters such as pH, BOD, COD, and oxygen demand help evaluate water quality and pollution levels. Advances in analytical methods, including biosensors and alternative BOD determination techniques, offer improved accuracy and real-time monitoring capabilities. Adhering to standardized guidelines and regulations ensures the safety and usability of water sources. Integrating water quality assessment methods into research on enzymatic dehairing ensures environmental sustainability and adherence to quality standards.

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CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and Reagent

The intracellular protease derived from *Escherichia coli* BL21 (DE3) pLysS harboring thermostable alkaline protease 50a (Ibrahim & Yusoff, 2013), Tris-HCl consists of Tris base (molecular biology grade), calcium chloride ($CaCl_2$), and concentrated HCl, phosphate buffer, trichloroacetic acid (TCA), sodium hydroxide, distilled water, sodium chloride, bovine serum albumin (BSA), azocasein, bovine albumin Fraction V, Bradford's Reagent, sodium sulphide, calcium oxide, and Hach COD vials (high range).

3.1.2 Apparatus

Petri dish, conical flask, micropipette, cuvette, microcentrifuge tube, beaker, gloves, mask, pipette tips, pH meter, knife, scissor, falcon tube, measuring cylinder, forceps, spatula, filter funnel, filter paper, test tube rack, parafilm, aluminium foil, rubber stopper, and magnetic stirrer bar.

3.1.3 Instrument

Vortex, shaking waterbath, digital thermometer (TP-300), UV-Vis spectrophotometer, lab oven, blender, orbital shaker, centrifuge, Scanning Electron Microscope (SEM), spectrophotometer, Hach DRB200 Reactor, Hach DR 2800 portable spectrophotometer, Testometric machine M500-50CT, analytical balance, angle grinder, band saw machine, and stirring hotplate.

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3.2 Methods

3.2.1 Curing and Drying Process

The cowhides obtained from the local slaughterhouse underwent a thorough washing using tap water to eliminate any traces of blood and prevent any unpleasant odours. The fatty layer, which was unnecessary, was removed, and salt was applied to the entire skin surface to prevent damage. The hides were then dried under sunlight at a sufficiently high temperature to prevent moisture and odours. This drying process typically took around 1 month for complete dryness. The hides could be cut into 5 cm × 5 cm pieces either after washing or while still wet. It was important to consider the moisture level during cutting, as excessively dry hides became hard and difficult to work with. After drying, the hides became thinner due to the removal of water. The dehairing process involved various treatments such as control, conventional treatment, enzyme-assisted treatment, and single enzyme treatment. The hides were soaked in the treatment solution to facilitate dehairing, and salt was applied to prevent microbial growth before the hides were fully dried (Maina et al., 2019).

3.2.2 Enzyme Assay

3.2.2.1 Proteolytic Activity Assay

The determination of protease activity involved a modified procedure. A solution containing 0.9 ml of 0.5% azocasein in 0.1 M Tris-HCl, 0.002 M $CaCl_2$ at pH 8.6 was prepared, and the reaction mixture was pre-incubated in a water bath shaker at 70 °C for 5 minutes. The reaction commenced by adding 100µl of either crude or purified protease, followed by incubation at 70 °C for 30 minutes. To stop the reaction, 1.0 mL of 10% trichloroacetic acid (TCA) was added in an equal volume. The mixture was then allowed to sit at room temperature for 30 minutes and subsequently centrifuged at 13,000 rpm for 10 minutes. From the resulting mixture, 1 ml of supernatant was collected and combined with 1 ml of 1 M NaOH. The absorbance at 450 nm was measured using a spectrophotometer. The same procedure was applied for the control group, with the addition of TCA to the crude enzyme before mixing with the azocasein solution. A blank consisting of distilled water was utilized. The enzyme activity was conducted in triplicate, and the results were presented as mean values with standard deviations (Heh et al., 2013). One unit (U) of azocaseinase activity was defined as the amount of enzyme activity that produced a change in absorbance (0.001 per min) at 450 nm at 70°C under the standard assay conditions. Alternatively, activities were also measured using the formula: $U = \Delta A_{450\ 23} \times 1000\ min^{-1}$ (Ceron et al., 2023).

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3.3 Preparation of Standard Curve

Initially, prepared microcentrifuge tubes (duplicate) for the standard curve. BSA was prepared by dissolving 0.008 g in 1 mL distilled water and was kept on ice because BSA must be freshly prepared before use. 0.1 M Tris-HCl buffer pH 8.6 was prepared by the addition of Tris-base, $CaCl_2$ and HCl. For the preparation of standard curve, the BSA were placed along with Tris-HCl buffer and Bradford's reagent in a different beaker.

Each 2 mL microcentrifuge tube had a different concentration of two-fold diluted BSA: 0.00 mg/mL, 0.08 mg/mL, 0.20 mg/mL, 0.40 mg/mL, 0.80 mg/mL, and 1.60 mg/mL. The specified amounts of PBS buffer were added to each tube, followed by the BSA protein standard at a concentration of 8 mg/mL or 0.008g. According to the sequence of different BSA concentrations, the volumes of BSA were 0.0 uL, 5.0 uL, 12.5 uL, 25.0 uL, 50.0 uL, and 100.0 uL, while the volumes of PBS buffer were 500.0 uL, 495.0 uL, 487.5 uL, 475.0 uL, 450.0 uL, and 400.0 uL. A table named 'Table 3.1: BSA Concentration Calculation Table' was utilized to calculate the BSA concentration for each tube using the given BSA stock concentration, BSA volume, and PBS volume with the equation $M_1V_1 = M_2V_2$.

The solutions in each tube were then vortexed for 3-5 seconds and allowed to rest for about 1 minute. Subsequently, 1 mL of Bradford reagent was pipetted into each cuvette. The absorbance at 595 nm was read, with Tube 1 used as the blank. A graph of the standards was prepared, with the dependent variable (mg/mL) on the X-axis and the independent variable (Abs 595nm) on the Y-axis. Rather than connecting the dots of the standard curve, a linear regression was performed on the data.

The regression 'r' value was shown in the graph's box, indicating the linearity of the data. Values closer to 1.0 suggested a stronger correlation, with 0.9 to 1.0 considered reasonable. The linear regression was then used to calculate the concentration in unknown samples.

For the preparation of a protein sample (thermostable alkaline protease 50a) was assayed. 100 µL of the sample was added to 1.0 mL of Bradford reagent, and the mixture was vortexed for 3-5 seconds. After 5 minutes, the absorbance value at 595nm was read. The protein content was calculated based on the earlier established standard curve.

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Table 3.1: BSA Concentration Calculation Table

Concentration of BSA (mg/mL)	BSA (uL)	PBS (uL)	Abs 595nm
0.00	0.0	500.0	
0.08	5.0	495.0	
0.20	12.5	487.5	
0.40	25.0	475.0	
0.80	50.0	450.0	
1.60	100.0	400.00	

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3.4 Method for Dehairing

3.4.1 Conventional Treatment for Dehairing

The cowhide underwent treatment by immersing them in a mixture of 100 ml of distilled water combined with 5% calcium oxide (w/w) and 2% sodium sulphide (w/w). The flasks containing the hide was then positioned on an orbital shaker, rotating at a speed of 150 rpm, and kept at a temperature of 37 °C for a duration of 24 hours. Following this, a blunt knife was used to remove the hair from the hide. Throughout the experiment, the progression of the cowhide was observed at various time intervals. The entire process was carried out in duplicate to ensure accuracy and consistency (Al Mamun et al., 2015).

3.4.2 Enzyme Assisted Treatment

In the enzyme-assisted method, the hide pieces were immersed in a solution containing 5% calcium oxide (based on the volume of distilled water) for a duration of 6 hours at a temperature of 37 °C. This process aimed to loosen the collagen fibres in the cowhides through the action of collagenase. Subsequently, the treated hides underwent multiple rinses with tap water. Following this, the hides pieces were placed in 250 ml conical flasks containing 100 ml of distilled water. Each flask contained varying concentrations (10%, 20%, 30%, and 40% v/v) of thermostable alkaline protease 50a. The protease activity was 119.889 U/mL. For example, a 10% concentration of thermostable alkaline protease 50a in 100 mL of distilled water consisted of 10 mL of the protease solution. The protease activity of the 10% concentration of thermostable alkaline protease 50a was calculated by multiplying 119.889 U/mL by 10 mL, and the activity of other concentrations of protease was calculated using the same method. The flasks were then positioned in an orbital shaker rotating at 150 rpm for 24 hours at 37 °C. This entire experiment was conducted in duplicate to ensure consistency and accuracy. To remove the hair, a blunt knife was used. The cowhides were monitored over time to track their progress (Al Mamun et al., 2015).

3.4.3 Single Enzyme Treatment

The dip method was employed for enzymatic dehairing using a thermostable alkaline serine protease. In this process, pieces of hides were immersed in 100 ml of water within 250 ml conical flasks. Each flask contained varying concentrations (10%, 20%, 30%, and 40% v/v) of thermostable alkaline protease 50a. The protease activity was 119.889 U/mL. For example,

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a 10% concentration of thermostable alkaline protease 50a in 100 mL of distilled water consisted of 10 mL of the protease solution. The protease activity of the 10% concentration of thermostable alkaline protease 50a was calculated by multiplying 119.889 U/mL by 10 mL, and the activity of other concentrations of protease was calculated using the same method. The purpose of these varying concentrations was to compare the effects of low and high enzyme concentrations on the structure of cowhides. To ensure thorough mixing, all flasks were placed in an orbital shaker rotating at 150 rpm for 24 hours at a temperature of 37 °C. The hair was removed using a blunt knife. This experiment was conducted duplicate to ensure accuracy and reliability (Khandelwal et al., 2015).

3.4.4 Control of Dehairing Method

For the control method, the hide was immersed in distilled water and placed in an orbital shaker at a temperature of 37 °C. The incubation period lasted for 24 hours while the shaker was set to a rotation speed of 150 rpm (Khandelwal et al., 2015).

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3.5 Characterisation of Treated Cowhides

3.5.1 Scanning Electron Microscope (SEM)

The samples underwent a drying process and were cut into small fragments. Then, double sided carbon tapes were used to securely mount the hide samples. To ensure effective conductivity, silver paint was carefully applied to the exposed surface area surrounding the sample. Additionally, a thin coating was applied through sputtering. The examination of the samples was carried out using an environmental scanning electron microscope (FEI Quanta series) at an acceleration voltage of 12 kV. The magnification levels utilized ranged from ×100 to ×300, allowing us to analyse the grain surface and follicles present in the cowhides (Khandelwal et al., 2015).

3.5.2 Tensile of the Treated Cowhides

The mechanical behaviours were determined by tensile tests, and samples were prepared by using the treated cowhides, 5 cm in length and 5 cm in width for testing in the testometric machine. The samples were dried in an oven. Prior to the tensile test, the width, thickness, distance between the jaws, and initial length of the cowhides were measured. For the tensile test, the testometric machine was designed with jaws that held the hide and prevented it from slipping off the sample. Tear resistance was measured based on tear initiation and tear propagation. A 1.000 N tensile force was applied at a speed of 5 mm/min. The samples for the tensile test had the following physical properties: Young's Modulus (N/mm^2), Stress @ Peak (N/mm^2), Stress @ Yield (N/mm^2), Stress @ Break (N/mm^2), Strain @ Break (%), and Force @ Peak (N) (Alhassan et al., 2020).

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3.6 Water Quality Assessment and Analysis Methods

After 24 hours of dehairing treatment, the pH meter was used to measure the pH value of each wastewater sample after dehairing treatment. Before measuring the pH value, the pH meter had to be calibrated using pH 4 buffer solution, distilled water, and pH 10 buffer solution. After measuring each sample, the pH meter was cleaned with distilled water. Then, the pH values of all samples were recorded (Springer, 2014).

The chemical parameter analysis for wastewater samples from the dehairing treatment involved the evaluation of parameters such as chemical oxygen demand (COD). Within 24 hours of collection, a wastewater sample was subjected to analysis using a spectrophotometer at wavelengths suitable for high-range COD (20-1500 mg/L), employing the DRB 200 for chemical oxygen demand (Roman et al., 2018). The COD determination process began with the collection of wastewater samples in clean Falcon tubes from the dehairing treatment. For reactor digestion, 100 mL of the sample was blended for 30 seconds, or until homogenized. In cases with substantial solids, homogenization time was adjusted accordingly. For the 200-15000 mg/L range, the homogenized sample was transferred to a 200 mL beaker and gently stirred. The DRB200 Reactor was then set to a preheat of 150 °C. Following preheating, a vial was prepared by adding 2.00 mL of the sample (or 0.20 mL for 250-15000 mg/L range) for testing. A blank vial was also prepared with 2.0 mL of deionized water (or 0.20 mL for 250-15000 mg/L range). Both vials were tightly closed, rinsed, and mixed before being placed in the preheated reactor for a 2-hour heating process. After cooling, the COD reading was measured using a spectrophotometer with suitable wavelengths for high-range COD (20-1500 mg/L). The subsequent colorimetric procedure involved initiating program 435 COD HR. A blank sample cell was cleaned, inserted into the cell holder, and zeroed. The prepared sample cell was then cleaned and inserted, and the reading was taken, providing results in mg/L COD. In the case of using High Range Plus COD digestion reagent vials, the result was multiplied by 10. For samples near 1500 or 15000 mg/L COD, a repeat analysis with a diluted sample was recommended for optimal accuracy. Additionally, blanks for colorimetric determination could be reused with the same lot of reagent vials, with the recommendation to prepare a new blank vial when absorbance changes were observed over time (Noguerol-Arias et al., 2012).

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CHAPTER 4

RESULTS AND DISCUSSION

4.1 Preparation of Thermostable Alkaline Protease 50a of Cowhides

The protease enzyme used in the experiment was derived from *E. coli* BL21 (DE3) pLysS strain harboring thermostable alkaline protease 50a gene (Ibrahim & Yusoff, 2013). This protease has been identified as a thermostable alkaline enzyme. Numerous reports in the literature had highlighted the application and efficacy of thermostable alkaline protease 50a in the dehairing process (Khambhaty, 2020). Consequently, this investigation utilized a locally isolated and produced thermostable alkaline protease 50a for its application in the dehairing process.

Before undergoing the dehairing treatment, it was necessary to assay the protease enzyme and protein content to obtain the specific activity of the enzyme through a spectrophotometric assay (Akhtaruzzaman et al., 2012). To assess protease activity, the enzyme's specific activity was determined through a reaction with the azoprotein substrate. This substrate, azocasein, is a chemically modified protein in which a sulphanilamide group is covalently linked to a peptide bond of casein (Coêlho et al., 2016).

The protease assay was heated to 70 °C in the water bath shaker because the enzyme exhibited its highest activity at this optimum temperature. This temperature also ensured the stability of the enzyme (Abusham, 2009). If the temperature was below this optimum, the enzyme activity would be low. Conversely, if the temperature was too high, the enzyme stability would decrease due to denaturation (Daniel et al., 1996). Trichloroacetic acid (TCA) was used to terminate the reaction because of its ability to precipitate proteins (Kim et al., 2012). It is commonly employed in determining protease assay, especially in diluting biological samples containing low quantities of protein. Sodium hydroxide (NaOH) was added to the protease assay as it facilitated the solubilization of membrane proteins and reduced the color yield, minimizing variations in protein-to-protein measurements (Senadheera, 2020).

The quantification of protein concentration was necessary, and the Bradford method was selected for this purpose. Several methods, including the Bradford and Lowry methods, were available for determining protein content (Seevaratnam et al., 2009). In the pursuit of purifying thermostable alkaline protease 50a, the Bradford method was applied to assess the relative concentration at 595 nm (Thakur et al., 2018). This technique facilitated the measurement of the ratio of protein-bound dye in the solution, with the dye binding to proteins. Under acidic conditions, the dye exhibited a red color in the protonated state (Friedman, 2004). The interaction with a protein molecule through electrostatic and hydrophobic forces led to the stabilization of the dye in the form of an anionic blue complex (Akagi et al., 2010). A calibration curve was established using a standard solution of BSA, plotting absorbance against mass concentration (Brady & Macnaughtan, 2015).

In determining the unknown protein, the analytical reaction of the target protein mirrored that of BSA in the standard curve, facilitating the assessment of relative protein concentrations in the sample. The standard curve, as shown in Figure 4.1, represents a linear least square fit line obtained by plotting absorbance against BSA concentration. The calculated standard concentration for the known sample was noted as 0.9746, serving as a crucial metric for future protein preparations. However, it was imperative to acknowledge that the absorbance per unit concentration, while collectively informative, might not have always accurately reflected assay sensitivity or response due to inherent protein-to-protein variability (Liu et al., 2016). This variability had the potential to lead to either over- or underestimation of analyte protein concentrations (Schiettecatte et al., 2012). The Bradford method, employed in this analysis, relied on electrostatic interactions in dye-protein binding (Moore et al., 2010). Notably, the hydrophobic factor of the dye introduces an additional consideration in the assay outcomes. It is important to recognize that the results of the Bradford method may have been influenced by the protein composition, thereby introducing potential bias into the analysis (Macart & Gerbaut, 1982). The successful establishment of a reliable standard curve was paramount for quantifying the purified protease enzyme, a critical step in the enzyme's characterization.

The absorbance values in protein analysis are indeed impacted by the hydrophobicity of proteins. Proteins characterized by a predominantly hydrophobic nature demonstrate higher absorbance values when compared to those with fewer hydrophobic characteristics and a diminished number of basic residues, resulting in discernible differences in absorbance even when the mass concentrations are the same (Chandrapala et al., 2011). It was crucial to

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acknowledge the discrepancy between the standard and the composition of proteins, as the presence of bovine serum albumin (BSA) in Bradford assays may lead to inaccurate concentration determinations (Olson & Markwell, 2007). The calibration curve of the Bradford assay relies on the mass concentration of BSA to determine unknown concentrations of proteins. Notably, the color intensity observed at absorbance 595 nm in the Bradford assay is composition-independent, indicating that the mass sensitivity or response of the assay remains consistent for every protein. The blue color produced in the Bradford assay was attributed to the stabilization of the anionic form of the dye through electrostatic and hydrophobic interactions (Brady & Macnaughtan, 2015). The Coomassie Brilliant Blue dye initially presents a reddish or purplish liquid color, which transforms to blue upon binding with proteins. The absorption of light by the dye-protein complex was measured spectrophotometrically at a common wavelength, often 595 nm, though the specific wavelength might have varied depending on the protocol. The intensity of the blue color was proportional to the amount of bound protein, and quantification was often achieved by referencing a calibration curve with known concentrations of a standard protein. This method is widely utilized in protein quantification assays, with the Bradford assay being a seminal reference (Chang & Zhang, 2017).

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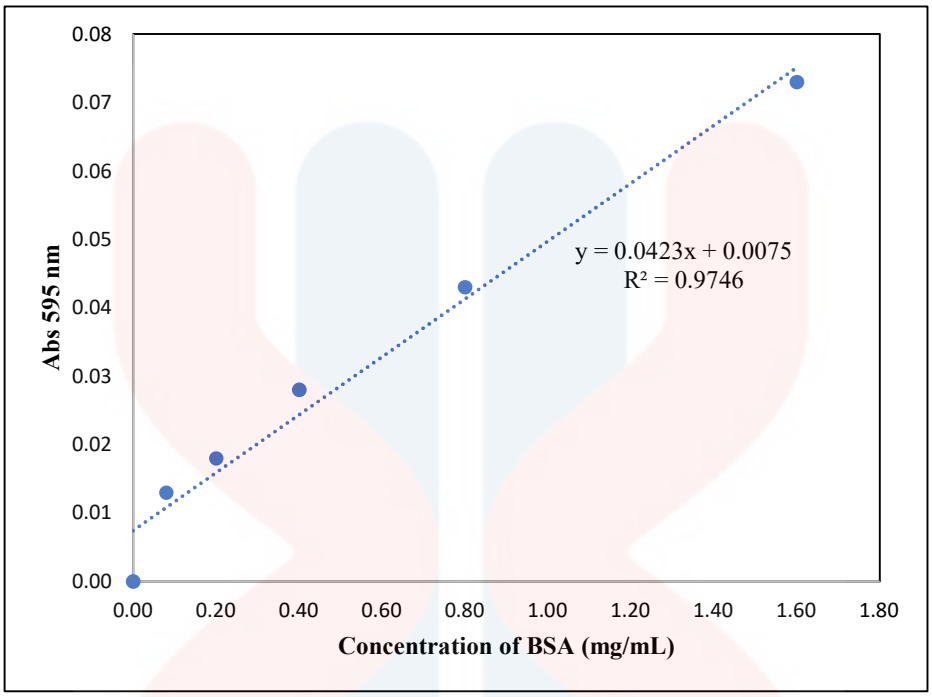

Figure 4.1: The Bradford standard curve using BSA as standard sample

In referencing Table 4.1, the efficacy of the purification process for thermostable alkaline protease 50a was assessed by determining the protein content. The quantification of total purified alkaline serine protease activity resulted in 23977.8 U, which was calculated in enzyme units. International Units (IU) were employed to measure enzyme activity under specific conditions, indicating the conversion of 1 µmol of a given substrate to a specific product per minute for quantifying enzyme amounts (Baltierra-Trejo et al., 2015). The total protein concentration in the samples was determined to be 0.662 mg, confirming the success of the purification method in eliminating contaminating proteins. The specific activity of thermostable alkaline protease 50a was determined to be 36220.24 U/mg, underscoring the effective removal of inactive proteins during the purification steps. Specific activity, representing the ratio of enzyme units to the total protein in the enzyme solution, is a critical parameter for evaluating enzyme purity. It is important to clarify that the enzyme's mass is not directly linked to its function and activity. The purity assessment relies on specific activity, calculated using the formula that involves enzyme activity per unit mass of protein, expressed in units/mL.

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Table 4.1: Protease activity, protein concentration, total protease activity, total protein and its specific activity

Method	Volume (mL)	Protease activity (U/mL)	Protein concentration (mg/mL)	Total protease activity (U)	Total protein concentration (mg)	Specific activity (U/mg)
Heat Treatment	100	119.889	6.615	23977.8	0.662	36220.24

Notes:

Total protease activity (U) = Total volume (mL) × Protease activity (U/mL)

Total protein concentration (mg) = Starting volume (mL) × Protein concentration (mg/mL)

Specific activity (U/mg) = Total protease (U) / Total protein concentration (mg)

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4.2 Preservation of cowhides

The essential raw material for the tanning industry was hides or skins. In environments with a temperature of 33°C, the urgency to preserve cowhides became paramount due to elevated temperatures accelerating bacterial activity. The preservation process began with the immediate flaying of hides or skins within 1 to 2 hours after the animal's demise to prevent bacterial growth (Verma et al., 2022). Natural or soil-originated bacteria can lead to protein putrefaction, rendering hides or skins unsuitable for high-quality leather production. Metabolic changes occur in hides or skins following the animal's death due to the absence of oxygen and essential nutrients (Nawaz et al., 2021). Consequently, toxic substances accumulate, resulting in the inactivation of certain coenzymes.

The decomposition process initiates with autolysis, breaking down proteins into peptides and ultimately into amino acids (Rawlings et al., 2011). Autolysis products undergo further breakdown through a secondary process facilitated by putrefactive bacteria (Skopp, 2004). The prevention of protein decomposition in hides or skins after the flaying process could be achieved through two viable methods: prompt initiation of the tanning process or the application of suitable preservation methods (Wanyonyi & Mulaa, 2020). In the absence of a tanning facility, the focus shifts to proper preservation methods. A common approach is immediate wet-salting after flaying, typically using 40%-50% sodium chloride. Sodium chloride acts by dehydrating cowhides and leveraging its bacteriostatic properties, limiting bacterial growth. The presence of salt induces dehydration, safeguarding the skin from conditions conducive to bacterial proliferation and leading to plasmolysis (Kanagaraj & Babu, 2002). After the salt curing process, cowhides reach the desired dry condition and undergo washing to eliminate residual salt.

Continuing the investigation into cowhide preservation methods, it was imperative to explore alternative techniques beyond wet-salting. Research suggests that other preservation agents, such as natural tannins derived from plant sources, may offer environmentally friendly and effective alternatives to traditional salt curing (Unango et al., 2019). Future studies could delve into the comparative effectiveness of various preservation methods, considering factors like cost, environmental impact, and the overall quality of the preserved hides.

In conclusion, the preservation of cowhides is a critical step in the leather production process. Understanding the biochemical processes involved and exploring alternative

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preservation methods contribute to the sustainable and efficient utilization of raw materials in the tanning industry.

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4.3 Dehairing Method

In this study, the dehairing methods were implemented, inspired by a previous study conducted by (Al Mamun et al., 2015). Their work initially classified dehairing into three categories: conventional treatment, enzyme-assisted treatment, and enzyme-mediated treatment. However, we expanded on this classification, introducing four different methods: conventional treatment (2% Na_2S + 5% CaO), single enzyme treatment (thermostable alkaline protease 50a solution), enzyme-assisted treatment (5% CaO pre-treatment, thermostable alkaline protease 50a solution), and a control treatment (distilled water). These methodologies were selected to enhance the comprehensiveness of our study and included the use of conventional treatment, single enzyme treatment, enzyme-assisted treatment, and a control treatment.

As referenced in a study, the dehairing methods employed in the experimental setup included the paste method for conventional treatment and enzyme-assisted treatment (Durga et al., 2017). According to the study results from Durga et al. (2017), the efficacy of the paste method in hair removal was limited or ineffective, particularly when compared to the dip method, which proved suitable for all dehairing treatments (Durga et al., 2017). The inefficiency of the paste method in hair removal may have been attributed to the low quantity of water used in this approach. Notably, the paste method relied solely on chemical treatment, lacking the application of physical treatment achieved through placement in an orbital shaker for enhanced hair removal. Consequently, for this study, the dip treatment method was exclusively selected.

The decision to focus solely on the dip treatment method was influenced by the shortcomings observed in the paste method, as indicated by the referenced study. Given the limitations in the effectiveness of the paste method, our study sought a more comprehensive approach that would be universally applicable to all dehairing treatments. By exclusively employing the dip method, we aimed to ensure a consistent and reliable dehairing process across all treatment categories.

In this study, it is essential to highlight the significance of the dip method in achieving efficient and uniform dehairing. The dip method, characterized by its immersion process, ensures that the entire surface of the samples undergoes treatment, leading to more effective hair removal. This method is not only practical but also aligns with the findings of

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(Saravanabhavan et al., 2005), supporting the notion that the dip method surpasses the paste method in terms of overall effectiveness.

4.3.1 The Effectiveness of Different Dehairing Treatments on Cow Hides

The study investigated the effectiveness of various dehairing treatments on cow hides, employing the dip method for a comprehensive and universally applicable approach. The treatments included conventional treatment (2% Na_2S + 5% CaO), enzyme-assisted treatment with different concentrations of thermostable alkaline protease 50a solution, single enzyme treatment, and control (distilled water). The results, as presented in the Table 4.2, highlighted the dehaired area obtained and the corresponding area yield for each treatment.

The conventional treatment (2% Na_2S + 5% CaO) exhibited a 100% area yield, indicating complete hair removal. In contrast, the control treatment (distilled water) demonstrated a significantly lower area yield of 14.72%, confirming the ineffectiveness of water alone for dehairing. The enzyme-assisted treatments showed varying degrees of effectiveness, with the best result achieved using a 20% thermostable alkaline protease 50a solution, producing an impressive 81.92% area yield. This suggested that enzymatic action played a crucial role in enhancing the dehairing process.

It is noteworthy that the concentration of the thermostable alkaline protease 50a solution significantly influenced the outcome of the enzyme-assisted treatments. While a 10% concentration resulted in a moderate 20.96% area yield, concentrations of 30% and 40% yielded 42.72% and 30.72%, respectively. Surprisingly, the 20% concentration outperformed all others, emphasizing the importance of optimizing enzyme concentrations for maximum efficiency.

The superior performance of the 20% thermostable alkaline protease 50a solution can be attributed to the delicate balance between enzyme activity and substrate interaction. Higher concentrations may lead to saturation or substrate inhibition, hindering the enzymatic dehairing process (Briki et al., 2016). Conversely, lower concentrations may not provide sufficient enzymatic activity for effective hair removal.

To promote eco-friendly dehairing methods, it was essential to consider alternatives to conventional treatments. Enzyme-assisted treatments, particularly with the optimal 20% thermostable alkaline protease 50a solution, showcased promising results and presented a

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sustainable and environmentally friendly option. Enzymatic dehairing not only demonstrated efficacy but also aligned with the growing emphasis on green technologies and reduced chemical usage in industrial processes.

In conclusion, the study highlighted the significant impact of different dehairing treatments on cow hides. The enzyme-assisted treatments, especially with a 20% thermostable alkaline protease 50a solution, proved to be highly effective, offering a potential alternative to conventional methods. Future research could explore further optimization of enzymatic concentrations and explore additional environmentally friendly dehairing methods for the leather industry.

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Table 4.2: Effect of different treatment on dehaired area yield of the cowhides

Treatment	Area of treated hides (cm^2)	Agents	Duration of treatment (hours)	Dehaired area obtained (cm^2)	Area yield (% of treated area)
Conventional	25	2% Na2S + 5% CaO	24	25	100
Control	25	Distilled Water	24	3.68	14.72
Enzyme Assisted	25	5% CaO + 10% Thermostable Alkaline Protease 50a Solution	30	5.24	20.96
Enzyme Assisted	25	5% CaO + 20% Thermostable Alkaline Protease 50a Solution	30	20.48	81.92
Enzyme Assisted	25	5% CaO + 30% Thermostable Alkaline Protease 50a Solution	30	10.68	42.72
Enzyme Assisted	25	5% CaO + 40% Thermostable Alkaline Protease 50a Solution	30	7.68	30.72
Single Enzyme	25	10% Thermostable Alkaline Protease 50a Solution	24	4.2	16.8
Single Enzyme	25	20% Thermostable Alkaline Protease 50a Solution	24	20.34	81.36
Single Enzyme	25	30% Thermostable Alkaline Protease 50a Solution	24	15.12	60.48
Single Enzyme	25	40% Thermostable Alkaline Protease 50a Solution	24	8.3	33.2

Notes:

$$\text{Area yield (\% of treated area)} = \frac{\text{Dehaired area obtained (cm2)}}{\text{Area of treated skin (cm2)}} \times 100$$

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4.4 Scanning Electron Microscopy

Scanning Electron Microscopy (SEM) was used to examine the smoothness of the grain surface of dehaired hides, which resulted from the removal of hair from the epidermis. It also showed the undamaged grained structure of hides.

4.4.1 Conventional Treatment

In the conventional dehairing treatment of cowhides, the use of hydrogen sulphide, classified as highly alkaline, raised concerns about environmental impact due to the release of toxic chemicals such as calcium oxide (lime) and sodium sulphide into the effluent (Sawalha et al., 2019). This not only posed a threat to aquatic ecosystems but also presented a significant health hazard to tannery workers.

The conventional treatment, using 2% Na_2S and 5% CaO, successfully achieved 100% dehaired area for cowhides, measured at 25 cm^2. However, this treatment resulted in low-quality leather as the method denatured and coagulated hide proteins at a high pH, causing the hide's surface structure to become wrinkled and less smooth showed in Figure 4.2 (a). The high pH denaturation and coagulation of hide proteins induced changes in the collagen fiber structure, impacting the leather's texture (Arfin & Mogarkar, 2018). The elevated pH caused the collagen fibers to lose their natural alignment, leading to a wrinkled and less smooth surface. Moreover, the hair follicles' openings were rough and varied in size, indicating the chemical treatment's impact on the hide's epidermal layer.

The application of calcium oxide and sodium sulphide resulted in the swelling of hides, reaching the maximum level in the chemical specimen. This swelling was likely a consequence of water absorption or changes in the osmotic balance within the hide, caused by interactions between the chemicals and hide proteins. The conventional treatment, also known as chemical treatment, produced significant hazardous waste, contributing to environmental concerns.

In the control treatment using distilled water, Figure 4.2 (b) clearly showed hair remaining on the hide, indicating ineffective dehairing. The hide surface exhibited slight wrinkling, possibly due to changes in collagen hydration and structure caused by distilled water. Hair follicles remained intact and of regular size, suggesting that distilled water alone is not effective in removing or loosening hair. Specific dehairing treatments typically involve chemicals or enzymes to break down the bonding structures between hair and hide.

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Comparing the conventional treatment to the control, the former completely removed the hair, resulting in a cleaner skin surface observed through SEM. However, the control treatment left most of the hair roots intact. The use of CaO and Na_2S in the conventional treatment led to a rougher skin surface than the control treatment with distilled water, indicating damage to the grain surface of cowhides due to the chemical treatment.

4.4.2 Enzyme Assisted Treatment

The combined use of calcium oxide with thermostable alkaline protease 50a at concentrations of 10%, 20%, 30%, and 40% exhibited varying effects on the efficiency of dehairing. The most favorable outcome was observed with a 20% thermostable alkaline protease 50a solution with a pre-treatment 5% calcium oxide. Conversely, the combination of 5% calcium oxide (pre-treatment) with 10% thermostable alkaline protease 50a exhibited the lowest efficiency in dehairing, whereas calcium oxide concentrations of 30% and 40% resulted in moderate dehairing efficiency.

Observations made through scanning electron microscopy revealed that the enzyme-assisted treatment, as depicted in Figure 4.2 (c, d, e, f), prevented damage to the grain surface compared to conventional treatments using CaO and Na_2S. Within the optimal enzyme concentration, hair removal was more effective. White residue contamination was observed in all four treatments depicted in Figure 4.2 (c, d, e, f), as a result of cowhides being soaked in the lime (calcium oxide) solution. The enzyme-assisted treatment demonstrated a clear opening of hair pores on the cowhide. This effect was facilitated by the action of CaO, which played a crucial role in opening up collagen fiber bundles. As a result, water absorption was enhanced during the treatment.

In Figure 4.2 (c), although the pores on the cowhide opened, the hair on the cowhide remained intact, and the majority of the hair did not fall out. The presence of white residue contaminants was clearly observed on the surface of the hide in the figure. In Figure 4.2 (d), despite the pores on the cowhide opening, the hair on the cowhide also remained intact, but most of the hair had fallen out. Similar to Figure 4.2 (c), white residue contaminants were visible on the hide's surface. The surface of the hide in this figure appeared smooth. Moving on to Figure 4.2 (e, f), the pores on the cowhide were open, revealing various enlarged pores. Some of the hair had fallen out, and the presence of white residue contaminants was clearly

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observed on the surface of the hide under a magnification of ×300. The surface of the hide in these figures appeared smooth and exhibited a linear texture.

The enzyme-assisted treatment represents a viable alternative for minimizing the reliance on chemicals in the dehairing process of skins and hides. This innovation not only enhances the quality of leather produced but also substantially reduces environmental pollution.

4.4.3 Single Enzyme Treatment

In Figure 4.2 (g), the hair structure on the cowhide remained intact, and the majority of the hair did not fall out, resulting in a very dense appearance. White residue contaminants were clearly observed on the hair in the figure. Moving to Figure 4.2 (h), the pores on the cowhide were open, the hair structure remained intact, but a significant portion of the hair had fallen out, and the hair follicles appeared damaged. Some white residue contaminants were visible on both the hide's surface and hair, and the surface exhibited a less smooth and wrinkled texture.

Figure 4.2 (i) reveals irregularly shaped and sized pores on the cowhide, with some pores enlarged. Although the hair structure remained intact, over half of the hair had fallen out, and both hair pores and follicles were damaged. A slight white residue contaminant was visible on the hide's surface and hair, and the texture appeared less smooth and more wrinkled. In Figure 4.2 (j), the pores on the cowhide were irregular in size, with some enlarged pores. The hair structure on the cowhide remained intact, but some hair had fallen out, and the hair pores and follicles were damaged. White residue contaminants were visible on the hide's surface. The texture of the hide in this figure appeared less smooth with a wrinkled texture.

The application of a single enzyme treatment for dehairing cowhides proved to be a viable alternative to traditional methods involving lime and sulphide. The enzymatic dehairing exhibited nearly the same effectiveness in hair removal while mitigating the use of sulphide. However, variations in the extent of hair loss and damage to hair follicles were observed across different treatments (Figures 4.2h-j). A critical observation was the presence of white residue contaminants on both hair and hide surfaces in enzyme-treated samples, impacting the overall texture.

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The enzyme-based dehairing process showcased environmental benefits through reduced chemical effluent decomposition and improved wastewater quality. Thermostable alkaline protease 50a emerged as a promising dehairing agent due to its stability, straightforward production process, and cost-effectiveness. However, it's essential to note that the enzymatic dehairing process necessitated a longer processing time compared to the conventional combination of sodium sulfide and lime.

While the SEM results indicated that the single enzyme treatment did not completely remove hair, the organoleptic properties, such as softness and grain surface, favored the enzyme-treated hides over conventional methods. The enzyme-treated hides exhibited a smoother texture and better overall quality, despite some hair remaining intact at the hair root. The clear visibility of the grain surface, particularly with alkaline serine protease, further highlighted the potential of enzyme-assisted dehairing in producing high-quality leather.

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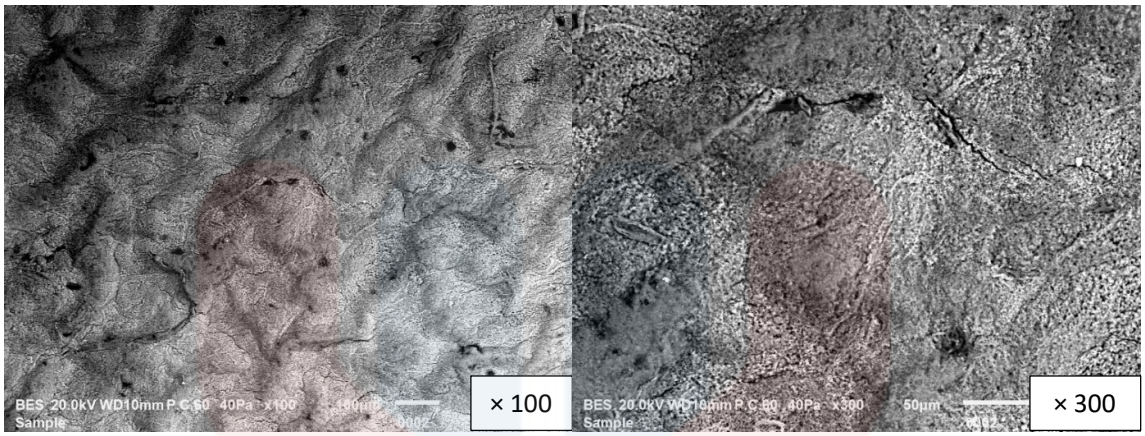
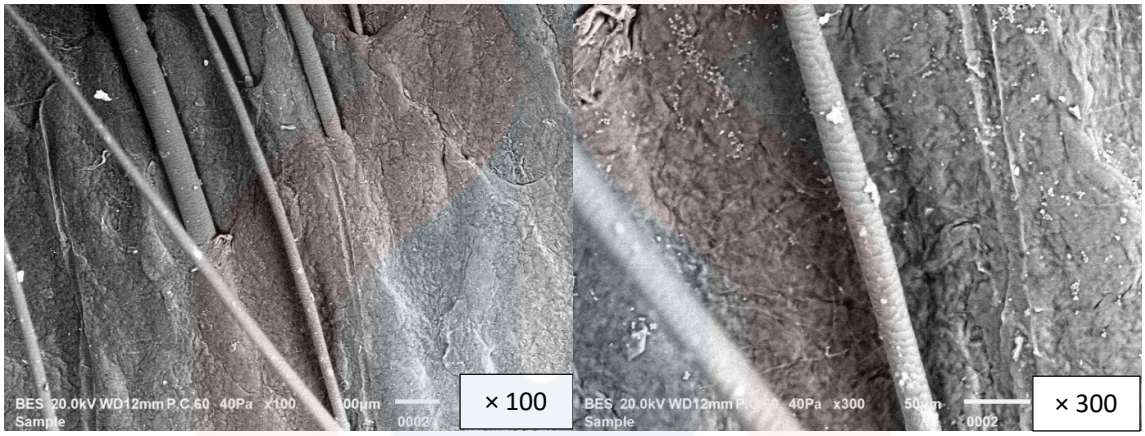


(a)



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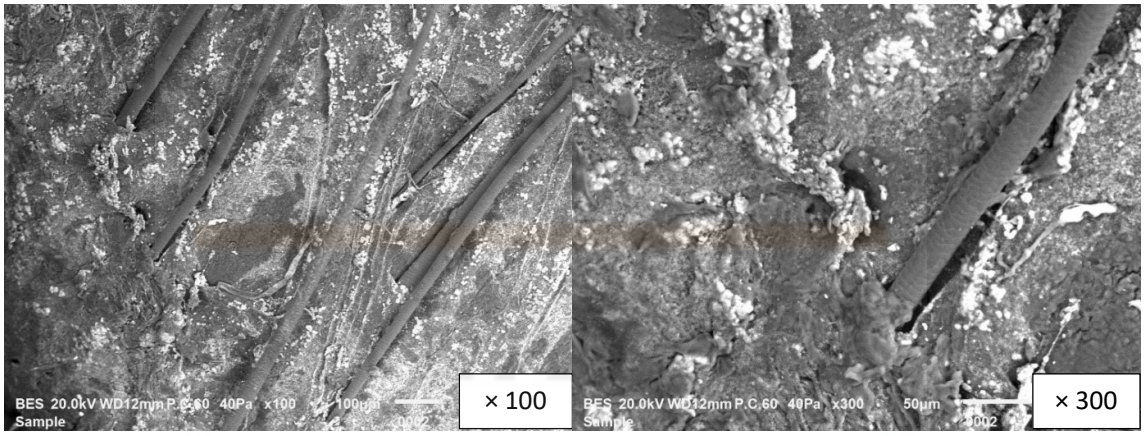

(c)

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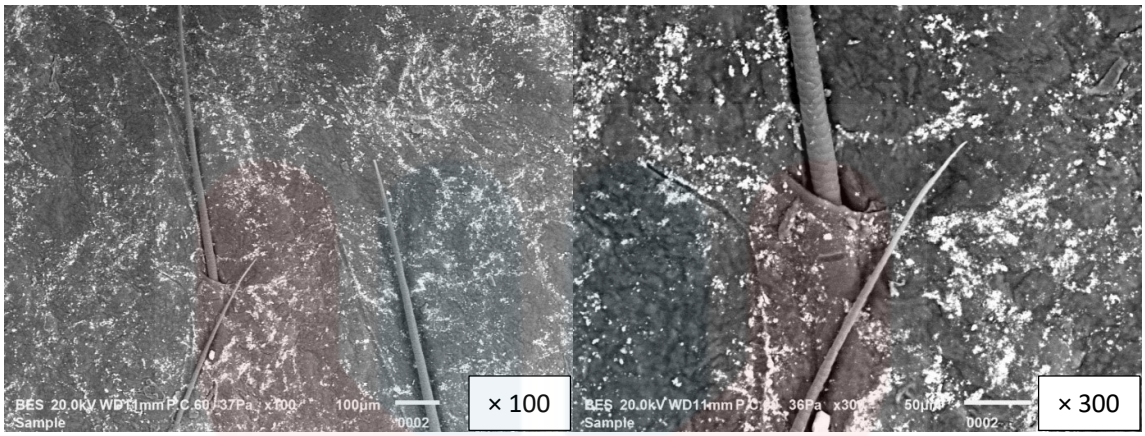
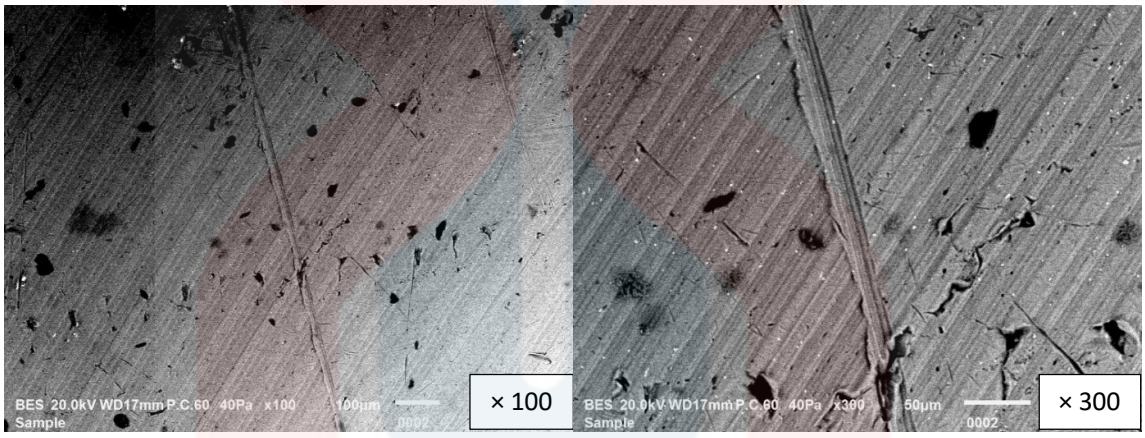
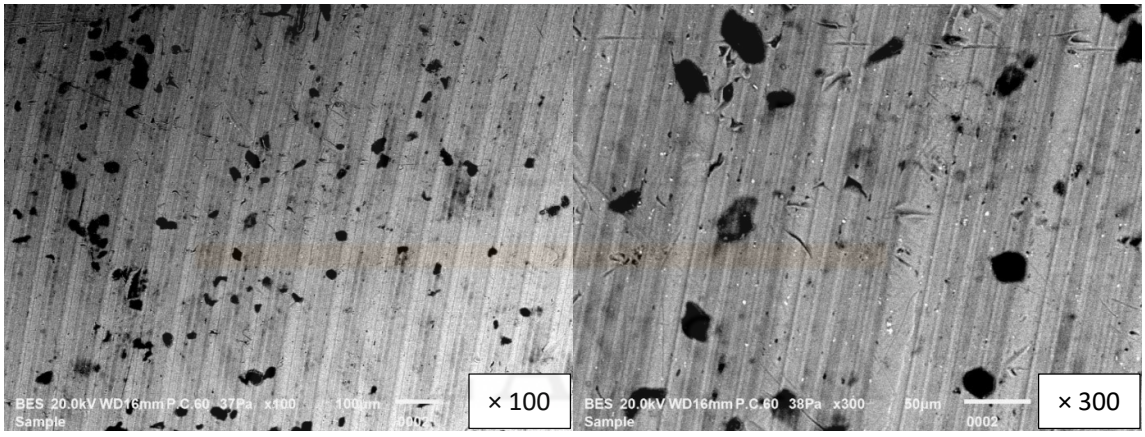


(d)



(e)



(f)

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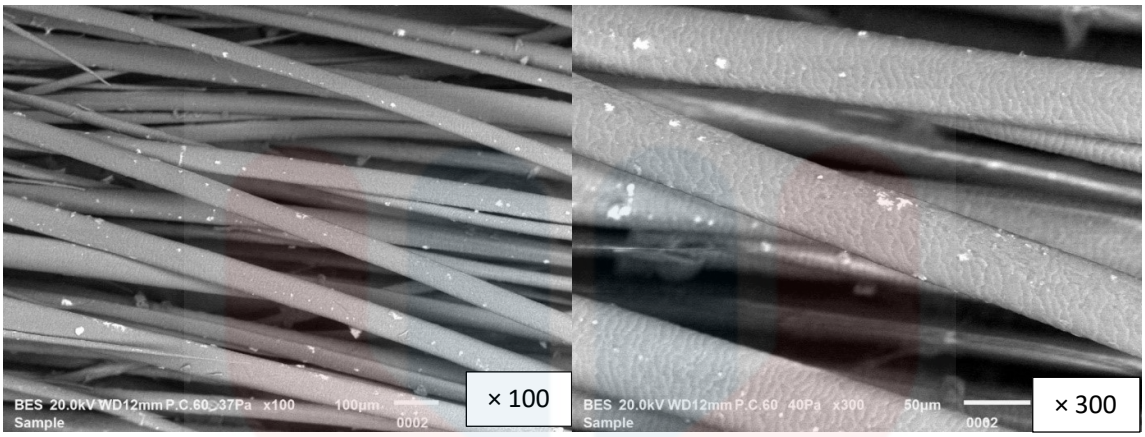
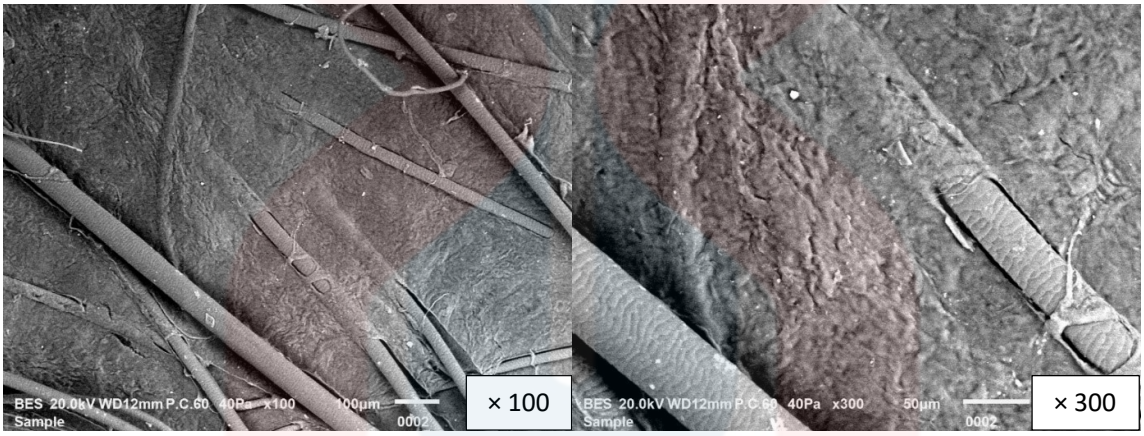
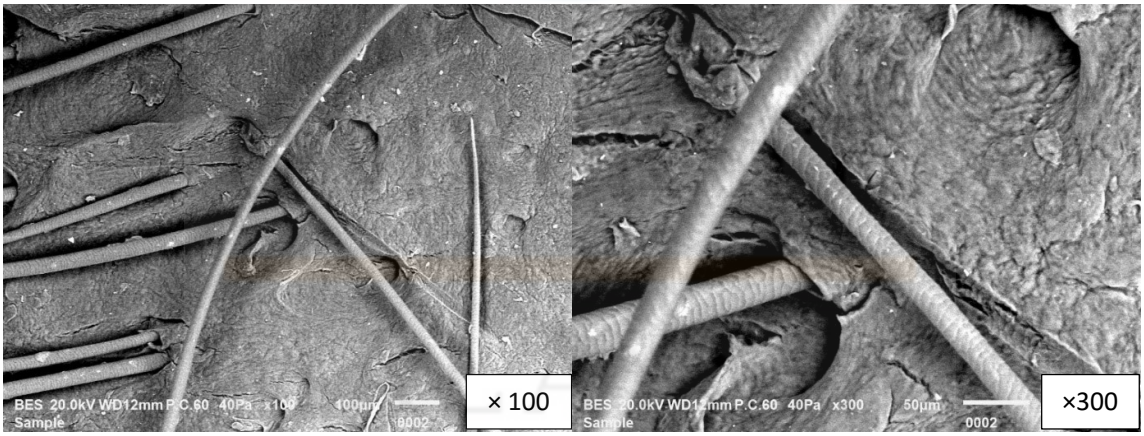


(g)



(h)



(i)

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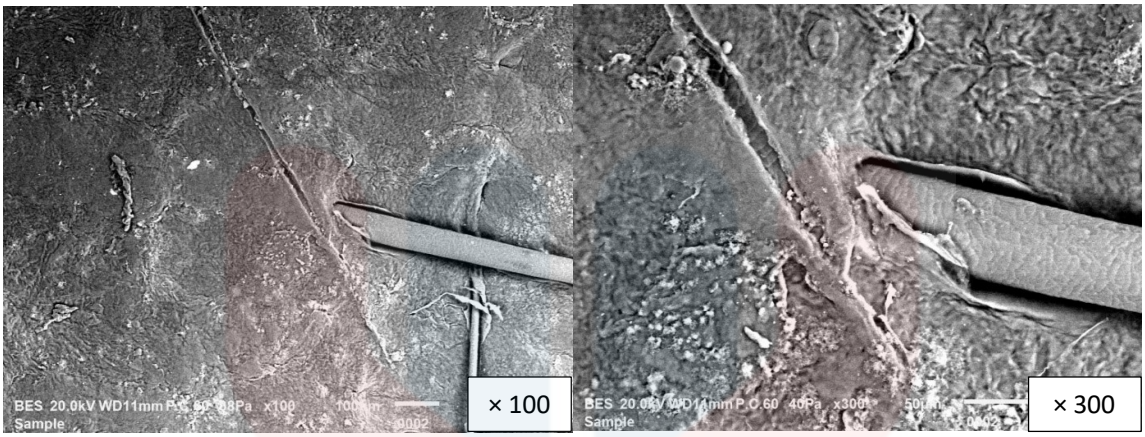

(j)

Figure 4.2: SEM showing the grain surface of cowhides from (a) Conventional (b) Control (c) CaO(6h) + 10% thermostable alkaline protease 50a (d) CaO(6h) + 20% thermostable alkaline protease 50a (e) CaO(6h) + 30% thermostable alkaline protease 50a (f) CaO(6h) + 40% thermostable alkaline protease 50a (g) 10% thermostable alkaline protease 50a (h) 20% thermostable alkaline protease 50a (i) 30% thermostable alkaline protease 50a (j) 40% thermostable alkaline protease 50a

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4.5 Tensile Strength of Treated Cowhides

In the investigation of the tensile strength of treated cowhides, various treatments were applied to assess the mechanical properties of the samples. Specifically, the study focused on evaluating Young's modulus, which measures the stiffness of the material (cowhide); stress @ peak, indicating the maximum stress endured by the cowhides during testing; stress @ yield, denoting the stress at which the material begins to exhibit permanent deformation; stress @ break, representing the stress at which the cowhides ultimately fracture; strain @ break, delineating the extent of deformation at the point of fracture; and force @ peak, quantifying the maximum force applied to the samples before failure (Von Hoven, 2002).

Starting with the conventional treatment, a combination of 2% Na_2S and 5% CaO yielded a Young's modulus of 4.939 N/mm^2, the minimum value observed. The control treatment, utilizing distilled water, exhibited a stress @ peak of 2.260 N/mm^2, the lowest among the treatments. The enzymatic treatments, both enzyme-assisted and single enzyme, demonstrated varying degrees of impact on the tensile strength of the cowhide samples.

Among the enzyme-assisted treatments, the 40% concentration of thermostable alkaline protease 50a Solution presented notable results. This treatment showcased the highest values for Young's modulus (10.875 N/mm^2) and force @ peak (360.200 N), indicating improved tensile strength compared to conventional and control treatments. However, it is essential to note that the strain @ break for this treatment was comparatively lower, suggesting increased stiffness.

In the single enzyme treatments, the 30% concentration of thermostable alkaline protease 50a solution exhibited the highest values for Young's modulus (89.948 N/mm^2), Stress @ yield (3.130 N/mm^2), and force @ peak (732.600 N). These results indicate that the single enzyme treatment at this concentration significantly influenced the mechanical properties of the cowhides, potentially enhancing their tensile strength.

The mean values for all treatments were calculated to provide a comprehensive overview. The overall mean Young's modulus was 27.517 N/mm^2, stress @ peak was 2.572 N/mm^2, stress @ yield was 0.823 N/mm^2, stress @ break was 0.080 N/mm^2, strain @ break was 58.540%, and force @ peak was 408.350 N. These mean values demonstrate the collective impact of the various treatments on the tensile strength of the cowhide samples.

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Furthermore, the standard deviation values were computed to gauge the variability within each parameter. Notably, the standard deviation for Young's modulus was relatively high at 31.929 N/mm^2, indicating considerable variability in the stiffness of the treated cowhides.

The applied tensile PWG50 test, conducted with a Testometric M500-50 CT Machine, specified a test speed of 5.000 mm/min, pretension of 1.000 N, and a sample length of 40.000 mm.

The results from the tensile strength analysis suggest that enzymatic treatments, both enzyme-assisted and single enzyme, have a discernible impact on the mechanical properties of cowhides compared to conventional and control treatments. The concentrations of the thermostable alkaline Protease 50a solution played a crucial role in determining the tensile strength outcomes.

A study by Otunga (2002), examined the effects of enzymatic treatment on the tensile strength of leather samples (Otunga, 2002). Their findings echoed the observations in this investigation, showing that enzymatic treatments, particularly those utilizing alkaline proteases, led to notable improvements in tensile strength parameters such as Young's modulus and stress @ yield. Almeida et al. also highlighted the importance of enzyme concentration, with higher concentrations typically resulting in enhanced mechanical properties (Otunga, 2002).

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Table 4.3: The Tensile Strength of The Cowhides

Treatment	Agents	Parameters					
		Youngs Modulus (N/mm^2)	**Stress @ Peak (N/mm^2)**	**Stress @ Yield (N/mm^2)**	**Stress @ Break (N/mm^2)**	**Strain @ Break (%)**	**Force @ Peak (N)**
Conventional	2% Na_2S + 5% CaO	4.939	2.883	0.577	0.118	69.947	361.500
Control	Distilled Water	50.094	2.260	1.068	0.042	47.133	455.200
Enzyme Assisted	5% CaO + 10% Thermostable Alkaline Protease 50a Solution	1.940	0.650	0.130		40.838	130.400
Enzyme Assisted	5% CaO + 20% Thermostable Alkaline Protease 50a Solution	22.557	0.920	0.624	0.129	35.931	179.800
Enzyme Assisted	5% CaO + 30% Thermostable Alkaline Protease 50a Solution	8.308	0.670	0.445	0.103	19.499	109.600
Enzyme Assisted	5% CaO + 40% Thermostable Alkaline Protease 50a Solution	10.875	2.751	0.639	0.299	50.794	360.200
Single Enzyme	10% Thermostable Alkaline Protease 50a Solution	104.943	2.659	2.475	0.408	29.004	413.600
Single Enzyme	20% Thermostable Alkaline Protease 50a Solution	97.298	3.430	1.886	0.330	47.870	533.500
Single Enzyme	30% Thermostable Alkaline Protease 50a Solution	89.948	6.723	3.130	0.584	59.367	732.600
Single Enzyme	40% Thermostable Alkaline Protease 50a Solution	66.794	3.548	1.462	0.269	59.343	578.600
Minimum		4.939	2.260	0.577	0.042	47.133	361.500
Mean		27.517	2.572	0.823	0.080	58.540	408.350
Maximum		50.094	2.883	1.068	0.118	69.947	455.200
Standard Deviation		31.929	0.441	0.347	0.054	16.132	66.256

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Note:

Minimum	: The smallest value in the dataset.
Mean	: The average value of the dataset, calculated by summing all values and dividing by the number of values.
Maximum	: The largest value in the dataset.
Standard deviation	: A measure of the dispersion or spread of values in the dataset around the mean.

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4.6 Water Quality Assessment and Analysis Methods

The water quality assessment after cowhide dehairing treatments, as presented in Table 4.4, aimed to evaluate the pH values and chemical oxygen demand (COD) of wastewater. The four types of treatments included conventional, control, enzyme assisted, and single enzyme treatments, each with varying concentrations. The reference standards for pH (5.5 - 9.0) and COD (≤ 200 mg/L) were considered based on the Environmental Quality (Industrial Effluent) Regulations 2009.

The conventional treatment, utilizing a mixture of 2% Na_2S and 5% CaO, yielded a pH of 12.6 and a COD of 7380 mg/L. This result exceeded the recommended pH range, indicating highly alkaline conditions, while the COD level surpassed the regulatory limit. In comparison, the control treatment with distilled water exhibited a pH of 7.7 and a COD of 610 mg/L, meeting the pH standard but exceeding the COD limit.

The enzyme assisted treatments, using varying concentrations of thermostable alkaline protease 50a, demonstrated a more favorable outcome. Notably, the 40% enzyme assisted treatment exhibited a pH of 11.5 and a COD of 1300 mg/L, falling within the acceptable pH range and meeting the COD standard. Interestingly, the single enzyme treatments with 10% and 20% concentrations also yielded acceptable results, while the 30% and 40% concentrations exceeded the COD limit.

Comparatively, the enzyme assisted and single enzyme treatments generally showed improved water quality parameters when compared to the conventional and control treatments. The enzymatic treatments demonstrated the potential to mitigate environmental impacts associated with conventional methods.

The pH values and COD results are interconnected, as pH influences the chemical speciation and reactivity of substances in wastewater. The highly alkaline conditions in the conventional treatment likely contributed to elevated COD levels. In contrast, the enzyme assisted treatments-maintained pH levels closer to the standard range, potentially minimizing the generation of organic pollutants, reflected in lower COD values.

The single enzyme treatment at 40%, with a COD of 10060 mg/L, raises intriguing questions. Enzymes are proteins susceptible to denaturation, and the high COD might indicate a breakdown of the enzyme, releasing organic residues. This could be attributed to the concentration-dependent nature of enzyme activity or potential limitations in the enzymatic degradation process.

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In conclusion, the water quality assessment elucidated the efficacy of enzymatic dehairing treatments in maintaining water parameters within regulatory limits. The 40% single enzyme treatment, while effective in dehairing, exhibited a notable increase in COD, necessitating further investigation into its underlying mechanisms. The findings underscore the potential of enzymatic dehairing methods to enhance the eco-friendliness of leather processing, aligning with the objectives of this research.

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Table 4.4: Water Quality of Wastewater After Cowhides Dehaired Treatment - pH Values and Chemical Oxygen Demand (COD)

Treatment	**Agents**	**Parameters**	
		pH Value	**COD (mg/L)**
Conventional	2% Na2S + 5% CaO	12.6	7380
Control	Distilled Water	7.7	610
10% Enzyme Assisted	5% CaO	11.5	1550
20% Enzyme Assisted	5% CaO	11.5	2560
30% Enzyme Assisted	5% CaO	11.5	1220
40% Enzyme Assisted	5% CaO	11.5	1300
10% Enzyme Assisted	10% Thermostable Alkaline Protease 50a Solution	10.5	2480
20% Enzyme Assisted	20% Thermostable Alkaline Protease 50a Solution	9.8	3890
30% Enzyme Assisted	30% Thermostable Alkaline Protease 50a Solution	10.0	3920
40% Enzyme Assisted	40% Thermostable Alkaline Protease 50a Solution	8.9	4410
10% Single Enzyme	10% Thermostable Alkaline Protease 50a Solution	8.5	3310
20% Single Enzyme	20% Thermostable Alkaline Protease 50a Solution	8.5	3970
30% Single Enzyme	30% Thermostable Alkaline Protease 50a Solution	8.5	5540
40% Single Enzyme	40% Thermostable Alkaline Protease 50a Solution	8.5	10060
Subregulations 11(1), 11(2), and 11(3) of the Environmental Quality (Industrial Effluent) Regulations 2009, under Standard B.		5.5 - 9.0	-
Regulation 12 of the Environmental Quality (Industrial Effluent) Regulations 2009, under Standard B		-	200 mg/L

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CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

In conclusion, the study "Eco-Friendly Enzymatic Dehairing of Cowhide Using Thermostable Alkaline Protease 50a" aimed to assess the effectiveness of enzymatic dehairing methods as an eco-friendly alternative to conventional treatments. The objectives were met through a comprehensive investigation into the preparation of thermostable alkaline protease 50a, preservation of cowhides, dehairing methods, scanning electron microscopy analysis, tensile strength evaluation, and water quality assessment.

The protease enzyme derived from *E. coli* BL21 (DE3) pLysS strain harboring TAP 50a gene, demonstrated its alkaline thermostable nature. The purification process yielded a specific activity of 36220.24 U/mg, affirming the success of the purification method in eliminating contaminating proteins. Preservation of cowhides using enzyme-assisted treatments showed promising results, with enzymatic dehairing methods, particularly the 20% thermostable alkaline protease 50a solution, outperforming conventional treatments. Scanning electron microscopy revealed the preservation of the grain surface and undamaged grained structure of hides in enzyme-assisted treatments.

The tensile strength analysis indicated a significant influence of enzymatic treatments on the mechanical properties of cowhides, presenting potential enhancements in tensile strength. Water quality assessment demonstrated that enzyme-assisted treatments-maintained pH levels within acceptable ranges and reduced COD levels compared to conventional treatments, aligning with the objective of developing an eco-friendly dehairing process.

Overall, the study concludes that enzymatic dehairing methods, particularly utilizing thermostable alkaline protease 50a, offer a promising avenue for sustainable and eco-friendly cowhide processing, with positive implications for both the leather industry and environmental conservation.

5.2 Recommendations

Based on the findings of the study titled "Eco-Friendly Enzymatic Dehairing of Cowhide Using Thermostable Alkaline Protease 50a," several recommendations emerge to enhance the application and potential impact of enzymatic dehairing in the leather industry. First and foremost, it is recommended to further optimize the enzymatic concentrations used in the dehairing process. The study revealed that the effectiveness of the enzymatic treatments was concentration-dependent, with specific concentrations showing superior results. Further exploration and experimentation with different concentrations could lead to the identification of an optimal enzyme concentration that balances efficacy, processing time, and environmental impact.

In addition, considering the observed variations in the tensile strength of cowhides based on the enzymatic treatments, it is advisable to conduct in-depth mechanical property analyses. Understanding the interplay between enzyme concentration and mechanical properties can contribute to tailoring enzymatic dehairing processes to achieve not only effective hair removal but also desirable tensile strength in the resulting leather.

Furthermore, the study highlighted the potential environmental benefits of enzymatic dehairing. To capitalize on this, it is recommended to conduct a comprehensive life cycle assessment (LCA) to evaluate the overall environmental impact of enzymatic dehairing compared to conventional methods. LCA can provide a holistic view, considering factors such as energy consumption, water usage, and waste generation throughout the entire leather production process.

Moreover, exploring alternative preservation methods for cowhides is recommended. While wet salting is a conventional approach, investigating the efficacy and environmental impact of natural tannins derived from plant sources could offer eco-friendly alternatives. This aligns with the broader goal of sustainable and environmentally conscious leather production practices.

Lastly, given the significance of water quality in the leather industry, continuous efforts should be made to refine and improve the enzymatic dehairing process to ensure that the wastewater parameters comply with environmental regulations. This may involve tweaking the enzymatic formulation, optimizing reaction conditions, or implementing additional treatment steps to reduce the chemical oxygen demand (COD) in the effluent.

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In conclusion, these recommendations aim to guide further research and development efforts toward the advancement of enzymatic dehairing methods, fostering a more sustainable and environmentally friendly approach in the leather processing industry.

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APPENDIX A

Figure A.1: Raw cowhide

Figure A.2: Raw cowhide after removed leftover meat and fats

Figure A.3: Drying cowhide under sunlight

Figure A.4: Cutting the hides by using angle grinder, band saw machine

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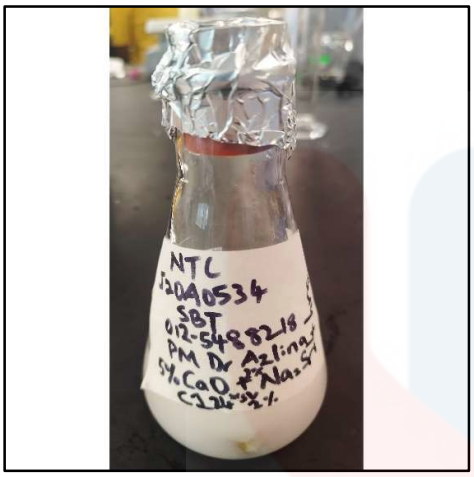

Figure A.5: Hides submerged in conventional treatment

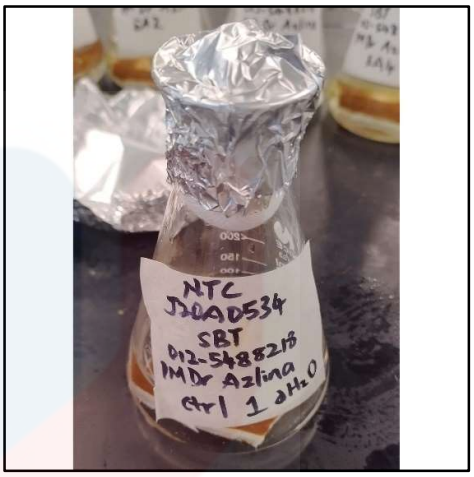

Figure A.6: Hides submerged in control treatment

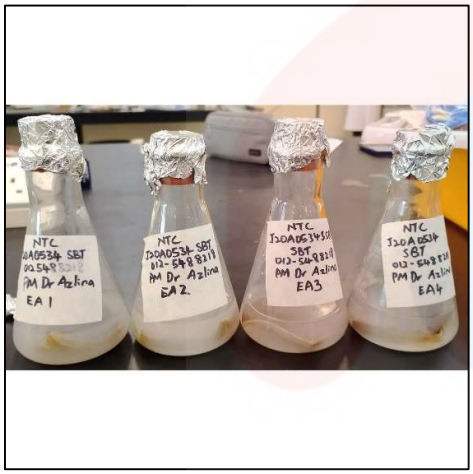

Figure A.7: Hides submerged in enzyme-assisted treatment

Figure A.8: Hides submerged in single enzyme treatment

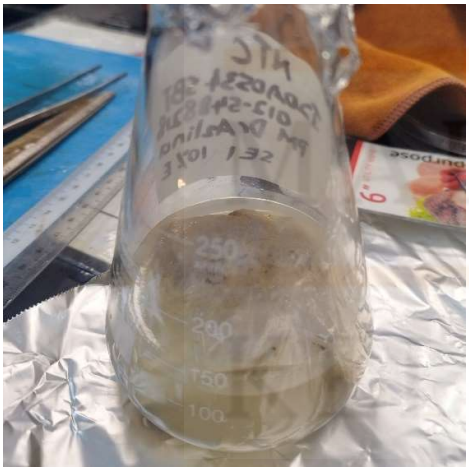

Figure A.9: Hairs being removed

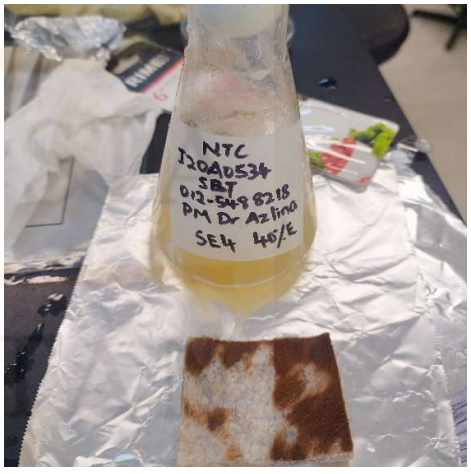

Figure A.10: Removed hair after treatment by using back of knife

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APPENDIX B

Table B1: Calculation for Concentration of BSA (mg/mL)

Concentration of BSA (mg/ml)	BSA (uL)	PBS (uL)
a	0.0	500.0
b	5.0	495.0
c	12.5	487.5
d	25.0	475.0
e	50.0	450.0
f	100.0	400.00

*Use an unknown to represent concentration of BSA for each tube (mg/mL)

Calculation for Concentration of BSA (mg/mL) = $M_1V_1=M_2V_2$

a) $8(0) = M_2(500)$

   $M_2 = 0$

b) $8(5.0) = M_2(500)$

   $M_2 = 0.08$

c) $8(12.5) = M_2(500)$

   $M_2 = 0.2$

d) $8(25.0) = M_2(500)$

   $M_2 = 0.4$

e) $8(50.0) = M_2(500)$

   $M_2 = 0.8$

f) $8(100.0) = M_2(500)$

   $M_2 = 1.6$

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Figure B1: The Bradford standard curve using BSA as standard sample

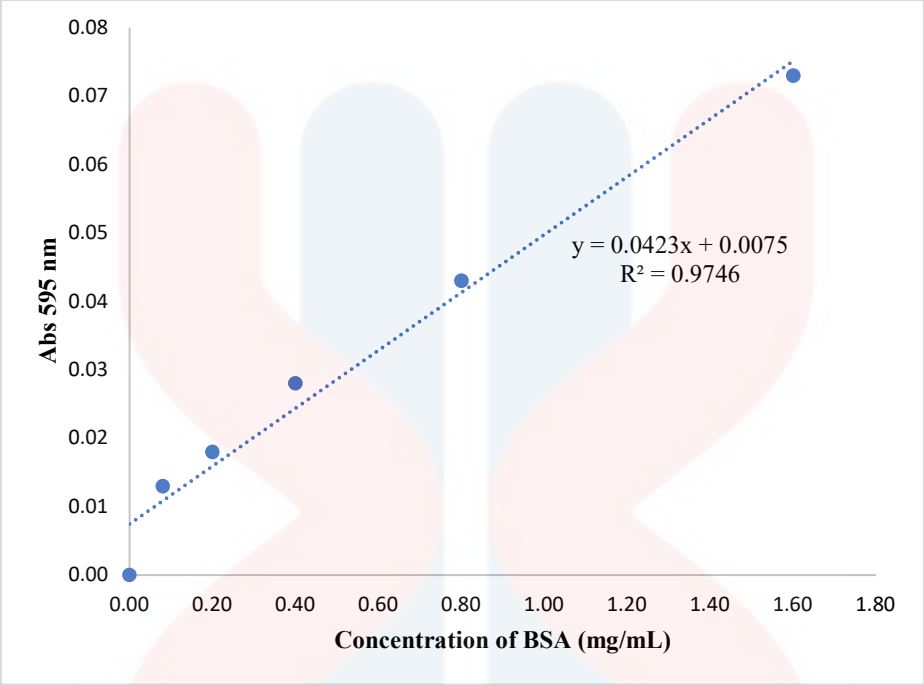

Table B2: Sample for protein concentration determination

Sample	Absorbance 595nm		Average
1	0.299A	0.297A	0.298A
2	0.274A	0.256A	0.265A
3	0.306A	0.291A	0.299A
Total average			0.287A

$$\text{Protein concentration (mg/mL)} = \frac{\text{Total average} - \text{y} - \text{intercept}}{\text{Gradient}}$$

$$\text{Protein concentration (mg/mL)} = \frac{0.287 - 0.0075}{0.0423}$$

Protein concentration (mg/mL) = 6.615 mg/mL

Total protein concentration (mg) = Protein concentration × protein sample (protease)

= 6.615 mg/mL × 0.1 mL

=0.662 mg

Table B3: Sample for protease activity determination

Sample	Absorbance 450nm
1	0.347A
2	0.443A
3	0.411A
Total average	0.400A

Table B4: Control for protease activity determination

Sample	Absorbance 450nm
1	0.041A
2	0.045A
3	0.036A
Total average	0.041A

$$\text{Protease activity (U/mL)} = \frac{(Total\ Average\ Sample\ -\ Total\ Average\ Control) \times 1000}{\text{Incubation time} \times \text{enzyme}}$$

$$\text{Protease activity (U/mL)} = \frac{(0.400\ A - 0.041A) \times 1000}{30 \times 0.1}$$

Protease activity (u/mL) = 119.889 U/mL

Total protease activity (U) = Protease activity × Total volume of protease used (include duplicate)

Total protease activity (U) = 119.889 U/mL × 200mL

Total protease activity (U) = 23977.8 U

Table B5: Protease activity determination

Total Volume of Treatment (mL)	Concentration of Protease (%)	Volume of Protease (mL)	Calculation of protease activity
100	10	10	119.889 U/mL × 10mL = 1198.89 U
100	20	20	119.889 U/mL × 20mL = 2397.78 U
100	30	30	119.889 U/mL × 30mL = 3596.67 U
100	40	40	119.889 U/mL × 40mL = 4795.56 U

Total volume of protease solution (mL): (10+20+30+40) mL = 100mL

Total enzyme activity (U): 1198.89 U + 2397.78 U + 3596.67 U + 4795.56 U = 11988.9 U

Total volume of protease solution (mL) (duplicate): 200 mL

Total enzyme activity (U) (duplicate): 23977.8 U

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Table B6: Protease activity, protein concentration, total protease activity, total protein and its specific activity

Method	Volume (mL)	Protease activity (U/mL)	Protein concentration (mg/mL)	Total protease activity (U)	Total protein concentration (mg)	Specific activity (U/mg)
Heat treatment	100	119.889	6.615	23977.8	0.662	36220.24

Notes:

Total protease activity (U) = Total volume (mL) × Protease activity (U/mL)

Total protein concentration (mg) = Starting volume (mL) × Protein concentration (mg/mL)

Specific activity (U/mg) = Total protease (U) / Total protein concentration (mg)

a) Total protease activity (U) = Total volume (mL) × Protease activity (U/mL)
= 200 mL × 119.889 U/mL
= 23977.8 U

b) Total protein concentration (mg) = Starting volume (mL) × Protein concentration (mg/mL)
= 6.615 mg/mL × 0.1 mL
=0.662 mg

c) Specific activity (U/mg) = Total protease (U) / Total protein concentration (mg)
= 23977.8 U / 0.662 mL
=36220.24 U/mg

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