HALIMAH KHALID

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DEVELOPMENT OF AN ECO-FRIENDLY ENZYMATIC DEHAIRING OF SKINS AND HIDES USING ALKALINE PROTEASE

HALIMAH BINTI KHALID

UNIVERSITI MALAYSIA KELANTAN

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DEVELOPMENT OF AN ECO-FRIENDLY ENZYMATIC DEHAIRING OF SKINS AND HIDES USING ALKALINE PROTEASE

by

HALIMAH BINTI KHALID

A report submitted in fulfilment of the requirements for the degree of Bachelor of Applied Science (Bioindustrial Technology) with Honours

FACULTY OF BIOENGINEERING AND TECHNOLOGY

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DECLARATION

I hereby declared that the work embodied in this report is the result of the original research and has not been submitted for a higher degree to any universities or institutions.

____

Student

Name: HALIMAH BINTI KHALID

Date:

I certify that the report of this final year students entitled “development of an eco-friendly enzymatic dehairing of skins and hides” by Halimah Binti Khalid, matric number F14A0077 has been examined and all the corrections recommend by examiners has been done for the degree of Applied Science (Bioindustrial Technology) with Honours, Faculty of Bioengineering and Technology, University Malaysia Kelantan.

Approved by:

____

Name: DR. NOOR AZLINA BINTI IBRAHIM

Date:

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Development of an Eco-friendly Enzymatic Dehairing of Skins and Hides using Alkaline Protease

ABSTRACT

This study was aimed to provide an alternative and eco-friendly method by using the alkaline protease (210.67 U/ml) in order to reduce the application of chemicals substance in leather manufacturing. Leather manufacturing creates environmental pollution regarded as a source of water pollution. In this research, alkaline protease was used specifically produced from Bacillus subtilis. The enzymes are used in the dehairing treatment of cowhides performed by single enzyme treatment and enzyme assisted treatment, meanwhile conventional treatment by using chemicals. The incubation time for dehairing of cowhides in single enzyme treatment within 24 hours under mild shaking condition at room temperature resulted in 60%-80% of hair removal and enzyme assisted treatment within 75% until 100% of hair removal. Meanwhile, the chemical treatment removed 100% under same condition within 2 hours. After dehairing treatment, scanning electron microscopy (SEM) is made to observe the grain surface of cowhides and histologic analysis to identify the epidermis and dermis layers. The grain surface of skin using enzyme treatment revealed by SEM was smoother and silkier than chemical treatment. Histologic analysis of the skin showed the quality of enzymatically dehaired was better compared with chemically treated one. The keratinized cell of the skin presence in enzymatically dehaired presence with non-collagenolytic activity in comparison with chemically treated skin. Keratinize cell represent the grain surface of leather determined the superiority good quality of leather. Collagen act as an important component of the hides. Based on the results, the application of enzyme assisted treatment in large scale dehairing skin trial is the alternative to reduce the application of chemicals.

Keyword: Alkaline protease, eco-friendly, enzyme assisted treatment, single enzyme treatment, histology

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Pembangunan Enzim Menanggalkan Bulu Mesra Alam untuk Kulit menggunakan Protease Alkali

ABSTRAK

Kajian ini bertujuan untuk menyediakan kaedah alternatif dan mesra alam dengan menggunakan protease alkali (210.67 U/ml) untuk mengurangkan penggunaan bahan kimia dalam pembuatan kulit. Pembuatan kulit mencipta pencemaran alam sekitar dianggap sebagai sumber pencemaran air. Dalam kajian ini, protease alkali digunakan khusus dihasilkan dari *Bacillus subtilis*. Enzim-enzim ini digunakan dalam rawatan menanggalkan bulu pada kulit lembu yang dilakukan oleh rawatan enzim tunggal dan rawatan enzim yang dibantu, sementara rawatan konvensional dengan menggunakan bahan kimia. Masa inkubasi untuk menanggalkan bulu pada kulit lembu dalam rawatan enzim tunggal dalam tempoh 24 jam di dalam keadaan gongcangan yang ringan pada suhu bilik menghasilkan 60% hingga 80% pembuangan rambut dan rawatan enzim dibantu dalam 75% hingga 100% penyingkiran bulu. Sementara itu, rawatan kimia membuang 100% bulu dalam keadaan yang sama dalam masa 2 jam. Selepas rawatan menanggalkan bulu, microskopi pengimbasan electron (SEM) dibuat untuk mengenal pasti permukaan corak kulit lembu dan analisis histologi untuk mengenal pasti lapisan epidermis dan dermis. Permukaan kulit oleh rawatan enzim kulit yang didedahkan oleh SEM adalah lebih halus dan berkilat daripada rawatan kimia. Analisis histologik pada kulit menunjukkan kualiti enzimatik bulu ditanggalkan lebih baik berbanding dengan rawatan kimia. Sel keratin pada kulit dalam menanggalkan bulu secara enzimatik yang tidak mempunyai kolagenolitik aktiviti berbanding dengan kulit yang dirawat kimia. Sel keratin mewakili permukaan corak kulit menentukan keunggulan kualiti kulit yang baik. Kolagen berfungsi sebagai komponen penting bagi kulit. Berdasarkan keputusan, penggunaan enzim yang dibantu dalam percubaan menggalkan bulu kulit pada skala besar adalah alternatif untuk mengurangkan penggunaan bahan kimia.

Kata kunci: Protease Alkali, mesra alam, rawatan enzim dibantu, rawatan enzim tunggal, histologi

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LIST OF ABBREVIATION AND SYMBOL

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
BOD	Biological Oxygen Demand
COD	Chemical Oxygen Demand
TDS	Total Dissolve Solid
DO	Dissolved Oxygen
ORP	Oxidation Reduction Potential
%	Percentage

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CHAPTER 1

Introduction

Research Background

In the manufacturing leather into the finished product, the tanning process involves changing the raw skin so the stable material of leather. The tanning process includes the chemical reactions, physical and mechanical process. This creates a high potential toward the environmental pollution caused by using chemical and it release residual waste, emission of gaseous liquids or solids and this will give bad environmental impact. Environmental pollution also generates raw materials of skin, energy and water. The waste from the tanning process includes chromium salts, sodium chloride, lime, sodium, proteins, chemicals, and solvents from wastewater. The salt chromium gives the largest impact on environmental pollution. The effluents containing chromium salts are rich in neutral salts these lead to the recovery of chromium from water for reuse again (Bondrea *et al.,* 2016).

The tanning agent that mostly use is chromium (III). The tanning process will be dangerous to the workers as the chemical use is a hazardous material. In addition, the effluent release from this chrome tanning will absorb into the soil and pollute the water body then into the source of water, this will lead to problem health to the people who live near the residential area (Bondrea *et al.,* 2016). To develop a sustainable leather manufacturing industry, a clean technology, eco-friendly in order to protect the

ecosystem and the environment by reducing the use of the chemical substance. This will generate an eco-friendly environment for the workers (Bondrea *et al.,* 2016).

The leather industry is the major in contributing pollution to the environment either it generates economy to the country. Environmental pollution occurs by the application of hazardous chemicals in the leather processing. During leather processing, the initial step is the removal of hair along the epidermis, non-collagenous proteins and other cementing substances from the skin (George *et al.,* 2013).

In the conventional method for dehairing animal skin, the chemical involve are lime and sulphide. This will generate pollution noxious gas and solid wastes such as hydrogen sulphide and lime. This method is by making lime and sodium sulphide into a paste then it is applying on the flesh side of skin/hide. This conventional dehairing method categorizes as the polluting method that gives serious impact on water sources and soil. The conventional dehairing method also has it the negative site to the workers as it exposes to the chemical environment, there will be skin damage effect from too much exposure of sulphide. Then, the process of dehairing is complicated as the removal of hair will release to the affluent and the costly. As consequences, it gives a bad impact on the environment. Thereby, an alternative to the environment-friendly method should be use (Khandelwal *et al*., 2014).

The enzyme is the alternative to replace the chemical method. Mostly, the enzyme used is protease enzyme along with small amount of sulphide or without sulphide for the enzyme-assisted method. The enzyme uses for dehairing derived from *Bacillus subtilis* as it has the capability to produce a sufficient amount of alkaline protease due to its proteolytic activity and stability at high pH and temperature.

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Alkaline protease removes the hair from the animal hide by swelling the hair roots and attack the hair follicle proteins (Mamun *et al*., 2016).

Problem Statement

Tanning is most highly pollutant contribution to the environment in the production of leather industry. The process of tanning used the chemicals as the major such as lime, sodium sulphide, salt and solvents. The manufacturer of leather includes curing, soaking, dehairing, bating and degreasing. This leather processing industry gives the crucial problem to the environmental pollution produces a substantial amount of chemical wastes as effluents. In the conventional method of pre-tanning process, dehairing of animal hides is preferable to use lime and sodium sulphide and give a bad impact of the overall pollution refers to the biological oxygen demand, chemical oxygen demand, total dissolved solids and total suspended solids (Mathew *et al*., 2017).

The pollutants from tannery effluents with high sulphide content produce alkaline nature of the surface water and corresponding to the groundwater sources. The impact causes severe health problem to the people living nearby and also tannery workers. The alternative method to dehairing animal skin and hides through bacterial and fungal strain produce from extracellular protease. Animal skin contains collagen provide strength, durability and flexibility to leather. The processing of leather needs to remove non-collagenolytic activity known as non-structural proteins such as albumins, globulins, mucins and mucoid. Mostly, protease and keratinase are used in enzymatic dehairing derived from bacteria (Mathew *et al*., 2017).

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By practising this eco-friendly method, it can minimize pollutant and become environmentally. The benefit using eco-friendly enzymatic dehairing method produces high quality of leather and ecologically safe environment to the workers. Protease is the good source of microbial enzymes and its application in the detergent, pharmaceutical, chemical and leather industries. The environmental pollutant cause by leather industry in conventional methods so enzymatic dehairing can replace this chemical technique (Mamun *et al*., 2016).

Objectives

1. To compare the dehairing method between enzymatic and conventional and the effectiveness of hair removal.
2. To observe the effectiveness of different concentration of protease through different dehairing by using single enzyme treatment and enzyme assisted treatment.
3. To determine the physical parameters and chemical parameters of wastewater from dehairing treatment.

Scope of Study

The study was carried out to get a better understanding of the enzyme activity based on single enzyme treatment and enzyme assisted treatment as an alternative to replace conventional treatment. The analysis of the dehairing efficiency was based on hair removal using enzymatic treatment and conventional treatment. The results of the dehairing treatment were observed using scanning electron microscope to observe

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the grain surface and histologic analysis to identify the opening of hair follicles and keratinized cell.

Significant of Study

This study was focussing on eco-friendly leather industry toward the environment. By using the enzyme, the application of chemical can be reduced and green technology of leather will be existing. Besides, the hair from the treatment must be filtered out to obtained the accurate reading of BOD and COD, as it can give a higher reading. This alternative will give a good impact, especially towards our health when the impact on the environment can be prevented. The speciality of leather identified by wrinkle present on the grain surface. The chemical treatment can be replaced with enzymatic treatment by which the bad odour from the chemical can improve the atmosphere within our environment.

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CHAPTER 2

Literature Review

2.0 Leather and its industry

The leather industry creates environment pollutant. Although it generates economy to the country. The pollutant gives harmful effect because it releases the hazardous chemical. In leather manufacturing, dehairing is the crucial step as it will produce leather. The animal hides consist of long hair with the layer such as epidermis, non-collagenous proteins and other strengthening substances are removed from the skin. Lime and sulphide are used in the conventional dehairing method will generate pollution. The environmental method to prevent pollution is by using the enzyme to replace the chemical method. Besides, the enzyme protease is the alternative method to replace the chemical dehairing. This method is unacceptable by the tanners because there is mild disadvantage cause the application need stability conditions such the pH and temperature also there will be costing in the production of the enzyme (George *et al.,* 2013).

The pollutant that creates from the leather industry started from the preparation of raw hides, the preservation of the hide, and the technology used to dehairing the skin and also tanning. Mostly, the conventional method uses lime and sulphide in the tanning process because it is effective and easily available technologies. The cost is cheaper than new technologies. The dehairing process cause the effluent pollute with

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sulphur derive from the organic matter and mostly hair is the waste from processing leather include compound from the dehairing process such as surfactants and dehairing agents such as sodium sulphide (Na_2S) (George *et al,*. 2013).

The hydrogen sulphide causes adverse effect as it causes the environmental problem. To prevent the accumulation of hydrogen sulphide in the effluent, the treatment used is by oxidized the sulphide and need to be further for wastewater treatment. The dehairing have many techniques include hydrogen peroxide and sodium hypochlorite. Thereby, the environmentally friendly method by using enzyme is better than the chemical process. Mostly protease and keratinase are used in leather industry because of the advantages are better than the conventional method. The role of protease is making the collagen reachable to water by hydrolysing the protein and loosening the attachment of the cementing layer. Keratinase help in the breakdown disulphide bonds of the molecule by hydrolyzing the keratin hair of animal skin and epidermis (George *et al,*. 2013).

Nasr *et al.,* (2017) state that waste from fats of sheep limed fleshing can be processed to become sulphated fat liquor in leather processing. The waste can be undergone neutralization in sulphation with ammonium hydroxide in order to obtain fat liquor. This fat liquor is used by applying leather during processing. Based on their result, the waste from the sheep limed fleshing containing fat constitute the original weight contain 20% and 60% for unsaturated fat and predominated known as oleic acid. As it achieves the suitability for the preparation of fat liquor for sheep leather processing, this technique can be modified to become fat liquor in the leather processing of cowhides. Sulphated fat liquor is used in the tannery process in leather processing to become an alternative to reduce pollution to replace traditional fat liquor and beneficial for the production cost.

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Zarlok *et al.,* (2014) research focus on the mechanical and technical process apply for skins or hides to produce leather in the tanning process. The primary process is pre-tanning, then undergoes with tanning and lastly post-tanning. In the primary process, the purpose is to separate the collagen by eliminating non-collagenous structure on the surface of the skin thus the leather can turn out to be rigid as the collagen fibres stick together. Then, it undergoes the tanning process where the skins will be coating with tanning agent to achieve desired resistance towards physical, chemical and biological factors. In post tanning process, fats, oils and dyes and also re-tanning agent will apply in order to avoid the skin to be sticking together during drying to enhances its softness, water resistance and physical strength. The consequences of tanning process will produce pollution as its various types of liquid, gaseous and solid wastes.

In contradiction with Kowalska *et al.,* (2014) research, during mechanical processing, the undesirable fats will be removed together with meats from the flesh sides to obtain raw hides in order to enable the chemicals to diffuse into the pelt. As this step is skipped during the tanning process, the impact is the excess of chemicals and the high quality of leather cannot be achieved. The researcher also states that the limed fleshing waste is categorized as non-tanned waste products from the process of the treatment of hides with lime and sodium sulphide. In the leather industry, the waste generates about 50-60%. The waste products from leather industry are not considered to undergoes treatment and it became the main problem when the waste just disposes into the landfill.

Nyamunda *et al.,* (2012) reported that the process of fat liquoring will diffuse oils and fats in dispersed form into leather processing matrix. The emulsification process enabling the fat liquoring process done by combining the sulphate and

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sulphonate group into the structure of oils and fats or done with adding surfactants to the structure of fat liquors. The challenge of this study is the ability to apply fat liquor obtained from bovine fat in preserving light leathers. The production of fat waste from leather processing is high and this waste is difficult to treat as there is no technique to recover this waste. The solution of bovine fat applies to waste management to produce liquor.

The specialities properties of cowhides are natural fire resistance leather compare to cloth and plastic materials. Thereby, benefit required for fire safety of the leathers are widely used in aircraft, airspace, automobiles, use in certain home and office where it can minimize the risk surely. The pre-tanning agents, fat liquors and flame retardants leather used to test its flammability. The investigation on flame or glow retardance of leather affects the flame-resistance of leather effect by the flammability of the leather finish coat when exposed to high temperature or flame. For further study, to minimize the damage of leather manufacturing could be done by exposing to flame for flammability test. This test could be done by vertical flame test and oxygen index (OI) test methods. The flammability test can measure the effect of finishing agents such as film-forming materials on the pigments of animal skin (Cheng *et al.,* 2013).

The compositions of finishing agents leather consist of hydrocarbons. The uniqueness of finishing leather where it exposed to flame or high temperatures could decompose hydrocarbons lead to non-combustible substance release carbon dioxide, water and some flammable volatile gases. The finishing coat of leather plays important role in prevention of circulation of the oxygen and these flammable volatile gases. This shows that the thicker the formulations of finishing coat, the higher the flammability resistance of the leather (Cheng *et al.,* 2013).

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Finish formulations derived from pigments and dyes because of their ability in covering power and pigments which is non-water-soluble. The pigments are covered by resin help to prevent flammable by spreading the fire toward the resin in the burning process of finished leather. So, the heat is consumed by the resin of pigment particles. The pigment particles will decrease the fire spreading velocity and improves the flame-resistance of the leather. To improve the quality of the leather product, they need a finishing process such as feel modifier, cross-linker and anti-tack agent (Cheng *et al.,* 2013).

2.1 Leather Processing

The manufacturing of leather involves pre-tanning process at the beam house operations, involves in tanyard operations, post-tanning by wet finishing and lastly finishing. At beginning of the process, the tanning process undergoes dehairing and remove the fat from the skin. The major part of this process is the removal of hair roots. The tanning process undergoes after pickling by using chrome tanning. The main of chrome tanning is to protect the hides from bacteria and resistant to high temperature. The post tanning process is done by wet tanning to change the properties of leather such as smoothness and colour. Finishing operations involve retanning and drying. The transformation of leather in this operation to obtain the softer hides when treated with organic solvent and varnish (Mondal A *et al.,* 2012).

The major chemicals used in the leather industry is sodium chloride (NaCl) for curing, soaking, and pickling process. In tannery wastewater, chloride is considering as a pollutant to the environment. NaCl changes the skins/hides by and induced the

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collagen molecules. Researcher state that a good quality of leather by which the collagen fibre is dispersed. By other words, the quality of leather depends on the fibrous network structure of resultant leather (Li *et al.,* 2016).

Chromium (III) is used tanning agent and act as stabilizing agent for collagen to create flexibility structure without deteriorating when it dries out and becomes suitable for many applications. The post-tanning process involves in softening the leather with oil and mixed with the other tanning agents to improve the surface quality and properties of leather become uniform physical properties by filling the looser and softer parts of leather. The last process is to enhance the appearance of leather and improve the performance of leather by finishing the leather with colour, gloss, flexibility as well as their properties such as lightness and others (Laurenti *et al.,* 2016).

2.2 Leather Dehairing

The dehairing process is the major step by which the removal of hair, non-collagenous protein and the other cementing substance. Lime and sulphide are the most useful in the conventional dehairing method. The effect of using this chemical method in leather manufacturing industries had released noxious gas followed with solid wastes for example hydrogen sulphide and lime. Many industries have replaced from chemical process to the enzyme-based process. This is an alternative to reduce pollution. This enzyme based dehairing process used the enzyme from dehairing animal hides/skins. Mostly enzyme used for dehairing is protease but this enzyme not widely used in the manufacturing leather. Tanners prefer to use chemical dehairing process rather than enzyme because of its stability conditions such as pH, temperature,

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cost of enzyme production, and the consistency of enzyme performance (George *et al.,* 2013).

Recently, efficient and environmentally dehairing protease microbial protease enzyme. Mostly the enzyme used in manufacturing leather is the alkaline protease. It is an important enzyme for the dehairing animal skin to convert into leather. By this method, the using of chemical dehairing can change to the enzyme-based dehairing process. The protease would give the environmental benefits by reducing or preventing the use of chemicals. By using protease in leather industries, it fulfils the requirement of industries when unwanted protein can be degraded by simple diffusion. Protease is a stable alkaline where it can act at pH range 8-12 and has a potential for dehairing of hides (Abrar., 2017).

2.3 Structure of Skins and Hide before and after treatment

Leather industries convert animal hides into leather become a stable material which can be used to manufacture a variety of product. The final product from tanning processing will perform specific characteristics toward stability, water, temperature resistance, elasticity and permeability to air. The leather can be derived from derma animal hides like cattle, sheep and goat. The structure of skin at the epidermis and the hypodermis tissue consist dermis. The connective tissue consists of two parts which are the shallow area called the papillary region and a bottomless area called reticular dermis. The dermis has the structural component which are collagen, elastic fibres, extra-fibrillary matrix and diverse types of cells. The cells are fibroblasts, immune cells, sensory and glandular cells (Montelli *et al.,* 2015).

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The important of dermal component serves a combination of elasticity strength to the skin. The part of leather which provides a good quality of leather located at the part of the dermis, it called grain leather which consists of a linkage of collagen and elastic fibres embedded in polysaccharides. On the contrary, the crust leather known as reticular dermis comprises of thin fibrous collagen bundles with a limited presence of elastin and it is used to produce a low quality of the product (Montelli *et al.,* 2015).

In meat industries, bovine can become a crucial by-product where tanner's intent to know where they can make some modification towards post-mortem dermal in order to achieve the high quality of leathers and generates a good production process by decreasing waste and pollution. Basically, the leather processing will produce waste during pre-tanning processes. The processing animal hides done by skins from the animal is removed then starts immediately the falling-off of fresh dermal tissue's properties (Montelli *et al*., 2015).

To prevent decay structure, the tanners use different curing method before the leather processing can be started. Within a week, the skin is preserved by cooling with crushed ice or store in the refrigerated room for short-term preservation. For long-term preservation, salting and drying and salt drying are used. The concern is to produce high specification leather by preventing the deterioration damages of the raw material. Biodeterioration caused by the microorganism needs some effort to give more attention in evaluation dermal tissue degradation over the time within different condition storage. The damages of dermal tissue deterioration can occur during the slaughterhouse of the animal to the next process of the tanning process of bovine hides, within the storage time in the refrigerators or by salting (Montelli *et al*., 2015).

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2.4 Hides Preservation

In leather manufacturing, preservation of raw hides is important and is a challenge to avoid the damage of the skin. Effective preservatives are needed because denaturation of the skin occurs within 5-6 hours after removal of skins from the animal. The preservation is crucial in order to protect the protein matrix and from the microbial attacks. Preservation can be done by killing the bacterial activity or inhibiting the bacterial contamination. In the conventional method, wet salting is used for curing then the salt will remove during soaking operation (Gudro *et al*., 2014).

After the animal died, the process of skin decay will start instantly. There is a need to preserve the skin quickly to prevent from the microorganism during storage before it converts into leather. The preservation commonly used salt (NaCl) to dehydrate the skin properties. Commonly the concentration of calcium chloride between 40% until 50%. The salt act as curing agent because of its dehydrating ability and bacteriostatic effects. The physical methods to preserve the hide/skin is drying. This method found similar to curing method (Ahmed., 2015).

2.5 Chemical Dehairing

Leather making use tanning method including dehairing the surface of the skin from the hair, epidermis, non-collagenous protein and other cementing substances. The application of the lime and sulphide involve in the conventional method of dehairing and the mixture is pasted on the skin/hides. The application of lime sulphide involves the chemical reaction to remove the hair which contains cysteine-rich fibrous keratin,

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mainly a protein is disposed to alkaline hydrolysis. The pollution problem comes from the conventional dehairing disturb the water source and soil. Nowadays, it becomes a challenge to provide the environmental-friendly method where cause such pollution. It is an alternative to reduce the chemical-based dehairing method for processing hides and leather (Khandewal *et al.,* 2014).

Currently, the protease enzyme is used in the enzyme-assisted method and reducing the chemical substances with a little amount of sulphide with or without lime. Protease is produced from the *Bacillus lichennoformis* strains and there need to prepare it in crude enzyme for enzymatic dehairing. The end product depends on the quality of leather, the collagenous activity in crude protease will deteriorate the collagen of the grain layer leading to denature of skin structure. In order to avoid the collagenase activity, there is need associated enzyme such as keratinase use with protease help in the dehairing process (Khandewal *et al.,* 2014).

2.6 Enzymatic Dehairing

Leather processing undergoes curing, soaking, liming, dehairing, bating, picking, degreasing and tanning and the application of enzyme for easing procedure and improving the leather quality. Mostly enzyme used is alkaline protease and it functions as removal of non-fibrillary proteins during soaking to create a soft structure of leather (Singh *et al*., 2016). The important of collagen is its act as protein in the leather so there will need the enzyme with non-collagenolytic activity like keratinase enzyme. The keratinolytic activity will swell the structure of skin and the non-fibrillary will dissolve. The use of this enzyme can give toughness to the skin to produce leather (Mathew *et al*., 2017).

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The preparation of crude protease can react with keratin and azocasein but it does not show collagenous activity. The reaction of protease involves in chondroitinase, laminarase, and chitinase. The removal of hair from skin/ hides complete within 16-18 hours. After the removal of hair, there will be white appearances and smooth of the epidermal layer. Besides, the enzymatic mixture paste on the skin gives the clean grain without damage by enzymatic dehairing. By using the enzymatic dehairing, it can support the fibre opening in the dermis and the corium region (Khandelwal *et al*., 2014).

The microbial enzyme has been widely used in industries according to their stability, catalytic activity and the process is easy rather than plant and animal enzymes. Enzyme mediates process as a choice in the leather processing due to the non-toxic, cost-effective and eco-friendly method can replace the chemical dehairing method. By fulfilling the demand of this microbial enzyme, advanced technology has been used to produce recombinant protease and the microbe can be manipulated by protein engineering and cultured it in large amount to fulfil the market demand. The leather industry will produce waste then its discharge comes from different level stages of leather processing have a risk of health hazards and environmental pollution (Singh *et al.,* 2016).

The alternative to solve this problem is by using the enzyme cause its biodegradable efficient way to improve the leather and help to dispose of waste. The major uses of the enzyme in the dehairing process need a high quantity of proteases such as protease and keratinase. The enzyme gives benefit by removing the hair and this organic waste can be released into the effluent. Harmful chemical like sulphide, lime and amines can be replaced with enzyme and become alternative reduced these harmful substances (Singh *et al.,* 2016).

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2.7 Enzymes involved in the Dehairing Method

Protease is categorized into three groups based on their characteristic's acid-base behaviour which is the acid, neutral and alkaline protease. (Sengupta *et al*., 2017). The optimum pH for alkaline protease amongst 9-11. It derives from the species of *Bacillus* which can produce alkaline protease. Mostly, this enzyme can obtain from the soil and water and some strains have the ability to resist harsh environmental conditions as well as the high alkaline protease. Alkaline serine protease composes of Aspartate(D) and Histidine(H) residues together with Serine(S) located at their active site making a catalytic triad. There is the different type of protease which acid, neutral and alkaline, but the main use is the alkaline protease as it mostly uses in the industrial enzyme because its stability and activity at alkaline pH (Furhan *et al.,* 2014).

The characteristics of alkaline protease are elastolytic, keratinolytic activities and low hydrolytic activity and these are an important role to be used as a dehairing agent which can be derived from the *Bacillus* strains as its speciality capable to release high activity of enzymes. Mostly, alkaline protease has wide application in the leather industry as their key characteristics are elastolytic and keratinolytic activity. This enzyme applies during the preparation stage of skins and hides in the process of soaking, dehairing and bating. By using the role of enzymatic treatment, it acts to destroy unwanted pigment as the hide can be clean will affect the increasing of skin area (Furhan *et al.,* 2014).

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2.8 The structure of hides

The strength of skin to produce the quality of leather can be determined by the skin characteristics to advance new materials including the elasticity and plasticity. The type of materials uses to distinguished the composite structure are carried up together. The good quality of leather-based on their reconstructive and elongation capabilities. The test for leather analysis based on the tensile test, the stresses, strains, forces of the cowhides. The forces and stresses correlate with tensile tests. The tensile tests results can be obtained by computer simulations (Yilmazcoban *et al.,* 2016).

2.9 Environmental impact

Water quality can be defined based on physical parameters and organic nutrient demand and parameters. Physicals parameters include temperature, turbidity, pH, conductivity and total dissolved solids. pH is commonly used to analyse water testing refer as standard to measure the acidity or alkalinity of the solution. The pH scale is between 0-14. There is an acidic range less pH 7, neutral is pH7, while alkali is greater than pH 7. Water quality can be defined based on physical parameters and organic nutrient demand and parameters (Sailjaja *et al.,* 2012).

The Biochemical oxygen demand can be determined after 5 days as it is a standard for the experimental procedure known as BOD_5. This relates to aqueous microbe require relative oxygen to consume organic materials in the water sources. Biodegradable organics compound in the water system can use BOD_5 as a good indicator to measure the number of organic pollutants. The time taken to obtain BOD_5

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take period 5 days, this method is not measured as the suitable parameter. The process control of water sources between real-time water quality monitoring system need direct feedback as it is vital to know the real condition of the water (Rahmanian *et al.,* 2015).

In order to improve ineffectiveness of this conventional BOD_5 test, it can be replaced by biosensors, UV-visible spectrophotometry, fluorescence measurements to obtain the BOD_5 of the water sample. The alternative of BOD_5 sensors can identify respiration activity of microbe cells by applying appropriate transducer. This biosensor is more comprehensive to obtain BOD_5 rather than conventional BOD_5 method. The inaccuracy of BOD_5 method depends on the activity of microbes affect by the changes of environmental condition which is concentrations of nutrient, temperature and pH (Rahmanian *et al.,* 2015).

The pollutant discharge into the water sample will deplete the oxygen contents. The oxygen depletion can be measured by biological oxygen demand (BOD), chemical oxygen demand (COD) or total oxygen demand (TOD). The parameters used are the oxygen demand in the sample of water required in the biodegradation organic matter. During the oxidation process, the oxygen corresponding is the chemical oxygen demand by using the chemical oxidizing agent such as potassium chromate/dichromate. The oxygen portion corresponding to the organic matter in the chemical oxygen demand of the water sample undergone oxidation by potassium dichromate and this parameter is faster to determine the pollution strength. Potassium permanganate ($KMnO_4$) also can be used to measure the biochemical oxygen demand give a better result from COD measurements. This result shows that potassium permanganate cannot oxidize effectively all the organic content in the water sample for COD test.

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Since then, potassium dichromate is most effective oxidizing agents that can be used to determine COD (Fathima *et al.,* 2014).

There is the relationship between COD and BOD are dependent. When the value of COD is determined, this data can result from BOD in relying highly upon wastewater. Both have advantage and disadvantage relying on numerous factors based on the reproducibility of the determinations, the time taken needed, the location of the test. The more precisely test is COD, it can determine the amount of organic matter within three to four hours differently with BOD_5 which lacking in the measurement of biodegradation of organic matter in the water sample. The value of COD commonly higher than BOD (Roman *et al.,* 2018).

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CHAPTER 3

Material and Method

3.1 Materials

3.1.1 Chemicals and Reagent

The intracellular protease obtained from *E.coli* BL21 pLysS harbouring alkaline 50a protease (Yusoff *et al.,* 2013), Tris-HCl consist of Tris-buffer, $CaCl_2$, and HCl, phosphate buffer, trichloroacetic acid (TCA) sodium hydroxide, hydrochloric acid, ethanol, distilled water, sodium chloride, bovine serum albumin (BSA), Coomassie Brilliant Blue G250, ortho-phosphoric acid, sodium sulphide, calcium oxide, BOD bottle, COD vials, BOD Nutrient Buffer Pillow, formaldehyde.

3.1.2 Apparatus

Petri dish, conical flask, micropipette, cuvette, micro centrifuge tube, beaker, gloves, mask, yellow tips, pH meter, knife, scissor, falcon tube, measuring cylinder,

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forceps, spatula, orbital shaker, centrifuge machine, UV-spectrophotometer, sonicator, filter funnel, filter paper, sonicator, dark bottle, test tube rack, killing jar, parafilm.

3.1.3 Instrument

Uv-spectrophotometer, orbital shaker, centrifuge, Scanning Electron Microscope (SEM), YSI 556 Multi parameter Probe, spectrophotometer (DRB 200), Harch DRB 200 Reactor, probe meter (HQ40d) and Testometer, Tissue Processor, light microscope, Slide Scanner.

3.2 Methods

3.2.1 Curing and Drying process

Cowhides obtained from the local slaughterhouse was washed thoroughly with tap water to remove the blood until clean to prevent smelly odor come from the blood. The fats layer was removed as this part was unnecessary. The salt was sprinkled to all the skin surface to prevent the skin from damage. For the drying process, the hides were drying under the sunlight and be sure the temperature was high as to prevent the hides to become wet so the hides do not have a smelly odour. The duration for the cow hides to drying completely within 3-4 weeks. For the cutting process, the hides were cut into 5 cm × 4 cm and it can be done whether directly after washing the hides or

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during the hides in wet condition. This is because when the hides were too dry, the hides are so hard and not easy to cut. After the drying process, the hides will become thinner as the water was removed. The dehairing process of the cowhides was carried out using several parameters which were control, conventional treatment, enzyme assisted treatment and single enzyme treatment. The method to treat the cowhides by soaking the hides into the treatment solution. To prevent unwanted microorganism growth at hides, it was sprinkled with salt and let it dried completely. After the hides were drying, the hides must keep at the dry place to prevent contamination during the dehairing treatment (Alagumuthu *et al.,* 2015).

3.2.2 Enzyme assay

3.2.2.1 Proteolytic activity assay

Protease activity was determined by a modification of the method. 1 ml of azocasein 0.5% w/v was dissolve in 0.1 M Tris-HCl, 0.002 M $CaCl_2$ pH9 and was pre-incubated for 5 minutes. The reaction was initiated by the addition of 100µl crude protease or purified protease then was incubated at 80 °C for 30 minutes. To terminate the reaction, an equal volume of 10% trichloroacetic acid (TCA) was added. The mixture was allowed to stand at room temperature for 30 min and was centrifuged at 13 000 rpm for 10 minutes. 1 ml of supernatant was taken from the mixture and mix with 1 ml of 1 M NaOH. The absorbance value was read at 595 nm using a spectrophotometer. For the control, it was treating the same technique but difference TCA was added to the crude enzyme before mixing with azocasein solution. The

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distilled water was used as the blank. The enzyme activity was carried out in triplicate and the data was represented by mean values with standard deviations (Yusoff *et al.,* 2013).

3.3 Bradford protein assay

For the Bradford protein assay, Bradford stock solution was prepared first. 50 ml of 95% (final concentration 5%) ethanol was added with 100 ml 85% ortho-phosphoric acid (final concentration 8.5%) and 100 mg of Coomassie brilliant blue G250 (0.01% w/v). Later, the mixture was diluted up to1 liter with distilled water. The solution was filtered until brown colour appears. The solution can be stored up to 1 month in dark at 4 °C in reagent bottle (Valipour Nouroozi *et al.,* 2015).

3.3.1 Preparation of standard curve

BSA was prepared by dissolving 0.00 1 g in 10 ml distilled water and was kept on ice because BSA must be freshly prepared before use. 0.1 M Tris-HCl buffer pH9 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 working buffer in a different test tube. Each test tube had different concentration of two-fold dilution BSA of 1000 mg/ml, 500 mg/ml, 250 mg/ml, 125 mg/ml, 62.5 mg/ml, 31.25 mg/ml and 13.62 mg/ml. The volume of Bradford working was 2500 µl for same each test tube. The final volume of each test tube was 3.5 ml. After all the solution was

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mixed together and incubated for 5 minutes. The absorbance reading was taken at 595 nm using a spectrophotometer. For protein sample assay, 1000 µl of the enzyme was added along with 2500 µl Bradford working buffer. Then, the mixture was incubated at room temperature for 5 minutes and the absorbance reading was taken at 596 nm (Valipour Nouroozi *et al*., 2015).

3.4 Method for dehairing

3.4.1 Conventional treatment for dehairing

The hides were soaked in in 100 ml water + 5% calcium oxide (w/w) and 2% (w/w) sodium sulphide. All the flasks were placed in the orbital shaker rotating at 120 rpm for 26 hours at 37 °C and the hair was removed by using a blunt knife. The cowhides were observed in the time series. The experiment will be done triplicate (Mamun *et al.,* 2016).

3.4.2 Enzyme Assisted Treatment

In enzyme assisted method, the pieces of hides were soaked in 5% calcium oxide (w/w of the skin) solution for 6 hours at 37 °C to open up the collagenase fibers of the cowhides. After treated with calcium oxide, the hides were washed multiple times by tape water until the pH of the skin release near 7.5. Next, the pieces of

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cowhides were dipped in 100 ml of water in 250 ml conical flask and contained 2.5% alkaline protease (v/v) (5 U/ml), 20% alkaline protease (v/v) (42 U/ml) and 40% alkaline protease (v/v) (84 U/ml) respectively. All the flasks were placed into the orbital shaker rotating at 120 rpm for 26 hours at 37 °C. The experiment will be done in triplicate and the hair was removed by using a blunt knife. The cowhides were observed in the time series. The experiment will be done triplicate (Mamun *et al.,* 2016).

3.4.3 Single Enzyme Treatment

Enzymatic dehairing by alkaline protease was done by dip method. The piece of hides was soaked in 100 ml water in the 250ml conical flask containing 2.5% alkaline protease (v/v) (5 U/ml) and 60% alkaline protease (v/v) (126 U/ml) respectively. The concentration was used because to obtain the comparison between a low concentration of enzymes and high concentration of enzyme toward the effect of cowhides structure. All the flasks will be placed into the orbital shaker rotating 120 rpm for 26 hours at 37 °C. The hair was removed by a blunt knife. The experiment will be done in triplicate (Khandelwal *et al.,* 2014).

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3.4.4 Control of dehairing method

As for the control method, the hides were soaked in distilled water and incubate for 24 hours within temperature 37 °C in an orbital shaker at 150 rpm (Khandelwal *et al.,* 2014).

3.5 Scanning Electron Microscope

The sample treated was dried and cut into small pieces. The carbon adhesive tabs were used to mount the hides samples with the glue. To obtain good conductivity, the silver paint was used to the exposed surface area around the sample and the sample was sputter-coated. The samples were observed by using FEI Quanta series environmental SEM at 12 kV was used to determine the grain surface and the follicle present at the cow hides. The magnification used to range from ×50 until ×200 (Khandewal *et al.,* 2014).

3.6 Histologic section of dehaired pelts

Soaked skin which a control treatment of cow hides and dehaired pelts from the enzyme only method, assisted method and conventional method were fixed in 5% (v/v) formaline solution for 24 hours for histological characterization. After the tissue process has finished in the tissue processor, the fixed tissue was embedded in a paraffin

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block and was cutting in 4 µm sections by using microtome. Then the tissue section was stained with hematoxylin-eosin (H&E) for histological examination. The skin tissue was observed under light microscope and a slide scanner (Gumilar *et al.,* 2017).

3.7 Tensile of the treated cowhides

Tensile tests used to determine the mechanical behaviours were carried out with sample treated were prepared by cutting the cowhides in the length of 10 cm and the width of 2 cm to undergo testometer machine. Then, the samples were dried by oven drying. The dimension of the cowhides for tensile tests was taken before the undergone the tensile test. It based on width, thickness, the distance between Jaws and the initial length. Testometer machine for the tensile test was set up with the jaws to grip the skin prevent from slipping off the sample. Tear resistance was measured in accordance to tear initiation and tear propagation. For the tensile force, 0.5 kN was used with the speed of 0.5 mm/s. The physical properties of the sample for the tensile test based on the tensile strength at yield (N/mm^2), tensile strength at break (N/mm^2), elongation at yield (%), and elongation at break (%) (Yilmazcoban *et al.,* 2016).

3.8 Water Quality Analysis of Wastewater from Dehairing treatment

3.8.1 Physical parameters analysis

In-situ water quality measurement was carried after 24 hours of dehairing by using YSI 556 Multi parameter Probe. It was dip into the wastewater from dehairing

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treatment to analyse the physical parameters based on the temperature, pH, salinity, total dissolved solid and dissolved oxygen. Distilled water was used to calibrate the measurement of each water sample by cleaned the probe (Kok Weng *et al.,* 2015).

3.8.2 Chemical parameters analysis

Each of wastewater sample from dehairing treatment collected within 24 hours. The parameters to be analysed which are biochemical oxygen demand and chemical oxygen demand. The analytical procedures were done by the wastewater sample from dehairing treatment was collected using spectrophotometer of a different wavelength which was low range COD (3-150 mg/L) and high range COD (20-1500 mg/L) by using DRB 200 for chemical oxygen demand and biological oxygen demand measure by Harch DRB 200 Reactor (Roman *et al.,* 2018).

3.8.2.1 Biological Oxygen Demand (BOD)

Plastics bottle used to store the water sample covered with aluminium foil in order to prevent light penetrates through the water sample. The BOD bottle was filled with 10 ml of the serological pipette. 10 ml of water sample was collected into a 300 ml BOD bottle. The bottle was filled with pre-prepared 290 ml of dilution water seeded with BOD Nutrient Buffer Pillow and the solution was allowed to mix. The water was added slowly flow down the sides of the bottle to prevent bubbles from forming and eventually closed the mouth of the bottle to prevent from the oxygen to enter into the

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sample. A stopper was put at the mouth of the bottle and carefully do not trap any bubbles. The solution was allowed to mixing properly by inverting the bottle several times. The initial dissolved oxygen was determined thrice using a BO demand kit to calculate the average value later on. The initial dissolved oxygen was determined thrice using a biological oxygen demand kit to calculate the average value later on. The dilution water was added enough to the lip of the BOD bottle to make a water seal. The aluminium foil was used to cover the bottle and it was placed in an incubator at 20 °C and it was left in the dark for 5 days. To obtain the blank for calibration, the distilled water filled in the BOD bottle. The initial reading of BOD was taken by using probe meter (HQ40d). After 5 days incubation, the reading of dissolved oxygen for each bottle was taken. The results determined in (mg/L DO remain) from each of sample the reading were taken thrice to obtain an accurate reading. The BOD_5 for each wastewater sample is then calculated using the equation:

$$\text{Use } BOD_5 \text{ (mg/L)} = \frac{D1 - D2}{p} \qquad \text{Equation 1}$$

Where:

D1: initial sample dissolved-oxygen (DO) concentration (in mg/L)

D2: sample DO (in mg/L) after 5 days

P: decimal volumetric fraction of the sample used

(Roman *et al.*, 2018)

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3..2.2 Chemical Oxygen Demand (COD)

The water samples were collected in a glass bottle from the wastewater of dehairing treatment. The water sample was homogenized using the blender to ensure the particles in the water samples were mixed well. The homogenized wastewater was put in the COD vials. Firstly, the reactor was turned on and pre-heat to obtain a temperature of 150 °C. The COD Digestion Reagent Vial was used for an appropriate range of the water sample. The wastewater sample collected in the vials placed in the pre-heated DRB 200 reactor and closed the protective lid. The wastewater sample was heated at 150 °C for 2 hours. After 2 hours, the reactor was turned off the water sample was cool down for 30 minutes before taking the COD reading. The reading of COD was measured using spectrophotometer with the wavelength of low range COD (3-150 mg/L) and high range COD (20-1500 mg/L). The COD reading was compared with the BOD reading. The determination of calorimeter was used to measure the COD mg/L (Roman *et al*., 2018).

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CHAPTER 4

Result and Discussion

4.1 Preparation of Protease for dehairing treatment of skins or hides

The protease enzyme used in the experiment obtained from *E.coli* BL21 (DE3) pLysS harbouring protease 50a gene (Yusoff *et al*., 2013). The protease identified as the alkaline thermostable protease. A lot report has been mentioned the application of alkaline protease in the dehairing process (Padmapriya *et al.,* 2012). Thus, this study used locally isolated and produced alkaline protease in the application of the dehairing process.

Before undergoing dehairing treatment, the protease enzyme and protein content were needed to be assayed first to obtain the specific activity of the enzyme with the measurement of the spectrophotometric assay. For protease activity, it involved the reaction of the azoprotein substrate to determine the specific activity of the enzyme reaction which chemically known as azocasein use to the altered protein comprising sulphanilamide group connected to a peptide bond of casein in covalently. The protease assay was heated at 70 °C because the enzyme shows the highest activity at optimum temperature. The stability of the enzyme can be achieved at this temperature. If the temperature is below, the enzyme activity will be low. Meanwhile, if the temperature was too high, the enzyme stability will also low as the enzyme was denatured. TCA used to terminate the reaction by its ability in protein precipitation practically used in determination protease assay to dilute biological samples

containing low quantities of protein. NaOH was added in the protease assay as it allowed the solubilization of membrane protein and were reduces the colour yield of the protein-to-protein variation (Charu Lata *et al.,* 2014).

Protein content was needed to measure the protein concentration by using the Bradford method. A lot of methods can be used for determining protein content such as the Bradford method and Lowry method (Kruger, N.J., 2002). The purification of *E. coli* Alkaline Protease attempt to use the Bradford method to measure the relative concentration at 595 nm. The ratio of protein-bound dye in the solution can be measured by this technique, as dye bound to protein. When the condition is acidic, the dye exists in red colour in the protonated state. The interaction through electrostatic and hydrophobic with a protein molecule exist in form of anionic blue form when it is stabilized. A calibration curve used the standard solution of bovine serum albumin (BSA) by absorbance versus mass concentration (Maldonado *et al*., 2018).

To determine the unknown of protein, the reaction of analyte protein same as the BSA of the standard curve for the determination of relative protein concentrations in the sample. From the standard curve obtained in Figure 4.1, the standard concentration of the known sample is 0.9711. This showed the linear least square method resulting in a fit line. To facilitate the analysis of future protein preparations, a successful reliable standard curve needed as the quantitative measure of the purified protease enzyme essential for characterization of the enzyme. The absorbance per unit concentration is collective is not always correct toward the assay sensitivity or response due to protein-to-protein variability may affect to an over- or under-estimation of the analyte proteins concentration. The results of the Bradford method can be biased with the protein composition. Bradford assay involves the electrostatic

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interactions of dye-protein interactions. In addition, there is another factor which is the hydrophobic factor of dye (Brady *et al.,* 2015).

Proteins that are basically hydrophobic will result in the higher absorbance values different with the same mass concentration of a protein with fewer hydrophobic character and/or smaller number of basic residues. There is the difference between the standard and the composition of the protein commonly BSA can affect the incorrect concentration determinations. The standard for Bradford assay used the BSA as the calibration curve depends on the mass concentration of BSA to apply in the determination of an unknown concentration of protein. The colour intensity of Bradford assay at absorbance 595 nm stands the composition-independent, for instance, the mass sensitivity or response of the assay is similar for every protein. Bradford assay produced a blue colour as the anionic form of the dye is stabilized over electrostatic and hydrophobic interactions. The Coomassie brilliant blue presence purple liquid colour then was changed to blue colour as the dye bunding with protein. The higher the blue colour intensity, it showed the highest amount of protein and it was measured spectrophotometrically at absorbance 595 nm and it was effective as the light can be absorbed by the sample (Brady *et al.,* 2015).

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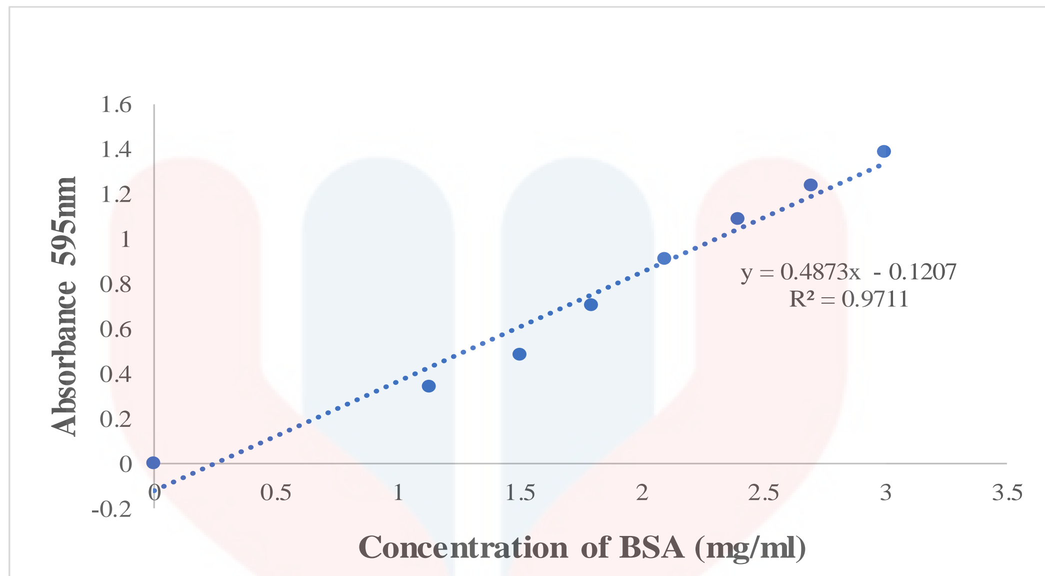

Figure 4.1: The Bradford standard curve using BSA as standard sample

By referring to the table 4.1, the purification of thermostable alkaline protease 50a has determined the effectiveness by protein content. Quantification enzyme activity of purified alkaline protease was 31600.5 U calculated by enzyme units. International Units (IU) used to measure the enzyme activity based on specific conditions as the conversion of 1 µmol of a given substrate to a given product per minute to quantify the amount of enzyme. The total protein of the samples was 115.95 mg indicates the target protein as the purification method eliminate the contaminating protein. The specific activity of thermostable alkaline protease 50a was 272.54 U/mg and the purification steps removed the inactive protein. The meaning of specific activity of enzymes is the ratio of the enzyme unit to the total protein of the enzyme solution. Enzyme mass is not related to its function and activity. The enzyme's purity is measured by specific activity as the formula are the enzyme activity per mass of protein and it is expressed by units/ml (Arutselvi *et al.*, 2012).

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Table 4.1: Protease activity, protein and its specific activity

Method	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)
Heat treatment	150	316000.5	115.95	272.54

Notes:

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

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

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

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4.2 Preservation of cowhides

The basic raw material for tanning industry is hides or skins. The process begins with flaying the hides or skins the prevent the bacterial growth within 5-6 hours after the animal dies. Bacteria exist naturally or originate from the soil which can lead to putrefaction of proteins and makes hides or skins inapt for the production of quality leather. There are metabolic changes in hides or skins during the animal death due to not supplying oxygen and nutritional components. As a consequence, the toxic substances will accumulate and leads to the source of inactivation of some coenzymes. It begins when decomposition of protein to the peptide by autolysis and finally amino acids. The process of autolysis product is further broken down over the secondary process by the reaction of putrefactive bacteria (Alagumuthu *et al.,* 2015).

Decomposition of hides or skins protein can be stopped after flaying with two possible option which is the tanning process started instantly and appropriately preservation. The tanning process is impossible because there is no tanning facility. Another option was proper preservation to protect the hide or skin. The conventional method to preserve fresh hides or skins was done immediately after flaying by wet-salting where commonly 40%-50% of salt (sodium chloride). The actions of sodium chloride were dehydrating the cowhides and the existence of bacteriostatic properties was being exploited in this preservation method (Ahmed., 2015). The presence of salt lead to dehydration action to preserve the skin from unfavourable condition towards the growth of bacteria, plasmolysis also happens and bacterial growth was limited. After the salt curing process of cow hides achieved the drying condition, the hides were washed to eliminate the salt (Alagumuthu *et al.,* 2015).

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4.3 Dehairing Method

Based on the previous work (Mamun *et al.,* 2016), four types of dehairing was done which the conventional treatment, enzyme assisted treatment, enzyme mediated treatment and single enzyme treatment. Some of the methods involved paste method in conventional treatment and enzyme assisted treatment. Based on the result, the hair removal of paste method was not effective compared to the dip method. The factor of inefficient hair removal based on the low quantity of water in the paste method. This method only used the chemical treatment while the application physical treatment was not done by which placed in the orbital shaker for easy hair removal. Thus, for this study, the dip treatment was only choosing.

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4.3.1 Effect of different dehairing treatment

The efficiency of different treatment of dehairing methods was calculated and it was determined in the percentage value tabulated in Table 4.2. The highest efficiency of dehairing treatment was the conventional method as the time taken to remove completely the hair intact with the skin surface within 2 hours the effectiveness of dehairing treatment could be seen in conventional treatment resulted in 100% as it faster than other treatments. Meanwhile for enzyme assisted method, the duration to remove the hair completely within 24 hours but with the combination of high concentration of enzyme with lime solution. The dehairing treatment of enzyme assisted method about 75% until 100%. The effectiveness of dehairing cowhides almost similar with conventional treatment without correlate with the duration of dehairing treatment. For single enzyme treatment, the lowest area of dehairing was a low concentration of protease. In others hands, the high concentration of protease can dehaired more surface area of the cowhides. The single enzyme treatment removed about 60% until 80% of hair from cowhides after 24 hours. All the treatments were conducted at room temperature within a mild condition. The incubation time for dehairing treatment between control, single enzyme treatment and enzyme assisted treatment within 24 hours and the enzyme assisted treatment was effective compared with single enzyme treatment. The dehaired area by enzyme assisted treatment was similar to the conventional treatment but this method more time-consuming. Based on the given results, enzyme assisted treatment has potential as an alternative to replace the conventional treatment (Mamun *et al.,* 2015).

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

Treatment	Area of treated skin (cm^2)	Agents	Duration of treatment (hours)	Dehaired area obtained (cm^2)	Area yield (% of treated area)
Conventional	16	2% Na_2S + 5% CaO	2	16	100
Enzyme assisted	16	5% CaO + 2.5% Protease	24	12	75
Enzyme assisted	16	5% CaO + 20% Protease	24	14	85
Enzyme assisted	16	5% CaO + 40% Protease	24	16	100
Single enzyme	16	2.5% Protease	24	10	60
Single enzyme	16	60% Protease	24	13	80

Notes:

$$Area\ yield(\ \%\ of\ treated\ area = \frac{dehaired\ area\ obtained\ (cm)}{area\ of\ treated\ skin\ (cm)} \times 100$$

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4.4 Scanning Electron Microscopy

4.4.1 Conventional Treatment

The dehairing method involves conventional treatment, enzyme assisted treatment and single enzyme treatment. In the conventional dehairing treatment of cowhides, comprises hydrogen sulphide categorize as a highly alkaline were not safe to release into the effluent. This is because the use of toxic chemicals such as lime and sodium sulphide which can give bad impact to the aquatic pollution and more serious problem is a health hazard to tannery workers. Conventional lime sulphide treatment showed the maximum area of dehaired animal skin was rapid. Nevertheless, this treatment showed low-quality of leather in case this method had denatured and coagulate the skin proteins at high pH and the structure of skin become wrinkled and less smoothness (Mamun *et al*., 2015). The application of sodium sulphide and lime detected the swelling of the hides was reached the maximum level in the chemical specimen (Cadirci *et al.,* 2016). The conventional treatment in leather manufacture also known as chemical treatments with that application of lime and sodium sulphide which release a huge amount of hazardous waste (George *et al.,* 2014).

There were differences between control in Figure 4.9(a) and conventional treatment in Figure 4.9(b). The conventional treatment removed the hair completely as referred with the control. There was also invisible of hair intact on the hair root instead of control. The conventional treatment by using CaO and Na_2S showed the surface of the skin was roughly presented in Figure 4.9(b). This chemical treatment eases the removal of hair intact as the SEM observation showed the clean surface of the skin. The chemical reaction of sulphide affects the skin to become black compared with

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other treatment. The damages of the cowhides were clearly seen as the chemical treatment damages the grain surface (George *et al.,* 2014).

4.4.2 Enzyme Assisted Treatment

On the other hand, skin treated with enzyme assisted treatment with the addition of 5% CaO treated for 6 hours along with enzymatic action formed area yield differently to the conventional treatment. This probably due to the soaking the cowhides into 5% CaO solution. The lime solution acted on the collagen fibers swelled osmotically by the absorption of water from the lime solution by changing of its structure aided removing the electrical charge from the basic groups in collagen and by which breaking hydrogen bonds. It contributes to the lessening of cohesive adhesion forces between the fibers triggering the fibers to become looser as the collagen fiber bundles can open up. Its aided the protease enzymes to across more easily into the skin by degrading the interfibrillar substances to open the fibrous structure. The benefit of using the enzyme-assisted treatment even though a short time of soaking in the lime solution, it contributes accelerating the penetration of alkaline protease through collagen matrix to act upon attaching proteins around the hair follicles without damage the collagen fiber structure and eventually facilitating the removal of hair (Mamun *et al*., 2015).

The combination calcium oxide with 2.5%, 20% and 40% protease had a different effect on the efficiency of dehairing. Calcium oxide with 2.5% protease showed low efficiency of dehairing. The efficiency of dehairing increase as the concentration of protease enzyme increase from 20% to 40%. Based on the appearance of the cow hides, the highest concentration of protease resulted in the smoothness of the skin. The low concentration of protease which was 2.5% protease after treated with

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5% calcium oxide showed the skin was very hard and the grain surface was deposited with calcium oxide. Meanwhile, the high concentration of protease within 60% protease after treated 5% calcium oxide showed the grain surface was silkier. Based on the experimental result, the higher the concentration of enzyme protease, the smoothness the skin surface and the calcium oxide aided for dehaired the cow hides (Mamun *et al*., 2015).

The observation was made by scanning electron microscope revealed that the enzyme assisted treatment based on Figure 4.9 (e, f, g), the combination of CaO and protease prevent the damages of the grain surface difference by using CaO and Na_2S in the conventional treatment. Within high concentration of enzyme, the hair can be removed completely. The contaminant of the white residue was seen on all the three treatment on Figure 4.9 (e, f, g) effect from soaking the cowhides in the lime solution. There was clearly seen the hair pore treated in the enzyme assisted treatment as the CaO opens up the collagen fibers bundle of the cowhides permitted the absorption of water. When the high concentration of protease applied, the hair pore becomes smaller. In Figure 4.9 (e), the hair still intact on the cowhides instead of concentration increased, the removal of hair increased. The smoothness of skin obtained from Figure 4.9 (g) based on the small hair pore. The enzyme assisted treatment is an alternative to reduce the use of chemicals related to dehairing of skins and hides. This will improve the leather processing industry as they can produce good quality of leather and also reduced the environmental pollution to the significant level (Zeng *et al.,* 2016).

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4.4.3 Single Enzyme Treatment

Single enzyme treatment for dehairing cowhides was an alternative rather than using lime and sulphide. This enzymatic dehairing can be a similar feature for the hair removal and limitation of the application of sulphide. The decomposition of the chemical in the effluent can be reduced as resulted improvement in the wastewater quality (Uddin *et al.,* 2015). Instead, the enzyme uses on its own, the skin appearance was clear as the leathers do not swell and the colour also attractive as the light colour close to white. Alkaline protease can be a potential source dehairing application because of its stability, simple production and using the cheap medium. The reduction of the dehairing process time by using the combination of sodium sulphide and lime differently by using alkaline protease in single enzyme treatment. The use of chemicals and reduced the effluent load of lime sulphide free or diminished concentrations of lime sulphide can be replaced by the enzymatic dehairing process. This will produce the quality of the final product by increasing the area yield (Cadirci *et al*., 2016).

The enzyme-based dehairing process is an alternative towards chemical dehairing to prevent the using of lime and sulphide because it develops environmental pollution. There are many studies apply the single enzyme treatment but its commercial application in leather industry is limited. There were a few factors to be considered for the enzyme to be used as dehairing agent depend on the efficient dehairing capability and inactivity on collagen (George *et al.,* 2014). The different concentration of enzyme resulted in different efficiency of dehairing treatment. The highest the concentration of enzyme, the more efficiency of dehaired area. The high concentration enzyme produced showed more smoothness of skin surface rather than low concentration of enzyme (Isaac *et al.,* 2016).

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The visual evaluation for single enzyme treatment based on the scanning electron microscopy was different with conventional treatment. It was noticed that single enzyme cannot remove the hair completely based on Figure 4,9 (c and d) different with the conventional treatment on Figure 4.9 (b). Based on the evaluation of the organoleptic properties such as softness, fullness and grain surface, single enzyme treatment more advantageous than conventional treatment. Alkaline protease showed there was no grain damage as its grain surface was clearly seen. Enzymatic treatment showed the skin presented was clean from hair pore and clear grain without any contaminants. Based on the removal of hair, there was still hair intact on hair root. But the cowhides were considered as a good quality of leather because it can be used as a saleable by-product (Isaac *et al.,* 2016).

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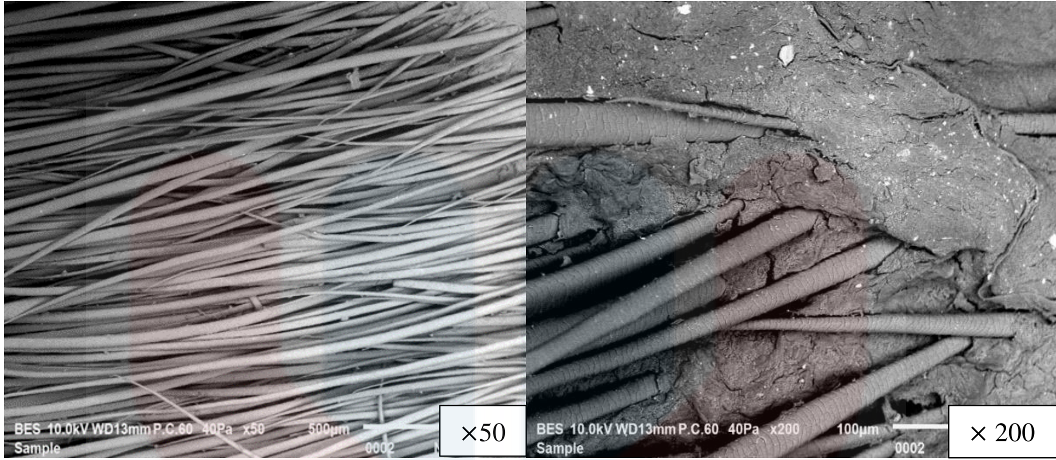
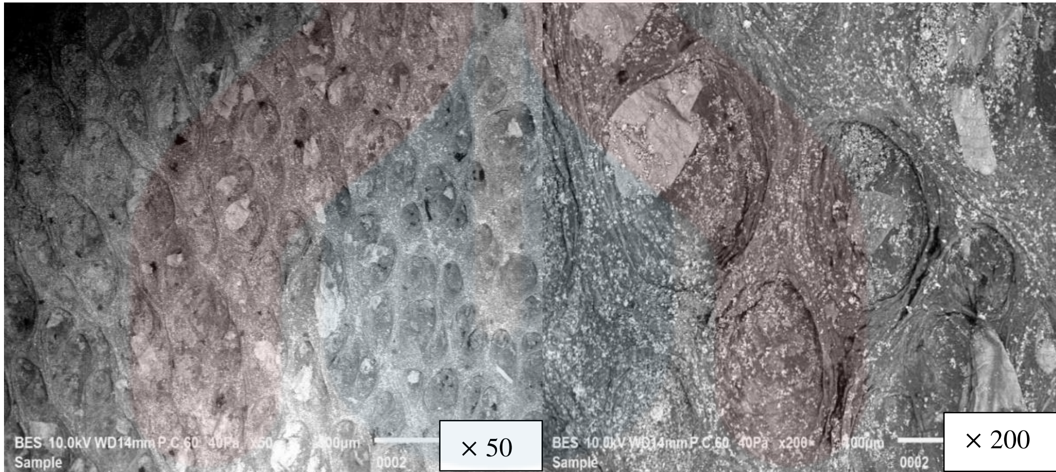


(a)



(b)

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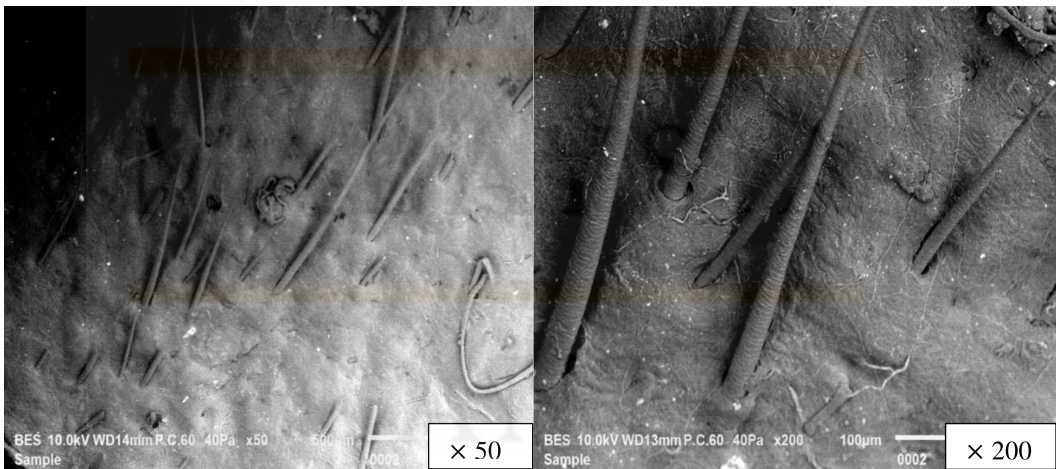

(c)

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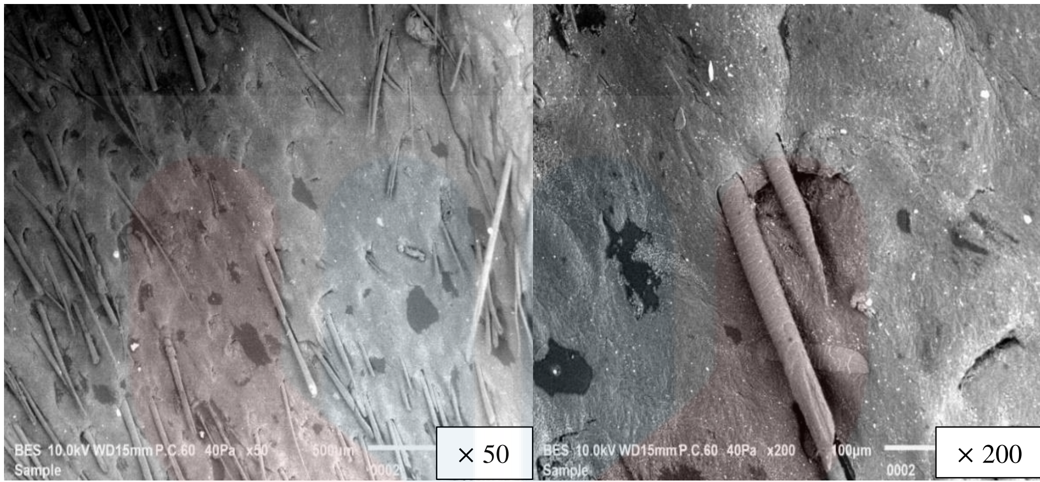
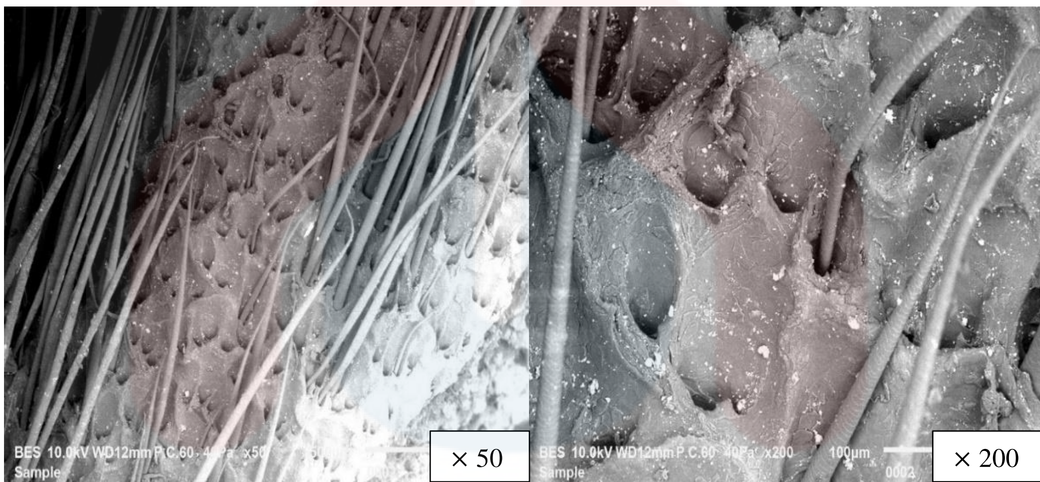


(d)



(e)

(f)

(g)

Figure 4.2: SEM showing the grain surface of cowhides from (a) Control (b) Conventional (c) 2.5% Protease d) 60% Protease (e) CaO(6h) + 2.5% Protease (f) CaO (6h) + 20% Protease (g) CaO (6h) + 40% Protease

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4.5 Histologic Analysis of Dehaired Pelts

4.5.1 Control Experiment

The dehairing treatment was based on the depilated area at the end of the treatment. The section of dehaired pelt was identified based on the histological analysis undergo H&E staining shown in Figure 4.2. The leather quality was specified by light microscope for the control treatment while the others treatment was identified by slide scanner. Figure 4.2 shown the stratified squamous epithelium, keratinized cell for the control treatment. The control treatment was used to compare the changing structure of untreated and treated cowhides. The keratinized cell can be identified on the control treated hide thereby if the dehairing treatment showed the presence of keratinized cell, it shown the best quality of leather. The keratinized structure can refer to the grain surface of the leather. The presence of grain surface play role in the strongest and durable part of the hides. The natural characteristics can be demonstrated based on the full grain surfaces. The best treatment of hides can be chosen to produce the highest quality of leather-based on the full grain surface (Eurell *et al.,* 2006).

The characteristic of leather can increase the added value by the presence of its natural marking. The strongest leather generates by maintaining its grain surface rather than diminish it as the advantageous can keep the leather last longer over time changes. The mechanism of quality leather shows the presence of pore and hair follicles and also the smoothness of the leather. Other characteristics make the value of leather-based on the natural line variation or the fat wrinkles existence in the grain surface. Based on Figure 4.2 (b), the presence of hair shaft shows the dehairing of the cowhides do not occur. Within the histologic analysis, life hair follicle structure was identified in Figure 4.2 (a). This structure was used to compare with the other treatment whether

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the treatment damaged the hair follicle of the cowhides. The damaged of hair follicle referred to the penetration of substance used in dehairing treatment. The collagenase activity absence as the control treatment does not damaged the collagen fiber. The smooth muscle show in Figure 4.2(c) is the linear structure as it close to the blood vessel of the bovine (Eurell *et al.,* 2006).

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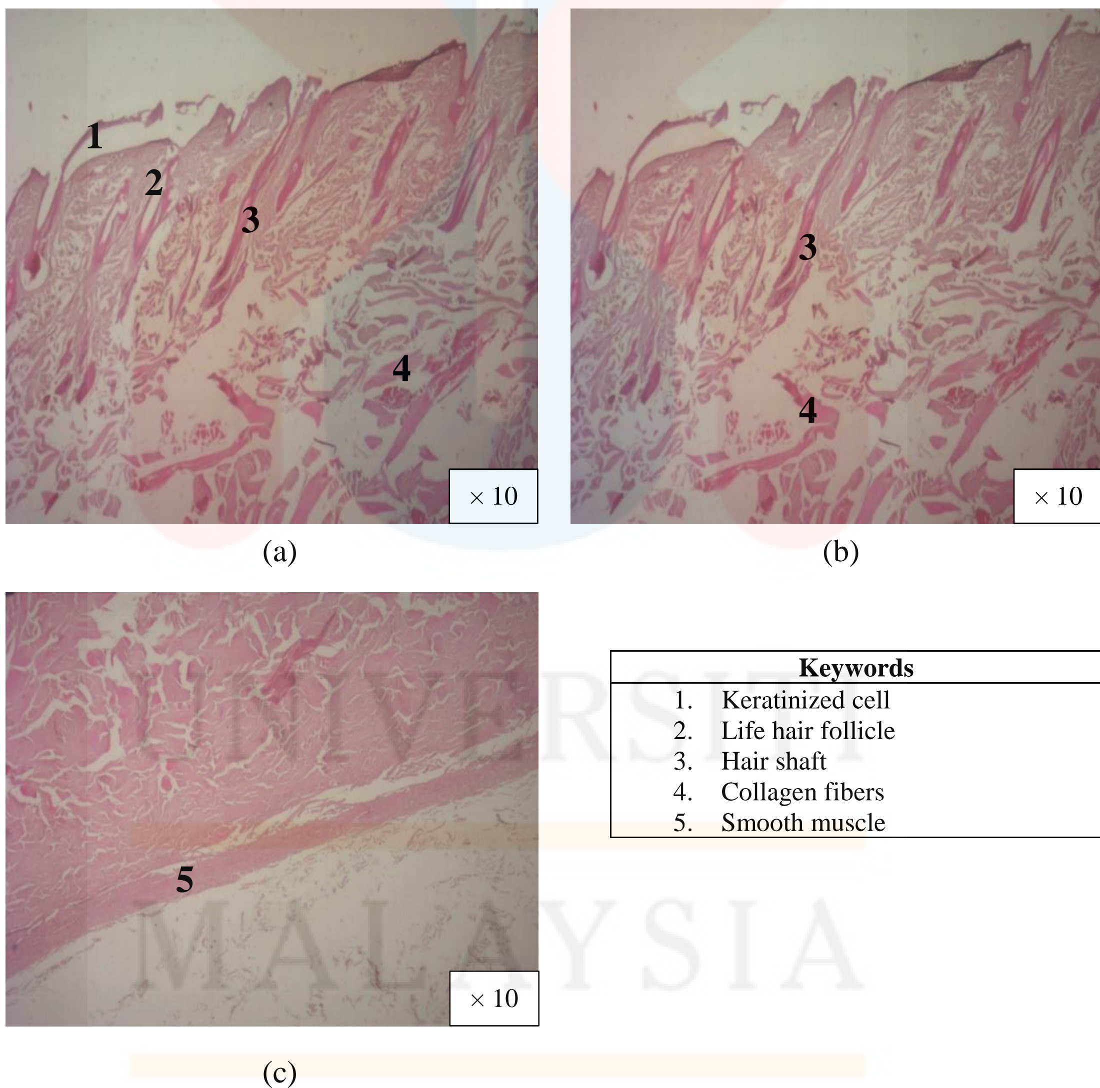

Figure 4.3**:** Histology analysis using control sample of cowhides: (a) Stratified squamous epithelium (b) Collagen fibers (c) Smooth muscle

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4.5.2 Conventional Treatment

The application of lime and sulphide in the conventional dehairing treatment contribute to hair removal. This action due to chemical reaction disturbed the hair root consisting cysteine-rich fibrous keratin categorize as the protein which presented by death hair follicle in Figure 4.3 (b). This treatment has a huge impact toward water sources and soil as it is the one of polluting treatment (Khandelwal *et al.,* 2015). The section of dehaired pelts by chemical treatment showed the absence of epidermis structure, and empty follicle appearance Figure 4.3(a). The collagenase activity does not occur as the cell tissue damaged by the chemical treatment when presented by red colour staining in Figure 4.3 (Hammami *et al.,* 2018).

The colour staining of the hides changes from control method (Figure 4.2) and single enzyme treatment (Figure 4.6) different with used of chemical in conventional treatment (Figure 4.3) or combination of a chemical with a higher concentration of enzyme in enzyme assisted treatment (Figure 4.5). As stated, the pink colour staining appeared in healthy tissue while darker red appeared showed the cell tissue was damaged due to the application of the chemical. Thereby, in the conventional treatment, the cell presence in purple staining due to the damaged cell refer to the non-keratinized cell. All the hair follicle can be seen clearly in red colour as it appeared as death hair follicle (Eurell *et al.,* 2006).

The skin treated in the conventional treatment cannot undergo to long as it can disturb the structure of the skin. The effect of the chemical dehairing effect the damage of epithelium tissue. Thereby, the period of conventional treatment was shortening than other treatment as the hair can be removed completely. The collagenase activity will presence because of the death of skin tissue. The application of lime

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and sulphide in the conventional dehairing treatment contribute to hair removal. This action due to chemical reaction disturbs the hair root consisting cysteine-rich fibrous keratin categorize as the protein which is susceptible. This treatment has a huge impact toward water sources and soil as it is the one of polluting treatment (Khandelwal *et al*., 2015). The section of dehaired pelts by conventional treatment showed the absence of epidermis structure and empty follicle appearance. The collagenase activity does occur as the cell tissue damaged by the chemical treatment (Hammami *et al.,* 2018). The skin treated in the conventional cannot undergo too long treatment as it can disturb the structure of the skin. The effect from the chemical dehairing effect the damaged of skin tissue. Thereby, the period of chemical treatment was shortening than other treatment as the hair can be removed completely (Cheville *et al.,* 2006).

The collagenase activity will presence because of the death of skin tissue. The structure of epidermis was digested by the application of 2% Na_2S because of the reaction of the chemical. The appearance of the of the cowhides treated was roughly and not compact. The structure of collagen was damaged as the penetration of chemical through the skin. The disadvantageous of chemical dehairing was the changing of hair into pulp presented by the death of hair follicle. This showed the epidermal structure was destroyed and reduced to the pulp when chemicals attack the hair (Gumilar *et al*., 2017).

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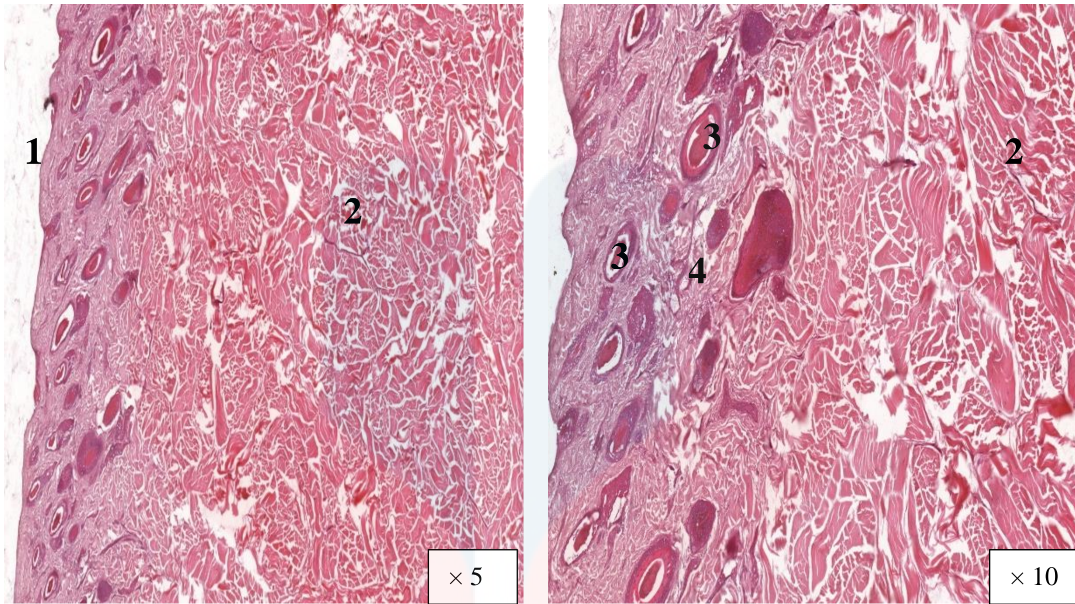

(a) (b)

Figure 4.4**:** Histology analysis using conventional sample of cowhides: (a) and (b) CaO (6h) + 2% Na_2S

Keywords	
1. Non- keratinized cell	3. Death Hair Follicle
2. Collagen fibers	4. Sebaceous Gland

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4.5.3 Enzyme Assisted Treatment

The effect of lime and protease upon cementing substance was relatively different. The skin undergoes lime will experience osmotic swelling presented by the opening of the hair follicle in Figure 4.5 and 4.5 (a and b) as the action of hydrolysing the amide side chains from the basic groups of collagens including asparagine and glutamine produce whether more or less collagenase. The distortion of collagen affects by negative charge produced from electrostatic repellency of identically charged area. The splitting of collagen bundles improves by the hydrostatic pressure built up in the collagen fiber structure. The impact of the splitting collagen bundles leads to shortening and thickening of each fiber and the whole fiber structure was increased in thickness (Sivasubramanian *et al*., 2008).

Based on Figure 4.4 (a and b), the enzyme assisted treatment at low concentration of 2.5% alkaline protease still protect the keratinized structure. As the concentration of protease increase, the damaged of the keratinized cell also increases. This action occurs when the concentration of 40% alkaline protease damaged completely the keratinized cell. The hair follicle of 2.5%, 20% and 40% protease in Figure 4.4 and Figure 4.5 (a and b) showed the swelling of hair follicle but in 40% of alkaline protease at a different section of hiding in Figure 4.5 (b) showed less opening. The life and death hair follicle consist in enzyme assisted treatment. The hair pore was unclearly seen in single enzyme treatment in Figure 4.6 while the enzyme assisted treatment and conventional, the existence of hair pore can be seen clearly. Thereby, the active chemical makes the hair follicle to swell and support the absorption of water into the skin. Whereby, the active enzyme can prevent the swelling of hair pore (Mamun *et al*., 2015).

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The colour of staining was different as the concentration of enzyme increased. The tissue cell in the lowest concentration of enzyme showed the pink colour of staining in Figure 4.4, as the concentration enzyme increased, the staining of the tissue cell appeared to be darker red in Figure 4.5. The colour of the staining also different with the tissue in control treatment and single enzyme treatment. The healthy tissue showed in pink colour while the damaged tissue showed a darker colour. This was due to the absence of keratinized cell which gives permeability from the harsh environment. Thereby, the chemical easily to penetrate into the tissue cell as the absence of keratinized cell (Aughey *et al.,*2001).

The cowhides treated in the higher concentration of enzyme showed the digestion of epidermis layer with the presence of death hair follicle. It specifies that the hair was loosened completely. The effectiveness of protease uses in this treatment showed its ability to break down the soft keratin consists of the hair follicle removal of the hair from the skin. The structure of collagen was damaged because the cow hides were soaked in the CaO solution. Based on the elastic tissue, there was still present and not being removed by the enzyme assisted treatment. The advantages of using the combination of CaO with chemical aided to loosen the hair by modified the epidermal tissue surrounding the hair bulb so the mechanical can remove the hair (Gumilar *et al*., 2017).

The sebaceous gland associated with hair follicle act as secretory product, sebum an oily substance through the holocrine mode. The degeneration of mature secretory cells to form sebum derived from vacuolated secretory cells synthesize lipid. The sebum release to the skin to develop good health of hair support by the smooth muscle of the hair follicle and the arrector pili undergo contracts (Aughey *et al.,* 2001).

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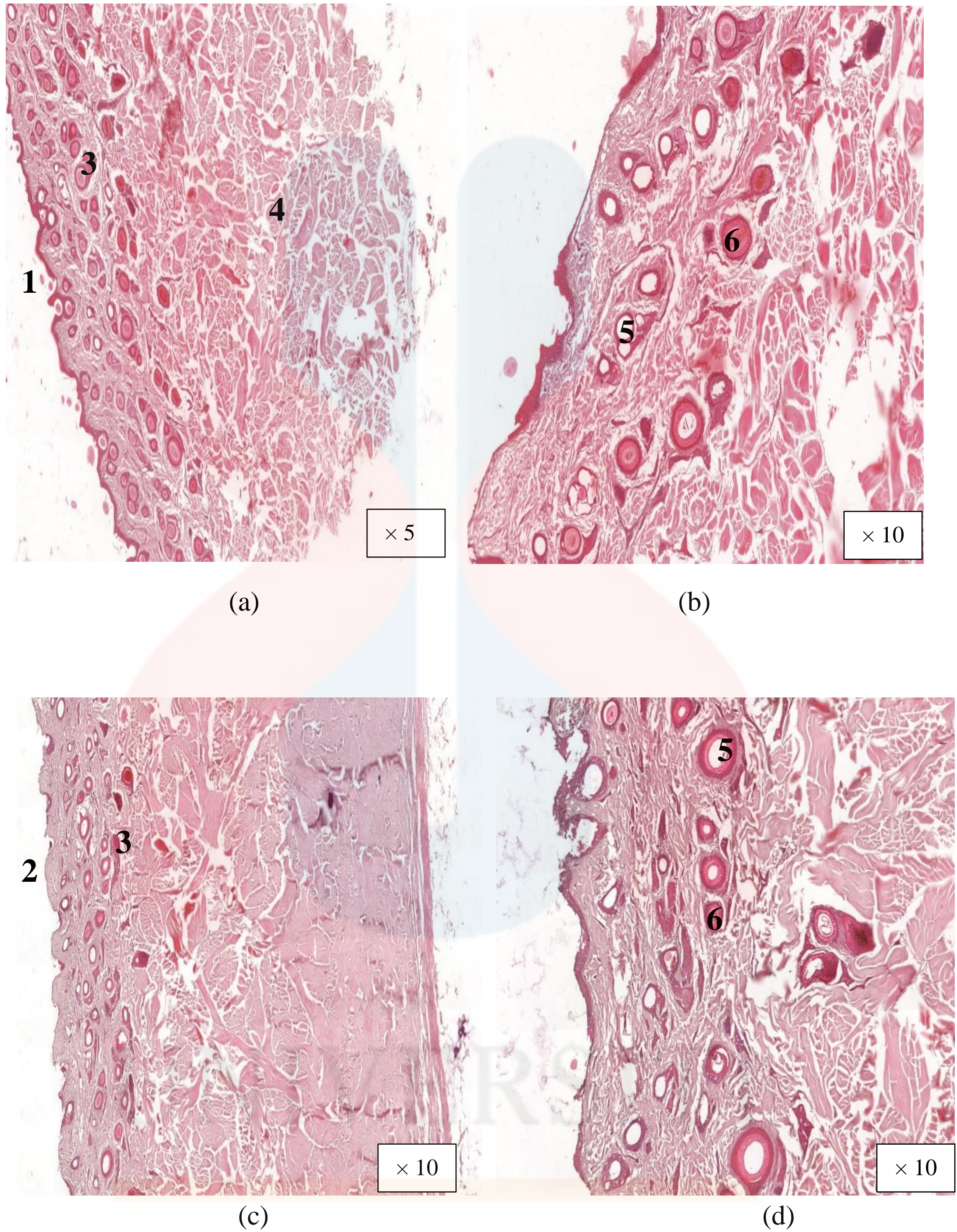
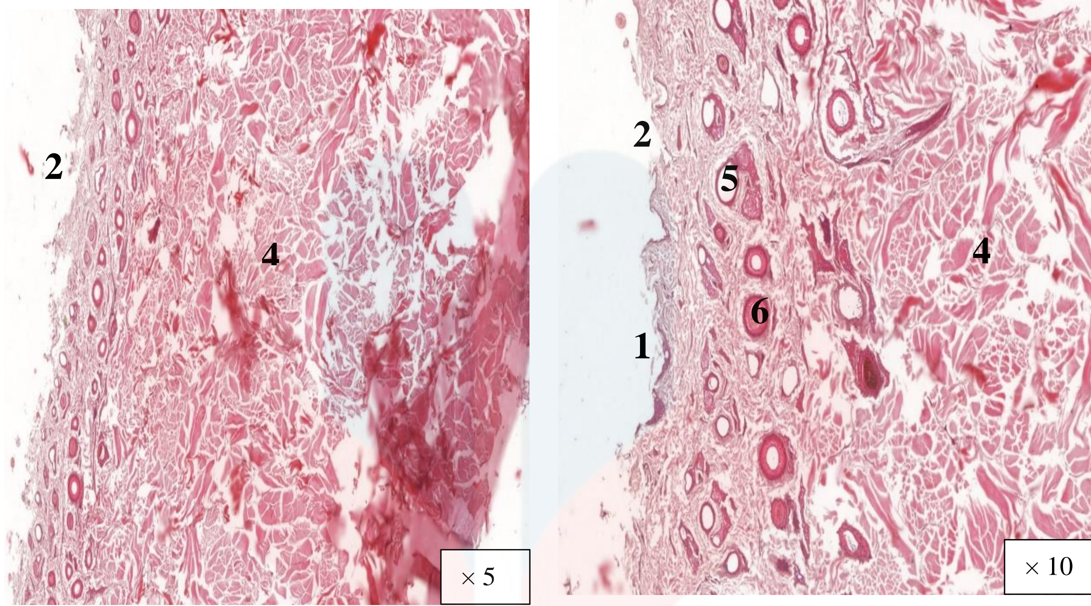
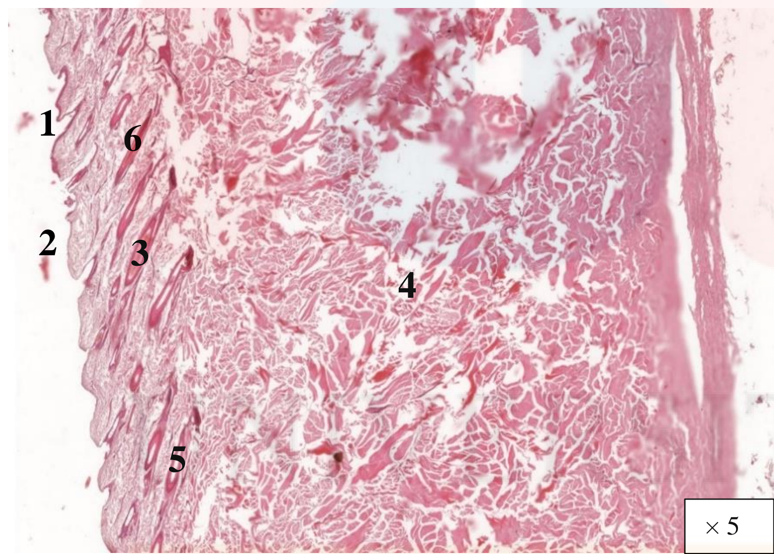


Figure 4.5: Histology analysis using enzyme assisted sample of cowhides (a) and (b) CaO (6h) + 2.5 % Protease (c) and (d) CaO (6h) + 20 % Protease

Keywords	
1. Keratinized cell	4. Collagen Fiber
2. Non-keratinized cell	5. Life hair follicle
3. Hair follicle	6. Death hair follicle



(a) (b)



(c)

Figure 4.6**:** Histology analysis using enzyme assisted sample of cowhides: (a) and (b) CaO (6h) + 40 % Protease (c) CaO (6h) + 40 % Protease (At different section of skin)

Keywords	
1. Keratinized cell	4. Collagen Fiber
2. Non-keratinized cell	5. Life hair follicle
3. Hair follicle	6. Death hair follicle

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4.5.4 Single Enzyme Treatment

The treatment based on the single enzyme treatment identified by H&E staining showed the effect on the skin collagen and the fiber opening (George *et al.,*2014). The treated section of the low enzymatic treatment does not damage the epithelium layer at 2.5% of alkaline protease (Eurell *et al.,* 2016). While the 60% alkaline protease show the keratinized cell was damaged. The leather generated from the single enzyme treatment protect the existence of the grain surface whereas the 60% alkaline protease diminish the existence of the grain surface. The presence of life hair follicle means the single enzyme treatment do not disturb the natural characteristics of the cowhides. The effect of damages the hair follicle demonstrates the leather will not last longer as time increases. The collagen was seen in the cowhides treated considered as non-collagenase activity (Hammami *et al.,* 2018).

The application of protease in this treatment based on the high catalytic activity and substrate specificity. The wide use of alkaline protease in the leather industry due to their non-collagenase activity. The valuable application of alkaline protease in leather dehairing as the hide produced was clean and increase the surface area thereby the treatment extinguishes the undesirable pigments of skins (Furhan *et al.,* 2014). The efficiency of alkaline protease was the capability to digest the cells of the Malpighian layer and the basal cells of hair bulbs while does not disrupt the native state of the cowhides. The activity of protease by attacking the outermost sheath to loosening the hair consequent the swelling the inner root sheath and also break down the part of it. The consequences application of single enzyme treatment is elimination the uses of sodium sulphide, generate the quality of leather-based on the complete removal of hair, provide the conducive environment, and within the surface area of the skin can

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generate better strength properties of leather. The single enzyme treatment was not damaged the hair follicle whether the different concentration of alkaline protease was used. The protection of hair follicle describes the cell tissue was not damaged by single enzyme treatment (Madhavi *et al*., 2011).

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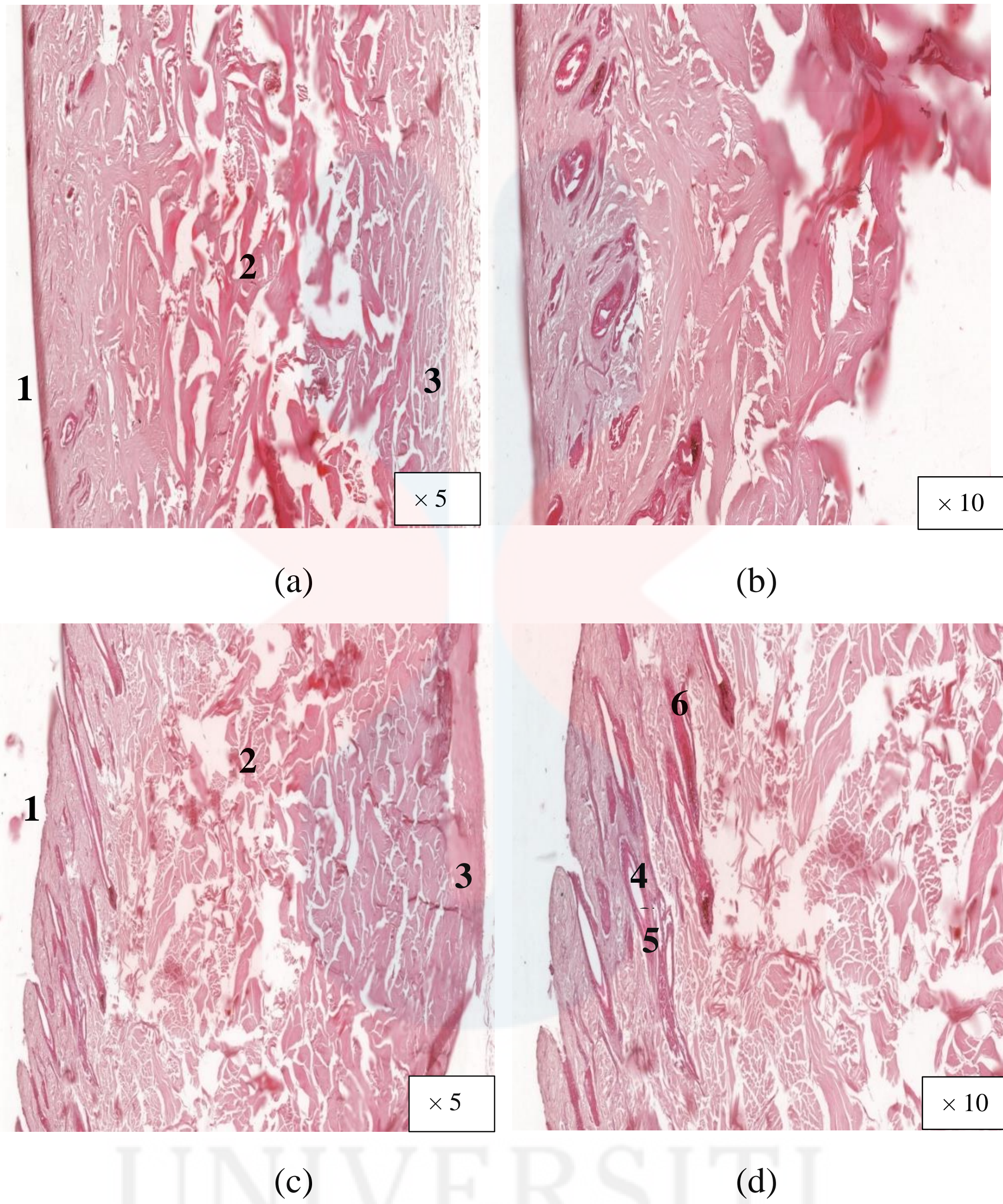

Figure 4.7**:** Histology analysis using single enzyme sample of cowhides: (a) and (b) 2.5% of alkaline protease enzyme (c) and (d) 60% of alkaline protease enzyme

Keywords	
1. Keratinized cell	4. Hair follicle
2. Collagen Fiber	5. Sweat glands
3. Smooth muscle	6. Sebaceous gland

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4.6 Tensile strength of treated cowhides

From the results collected by scanning electron microscope in Figure 4.9, larger grain region can be observed at tight leather than loose leather. The control and the single enzyme treatment of cowhides treated can be classified as tight leather meanwhile loose leather were classified as the cowhides treated in conventional and enzyme assisted treatment based on the existence of pore. The appearance of the grain layer structured with densely packed fibrils within tight in Figure 4.9(a) and loose samples in Figure 4.9 (b) while the loose sample seems to have a region under the grain by which the larger gap consists among fibre structure. The differences of the grain-corium junction area between loose and tight can be seen with higher magnification (Wells *et al*., 2016).

The observation showed the grain-corium junction area at loose leather has larger cavities around it while there was the absence of larger cavities but comprising more uniform densely packed fibres. For determination of tear and tensile strength, the loose leather was stronger than tight leather with data tabulated in Table 4.4. Based on the result, the control treatment measured in the stress break was -0.018 N/mm^2 classified as tight leather. Meanwhile, the enzyme assisted treatment showed the highest stress break by 8.311 N/mm^2 classified as loose leather. This is because the looser packing arrangement has a larger gap structure so they are not held together while the grain structure at the tight sample come apart slightly from the corium. Break scale test of loose leather resulted in the separation of the two layers with the appearance of the wrinkled (Wells *et al*., 2016).

The strength of leather act on collagen materials known as fibril orientation resulting in strong collagen fibrils while the bonding strength appears between fibrils is weak. The characteristics of strong leather were consistent align fibrils in the plane

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of leather along with the direction of stress in a tensile test. The loose leather affects by the connection of fibrils while too align resulted in the fibrils impotent to keep connections between each other and the gap will form. Hence, when the collagen fibres are aligned, the optimal leather structure exists give sufficient strength. In other hands, not so aligned collagen fibre structure able to form looseness by the interconnecting layers cooperated and gaps. The structural leather defect due to the appearance of looseness by which poor connectivity between layers of collagen fibres close to the grain-corium boundary of the leather. The loose packing arrangement effect by the lateral connections between fibre layers when the higher alignment of the fibres in loose. Based on the experimental of break test, when the loose leather is folded, the appearance of the grain and corium layers become separated and there is wrinkled exist on the surface giving poor break score (Basil-Jones *et al*., 2012).

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Table 4.3: The tensile strength of the cowhides

Treatment	Agents	Stress Break (N/mm^2)	Strain Yield (%)	Elongation Yield (mm)	Elongation Break (mm)	Youngs Modulus (N/mm^2)
Control	-	-0.018	1.206	0.796	12.714	344.966
Conventional	2% Na_2S + 5% CaO	0.103	1.685	1.112	34.437	9.763
Enzyme assisted	5% CaO + 2.5% Protease	8.311	1.272	0.840	10.714	247.134
Enzyme assisted	5% CaO + 20% Protease	7.497	1.934	1.277	9.379	197.753
Enzyme assisted	5% CaO + 40% Protease	0.246	2.391	1.578	8.885	245.634
Single enzyme	2.5% Protease	2.402	1.685	2.683	8.572	10008.481
Single enzyme	60% Protease	2.456	1.076	0.710	11.663	3780.549
Minimum		-0.018	1.076	0.710	8.572	9.763
Mean		3.50	1.95	1.28	13.77	2119.18
Maximum		8.311	4.064	2.683	34.437	344.966
Standard Deviation		3.56	1.04	0.69	9.24	3726.15

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4.7 Water Quality Analysis

4.7.1 Physical parameters of water quality

The treatment of dehairing cowhides releases wastewater effluents were analysis by physico-chemical parameters such as total dissolved solid, dissolved oxygen and pH based on the National Environment Quality Standards as shown in Table 4.5. The wastewater from the dehairing process based on the control method, single enzyme treatment, enzyme assisted treatment and conventional treatment. pH depends on oxidation-reduction potential (ORP) whereas low pH will give positive ORP while the high value of pH will give negative ORP. ORP relate with the degree of substance have potential as an oxidizing/reducing agent to another substance. The positive value of ORP reading specifies the element in the wastewater is an oxidizing agent while reducing agent by which element in the substance has the negative value of ORP (Yu *et al.*, 2014).

When there was low reading, the element categorizes as an anti-oxidizing agent. This ion exchange process occurred as the exchange of electron to achieve the stable state. When the element was lacking the electron, so the element stated as oxidizing agents contradict with element have surplus electron have the potential to donate their electrons indicate the element as reducing agents/oxidizing agents. The standard quality of water will give the positive ORP by which it referred as oxidizing agents. When the pH of water gives an alkaline reading, it referred as a reducing agent by which their electron uses to donate or to neutralize the water body from the harmful effects (Yu *et al.*, 2014).

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The control method in Table 4.4 showed pH 8.44 indicate low pH, related with positive ORP reading. After 24 hours of dehairing treatment, the positive value of ORP indicates the wastewater was safe to release into the effluent. The temperature was ranging from 24.82 °C until 26.97 °C and it changed depending on the condition of room temperature. After 24 hours of dehairing treatment, the total dissolved solids were decreased as associated with the salinity and dissolved oxygen. In single enzyme treatment, it showed the pH value was 8.38 and the final ORP reading positive. This treatment was close to the control method. Single enzyme treatment is a combination of CaO treatment with different concentration of enzyme. The ORP reading showed positive value. This means the effluent of this treatment was consider as safe because the ORP reading of standard quality of water is positive (Yu *et al*., 2014).

Conventional treatment was incubated within two hours as the hair completely removed from the outer surface of the cowhides. After 2 hours of dehairing treatment, the total dissolved was the highest correlate with the salinity and dissolved oxygen. The pH reading of the conventional treatment was highly related to negative reading and the effluents considered as unsafe to release without treatment first. The conventional treatment was the highest total dissolve solid and salinity in comparison with the lowest dissolved oxygen (Seng *et al*., 2018).

The range of total dissolved solids (TDS) within the range 0.001-15.60 mg/L and the average value of total dissolved solid 3 mg/L. The pH value ranging from 8.40 to 13.49. The wastewater from the conventional treatment and enzyme assisted treatment were exceeded the permissible limits. The lowest pH was the control method and the single enzyme treatment which was an alkaline state. This value was still in the permissible limits (Rouf *et al*., 2013). The reading of dissolved oxygen within the range of 1.31 mg/L until 1.91 mg/L and these respectively far below the standards

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value (Chowdhury *et al*., 2015). The total dissolved solid represented the total solid concentration released from the waste effluent by which consist the colloidal form and dissolved species. The increasing of total solid content based on the value of dissolved solids as the colloidal particles undergone content collision. pH also plays important role in the rate of collision of the aggregated process of the effluents (Kavitha *et al*., 2012).

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Table 4.4: The Physio-chemical characteristic of wastewater from the collected sample

Treatment	Agents	Parameters						
		Temperature (°C)	TDS (mg/L)	Salinity (%)	DO (%)	DO (mg/L)	pH	ORP (Eh)
Control	-	25.64	0.006	0.00	16.8	1.37	8.40	98.1
Conventional	2% Na_2S + 5% CaO	26.97	15.60	14.5	19.7	1.44	13.49	-196.6
Enzyme assisted	CaO(6h) + 2.5% Protease	24.87	0.368	0.27	21.0	1.74	9.67	53.3
Enzyme assisted	CaO (6h) + 20% Protease	24.82	0.001	0.00	23.0	1.91	10.21	32.4
Enzyme assisted	CaO (6h) + 40% Protease	25.15	0.377	0.28	22.8	1.88	10.39	23.2
Single Enzyme	2.5% Protease	25.92	2.883	2.36	17.6	1.41	8.19	99.7
Single Enzyme	60% Protease	25.75	1.760	1.40	19.7	1.59	8.38	88.5
NEQS (2000) Source (Kabir *et al*, 2017)		-	3500	-	-	4-6	6-9	-

Notes:

- NEQS= National of Environmental Quality Standards
- TDS=total dissolve solid
- DO=dissolve oxygen
- ORP= Oxidation Reduction Potential

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4.7.2 Chemical parameters of water quality

4.7.2.1 Chemical oxygen demand and biological oxygen demand

The value of BOD and COD are various as presented in Table 4.6. BOD of the different dehairing treatment ranging from 119.4 mg/L until 224.4 mg/L. This value was considered as high based permissible limits of ISO 9001. The high BOD can be attributed to the decomposition and mineralization of organic and inorganic compound. Higher BOD due to the of waste material loading which was hair derived from dehairing treatment from the cowhides. The COD reading various ranging from the lowest 105.33 mg/L from the control until 6294.33 mg/L from the conventional treatment (Vasudevan *et al*., 2012).

Chemical oxygen demand based on the COD test to determine the chemical oxidation of organic matter required oxygen based on the strong chemical oxidant. This COD test used to measure the effluent whether from domestic and industrial waste. Wastewater can be measured by the oxygen required for the oxidation of organic matter to produce oxygen and water. Organic compounds can be referring as oxidizing agents under acidic condition. BOD based on the 5 days of water has been processed to determine the initial oxygen when incubating at 20 °C. Based on Table 4.6, the value of BOD had been reached the permissible limits (Kavitha *et al*., 2012).

The COD determination in the measurement of oxygen consists in the water sample correspond to the content of organic matter in the water sample by strong chemical oxidant that was disposed to oxidation. COD test also can be determined the oxygen demand value stands as beneficial in stipulating the toxic condition and the

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existence of biologically resistant substances. The COD and BOD value can be related to the oxygen as depletion of dissolved oxygen occurs. As the DO content from the wastewater collected declined, there will be increased COD and BOD. The higher the value of BOD and COD contributed to the faster depletion of oxygen levels in the water samples. This will affect contamination to the water contributed to the pollution. COD test used to measure the amount of pollutant of organic material in wastewater similar with BOD but COD test was more accurately as it uses strong oxidant and time have taken to obtain the reading the also less different with the BOD test. Both COD and BOD test used to measure the organic compound in the wastewater (Woldeamanuele *et al*., 2017).

The effluent from the wastewater of dehairing treatment can be hazardous to the environment as it released the large quantities of protein and it degrades products can be measured by complex parameters such as BOD. The measure of BOD was the oxygen-consuming capacity of the water sample with the presence of organic matter such as material was dissolved in it. The conventional treatment gave higher BOD values as it considered as higher efficiency in dehairing resulted in the higher dissolving of protein leather. Although there was no harmful towards the aquatic environment by which released the organic matter from the treatment. It indirectly effects decreased of dissolved oxygen in the water body (Badar *et al*., 2016).

The quality of water referred to as the parameter by which oxygen-containing in the water and the causes of oxygen reduction can naturally lead to the serious cause of stress on the ecosystem. For instance, high BOD lead to the total deficiency of dissolved oxygen and the impact was all-natural life die within the affected area. The conventional treatment of dehairing caused the high value of COD reading. As the concentration in the tannery industry was high, it was not suitable to be released to the

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stream or lake without treatment first. The main problem of the tannery industry with the used of sodium sulphide can lead to the health effect of the workers (Badar *et al*., 2016).

The comparison between chemical treatment and the single enzyme treatment was the reduction of COD reading by 6294.33 mg/L to 139.33 mg/L. The BOD reading for both conventional and single enzyme treatment was slightly different between 199.2 mg/L and 199.5 mg/L presented in Table 4.6. These occur because the conventional treatment removed the hair outside the treatment solution and the organic matter was not put back into the solution treatment so that the BOD reading was near with single enzyme treatment dehairing. The sulphide reaction was very high as it destroyed the hair completely. The high concentration of single enzyme treatment showed high COD reading. This was due to the pH related to high concentration enzyme in alkaline condition. The reaction of the enzyme can be toxic when there was high concentration used as it was not environmentally friendly. The BOD reading of wastewater from single enzyme treatment was not too high compared with enzyme assisted treatment. This causes by the high amount of organic matter in the solution. As the concentration in enzyme assisted treatment increased, the BOD reading of wastewater treatment will also increase (Gumilar *et al*., 2017).

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Table 4.6: The reading of biological and chemical oxygen demand of waste water sample

Method	Agents	COD (mg/L)	BOD (mg/L)
Control	-	105.33	212.1
Conventional	2% Na_2S + 5% CaO	6294.33	199.2
Enzyme Assisted	CaO(6h) + 2.5% Protease	1159	214.2
Enzyme Assisted	CaO (6h) + 20% Protease	1243.67	216
Enzyme Assisted	CaO (6h) + 40% Protease	2101.67	224.4
Single Enzyme	2.5% Protease	139.33	199.5
Single Enzyme	60% Protease	2125.66	213
The Standard ISO 9001:2008 Certified Source (Patel *et al,* 2017)		100mg/L	30mg/L

- COD = chemical oxygen demand
- BOD = biological oxygen demand

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CHAPTER 5

Conclusion and Recommendation

5.1 Conclusion

The leather industry gives a bad impact on the environment. The enzyme is the alternative to replace the chemical method due to the environmental-friendly method. the enzyme used for dehairing treatment was alkaline protease derived from *Bacillus subtilis* due to its proteolytic activity and stability at high pH and temperature. The application of the protease enzyme in dehairing cowhides consist of single enzyme treatment and enzyme assisted treatment in comparison with conventional treatment by using chemical substances. The effectiveness of enzymatic dehairing was smooth fell with white appearance without disturbing the grain structure. Based on the histological analysis, enzymatically dehaired cowhides exhibits well-opened fiber structure without damaged grain structure presented by the keratinized cell. The enzymatic dehairing can produce a good quality of leather as the enzyme-free from collagenase activity. The advantages of eco-friendly dehairing treatment can reduce the application of chemical and importantly to provide a better environment.

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5.2 Recommendation

As a suggestion, the concentration of purified protease use in single enzyme treatment and enzyme assisted method need to be standardised to compare the effectiveness of dehairing treatment between both methods. For wastewater analysis obtained after dehairing treatment, this sample water must undergo filtration first to eliminate the organic matter from this treatment. Thereby, the accurate reading of chemical oxygen demand and biological oxygen demand can achieve. The property of cowhides is natural to fire resistance. The speciality of cowhides is natural to fire resistance. To improve the speciality of leather, the test of flammability to evaluate the limitation of fire resistance could be done based on the oxygen index (OI) test. The alternative to preserve cowhides rather than formaline is ethanol to prevent denaturation. It also can remove smelly odour from dehairing treatment. The concentration of formalin to prepare of cowhides for histologic analysis need to correspond with its size. When the use of higher concentration of formaline can lead to the hard surface of cowhides. So, the preparation of tissue sample cannot undergo as the tissue cannot be cut. This mistake needs to properly avoided as for further histologic analysis cannot be done.

Precaution need to take when handling the formaline as its carcinogen chemical. Avoid to smell this liquid and also untouched by uncovering hands. When the sample was taken out from the formalin solution in the preservation process, the tissue needs to be wash first. For cutting process cowhides, the sample must be cut vertical by the layer of tissue cell manually and before continuing into the tissue processor automatically. The tissue layer needs to be cut in a different section of the cow hides

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to identify the chemical reaction at this section. In the histologic analysis, the structure to undergoes this analysis need to identified properly. This analysis is different with scanning electron microscope because this analysis based on the surface of cowhides while the histologic analysis identified the tissue layer.

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APPENDIX A

Figure A.1: Raw hides

Figure A.2: Hides after cut

Figure A.3: Hides submerged in Calcium oxide

Figure A.4: Hides submerged in Conventional treatment

Figure A.5: Hair being removed

Figure A.6: Removed hair after treatment

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APPENDIX B

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Table B1: Control for protease activity determination

Control	Absorbance 596nm			Average
1	-0.326A	-0.325A	-0.326A	-0.326A
2	-0.335A	-0.337A	-0.147A	-0.273A
3	-0.340A	-0.340A	-0.340A	-0.340A
Total average				-0.313A

Table B2: Sample for protease activity determination

Sample	Absorbance 595nm			Average
1	0.331A	0.329A	0.331A	0.3310A
2	0.386A	0.387A	0.360A	0.378A
3	0.321A	0.212A	0.211A	0.248A
Total average				0.319A

Calculation:

$$= \frac{\Delta\,\text{Sample} - \Delta\text{Control}}{0.001 \times 30 \times 0.1\text{mL}}$$

$$= \frac{0.319\text{A} - (-0.313\text{A})}{0.001 \times 30 \times 0.1\text{mL}}$$

$$= 210.67\text{U/ml}$$

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Table B3: Standard curve preparation

Log $_{10}$	BSA concentration (µg/ml)	Tris- HCl Buffer (µl)	Bradford Working Buffer (µl)	Absorbance			Average
				Reading 1	Reading 2	Reading 2	
0	0	1000	2500	0.600A	0.611A	0.625A	0.459A
1.13	13.62	1000	2500	0.803A	0.805A	0.805A	0.804A
1.5	31.25	1000	2500	0.946A	0.948A	0.947A	0.947A
1.8	62.5	1000	2500	1.150A	1.167A	1.167A	1.161A
2.1	125	1000	2500	1.357A	1.373A	1.376A	1.369A
2.4	250	1000	2500	1.540A	1.549A	1.566A	1.552A
2.7	500	1000	2500	1.692A	1.698A	1.698A	1.696A
3	1000	1000	2500	1.851A	1.848A	1.845A	1.848

Calculation:

$y = 0.4873x - 0.1207$

$R^2 = 0.9711$

$y = 0.256A$

$0.256A = 0.4873\,x - 0.1207$

$$X = \frac{(0.256 + 0.1207)}{0.4873}\ \text{mg}$$

$= 0.773$ mg/ml

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Table B4: Protease activity, protein and its specific activity

Method	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)
Heat treatment	150 ml	150 ml × 210.67 U/ml = 31600.5 U	150 ml × 0.773 mg/ml = 115.95 mg	$\frac{31600.5\ \text{U}}{115.95\ \text{mg}}$ = 272.54 U/mg

Notes:

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

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

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

Biological Oxygen Demand

Formula BOD:

$$= \frac{(\text{Average of } DO_1 - \text{Average of } DO_5)}{\text{Volume of sample / Total volume}}$$

1) Control

$$\frac{(7.53 - 0.46)}{10/300} = 212.1 \text{ mg/L}$$

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2) Conventional treatment (Na_2S + CaO)

$$\frac{(6.89 - 0.25)}{10/300} = 199.2 \text{ mg/L}$$

3) Enzyme assisted treatment (CaO(6h) + 2.5 % Protease)

$$\frac{(7.47 - 0.33)}{10/300} = 214.2 \text{ mg/L}$$

4) Enzyme assisted treatment (CaO(6h) + 20 % Protease)

$$\frac{(7.57 - 0.37)}{10/300} = 216 \text{ mg/L}$$

5) Enzyme assisted treatment (CaO(6h) + 40 % Protease)

$$\frac{(7.78 - 0.3)}{10/300} = 224.4 \text{ mg/l}$$

6) Single enzyme treatment (2.5% Protease)

$$\frac{(7.09 - 0.39)}{10/300} = 199.5 \text{ mg/L}$$

7) Single enzyme treatment (60% Protease)

$$\frac{(7.47 - 0.37)}{10/300} = 2.3 \text{ mg/L}$$

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