ADDIS ABABA UNIVERSITY

ADDIS ABABA INSTITUTE OF TECHNOLOGY

SCHOOL OF CHEMICAL AND BIO ENGINEERING

STUDIES ON DEVELOPMENT OF CLEANER BEAMHOUSE PROCESS, EXTRACTION AND CHARACTERIZATION OF COLLAGEN FROM FLESHING WASTES

BY
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A Thesis Submitted to the School of Chemical and Bio Engineering, Addis Ababa Institute Technology (AAiT), Addis Ababa University Presented in Partial Fulfilment of the Requirements for the Master of Science in Chemical Engineering (Leather Technology)

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This is to certify that the MSc thesis prepared by Demissie Tsegaw, entitled: Studies on Development of Cleaner Beamhouse Process, Extraction and Characterization of Collagen from Fleshing Wastes and submitted in partial fulfilment of the requirements for the degree of master of science in chemical Engineering (Leather Technology) compiles with the regulations of the university and meets the accepted standards with respect to originality and quality

Signed by the Examining Committee

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Abstract

The study presents the development of cleaner beamhouse processes to facilitate the extraction of collagen from fleshing waste. 0.8% offer level of alkali protease in soaking process is observed to be optimum for opening up the fibre texture of the hide, and to provide good yield of fleshing waste. In addition to this, complete removal of hair is still an issue of concern for unhairing using enzymes. CLRI had developed a novel protease for unhairing and the effectiveness of this protease for unhairing in the presence of lipase as co-enzymes was study and presented in this thesis. Experimental results indicated that 1% lipase followed by 4% protease treatment improved the unhairing efficacy. The enzymatically dehaired hide was further analyse by SEM, and the complete removal of hair in the experimental process (1% lipase followed by 4% protease) is further established. The reduction in pollution load parameters viz., BOD, COD, TOC, TDS and TSS is 89.375%, 88.57%, 85.79%, 47.37% and 48.57%, respectively with reference to conventional unhairing process. The leathers processed from optimized soaking and unhairing system showed comparable strength characteristics with reference to leather processed with chemical unhairing system. Hence a total bio-based beamhouse processing in leather manufacture was feasible. Fleshing from conventional lime and green fleshing wastes were used for the extraction of collagen, which is high value protein that can be used for a wide range of applications viz., tissue engineering, cosmetic, food, and wound healing and pharmacy application. Extraction of collagen had been carried out using acetic acid solubilisation and it is observed that collagen yield obtained from green fleshing (96.53%) is higher than lime fleshing (84.87%). FTIR, SDS-PAGE, CD spectra and thermal melting experiments are used for the characterization of collagen. It was confirmed that the extracted collagen from lime/green fleshing wastes were type I and also found to have similar properties. This study clearly establishes the development of cleaner beamhouse process and effective recovery of collagen from fleshing waste.

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Acronyms

HP- Hydroxyproline

CD – Circular Dichroism Spectra

SDS-PAGE- Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

FTIR- Fourier Transform Infrared Spectroscopy

CHNS- Carbon Hydrogen Nitrogen and Sulphur Analysis

LIDI- Leather Industry Development Institute

CLRI- Central Leather Research Institute

PDAB - P-dimethyl Amino Benz Aldehyde

M- Protein marker

Lc- Liming collagen

Gc- Green collagen

C- Control

E- Experiment

PPII- Poly Proline II

SEM - Scanning Electronic Microscope

BOD – Biological Oxygen Demand

COD – Chemical Oxygen Demand

TOC – Total Organic Carbon

TDS – Total Dissolved Solid

TSS – Total Suspended Solid

Es – Experiment on soaking process

Eu – Experiment on unhairing

CCD – Central Composite Design

RSM – Response Surface Method

CHAPTER ONE

1. Introduction

1.1 Background of the Study

Leather industries have a great role in economic growth and account as a cornerstone for one country development. It produces different types of article. The raw material of the leather industries is hides and skins. Raw hides and skins were obtained from sluagthery house which supllied meat to meat industry and used as raw material sources for leather industry (Langmaier, Kolomaznik, Sukop & Mladek , 1999). Hides are the body cover of big animals such as cow, ox, and buffalos whereas skins are the outer shell of small animals like sheep, goat, and pig. Hides and skins have found in domestically animals which regularly supply for leather sector. Hence leather industries connect the rural farmer to the fashion world. The leather industry is a labor intensive and critical area for the inclusive growth of several developing economies. However leather industry especially the tanning industry is associated with a significant social stigma given the pollution emanated from them.

In tannery, a series of unit processes/operations are involved in the leather manufacturing. They are broadly classified as beamhouse, tanning, post tanning and finishing processes. In conventional beamhouse process especially liming/unhairing contribute to high pollution load due to the use of sodium sulphide and lime. Sodium sulphide is used as a sharpening agent to break the cysteine disulphide bridge of the hair keratin leading to pulping of hair (Ramasami & Prasad, 1991). The tanning industry is water intensive and consumes about 35-40 liters of water per Kg of skins/hides processed. Therefore an enormous amount of liquid and solid wastes are produced (Kanagaraj, Senthilvelan, Panda & Kavitha , 2015) . One metric ton of wet salted hides/skins yield about 180kg of end product, along with about 250kg of chromium-containing solid waste, about 350kg of chromium-free waste and about 100kg lost in wastewater. Due to large amount of wastes, tanneries face a problem to properly manage and treat (Alexander, Donohue, Cory & Sykes , 1992).

Tanning industries process generate different quantity of waste which is polluted the environment. About one metric ton of raw hides and skins processed, approximately 850kg is generated as solid wastes in leather sector. Only 180kg of the raw material is change to leather. Solid waste generated from tanning industries are raw hide/skins trimming, lime trimming, fleshing waste, chrome shaving, crust trimming and buffing dust (Kanagaraj, Velappan, Babu & Sadulla, 2006). Composition of solid waste generate from tanneries: ~20% fleshing, 25% (chrome shaving, chrome splits and buffing dust), ~40% is primary and secondary sludge (from ETP), ~10% of skin trimmings, ~5% of hair waste. Solid wastes cover 75-80% of beamhouse, 20-23% of tanning and 1-2% finishing. It can be convert hydrolysed and usable product (Ramamurthy & Mahendrakumar, 1989). Amongst the solid waste, huge amount of fleshing waste is disposed into the environment. Worldwide, approximately 800,000 metric tons of non-chrome waste solid waste, huge amount of waste which is fleshing waste, is processed annually (Li., Yang & Li, 2008). Extensive use of sodium sulphide bears unfavourable consequences on environment and also the efficacy of effluent treatment plants. The use of sodium sulphide is the only potential source for the formation of the toxic hydrogen sulphide gas. The H_2S gas had caused several fatal accidents in tanneries all over the world. Hence there is an imminent need to look out for alternative unhairing system.

Bio-based process is an alternative promising method to decrease the pollution load in pretanning processes. In recent years, there had been several attempts to develop a bio-based pretanning process using enzymes (Kamini, Hemachander, Mala & Puvanakrishnan, 1999). In such attempts significant reduction in pollution load has been observed (Dayanandan, Kanagaraj, Sounderraj, Govindaraju & Rajkumar, 2003; Thanikaivelan, Rao, Nair & Ramasami, 2004). Though options of enzymatic soaking and unhairing exist, commercial practice of enzymatic unhairing is still eluding due to the inefficiency that occurs either in the stringent process controls or the efficacy of enzyme used for the soaking and unhairing. Hence there is an imminent need to develop enzymatic assist and optimize them for enzymatic soaking and unhairing for effective hair removal. We hypothesized that fibber opening facilitate soaking process and removal of fatty materials during unhairing process was augur well for the efficacy of unhairing. Hence we chose to use a lipase followed by protease during the unhairing process and determine its effect for the removal of hair. In addition to this, green process wastes contain proteins and fats which are also less contaminated when we compare to conventional process wastes. Fleshing wastes is one type of solid wastes that contain useful chemical compound which is made major organic compound that obtained from beamhouse process.

Fleshing wastes contain different types of organic compound such as proteins, lipids and some inorganic compounds. One of the essential basic proteins is collagen. Within animal hides and skins, Type-I is the most dominant from other types of collagen and also a major structural component of tendon, bone and connective tissue (Ricard-Blum & Ruggiero, 2005). Until now, 29 types of collagens have been segregated (named types I-XXIX) of which type-I is the most abundant (Shoulders & Raines, 2009; Matmaroh, Benjakul, Prodpran, Encarnacion, & Kishimura, 2011; Chi, et al, 2014). The known structural motif of all collagens is triple helix structure in which each of the three parallel polypeptide strands with left-handed poly proline (PPII) II-type helical conformation coil to surrounding of other to form right-handed triple helix. The tight packing of PPII helices within the triple helix mandates that every third residue be glycine (Gly), resulting in a repeating X-Y- Gly sequence, where X and Y can be any amino acids. This sequence of pattern is common in all types of collagen. Even though at certain locations within the triple-helical domain of non-fibril collagen (Brazel, et al, 1987), X and Y positions of collagen are containing proline and hydroxyproline respectively. Pro-Hyp-Gly is the triplet (10.5%) in collagen (Ramshaw, et al, 1998). In some countries, fleshing wastes can be taken for raw materials for the viable products such as glue, collagen peptides, industrial gelatin, feed and fertilizers (Ollé, Sorolla, Casas & Bacardit, 2013).

Recently, the researchers focused on bioprocess which is not contaminated with polluted chemicals and collagens are extracted from solid waste that generated from tannery wastes. Method for extraction is acid solubilisation by acetic acid (Tanaka, Avigad, Eikenberry & Brodsky, 1988). Even though collagen present in the fleshing waste, Collagen proteins have a different viable application such as biomaterials for Medicine, a component of cosmetic, part tanning agent, making gelatine, tissue engineering, nutrition product, ability of inducing coagulation of blood platelets, food and agriculture, highly tensile strength, effectiveness cell differentiation, effectiveness in wound healing (Lee, Singla & Lee, 2001) , to prepare syntan for tanning/post-tanning and used as raw material for leather industry. Cheapest cost biomaterial has been obtained from fleshing waste.

Therefore, fleshing waste could be an option to make a viable product apart from avoiding its impact on to the environment. Hence, taking the overall concerns, the objective of the current study was design to develop cleaner beamhouse process and effective utilization of fleshing waste which not only reduces pollution but also enhances economic value and presents a roadmap for the commercialization of refine products from fleshing waste.

1.2 Statement of the problem

Conventional beamhouse process urgently needs to find solutions for reduction pollution; if it has to sustain in an environment of increasing pollution control regulations. Bioprocess is an alternative promising method to minimize the pollution load in beamhouse processes. There had been several attempts to develop a bio-based beamhouse process using enzymes. In such attempts significant reduction in pollution load has been observed (Dayanandan, Kanagaraj, Sounderraj, Govindaraju & Rajkumar, 2003; Thanikaivelan, Rao, Nair & Ramasami, 2004). However, options of enzymatic soaking and unhairing exist, commercial practice of enzymatic unhairing is still eluding due to the inefficiency of unhairing because of the requirement of stringent process controls. Hence there is an imminent need to develop and optimize enzymatic assist soaking/unhairing for effective fibber opening, hair removal and utilize fleshing wastes. We hypothesized that alkali protease enhances soaking process and facilitate green fleshing followed by the use of lipase and protease for an effective unhairing. Particularly for conventional method of unhairing, diffusion of protease at the site of hair bulb to disintegrate proteoglycan could be potentially hindered by the presence of fatty substances. Hence we hypothesis the use of lipase followed by protease could enhance the efficacy of unharing. One of the effective ways to handle pollution issue is to adopt as cleaner beamhouse process (green process) and utilize the fleshing waste as resource that can be generate revenue.

Removal of loose fibrous adipose from hides and skins is undesirable for leather and an imperative process in leather manufacture. Fleshing wastes generated from tanneries contribute to more than 60% of the total non-chrome containing solid wastes. The major drawback of conventional beamhouse process is the generation of fleshing with the presence of lime, which consequently is a hurdle for easy utilization of fleshing waste. It was easy to utilize the constituents if the fleshing wastes are devoid of lime. Hence green fleshing was an effective option for the recovery of valuable collagenous constituent present in the fleshing. The estimation of fleshing wastes produced 26 ton/day through Ethiopian tanning industries; a little amount of fleshing wastes was used for glue production whereas large amount fleshing wastes are disposed to the environment. We hypothesized that extract high valuable by-product from fleshing wastes which we follow a procedure is solubilisation using acetic acid

(Tanaka, Avigad, Eikenberry & Brodsky, 1988). Extraction of collagen from fleshing waste is a new opportunity that was lead to high value realization. Collagen can be utilised for food, tissue engineering, wound healing, cosmetic and pharmacy applications.

1.3 Objective of the study

1.3.1 General objective

The general objective of this study is to development of cleaner beamhouse process, extraction and characterize of highly purifed valuable by-products of collagen from fleshing wastes which was lead to sustainable development in tanning industies.

1.3.2 Specific objectives

The specific aims of the present study is :

- To develop and optimise enzymatic (soaking and unhairing) process for optimal yield of green fleshing
- To assess the pollution load from optimized enzymatic unhairing process and compare its effectiveness with liming processes
- To characterize green and lime fleshing wastes using proximate analysis method
- To extract and characterize collagen, a product of high value from green and lime fleshing wastes

1.4 Scope of the Studies

This study is focus on the development of cleaner beamhouse process especially focuses on enzymatic assisted soaking process and enzymatic unhairing process with effective utilization of fleshing wastes. Optimize enzymatic assisted soaking process utilizes alkali protease to minimize the rehydration time and provide non-contaminated solid wastes which called as green fleshing wastes. One of the activities is extract collagen from green fleshing and compared with reference of the extract collagen from lime fleshing wastes. Optimize enzymatic assisted unhairing process using lipase followed by 4% of protease. Finally, the study targets development of cleaner beamhouse, utilize of fleshing waste, which not only reduces pollution but also enhances economic value and presents a roadmap for the commercialization of refine products from fleshing waste.

1.5 Significance of the Studies

In Ethiopian context, leather manufacturing process utilize different chemical that polluted the environment. Among them, Conventional beamhouse process contributes more than 60% of the pollution load in leather manufacture process in the form of solid, liquid and gases where disposed to the environment. In order to reduce the pollution load from the environmental, tanning industry look new technology that environmental friendly to be achieve specific standard of requirement. Currently, the awareness of environmental issue increase time to time. Due to this reason, this work looks another option which is free from toxic chemicals that utilize for leather manufacturing. Bio-process is one of the effective strategies to address the promising of full fill the requirement of environmental regulation by reduction of pollution. This study presents a roadmap for cleaner beamhouse process, waste management and revenue generation by recovering valuable products from the solid wastes, which otherwise is dump.

1.6 Framework of the Studies

The thesis is organized into five sections. In the first section: optimized enzyme assist soaking process based on the assessment of yielding and hydroxyl proline content of green fleshing with benchmarking the lime fleshing wastes were discussed. Second section: preparation and quantification of green fleshing from optimized enzymatic assist soaking compared to deliming fleshing wastes presented in section two. Third section: optimize enzymatic unhairing based on enzyme activity with checking parameter of efficacy such as index analysis (subjectively), SEM and pollution load analysis. Fourth; extract collagen from green and lime fleshing wastes. Fifth: Characterize the collagens that obtain from green/lime fleshing wastes.

CHAPTER TWO

2. Literature Review

2.1 Beamhouse Process

Leather manufacturing is the process of transforming skins/hides into leather by sequential mechanical and chemical steps. In this process, skins/hides pass through four consecutive stages which are beamhouse process, tanning process, post tanning and finishing. Beamhouse process is the first steps of operation section in leather processes which used for devoid unnecessary material from raw hides/skins. It contains serious operation viz., soaking, unhairing, liming, deliming, bating and pickling process. Hence our research concentrated on selected operation. Among of them, soaking and unhairing were selected for study purpose which provides literature survey as follows:

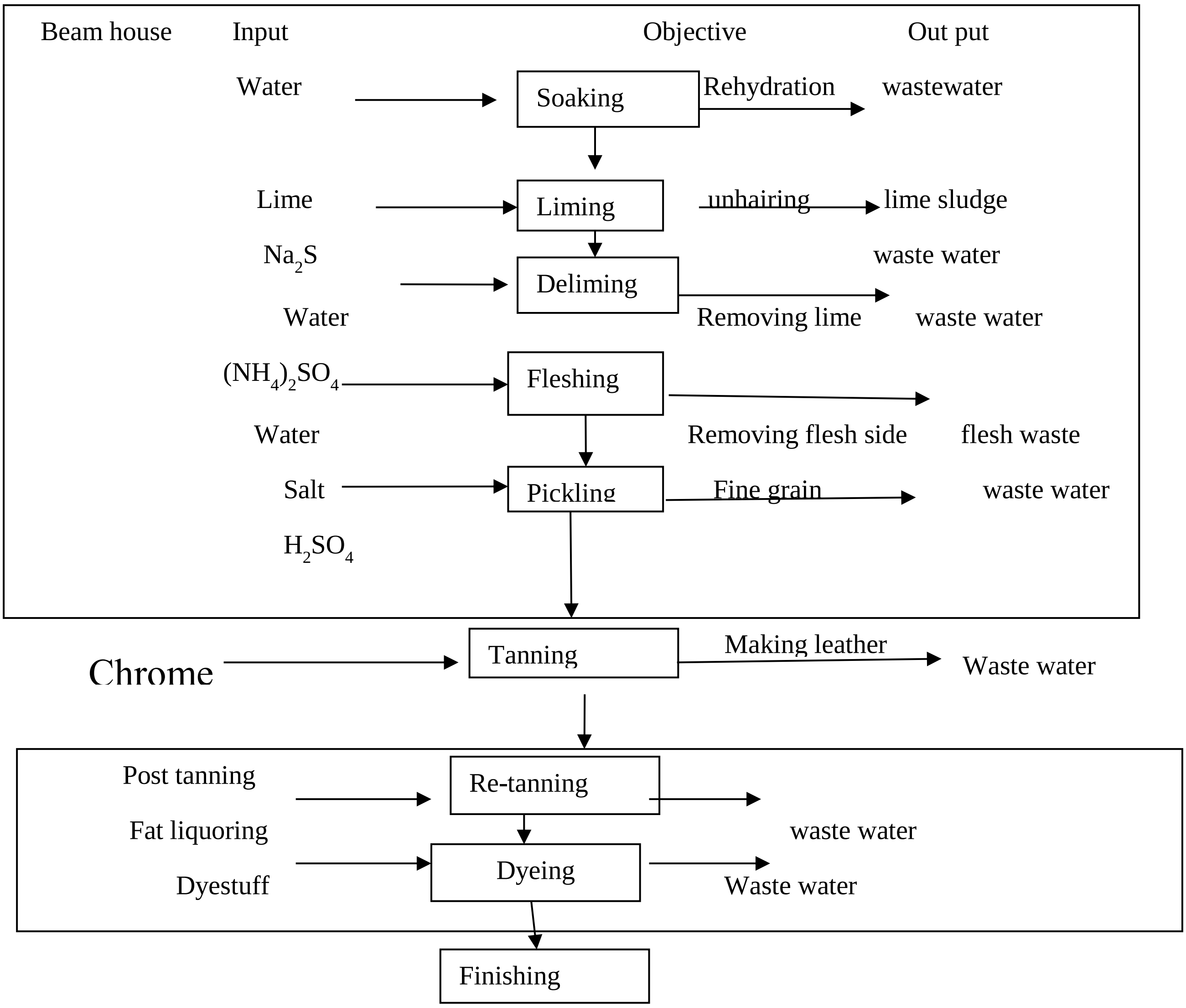

Figure 1: Mechanical and chemical application unit operation of leather process

2.1.1 Soaking Process

Soaking is the primary stage and basic requirement in leather processing to precede the next process. There are two main objectives for the soaking process (Thanikaivelan, Rao, Nair & Ramasami, 2005):

- Rehydration of hides and skins to bring the original condition of fresh hides or skins after flaying takes place
- Remove dirty material from hides and skins such as blood, urine, soil, dung, salt and other extraneous substances.

A soaking process also helps to avoid of non- collagenous proteins, water soluble albumins and salt-soluble globulins, constituents of the interfibrilary fluid and fibroblasts (Heidemann, 1993).

In general, soaking process is carried out into two or three stages depend on the condition of raw materials, the first or second steps is called as dirty or pre-soaking, which perform for short period of time. The last step is known as main-soaking (IPPC, 2001). The amount of water needed for soaking per tonne dried hide can be as high 20m^3 water (Ludvik & Buljan, 2000). As usual it needs 5 to 24 hours to complete the soaking process depending on the status of raw materials. Alkalis such as sodium hydroxide or sodium carbonate, wetting agent, preservatives and proteolytic enzymes are usually used as additives during soaking, and are depend on the condition of skins or hides, temperature condition affects (Heidemann, 1993; Thanikaivelan, Rao, 2005).

2.1.2 Unhairing Process

Unhairing processes are carried out to remove hair and some parts of non-structural proteins such as proteoglycans from hides and skins. The major structural protein of hair is keratin. Keratin mainly contain of amino acid known as Cystine. Reduction or oxidation can break down the disulphide bonds (S-S) of Cystine causing keratin degradation, without affect collagen. Sodium sulphide is used as sharpening agent to break the cysteine disulphide bridge of the hair keratin leading to the pulping of hair (Ramasami & Prasad, 1991). In pretanning processes particularly liming/unhairing operations contributes significantly to the pollution load generated. The conventional dehairing process is carried out with the use of lime and sodium sulphide, which contributes to high load of BOD, COD, sulphide and solid wastes. Extensive use of sodium sulphide bears unfavourable consequences on environment and the efficacy of effluent treatment plants. The use of sodium sulphide is the only potential source for the formation of the toxic hydrogen sulphide gas. The H_2S gas had caused several fatal accidents in tanneries all over the world. Hence there is imminent need to look out for alternative unhairing system.

2.1.3 Cleaner Beamhouse Process

Cleaner beamhouse process (bio-process) is an alternative method to minimize the pollution load in pretanning processes. In recent years, there had been several attempts to develop a bio-based pretanning process using enzymes Kamini, Hemachander, Mala & Puvanakrishnan, 1999). In such attempts significant reduction in pollution load has been observed (Dayanandan, Kanagaraj, Sounderraj, Govindaraju & Rajkumar, 2003; Thanikaivelan, Rao, Nair & Ramasami, 2004). In such attempts significant reduction in pollution load has been observed. However options of enzymatic for soaking and unhairing process were exist, industrial practice of enzymatic unhairing and soaking still eluding due to the inefficiency that occurs either in the stringent process controls or the efficacy of enzyme used for the unhairing. Hence there is an imminent need to develop protease assist and optimize them for enzymatic unhairing for effective hair removal. Earlier literature clearly states that the use of lipase remove the fat from the flesh side of skin/ hide matrix, and hence the use of lipase in our studies is well justified. We hypothesize that the removal of fatty materials during unhairing process was help well for the efficacy of unhairing. Hence we chose to use a lipase during the unhairing process and determine its effect for the removal of hair.

2.2 Leather Industries Solid Wastes

Leather industries can be catagorize as one of the polluting industries, and there are concerns that leather-making activity which affects the nature of the environment. The global production of about 24 billion meter square of leather by 2005 presents a considerable challenge to the industry considering the polluted character of some chemicals used in leather processing. The tannery effluents are characterized by high contents of dissolved, suspended organic and inorganic solids giving rise to high oxygen demand, toxic substance and chromium metal ions.

Moreover, solid waste generates from tanning industries which contain raw hide/skin trimming, keratin waste, lime pelt trimming, fleshing waste, chrome shaving wastes, buffing and finishing trimming. The major components of solid wastes are a fatty acid, protein and other chemicals, which are present in the waste generated from the tannery and not utilized correctly, it posed hazardous pollution problem to the environment (McLaughlin & Theis, 1945).

From 1000 kg of rawhide, nearly 850 kg is generated as solid wastes in leather processing, and the remaining of raw material is changed into leather. The major component of solid waste are described as follows: fleshing 50-60%, (chrome shaving, chrome splits and buffing dust together account for 35-40%), skin trimming 5-7% and hair 2-5%. 80 % of solid waste covered by beamhouse process, 19 % of solid waste contain by tanning waste and the rest 1 % is finishing solid waste (Ramamurthy & Mahendrakumar, 1989).

2.2.1 Chrome Tanned Solid Wastes

During tanning process take places, solid waste generates from it. Such as chrome shaving, tan trimming, and wet blue splits. These wastes have been given a useful product like glue, gelatine, protein and reconstituted collagen. The above products can be manufactured by using H_2O_2 to bring a remarkable degree of maceration, then grinding and extraction to yield by-product 95% (Kumaraguru, Sastry & Rose, 1998).

Chrome shaving waste hydrolysed using such chemicals magnesium oxide alone or magnesium mix with calcium hydroxide, sodium hydroxide or sodium carbonate to increase solubility efficiency, and also reduce the amount of enzyme needed and thus making the treatment more cost effective. Tan trimmings, shavings, buffing dust and trimming from the crust and finished leather have been converted into glue, food, and fertilizers (Taylor, Diefendorf & Na, 1990). Gels, adhesives product and high grade of gelable protein fraction have been obtained from chrome shavings under mildly alkaline conditions. However, characteristics of the products depend on the composition of raw solid chrome shaving waste and the specific treatment conditions (Prentiss & Prasad, 1981). Chromium cake is obtained from chrome shavings have been treated with the proteolytic enzyme at 60-65°C in the presence of lime (5-6%), and the protein has been separated by filtration. The protein hydrolysate (<4.5 ppm chromium) is potential as a feed, fertilizer, or as an additive in the cosmetic industry (Lollar, 1981).

2.2.2 Keratin Waste and Utilization

Keratin is solid wastes that generate from tanning industries, contain protein and present in the hair, nail, corn and hoofs of animals, which is rich in amino acid, cysteine. Keratin can be used for preparation of hydrolysate by concentrated NaOH or HCl, and it helps to enhance chrome tanning exhausted and re-chroming bath (Chakraborty & Sarkar, 1998).

2.2.3 Fleshing Wastes

Fleshing waste is one of solid waste that generate from beamhouse process which contain protein and fat. It is carried out to avoid excessive organic materials (fats & tissues) and to facilitate the penetration of chemicals into the hides. Fleshing takes place at different stages such as after soaking, after unhairing and pickling. The fleshing operation may carry out before unhairing is known as green fleshing and lime fleshing if the operation is carried out after liming (IPPC, 2001). Green fleshing reduces the chemical uptake during liming and helps in achieving a uniform liming effect (Thanikaivelan & Rao, 2005) to improve leather quality (Ludvik & Buljan, 2000).

The purpose of limed fleshings used to stabilizing the delimed pelt, which prepared active site of protein on the pelt. This pelt stabilizes by a product which is developed from amino acid change to aldehyde in the form of tanning agent. Reactive protein has been treated to the delimed pelt and tanned (Kanagaraj, et al., 2001).

Huge amount of solid waste produced in beamhouse operations which are covered by fleshing waste, and it is hydrolyzed using a pancreatic enzyme. The proteolytic activity of pancreatic homogenate showed a 6-fold increase in proteolysis against the control at the end of 7 days. Total protein content, collagen and free fatty acids in hydrolysate supernatant were 80, 10.64 and 72.86 mg/ml, respectively. The pH of the enzyme is 8.5. The physical property of hydrolysis is entirely liquefaction, and fleshing was dried. The hydrolysate is a source of protein and could be used as a feed formulation by mixing with other feed ingredients (Cot, Aramon, Baucells, Lacort & Roura, 1986). The major components of fleshing wastes were protein and fat.

2.3 Collagen

Collagen is a major structural protein of mammals which is present in the skin, bone, cartilage and tendon. The name ‘collagen’ is derived from Greek word ‘glue producer.' Collagen heated by high temperature which gives a product called glue.

2.3.1 Chemical Component and Structure of Collagen

Collagens are the most abundant in animal taxonomy and mainly found in the extracellular matrix. Collagen is generic term form of proteins a characteristic triple helix of three polypeptide chains and all members of the collagen family in the shape of supramolecular structures in the extracellular matrix even if the size, function and tissue distribution vary considerable, have 26 genetically different types of collagen are available (Brown, Thompson & Taylor, 1994). Now a day, there are 29 types of collagen in animals.

Collagen made up from three α chain which coils into a left-handed helix with about 18 amino acids per turn. The helical region of collagen is highly conserved among species. The amino acid within each chain displaced a distance of 2.91A^o and twist of 108A^o which contain the residues per turn 3.27A^o and the distance between each glycine is 8.7A^o.

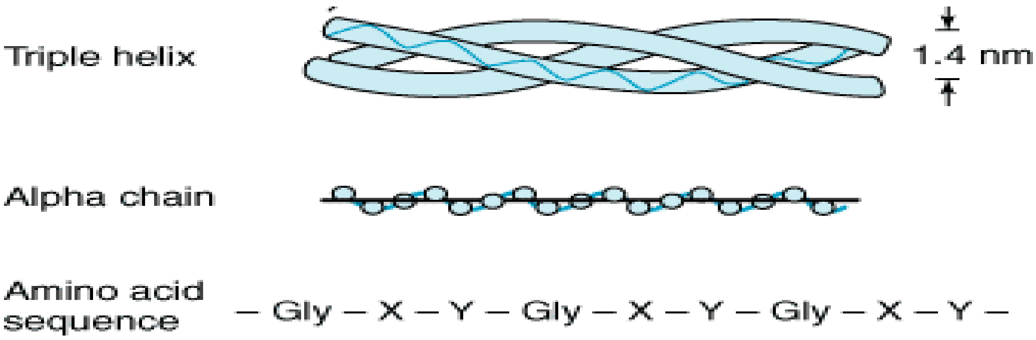

Figure 2: Molecular structure of a collagen triple helix in type I collagen molecule

Type I collagen has a component of triple helix and connected by nonhelical telopeptides. The length of collagen is 300nm and diameter is 1.5nm. The collagen contains a high amount of proline, hydroxyproline and hydroxylysine. Glycine is available every third position which responsible for triple helical folding. Triple helix has a repeat sequence of Gly-X-Y in which x and y can be any amino acid, but they are frequently occupied by the amino acids proline (about 100 of the x positions) and hydroxyproline (about 100 of the y positions). Each polypeptide chain has 1052 amino acid which Gly-x-y triplets occur 338 times. The most common triplet is Gly-Pro-hyp, which contributes to 12 % of the total triplets, Gly-Pro-Ala contributes to 9 %, and Gly-Ala-Pro is 6%. Proline and hydroxyproline have a significant role on the stability of triple helix. Due to rigid, cyclic amino acid, they limit the rotation of the polypeptide backbone. The side chain that attaches to amino acid gives rise to hydrophobic and polar cluster in collagen (Kuhn, 1986).

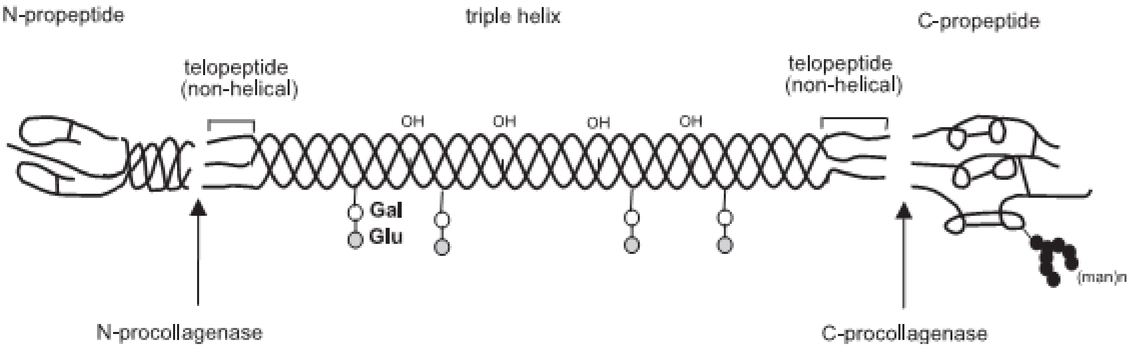

Figure 3: The structure of febrile collagens with the various subdomains as well as the cleavage sites for N- and C- pro-collagenases (shown is the type I collagen molecule).

Five collagens arranged them in the form of longitudinally with an overlap of approximately one quarter which forms micro-fibril of diameter 3.6nm. This method is called quarter-stagger combined with the gap between successive macromolecules which is responsible for the characteristic 64nm banding pattern observed in the electron microscope and by x-ray diffraction (Ramachandran & Kartha, 1955)

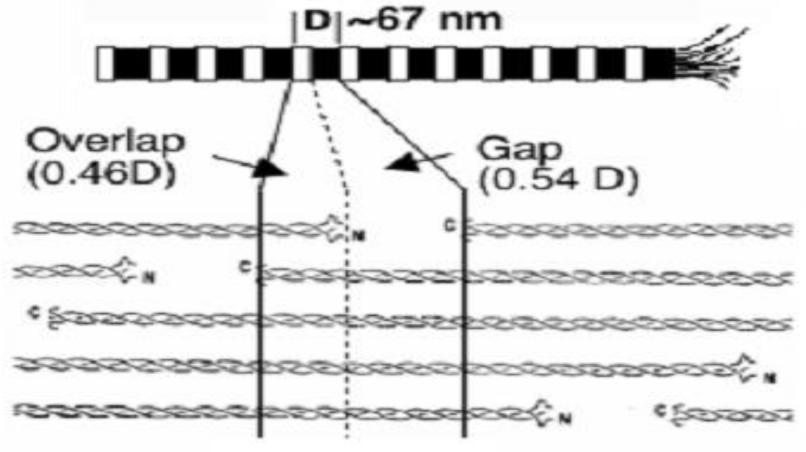

Figure 4: Quarter staggered structure of collagen with D-periodicity

2.3.2 Category of Collagen Types

There are 29 different kinds of collagen. Almost all collagens can be category according to the nature of their aggregate forms. The different kinds of collagen are originate from the assembly of core polypeptide chains, various length of the helix, according to a different function of helix and differences in the termination of helix component. Collagen can be classified into four divisions due to the nature of fibrils arrangement. These are fibrous collagens, network collagens, filamentous collagens, and fibril-associated collagens (Baer, Cassidy & Hiltner, 1991).

Fibrous collagens have 300nm length and rod-like molecules which are self arrangeable in a parallel, quarter-staggered end overlap assemble to form fibril bundle possessing a characteristic band pattern and have 67nm of identifiable periodicity in the electron microscope. Under this type of collagen present are Types I, II, III and some minor collagens V and XI (Eyre & Wu, 2005)

Network collagens are not rigid due to irregularities in the Gly-x-y sequence which have 400nm lengths. The structures are in the form of 'chicken-wire' system which used for the framework of basement membranes of vertebrates and invertebrates. Such as Types IV, VIII and X are classified under network collagens. The structure of filamentous collagens look like loosely packed, and 100nm length which is repeated occur to form by end-to-end alignment of tetramers. Example Type VI (Eyre & Wu, 2005). Fibril-associated collagens are formed by associated with other fiber forming collagens. Such as type IX collagen decorates the surface of the Type II collagen fiber.

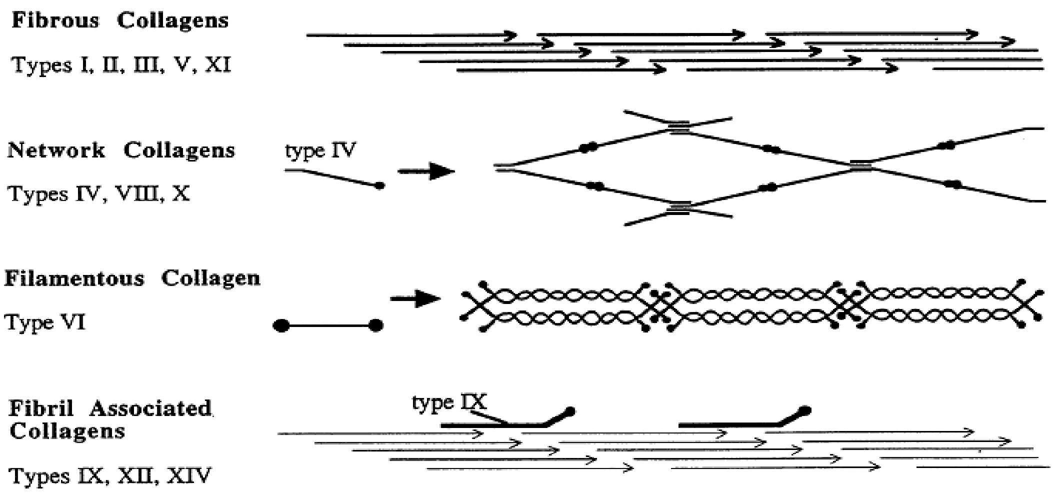

Figure 5: Diagrammatic representation of the aggregated forms of the collagen superfamily of proteins.

2.3.3 Contribution of Hydrogen Bonding in Collagen

Hydrogen bonding plays a great role in the stability of triple helix. The hydrogen atom link covalently with an electronegativity atom (such as N or O), and weakly bind another electronegative atom. The common types bond that found in protein mainly collagen is hydrogen bond which is NH…O, OH….O, and NH…N. Among this bonds NH….OC is high and occurs within a sequence of Gly-x-y triplet units (hydrogen bond between the NH of Gly in one chain and the C=O of the residue in the X position of the neighbouring chain) (Ramachandran & Sasisekharan, 1968)

2.3.4 Methods of Collagen Extraction

Collagens are proteins that found in the extracellular matrix. It exists with another component of in animals' body. So in order to separate from another part, it needs a protocol. The preparation of collagen protein includes a selection of raw materials, development of process, extraction, separation, purification and characterization. There are five kinds of collagen extraction methods; those are salting out method, alkali method, acid method, and enzyme and combination method.

A. Salting Out Method

One of collagen properties is soluble in salt solution. The salt solution which is prepared from a solution of NaCl, Tris-HCl, Phosphate or citrate. Controlling the concentration of salt in the solution is the primary condition. For example, when the concentration of sodium chloride is less than 1mol/L in the natural solution, it is suitable for the dissolution of type-I collagen, when the level bigger than 1Mol/L, it is good for the precipitate of type-II collagen (Wang, et al, 2006).

B. Alkali Method

Alkali method used to extract collagen which chemicals bind on firmly on its fiber. Such as chrome shaving and finishing leather. Collagen can be hydrolysis by the following compounds: $Ca(OH)_2$, NaOH, Na_2CO_3 and MgO which treats with alkali method which produces a collagen product and remove chrome chemicals from chrome shaving (Zhiwen, Guangyu, Zhaoguo & Xiaoyan, 2009).

C. Acidic Method

Acids with low concentration that used to extract collagen are acetic acid, citric acid, and hydrochloric acid which dissolve in the concentration 0.5mol/l with pH 2-3 range. An organic acid such as oxalic acid and citric acid which is high coordinate ability with chrome than carboxyl group. The removing of chromium is in a dissolved state under the acid condition and difficult to be completely withdrawn from the collagen. So dechroming rate under the acid method is relatively small. To get collagen protein with the acidic way is better than alkali methods.

D. Enzyme Method

The extraction method provides gives good quality of triple helix structure without damage other parts. The resulting products have been a high purity, stable physical and chemical properties. Enzymes that used to extract collagen proteins are pepsin, papain, and tryptase. Different kinds of proteins in animal tissues have an optimal temperature between 35 to 40 ^{o}c. Almost all enzymes have an optimal pH 5 to 8. For example, papain’s = 5 to 5.5; tryptase's=7.5; pepsin has a lower optimal pH between 1.5 and 2.5 (Ricard-Blum and Ruggiero 2005).

E. Combination Types of Method

The process of extraction which is lists above have not extracts completely and provides the right quality of the result collagen protein by combination methods provide a chance to minimize the risk of extraction collagen protein. Such as alkali with combine acidic methods, acidic mix with enzyme method and finally alkali combine with enzyme method.

2.3.5 Optimization

Optimization studies the effects of various factors on the process of an experiment to determine the optimized conditions for optimal results. An appropriate experimental design is therefore required for complex process where the targeted response is influenced by many factors. The efficiency of the collagen extraction process from fleshing waste is affected by several process factors such as acid concentration, extraction time and temperature. Thus, a suitable experimental design to analyse multiple factors is required. In an experimental design, once the region of optimal response is detected by preliminary studies, it is often required to characterize the response in that region. Box-behnken design and central composite design (CCD) are mainly used in experiments for response surface methodology to approximate a second order polynomial estimation to a response in that region (Wang & Wan, 2009). These designs were used to investigate optimum collagen extraction condition of fleshing wastes.

I. Response Surface Methodology

Response surface methodology (RSM) is a technique using mathematics and statistics to model problems where variables have an effect on response of interest, and the main goal is to obtain an optimized response (Montgomery, 2005). When the independent variables has a linear function that affects a response, then the first-order model is the approximate function of it. The advantages of using RSM are reduced number of experimental attempts to assess multiple parameters and the capability of the statistical tool to recognize interactions.

II. Box-Behnken Design

Box-Behnken design is a three-level fractional factorial design built (Box & Behnken, 1960). The design may be considered as a mixture of a two level factorial design with a partial block design. In every block, a definite number of variables set for all combinations for the factorial design, whereas other factors are maintained at the central levels. Box –Behnken design is slightly similar to the CCD but has fewer factor levels and does not have extremely high or extremely low levels (Wang & Wan, 2009).

2.3.6 Factor Affecting Collagen Extraction

A. Temperature

Collagen is a protein that is termed thermo-instable as it easily denatures at room temperature. The susceptibility to temperature is related to its chemical structure as there is a difference in the amino acid composition, particularly the varying amino acid (proline and hydroxyproline) content among fish species (Gudmundsson, Hafsteinsson, 1997; Wang, et al, 2008). Fish species with lower levels of hydroxyproline have lower denaturation tempratures as compared to those with higher levels of hydroxyproline (Muyonga, Cole & Duodu, 2004). This is due to the hydroxyproline in the inter-chain is able to form hydrogen bonding within the collagen molecule creating a more stable molecular structure. Denaturation temperatures of collagen from cold water fish such as cod, ayu and chum salmon have very low denaturation temperatures, which are 15^0C, 29.7^0C and 19.4^0C, respectively (Rigby, 1968; Kimura & Ohno, 1987; Nagai & Suzuki, 2000a). Therefore, the manipulation of the temperature throughout the extraction of collagen is vital in order to maintain the collagen's native structure.

B. Acid Extraction Time

Isolation of collagen from the backbone of Baltic cod, it showed that by increased the extraction time from 48 hour to 72 hours, the collagen solubility increased by 5 to 10% (Zelechowska, Sadowska & Turk, 2010). Extraction of collagen from skin and bone of bigeye snapper, in a repeated process of 24 hour extraction in 0.5M acetic acid solution, showed that only about 1.9% of collagen was dissolved (Kittiphattanabawon, Benjakul, Visessanguan, Nagai & Tanaka, 2005). It is generally known that mass transfer rate of analytes from the matrix is a significant factor in the extraction efficiency (Bartle et al., 1991; Wang, 2008). In convention extraction, the diffusion process controls the mass transfer rate which is dependent on the extraction time. The amount of analytes retrieved amplifies with increasing of time in the diffusion controlled process. Therefore, it is suggested that the extraction time affects the amount of collagen yield (Wang, et al., 2008).

C. Acid Concentration

The solubility of collagen in acid solutions is a significant factor in the extraction effectiveness (Kiew & Don, 2013). When collagen is in an extracting medium with sufficient amounts of proton (H^+), its solubility is increased. Sufficient quantities of H^+ assist the entry of water into the collagen fibbers. Water is obtained in the collagen by electrostatic swelling that is electrostatic forces among charged polar groups. As a result, the collagen solubility in the extracting medium is significantly affected by the acid concentration used due to the effect of swelling properties (Kiew & Don, 2013).

Partial solubility of skins indicates the presence of inter-molecular cross-links in collagen molecules. The first phase of solubilisation of collagen is the continuation of hydration of the fibrous collagen due to its contact with acids (Skierka & Sadowka, 2007). The structure of protein and its electrostatic infractions would change when the acid concentration changes, this is due to the pH value affects the charge of the protein's charge density of protein (Wang, et al., 2008).

In an experiment on the extraction of collagen from hybrid skin, various concentrations of acetic acid were used, which was ranging from 0.1M to 0.9M. When an increase of the acetic acid's concentration from 0.1M to 0.7M happened, the yield was increased to a maximum of 26%. However when the acetic acid concentration increased to 0.9M, an opposite trend was observed and the yield was decreased to 20.4% (Kiew & Don, 2013; Wang, Yang & Du, 2009) reported a similar trend of up to 0.5M acetic acid was used, and the yield was found decreasing thereafter. Other studies showed that optimal acetic acid concentration was 0.54M for the extraction of grass carp collagen (Wang & Wan, 2008).

When the extracting medium has a low P^H for example 0.9M acetic acid (P^H =2.39) it will cause a decrease in the water absorption ability of collagen. A low P^H would trigger the positively charged amine groups of proteins to form bonding with anions (CH_3COO^- in acetic acid aqueous solutions), creating more fragile electrostatic repulsive forces among the one nominal charge group. Consequently, loosening of the collagen fibbers structure and decrease in the ability to bond with water causes a reduction of the collagen solubility in the medium

(Skierka & Sakowska, 2007). Besides, collagen denatures at extremely low P^{H} values which is below 2.0 cause collagen fibers reduce in size, makes the hydration of protein become unachievable (Carvalho, et al., 2003; Wang, et al , 2008).

2.4 Literature Review on Fat

The major constituent of hides and skins are protein and fat, through leather manufacturing process which aims is to prepare for the next process, removal of non-structural protein. Beamhouse operation, after liming process completed and passes through fleshing mechanical operation to remove the flesh, adipose layer and natural fat from the flesh side of skin/hide where a substantial amount of solid waste of fleshing which is estimated 50-60% of total solid wastes (Kanagaraj, Velappan, Babu & Sadulla, 2006).

2.4.1 Source of Fat and Oil

Original source of fats derived from the animals, plants, and marine are composed of triglyceride. Almost they are the similar to oil in chemical nature and their composition, but there are some differences because of the degree of unsaturation and the carbon chain length of the fatty acid in the triglycerides. Oils which are obtained from the origin of minerals are entirely different from the oils from vegetable sources. The mineral oils are basically a mixture of long chain hydrocarbons and have no triglycerides.

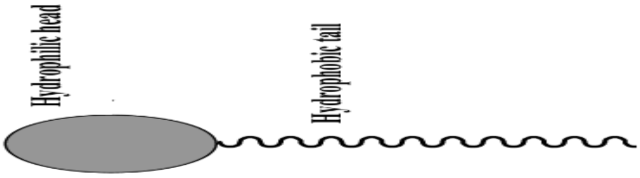

Figure 6: Molecular structure of fats and oils, hydrophilic=‘head’ and hydrophobic = ‘tail.'

Glyceride is an ester formed by the reaction of fatty acids with glycerol. Since glycerol has three hydroxyl groups, it reacts with one, two or three carboxylic acid group depending on the quantity of the fatty acid as also on the conditions of the reaction.

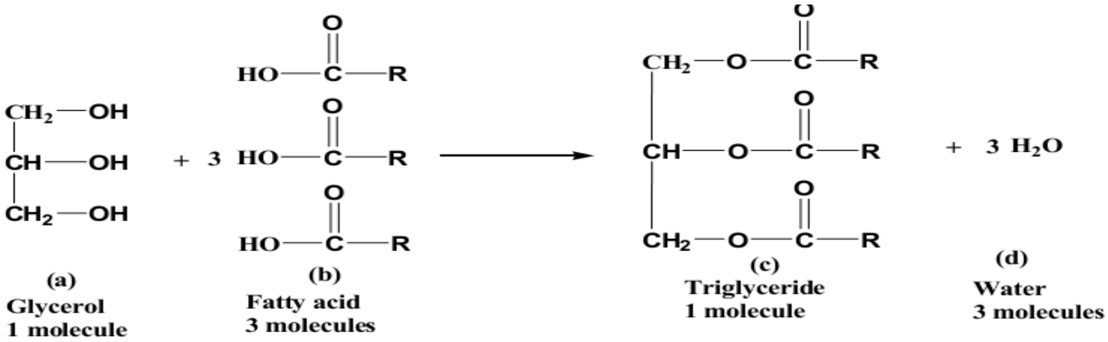

Figure 7: Formation triglyceride (c): glycerol (a), (b) three moles of fatty acid and water (d)

2.4.2 Ways of Determination of Fats

The fat purity can be analysis in terms of the amount, quality, characterization and purity within the compound which are Saponification value, iodine value, acid value and fat content.

a. **Saponification Value** is the process of hydrolysis of triglycerides into glycerol and the potassium salt of the fatty acid by applying an alcoholic solution of potassium hydroxide. The amount of alkali required to combine with the fatty acids liberated by the hydrolysis of the fat on saponification gives the equivalent weight as well as the molecular weight of the fatty acids. Saponification value is expressed in milligrams of the potassium hydroxide required to saponify one gram of fat.

b. **Iodine Value :** The number of iodine was calculated by the number of grams of iodine absorbed from 100g of the sample. It is a measure of the degree of unsaturation in the fatty acid chain. The test measures the amount of iodine which can be absorbed by fat. As the addition of iodine takes place at the double bond, measurement of the quantity of iodine consumed gives the idea about the number of the double bonds presents.

c. **Acidic Value** is a measure of the amount of free fatty acid present in fat. The number of mass in potassium hydroxide in milligrams is required to neutralize one gram of oil.

CHAPTER THREE

3. Materials and Methods

Within this chapter, the research methodology has been planned to be feasible with bounded time, described the necessary step of work and provide an effective strategies of the study. In order to manage the flow of study activity, the study work segregate into subsection and supported by flow diagram (Figure 8) and leather process (Figure 7).

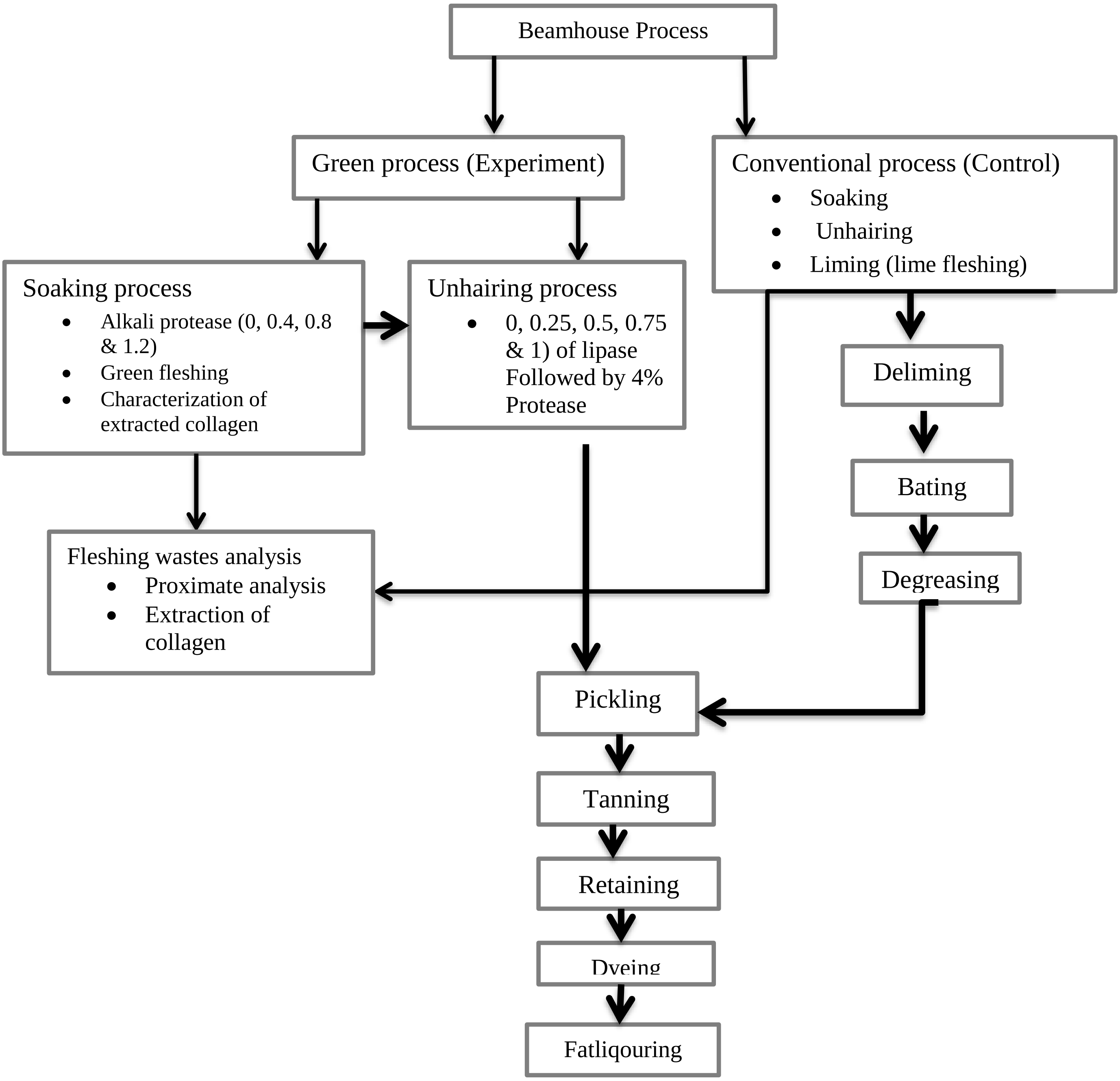

Figure 8: Experimental design of cleaner development beamhouse process with effective utilization of fleshing wastes

3.1 Materials

Wet salted cow hide were obtained from LIDI. The fleshings used for this study was collected from beamhouse process. Commericial chemicals and auxilaries were used for pretanning and post tanning process to make upper crust leather (sodium hydroxide, preservative, wetting agent, sodium sulphide, and lime powder). Analytical grade chemicals were used for the determination of hydroxyproline and collagen (sodium chloride, acetic acid, hydroxyl proline reagent tests (Per-chloric acid, Chloramine-T, Hydroxyproline, hydrochloric acid, PDAB - P-dimethyl amino benzaldehyde, citric acid monohydrate, glacial acetic acid, sodium acetate tri-hydrate), protein marker, Na_2HPO_4 - sodium hydro phosphate and potassium bromide). Enzymes were utilized for cleaner beamhouse process (CLRI develop protease enzyme from *Bacillus Subtillus*. The activity of protease and lipase used for the study is 100u.

3.2 Equipment

Tannery machine (drum, fleshing machine, pallet, samming, setting out, shaving and dryer), round bottom flask, graduate cylinder, volumetric flask, deep freeze, water bath, shaker with heating incubator, funnel, beaker (5L), thermometer, centrifuge tube, lyophilizer, refrigerator, heat incubator, hot oven, different size test tube, digesting tube, analytical instrument (UV spectrophotometer, CD spectra, micro-shrinkage tester, FTIR, P^H electrode, SDS-PAGE, weighing balance).

3.3 Methods

3.3.1 Cleaner Beamhouse Process Design

Beamhouse process is one of the essential and first stages of processes in leather industries which used for ready tissue matrix for next process. This study was planned on alternative of beamhouse process which was called as bioprocess (cleaner). Among beamhouse process, study was focus on enzymatic process design of soaking and unhairing with utilization fleshing waste. The bioprocess design on soaking and unhairing process was described as follows.

I. Enzyme Assisted soaking Process

Eight wet salted cow hides split into two halves sides were used for this study. Left sides have been taken for control and the corresponding right sides for experimental analysis. Experiments were carried out from 0 to 1.2% alkali protease enzymes by increase 0.4% and 0.1% of sodium hydroxide pour on soaking process whereas control processes follow conventional process. The use of sodium hydroxide was adjusted the pH of soaking solution which assists the efficiency of enzyme to rehydrate the fibre by open up the hide matrix. The control analysis was carried out based on conventional processes and lime fleshing provide after liming completed. Generally, the recipe of experiment process of enzymatic assisted soaking process is tabulate as follows.

Table 1: Cow hides sides for experiment (right side)/control (left side) on soaking process

Raw material: Cow hides

Match Pair Comparison Experiments

Conventional– Left side (liming fleshing)

Experimental- Right side (Green fleshing)

Control(Conventional Process)	Experiment(Enzymatic soaking-% protease)
Cs_1	Es_1 (0)
Cs_2	Es_2 (0.4)
Cs_3	Es_3 (0.8)
Cs_4	Es_4 (1.2)

Table 2: Experimental design of enzymatic assisted soaking process

Raw Materials	Types of process	Chemicals	% Concentration	Comment
Wet salted Cow Hides	Soaking I	Water	200	Leave in pit at 32-35^{o}c for 2 hrs.
		Wetting Agent	0.1	
		Preservative	0.1	
	Soaking II	Water	200	Leave in pit at 32-35^{o}c for 1 hrs.
		Wetting agent	0.1	
	Soaking III	Water	200	Leave in pit at 32-35^{o}c for overnight
		Sodium hydroxide	0.1(1:10)	
		Alkali protease	0, 0.4, 0.8 & 1.2	
		Preservative	0.1	

II. Analysis the Performance of Enzyme on Soaking Process

Based on the different concentration of alkali protease enzyme, it was attempted to optimize soaking process and quantify the yield of green fleshing. After soaking process completed, green fleshing was collected from fleshing machine operation. Then, compared the amount of green fleshing yield with lime fleshing which was obtained from conventional process. In addition to this, the amounts of collagens were quantified in green and lime fleshing wastes.

III. Determination of Collagen from Fleshing Wastes

Fleshing wastes were collected and cut into small pieces, grind and mix uniformly until homogenised samples were obtained. 1mg of fleshing waste sample and 1mL of 6N HCl for hydrolysis was taken in hydrolysis tube and the tube was sealed. The sealed tube was kept in an oven at 120^0C for overnight. Next day, the sealed cover of hydrolysis tube was removed and the content was poured in silica crucible and kept in a water bath at 80^0C for 3 hours which used to remove HCl. Then, the digested sample was collected by washing with distilled water three times on 50mL of volumetric flask. The samples were poured in 50mL volumetric flask and added distil water till the mark. Finally, it was followed the standard procedure by using hydroxyproline estimation which determine based on hydroxyproline content according to the method (Woessner, 1961) using a UV-viz., spectrophotometer (Cary win 100) and λ = 557nm which presented appendix C.

$$\begin{aligned}&Amount\ of\ Hydroxyproline\ in\ fleshing\ wastes\\&\qquad = concentration\ in\ \mu g\ x\ Dilution\ factor\end{aligned}$$

$$\begin{aligned}&Amount\ of\ Collagen\ in\ Fleshing\ Wastes\\&\qquad = Concentration\ of\ Hydroxyproline\ by\ UV\ \left(\mu\frac{g}{ml}\right) X\ 7.4\end{aligned}$$

IV. Enzymatic Unhairing Process

After the soaking process completed, enzymatic unharing process was continued by taking the left side as control and the corresponding right side for experiment. Lipase followed by 4% protease was carried out on experimental unhairing process. This experiment was carried at a different lipase offer level (0, 0.25, 0.5, 0.75 & 1%) followed by 4% of protease and using 4% of protease as control. After set the cleaner beamhouse process, the next steps of leather manufacturing process was followed conventional process till upper natural crust leather. Generally, the recipe of experiment process of enzymatic unhairing process was tabulated as follows whereas conventional process from soaking to natural crust was stated at appendix.

Table 3: Cow hides sides for experiment (right side)/control (left side) on unhairing process

Raw materials: Pelts

Matched Pair Comparison Experiments
Control (left side) – protease (4%)
Experimental (right side) -lipase + protease (4%)

Control (4% protease)	Experiment (%lipase + 4% protease)
Cu_1	Eu_1 (0)
Cu_2	Eu_2 (0.25)
Cu_3	Eu_3 (0.5)
Cu_4	Eu_4 (0.75)
Cu_5	Eu_5 (1.0)

Table 4: Enzymatic unhairing process (experiment)

Process	**Chemicals**	**% concentration**	**Duration**	**Remark**
Enzymatic Unhairing	Water	30	6 hrs.	Run in drum for 10 min. every one hour @ 4 rpm, 35^{o}c;
	Lipase	0, 0.25, 0.5, 0.75 &1		
	Protease	4		
Washing	Water	150	30 minute	Drain out

V. Unhairing Index

Hair removal efficacy was assessed subjectively by three CLRI scientists and pulling the hair with gentle force. Rating scale was given accordingly on a scale of 0-10 points, where higher points indicate a higher hair removal i.e. easy and completely hair removal of hair which is remove of hair in all the parts of the skin.

VI. Enzyme activity

The samples of spent liquor were drawn during the unhairing processes to understand the trends of enzyme activity with time by measuring the protease activity. Protease activity was measured by using the method (Kunitz, 1947). The spent liquor was filtered. About 0.1mL of sample was incubated with 1.9mL of Hammerstein casein solution for 10min at 60°C. Hammerstein casein solution was prepared by dissolve the Hammerstein casein (1% w/v) with 0.05M carbonate buffer. After incubation, the reaction was terminated by adding 3.0mL of 5% w/v tri-chloroacetic acid. Then, the reaction mixture was filtered. Finally, the release tyrosine content was measured by taking the OD at 280nm spectrophotometrically using JASCO V-220 spectrophotometer. One unit of protease activity of enzyme is defining as the amount of enzyme required to release of 1mg of tyrosine/mL/min.

VII. Scanning Electronic Microscope Studies (SEM)

SEM analysis was conducted to ascertain the hair removal from the root. Samples were taking from the optimized experiment and control process of unhairing for SEM analysis. Samples were washed with distilled water and then dehydrated stepwise using 10-100% absolute ethanol. The sample was then mounted on an aluminium specimen stubs, and coated with a thin layer of gold by Edwards E306 sputter coater. The samples were view microscopically using JOEL JSM5300 scanning electron microscope.

VIII. Analysis of Wastewater

Environmental parameters were studied by measuring the pollution parameters of wastewater. After unhairing process (both optimized experimental and conventional), wastewater samples were collected and analysed. Pollution parameters such as BOD, COD, Total Dissolved Solids (TDS) and Total Suspended Solids (TSS) were measured as per the standard procedure as reported by American Public Health Association (APHA-AWWA-WPCF 1989). Total Organic Carbon (TOC) was determined by TOC analyser (Model: Vario TOC select, Make: M/s Elementer, Japan). Samples of 0.1-0.5mL were combust at 850°C with moisture free air blown at a rate of 200mL min^{-1}. Triplicate analysis was done and the concordant values are present.

IX. Physical Testing and Organoleptic Properties of Leather

The crust leather samples for various physical tests from conventional and experimental beamhouse processes was obtained as per the IULTCS methods. The samples were conditioned at 27 ± 2 °C and 65 ± 2% RH for 48 hours as per the IUP 2 methods. After conditioning, samples were prepared for each test as per IULTCS standard method. Physical properties such as percentage elongation, grain crack, tensile and tear strength were examined as per the standard IUP procedures IUP 6, IUP 8, IUP 9 and IUP 10 respectively (IULTCS, 1996). Experimental and control leathers were assessed the organoleptic properties by three CLRI scientist experts, rated (0-10 scale) which lower rate scale indicated poor organoleptic properties whereas higher rate scale pronounced good organoleptic properties (fullness, softness, grain tightness, smoothness and general appearance).

3.4 Preparation of Fleshing waste for Collagen Extraction

3.4.1 Preparation of Green Fleshing Wastes

Samples preparation procedures were the crucial and basic desire for extraction collagen. Enzymatic assisted soaking process was carried out for opening up of texture of skin and yield of green fleshing wastes benchmarking lime fleshing wastes. Green fleshing wastes were collected from 0.8% offer level of enzymatic assisted soaking process. Sample preparation of the green fleshing wastes that precede the next for the analysis of proximate, extraction and characterization of collagen which was procedure as follows:

- Samples were washed the green fleshing wastes three times with normal water
- In order to clean more, put the samples with 0.9% sodium chloride salt for twenty minute followed by distilled water
- Samples of green fleshing wastes were cut into small size
- Ground and homogenise the green fleshing samples to achieve the uniform sample
- Stored the green fleshing samples in fridge at 4^{o}c for further work.

3.4.2 Preparation of Lime Fleshing Wastes

Lime fleshing wastes samples were collected from conventional process. These wastes were contaminated with sulphide which polluted the environment. Beamhouse process was produced 70% of pollution load from leather process. Lime fleshing wastes were contributing

30-40% of pollution load in tanning industries (Kanagaraj, eta al, 2015). Due to this reason, technologies were looking an alternative process which used to maintain sustainable of leather industries. Therefore, lime fleshing wastes utilise as benchmarking of green fleshing wastes. The wastes samples were prepared as follows:

- Samples of lime fleshing wastes washed three times to avoid bind chemicals from the surface of fleshing wastes
- Delimed fleshing wastes were done by 3% NH_4Cl in drums for 2hr and half hours
- The samples were checked whether the lime completely removed or not from during deliming process
- Delimed fleshing samples were washed with normal water for 20 minute
- Again washed with 0.9% of sodium chloride for 20 minute followed by distilled water
- Cut the delimed fleshing wastes samples into small size
- Ground and homogenise the samples to provide uniformly samples
- Store the prepared samples into fridge at 4^oC

The above sample preparation procedure supported the easy way of extraction of collagen from green and delimed fleshing wastes. This procedure helps to reduce the extraction time and increase the yield of collagen. Therefore, sample preparation of fleshing wastes can be used as standard. The procedures were supported by flow chart (Figure 9).

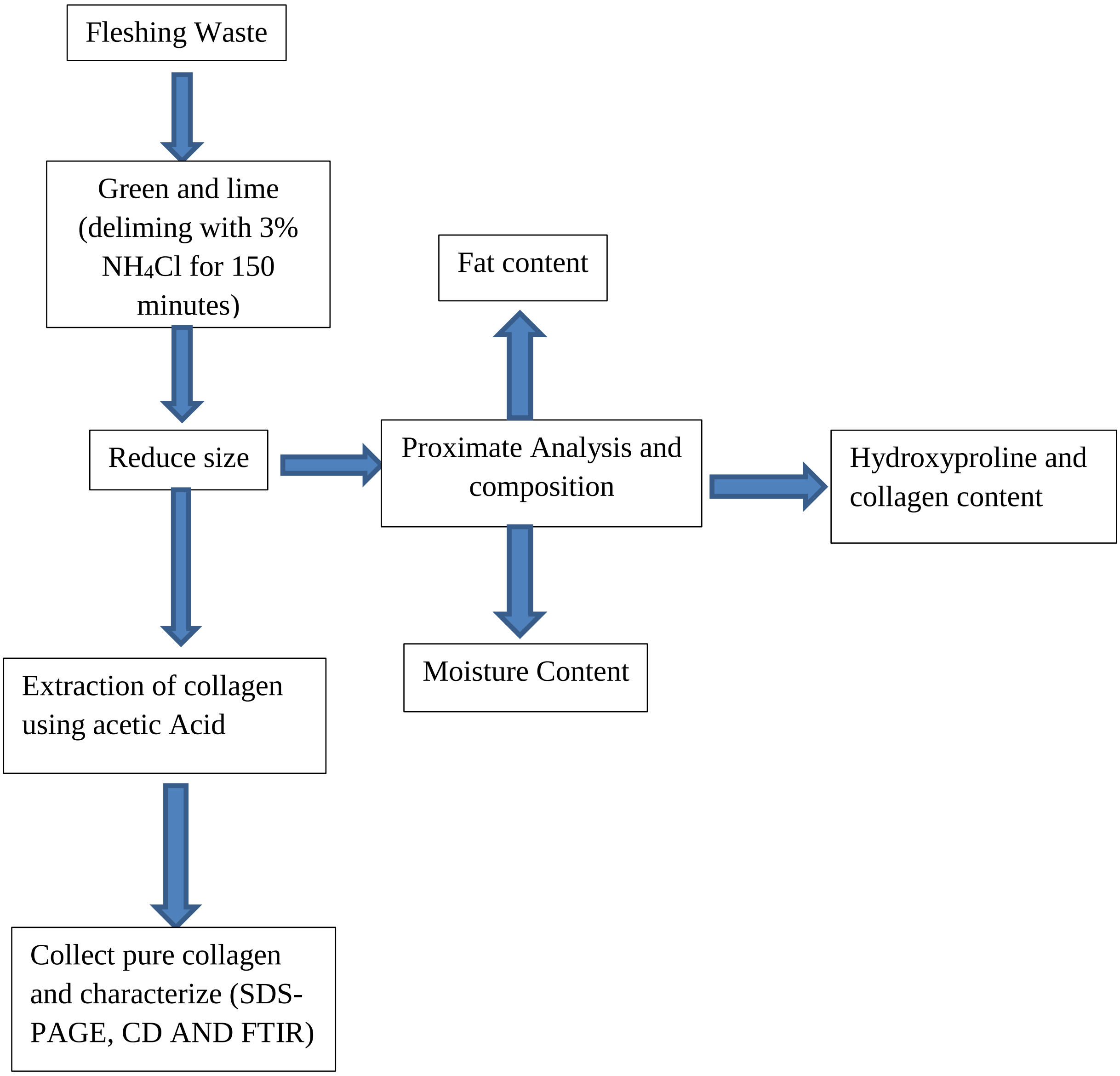

Figure 9: Flow chart of preparation of fleshing for characterization, proximate and extraction collagen from fleshing wastes

3.5 Proximate Analysis and Collagen Content of Fleshing Wastes

Proximate analysis of delimed and green fleshing wastes was carried out by standard methods (AOAC 1995). Moisture content was determined by hot air oven method. The fat content was determined by soxhlet method using petroleum ether (60-80 ^{o}C). The amount of extract collagen from deliming and green fleshing wastes were determined based on hydroxyproline content according to the methods (Woessner, 1961) as outlined in section III.

3.6 Optimization of Collagen Extraction

Response Surface Methodology was used to create an experimental model, evaluate the data, and design construction with the assistance of the software Design Expert@ version 6.0.8 (state-Ease, Inc., USA). The central composite design (CCD) determines the response three independent variables were used to determine the response design and to create a model. The extraction time, acid concentration and temperature were optimized to provide maximum yield of collagen from fleshing wastes.

Three factors at three levels; a total of 27 experiments were done in the optimization study and statistical analysis was made.

Table 5: Complete experimental design matrix of collagen

Variables	Factor Coding	Units	Levels		
			-1	0	+1
Acid concentration	A	Molar	0.3	0.5	0.9
Extraction time	B	Hours	24	72	96
Temperature	C	^{0}C	4	25	38

The summary of central composite design arrangement of main and interaction effect and the responses/ yields of collagen/ was presented on Table 6.

Table 6: Summary of central composite design arrangement of main and interaction effect

Run	Acid Concentration (M)	Extraction Time (hrs.)	Temperature (^oC)	Collagen yields (%)
1	0.3	24	38	
2	0.3	96	25	
3	0.5	24	38	
4	0.9	24	4	
5	0.9	96	4	
6	0.5	96	38	
7	0.5	72	4	
8	0.3	72	25	
9	0.5	96	25	
10	0.9	24	25	
11	0.5	96	4	
12	0.3	72	38	
13	0.3	72	4	
14	0.9	72	38	
15	0.9	96	25	
16	0.5	72	38	
17	0.9	72	25	
18	0.9	96	38	
19	0.9	72	4	
20	0.9	24	38	
21	0.3	96	4	
22	0.3	24	25	
23	0.5	72	25	
24	0.3	24	4	
25	0.3	96	38	
26	0.5	24	4	
27	0.5	24	25	

3.7 Extraction of Collagen from Fleshing Wastes

Delimed and green fleshing wastes were obtained from beamhouse processes. Delimed and green fleshing’s were washed three times with distilled water and its size was reduced using a cutter. Grinding and homogenisation of the green/deliming fleshing wastes was carried out which provide a uniform sample. It was washed with 0.9% of sodium chloride solution followed by distilled water two times at 20 minutes intervals. 50gm of green/delimed fleshing wastes were added into 600ml of acetic acid solution (0.5M) and stirr overnight at 4°C. The solutions were then homogenized at 18,000 rpm for 20 minutes and pour 1400ml acetic acid for 24 hours with continuous stirrer at 4°C. Add 3L of acetic acid solution and keep with stirrer 48 hours which was followed by centrifugation at 6000 rpm for 15 minutes with 4^{o}c and collected the supernatant for collagen extraction. In addition, 5% (w/v) of salt (NaCl) was added into this supernatant and kept for 5 hours which separated supernatant, collect residue followed by centrifuge at 10,000 rpm with 15 minute interval. Remove the supernatant and collect the pellets/ residual for the further extraction with 0.3M acetic acid solution and discard the supernatant, collect the residue follow by 5% (w/v) of salt (NaCl) precipitation. Remove the supernatant and collect the pellets/residual for the further extraction with 0.05M acetic acid solution by dialysis against distilled water three times changes within 12 hours intervals which followed by dialysis against 0.02M Na_2HPO_4 buffer solution with three times changes. After this again dialysis against 0.05M acetic acid and the collagen dissolve in 0.05M acetic acid solution and store at 4^{o}C for further use.

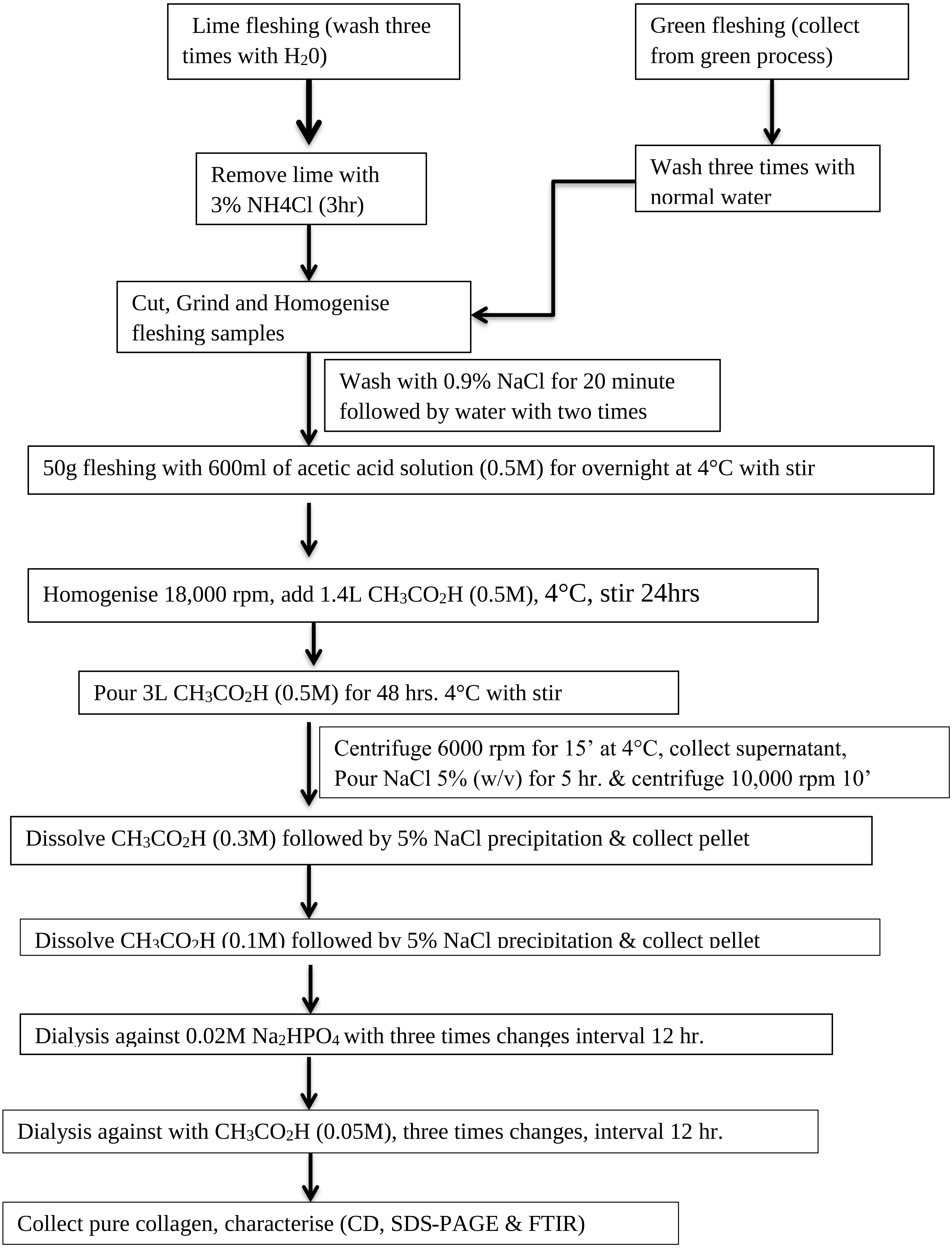

Figure 10: Flow diagram of extraction collagen from fleshing wastes

3.8 Characterization of the Extracted Collagen from Fleshing Wastes

The collagen extracted from lime and green fleshing wastes using acetic acid was characterize by using CHNS-Elemental analysis, SDS-PAGE, FTIR, CD spectra and thermal behaviour.

3.8.1 CHNS – Elemental Analyser

The extracted collagen was subjected to elemental analysis using a CHNS-Elemental Analyser EURO VECTOR EA-3000 (Germany). It was operated at 40% of humidity; 950^{o}C of furnace, helium or oxygen gases and at a pressure of 102kPa.

3.8.2 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The purity of the extracted collagen was estimated using SDS-PAGE. A 7% seperating gel was used for this purpose, and the gel was run at a constant voltage of 50V for 150 minutes. Coomassie brilliant blue R250 was used to stain the gel. A standard protein molecular weight marker ranging from 20kDa to 220kDa (Sigma-Aldrich) was used.

3.8.3 Fourier Transform Infrared Spectroscopy of Collagen (FTIR)

FTIR spectrum was measured using PerkinElmer (Spectrum two) spectrometer at room temperature. Lyophilized collagen (purified) was used for this purpose. The spectra were measured in the 400-4000cm^{-1} region by employing standard KBr pellet method. The spectrum was measured in transmission mode at a resolution of 4cm^{-1} and with an accumulation of 32 scans.

3.8.4 Circular Dichroism Spectra (CD)

CD spectra of collagen solution was analyzed immediately after acid digestion (before salting out and purification), and after purification, using a JASCO J715 spectropolarimeter (JASCO Inc., Japan) at 25^{0}C. CD spectra analyzer was between 190 and 250nm with a scan speed of 50nm/min. The sample concentrations of collagen were 0.5mg/mL while the path length of the rectangular quartz cell was 0.1cm. Every CD spectra represents an average of three individual scans. A slit width of 1nm and a time constant of 1 second were applied.

3.8.5 Thermal Melting Temperature of Collagen

Thermal melting behaviour of collagen was studied immediately after the CD spectrum scanning measurement of the sample. The temperature of the cell holder was control using

Peltier system provides by JASCO Inc. Temperature ramp rate was fixed at 1°C/min and ellipticity at 222nm was measured between 10 and 60^{o}C. All other parameters were same as mentioned in section 3.7.4. The melting temperatures of samples were calculated from the first derivative curve of the CD thermal transition spectrum.

CHAPTER FOUR

4. Results and Discussions

4.1 Enzymatic Assisted Soaking Process for Effective Fleshing Waste

Eight wet salted cow hide was selected for this study. Wet salted cow hide split into halves sides which were taking the left side for control whereas the corresponding right side was for experiment. Enzymatic assisted soaking process was carried out to rehydrate the raw cow hide and collect green fleshing waste with benchmarking lime fleshing wastes from conventional process. Different concentration of alkali protease (0, 0.4%, 0.8% and 1.2%) was feed in the soaking process which was presented in Table 2. Yielding of fleshing wastes and composition were presented in section 4.2 (Table 8). Among different concentration of alkali protease, 0.8% was an effective soaking or rehydration process which was carried out on E_3 and C_3 samples with confirmed by compared to their composition and yield of fleshing waste. In addition to this, the amount of collagen extracted from green fleshing (E_3) with benchmarking lime fleshing (C_3) were given as a result 14.87% and 12.58%, respectively. Therefore, enzymatically assisted soaking process was optimized at 0.8% which supplied high yield of green fleshing and collagen compared to lime fleshing.

Table 7: Enzymatic assisted soaking process recipe

Raw Materials	**Types Process**	**Chemicals**	**% Concentration**	**Remarks**
Wet Salted Cow Hides	Soaking I	Water	200	Leave for 6 hrs.
		Wetting Agent	0.1	
		Preservative	0.1	
	Soaking II	Water	200	Leave for 3 hrs.
		Wetting Agent	0.1	
	Soaking III	Water	200	
		Sodium Hydroxide	0.1(1:10)	pH=8.5-9.0
		Alkali protease	0,0.4,0.8 & 1.2	**0.8% effective offer level**
		Wetting Agent	0.1	
		Preservative	0.1	Leave for overnight

4.2 Quantification and Composition of Fleshing Wastes

Eight samples were conducted to obtain the exact optimized offer level of soaking process and high yield green fleshing waste. These samples were represented experiment as symbol of ‘E’ and control as symbol of ‘C’. Experiment one (E_1) and control one (C_1) were showed the result analysis of 24.4% with 39.4 % amount of fleshing yield, 1.2±0.3% with 1.4±0.2% of Hydroxyproline content and 8.88±0.3% with 10.36±0.2 % of collagen content respectively. 0.4% offer level of alkali protease carried out in soaking process which assigned by experiment two (E_2) and control two (C_2) were given an analysis of 19.6% with 25.8% yield of fleshing, 1.2±0.8% with 1.5±0.4 % of Hydroxyproline content and 8.88±0.2% with 11.1±0.2% of collagen content respectively. Experiment four (E_4) and control four (C_4) with offer level 1.2% were delivered the analysis of 36.8% with 45.2% of fleshing yield, 1.5±0.4% with 1.8±0.3% of Hydroxyproline content and 11.1±0.3% with 13.32±0.4% of collagen content respectively. Experiment three (E_3) and control three (C_3) were pronounced the effective rehydration of soaking process using 0.8% of alkali protease to assist the rate of penetration water entered into the skin matrix. Experiment three showed that 22.1% of green fleshing yield, 2.01 of hydroxyproline and 14.87% of collagen content whereas control three indicated that 20.2% of lime fleshing yield, 1.7% of hydroxyproline and 12.58% of collagen content. Then, analysis come to across conclusion that amount yield of green fleshing, hydroxyproline and collagen content of experiment was confirmed highest production from control.

Table 8: Quantification and Composition of fleshing wastes

Samples	Weight of side (kg)	Weight of fleshing wastes (kg)	% yielding	% of deviation from control	HP Analysis (%)	% of deviation from control	Collagen content (%)	% of deviation from control
C1	5.8 ± 0.1	2 ± 0.1	39.4	-15	1.4±0.2	-0.2	10.36±0.2	-1.48
E1	4.91±0.5	1.2±0.09	24.4		1.2±0.3		8.88±0.3	
C2	4.27±0.4	1.1±0.2	25.8	-6.2	1.5±0.4	-0.3	11.1±0.2	-2.22
E2	4.07±0.9	0.8±0.1	19.6		1.2±0.8		8.88±0.2	
C3	3.66±0.7	0.7±0.08	20.2		1.7±0.1	+0.5	12.58±0.1	
E3	3.62±0.8	0.8±0.07	22.1	+1.9	2.01±0.2		14.87±0.1	+2.29
C4	3.98±0.6	1.8±0.3	45.2		1.8±0.3	-0.2	13.32±0.4	
E4	4.08±1	1.5±0.4	36.8	-8.4	1.5±0.4		11.1±0.3	-2.22

4.3 Determination of Protease on Enzymatic Assisted Soaking Process

The active content of protease was 92% which help to calculate the actual protease offered. Based on this information, the amount of actual protease offered in grams of E_2, E_3 and E_4 were 140, 191.6 and 222.53, respectively. In addition to this, the total amounts of protease in spent liquors in grams were 38.7, 29.4 and 42.28, respectively. Data analyses were described in Table 9 as follows.

Table 9: Amount of protease in actual offer and spent liquor

Experiments	General amount of protease	Actual Protease	Protease (g/ml)	Volume of spent liquor	Total protease in spent liquor
	offered(g)	offered(g)		collected (ml)	(g)
E_2(0.4)	152.2±1.5	140.0	0.018	2150±1.0	38.7
E_3(0.8)	208.3±1.4	191.6	0.014	2100±0.8	29.4
E_4(1.2)	241.9±0.9	222.5	0.019	2225±1.2	42.3

From Table 9, we attempted the conclusion of the three offer level of process which pronounced that the amount of protease absorbed by E_2, E_3 and E_4 of hides were given a result of 72%, 85% and 81%, respectively. The results of protease absorbed by cow hides were demonstrated in Figure 11. From this data, the highest amount of protease that absorbed by cow hides was obtained 85% that was experiment three. The rate of penetration was increased at 0.8% offered level and provided high yield of green fleshing wastes with compared to the conventional output of fleshing wastes including hydroxyproline content and collagen content.

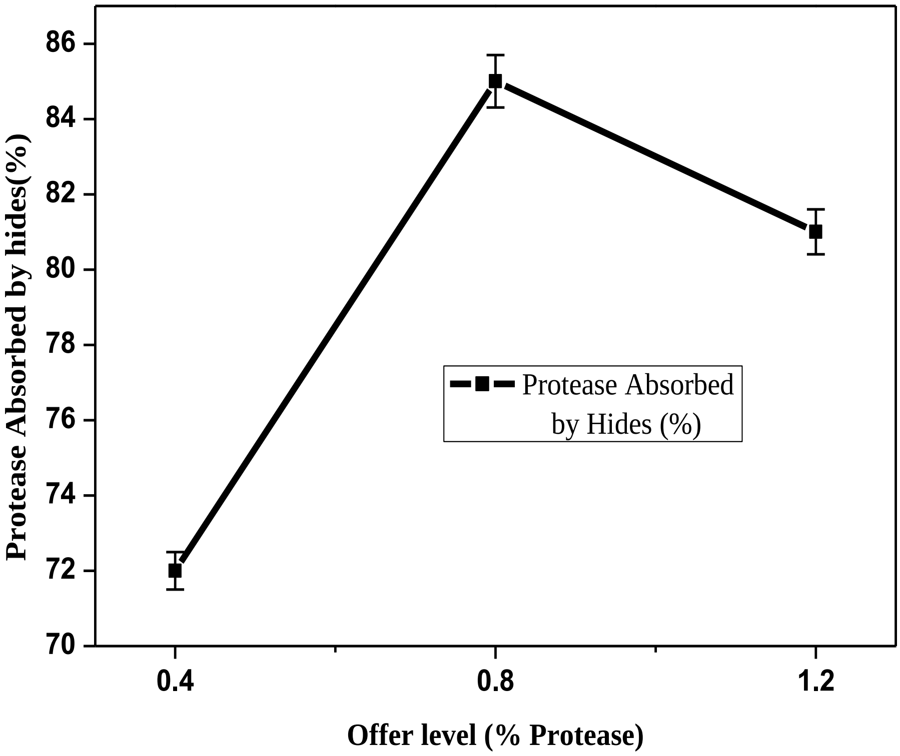

Figure 11: Amount of protease absorbed by hide

4.4 Enzymatic Unhairing Process

After optimized enzymatic assisted soaking process completed, enzymatic unharing process was continued by taking the left side as control and right side for experiment. Different concentration of lipase followed by 4% protease was carried out on an experiment unhairing process. This experiment was optimized lipase offer level (0, 0.25, 0.5, 0.75 & 1%) followed by 4% of protease with 4% of protease as control. From this offer level, 1% of lipase followed by 4% of protease pronounced completely removed the hair from skins. The main objective of lipase enzyme was to increase the penetration of alkali protease into the skin by degrading of fat from fleshing side. Generally, the recipe of experiment process of enzymatic unhairing process tabulate as follows (Table 10) whereas conventional process from soaking to natural crust presented at appendix A.

Table 10: Enzymatic assisted unhairing process (experiment)

Process	Chemicals	% concentration	Duration	Remark
Enzymatic Unhairing	Water Lipase Protease	30 0, 0.25, 0.5, 0.75 & **1** 4	6 hrs.	Run in drum for 10 min. every one hour @ 4 rpm, 35^{o}c;
Washing	Water	150	30 minute	Drain out

Note: From Table 10, 1% of lipase offer level followed by 4% of protease were assisted the unhairing from the rest different offer level of lipase.

4.5 Unhairing Index

The efficacy of unhairing process which was lipase followed by 4% protease evaluated subjectively pulling at each time with different concentration (0, 0.25, 0.5, 0.75 and 1.0). The efficacy of unhairing was described in terms of low rating scale was indicating that poor unhairing efficacy whereas highest rating scale completely unhairing. As a result, 1% concentration of lipase was effectively removed the hair at 6 hours which means the penetration of protease increased and devoid hair with short period of time from other offer level lipase and the result presented in Figure 12.

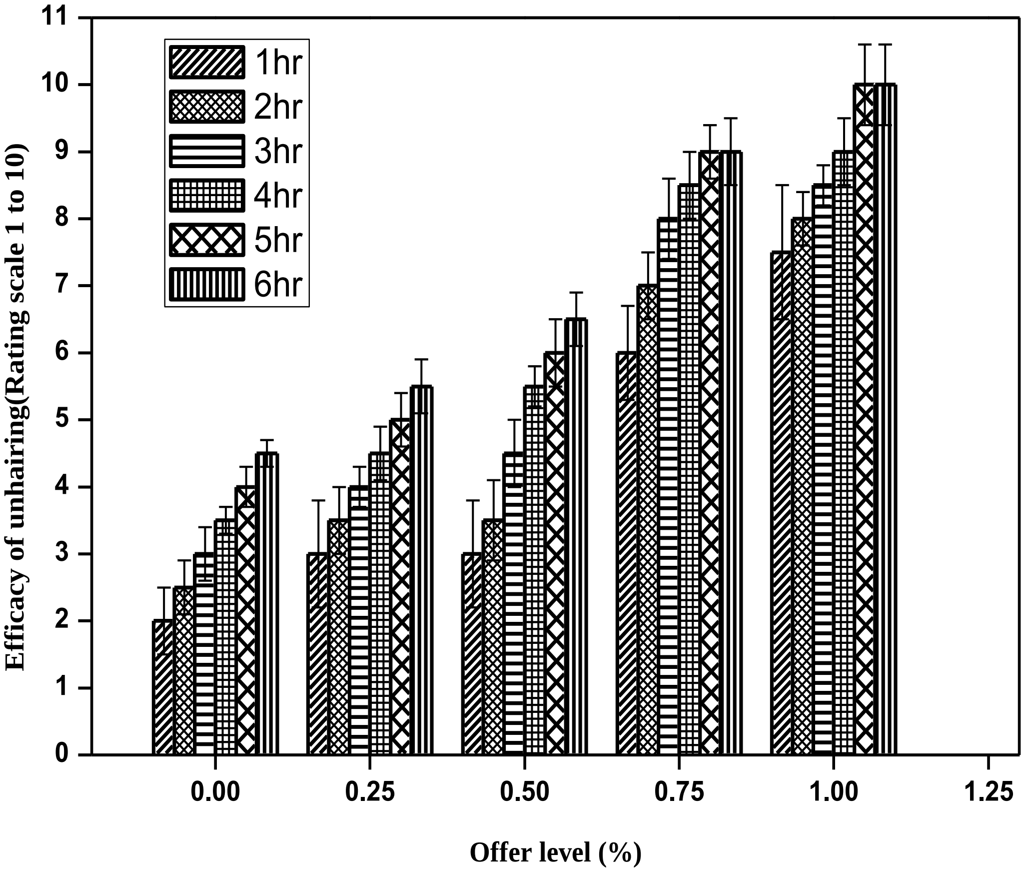

Figure 12: Efficacy of unhairing lipase followed by protease using unhairing index

4.6 Unhairing Enzyme Activities

The activity of protease was measured from spent liquor of the unhairing process. The spent liquor collected with two hour difference until unhairing completed for 6 hours. The effect of lipase that facilitates the unhairing process was 1% of concentration with 4% of protease. The amount of protease in spent liquor was decreased where the efficacy of removing hair was increased in the hide matrix. If the amount of protease enzyme is high in the spent liquor, the performance of protease is decreasing during unhairing process. The Figure 13 showed that the influence of lipase and protease on unhairing process. The amount of protease in the spent liquor was decreased at 1% of lipase followed by 4% of protease.

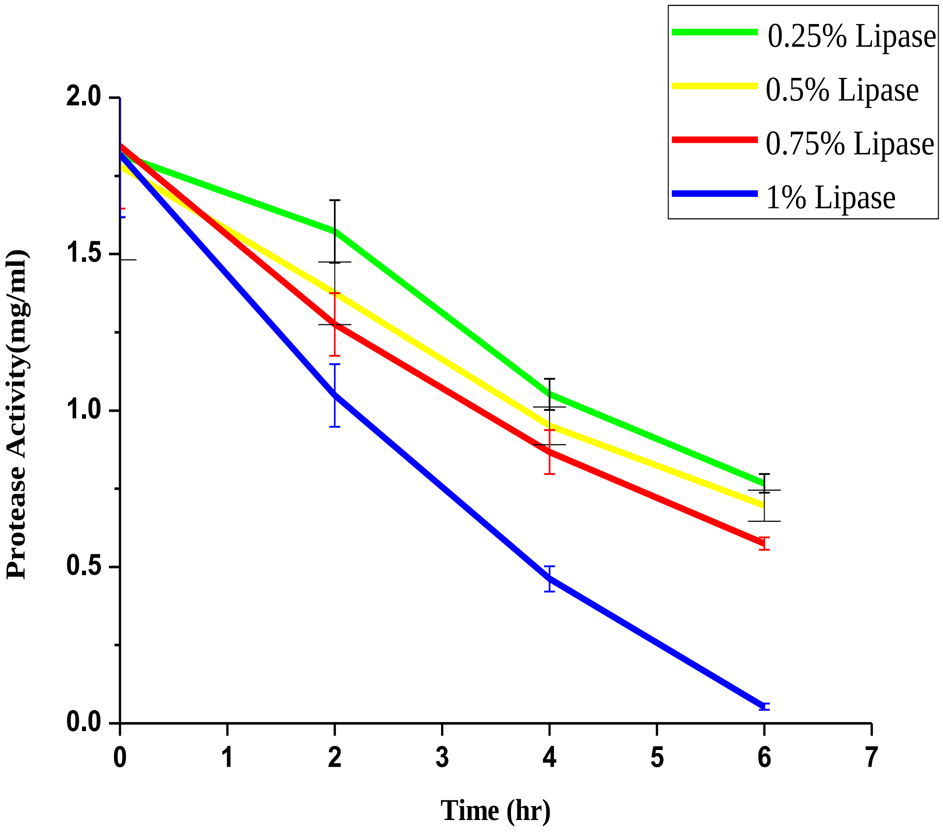

Figure 13: Activity of protease measure from spent of liquor lipase followed by protease

4.6.1 Determination of Protease on Enzymatic Unhairing process

From Table 11, different concentration of lipase followed by 4% of protease which were 0, 0.25%, 0.5%, 0.75% and 1% of lipase with the result of protease absorbed by hides were 60%, 80%, 81%, 83.58% and 90%, respectively. Therefore, highest amount of protease absorbed by hides was 90% and presented in spent liquor at lowest in the coenzyme combination of 1% of lipase followed by 4% of protease in unhairing process when we compared to another percent offer level of lipase.

Table 11: Amount of protease absorbed by cow hides on enzymatic unhairing process

Experiments	Protease offered (g)	Protease in spent liquor(g)	Protease absorbed by hides (%)
E(0)	70.23±0.5	28.1±0.2	60.0
E(0.25)	80.6±0.4	16.12±0.1	80.0
E(0.5)	75.47±0.3	14.34±0.3	81.0
E(0.75)	77.94±0.5	13.25±0.4	83.6
E(1.0)	107.25±0.9	10.87±0.6	90.0

4.7 Scanning Electronic Microscope Studies (SEM)

The structure of pelt was assessed through SEM which concentration was 1% lipase followed by 4% protease. Its analysis was provided by compared to the performance of enzymatic assisted unhairing process from control and experiment with identified the best performance combination of lipase with protease which keeps other parameter constant. The result was pronounced that the combination of 1% lipase followed by 4% protease completely removed the hair from skin better than 4% protease. The pelt structure easily open up and proteoglycan devoid with hair. The image A showed before unharing, image B indicated that 4% of protease offer level was not completely remove the hair from the skin whereas image C showed that completely removed the hair from the skin (1% lipase followed by 4% protease). Generally, the images were presented in Figure 14.

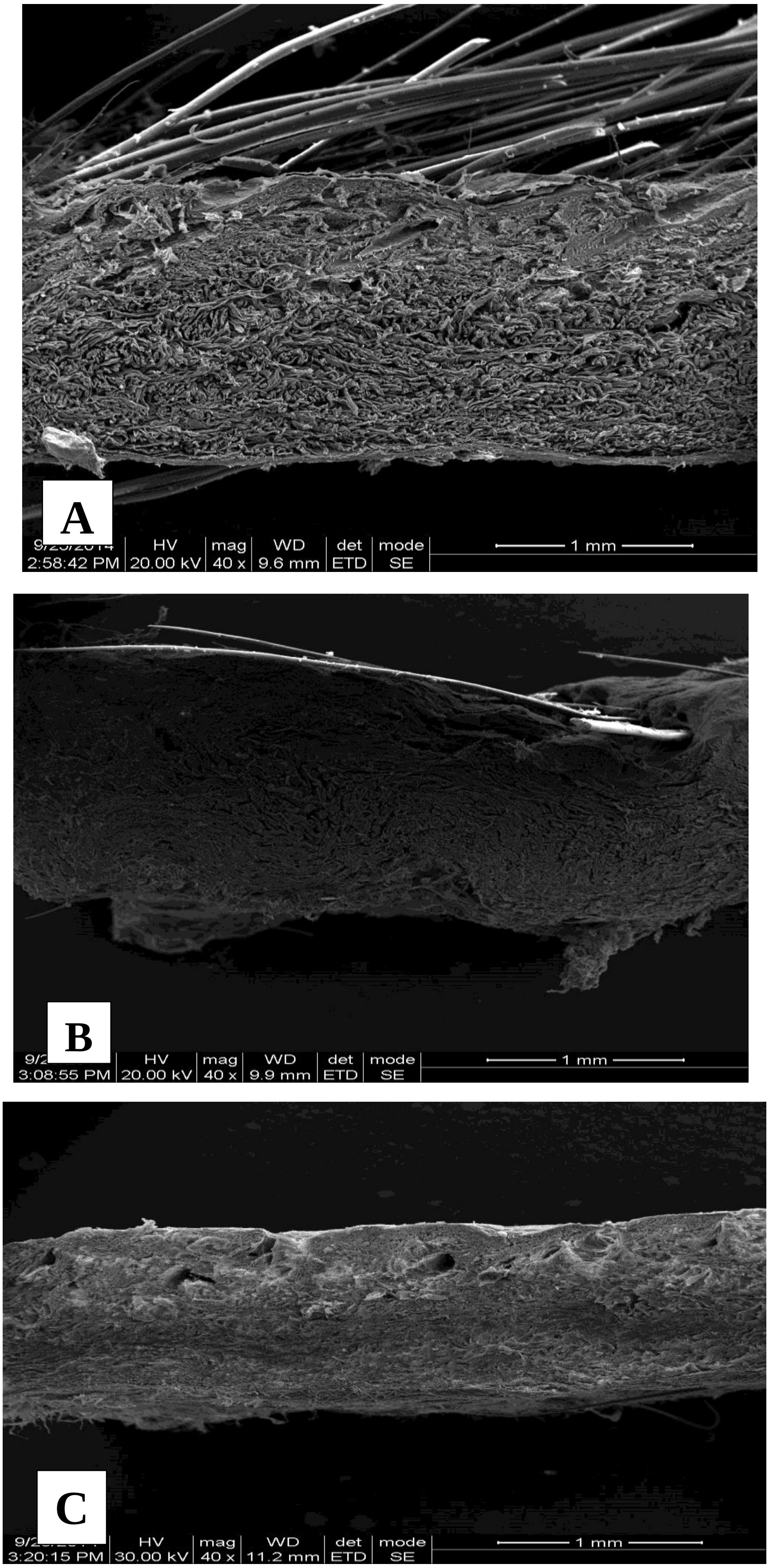

Figure 14: SEM analysis of enzymatic unhairing (A) Before unhairing; (B) Unhairing with only 4% protease and (C) Unhairing with 1% lipase followed by 4% protease

4.8 Wastewater Analysis

The amount of polluted chemical that generated from unhairing process was measured in terms of BOD, COD, TDS, TSS and TOC with compared to conventional process. There was a reduction in pollution loads from conventional process such as BOD, COD, TOC, TDS and TSS at the level of 89.375%, 88.57%, 85.79%, 47.37% and 48.57%, respectively. The unhairing process didn’t used sodium sulphide but involves lime for additional. It has performed effectively unhairing on cow hide by using protease with lipase than protease. Table 12 showed the pollution load generated in unhairing process.

Table 12: Pollution load (ppm) of wastewater from chemical and enzymatic unhairing process

Pollution loads(ppm)	Chemical	Bio-Process	Percentage reduction of pollution (%)
BOD	80±0.8	8.5±0.55	89.375
COD	28±1.5	3.2±0.25	88.57
TOC	38 ±1.2	5.4 ±0.28	85.79
TDS	190 ± 0.5	100 ± 0.64	47.37
TSS	70 ± 1.7	36 ± 1.2	48.57

4.9 Physical Testing and Organoleptic Properties of Leather

The physical test of upper crust leather resulted was confirm according to the standard parameter of crust leather. The result of physical analysis obtained from all the experiments and controls which compared to the value of percentage elongation, tensile and tear strength. The tensile strength of crust leather which have been done for experiment pronounced better than control except experiment one and control one. Both experiments and controls were fulfilled the standard requirement was presented in Table 13. The organoleptic properties of crust leather were assessed, in the form of grain tightness, smoothness, softness, fullness and general appearance. In all samples was pronounced that experiment better properties than control. The organoleptic properties were presented in Table 14.

Table 13: Physical testing result of crust leathers

Sample	Tensile strength (N/mm)		Percentage Elongation (%)		Tear Strength (N/m^2)	
	Average	% Deviation from control	Average	% Deviation from control	Average	% Deviation from control
C_1	21 ± 1.2	-3.33	43.5 ± 1.3	+22.07	113 ± 1.5	+9.73
E_1	20.3 ± 1.0		53.1 ± 0.9		124 ±1.3	
C_2	16.9 ± 1.3	+ 8.87	37.6 ±1.3	-4.52	107 ± 1.3	+5.61
E_2	18.4 ± 1.0		35.9 ± 1.0		113 ± 1.4	
C_3	18.3 ± 0.8	+ 7.1	49 ± 0.7	+4.1	105 ± 0.8	+0.95
E_3	19.6 ± 0.8		51 ± 0.8		106 ± 1.0	
C_4	17.4 ± 0.6	+20.11	41.5 ± 0.9	+0.96	101 ± 1.1	+1.0
E_4	20.9 ± 0.8		41.9 ± 1.0		102 ± 1.0	

Table 14: Assessment of organoleptic properties for crust leathers

Properties	Samples of experiments and controls							
	C_1	E_1	C_2	E_2	C_3	E_3	C_4	E_4
Fullness	5	6	5	6	6	8	6	7
Softness	4	7	6	6	7	9	4	5
Grain tightness	7	6	4	7	8	7	3	4
Smoothness	4	8	5	8	6	8	6	5
General appearance	5	6	6	7	6	8	4	5

4.10 Proximate Analysis and Composition of Fleshing Wastes

Within this study, green and lime fleshing wastes were utilized for extraction of collagen. Lime fleshing wastes are a critical issue for the environment because they contaminate with sulphide chemicals that used for unhairing whereas green fleshing wastes are non-contaminated of sulphides. So lime fleshing wastes were completely removing the lime before extraction of collagen. The homogenised fleshing wastes were used to characterize moisture, fat, hydroxyproline and collagen content. The proximate and composition analysis data were given in Figure 15. The moisture contents of lime fleshing wastes were determined to be 82.5%. Fat content of lime fleshing were 6.67% (w/w). The amount of collagen estimated to be 9.5% (w/w) whereas moisture content of green fleshing was 77.03%, fat content was 9.25% and collagen content was 14.87% (w/w) by hydroxyproline analysis of the completely hydrolysed collagen solution. We could draw a conclusion that total amount of collagen and fat content increase in green fleshing than lime fleshing waste due to utilize of chemicals during unhairing process which reduced the amount of collagen obtained from lime fleshing wastes.

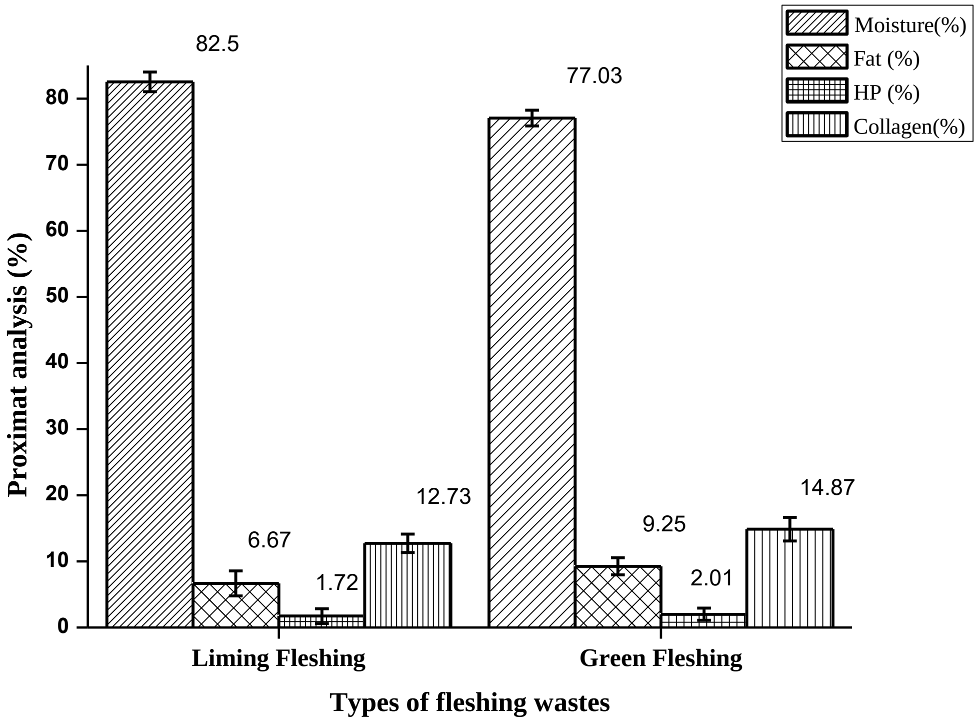

Figure 15: Proximate analysis of green and liming fleshing waste

4.11 Optimization of Collagen Extraction

Preliminary studies were carried out to identify the suitable extraction parameters of collagen extracted from fleshing wastes. The results obtained were used as a guideline to determine the input range of each independent variable for RSM analysis. The optimization study was conducted based on an experimental design using design expert 6.0.8 software application. Three process parameters or factors such as acid concentration, extraction time and temperature were investigated in three levels by keeping stirring speed at 4000 rpm with acetic acid solvent. Central Composite Design (CCD) was selected for the statistical analysis of the study and a total of 27 experiments were run. The yield of collagen was measured as response. The surface plots were generated from the regression analysis and analysis of variance (ANOVA). The central composite design arrangements and results of the experiment and results of statistical analysis of the ANOVA are given in Table 16.

As it was shown from the table the maximum yields of collagen was 82.9% w/w and the minimum yield was 45.5% w/w obtained from experimental run 27. The maximum collagen yield was obtained at 0.5M of acetic acid, 72 hours of extraction time and temperature at 4^{o}C. The minimum yield was obtained 0.9M of acetic acid, 24 hours of extraction time and temperature at 38^{o}C.

Table 15: Experimental design matrix by CCD and yield of collagen

Run	Acid Concentration (M)	Extraction Time (hrs.)	Temperature (^oC)	Collagen yields (%)
1	0.3	72	38	50.2
2	0.9	72	25	52.3
3	0.9	24	38	50.4
4	0.3	96	38	49.7
5	0.9	96	38	45.5
6	0.5	24	4	57.6
7	0.3	96	4	72.0
8	0.5	72	25	62.1
9	0.5	96	4	65.7
10	0.5	24	38	60.2
11	0.3	24	4	50.6
12	0.9	24	25	52.4
13	0.3	72	25	62.6
14	0.3	72	4	67.3
15	0.5	72	4	82.9
16	0.5	96	25	70.8
17	0.9	72	4	61.2
18	0.5	24	25	55.0
19	0.5	72	38	53.7
20	0.3	24	25	48.2
21	0.9	24	4	63.2
22	0.3	96	25	59.5
23	0.9	72	38	56.4
24	0.9	96	4	53.7
25	0.5	96	38	52.0
26	0.3	24	38	61.7
27	0.9	96	25	52.8

4.11.1 ANOVA Analysis

Response: Collagen Yeild

ANOVA for Response Surface polymonial Model

Table 16: Analysis of variance [Partial sum of squares]

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F
Model	1103.15	9	122.57	3.50	0.0125 significant
A	*32.89*	*1*	*32.89*	*0.94*	*0.3458*
B	*9.90*	*1*	*9.90*	*0.28*	*0.6016*
C	*371.05*	*1*	*371.05*	*10.61*	*0.0046*
A^2	*163.89*	*1*	*163.89*	*4.69*	*0.0449*
B^2	*115.43*	*1*	*115.43*	*3.30*	*0.0869*
C^2	*0.65*	*1*	*0.65*	*0.019*	*0.8932*
AB	*85.84*	*1*	*85.84*	*2.45*	*0.1356*
AC	*0.068*	*1*	*0.068*	*1.931E-003*	*0.9655*
BC	*151.26*	*1*	*151.26*	*4.33*	*0.0530*
Residual	594.51	17	34.97		
Cor Total	1697.66	26			

The Model F-value of 3.50 implies the model is significant. There is only a 1.25% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case C, A^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

Final Equation in Terms of Actual Factors

$$\begin{aligned}\text{Collagen Yeild} &= 19.74444 + 89.29716 * \text{Acid Concentration} + 0.74796 \\ &* \text{Extraction Time} + 7.30287E-003 * \text{Temperature} - 66.52778 \\ &* \text{Acid Concentration}^2 - 3.87731E-003 * \text{Extraction Time}^2 \\ &+ 1.21621E-003 * \text{Temperature}^2 - 0.23880 * \text{Acid Concentration} \\ &* \text{Extraction Time} + 0.014311 * \text{Acid Concentration} * \text{Temperature} \\ &- 5.64476E-003 * \text{Extraction Time} * \text{Temperature}\end{aligned}$$

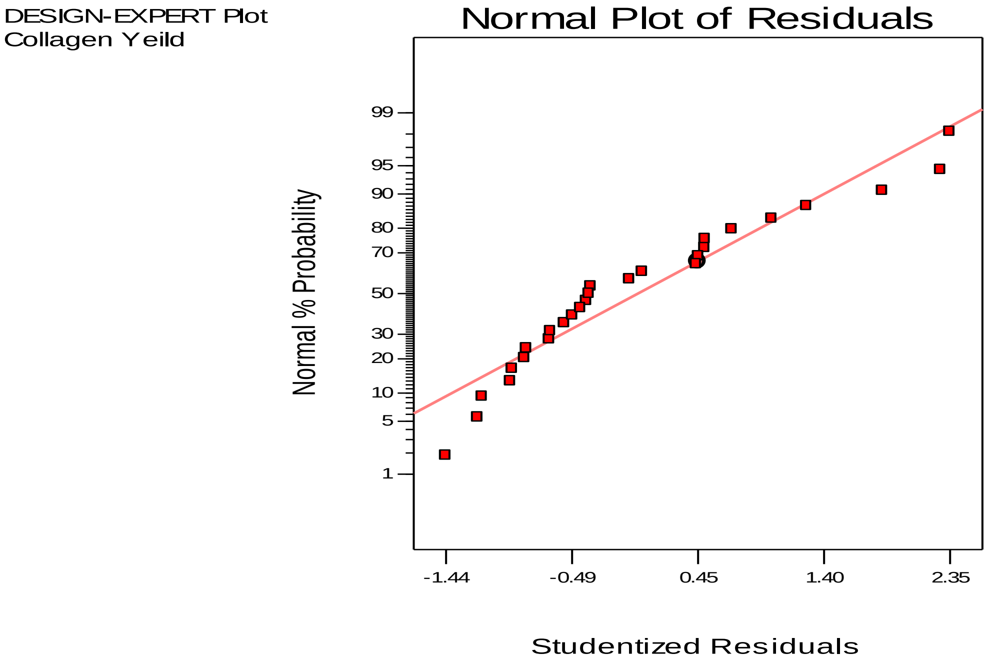

Figure 16: Normal probability plot

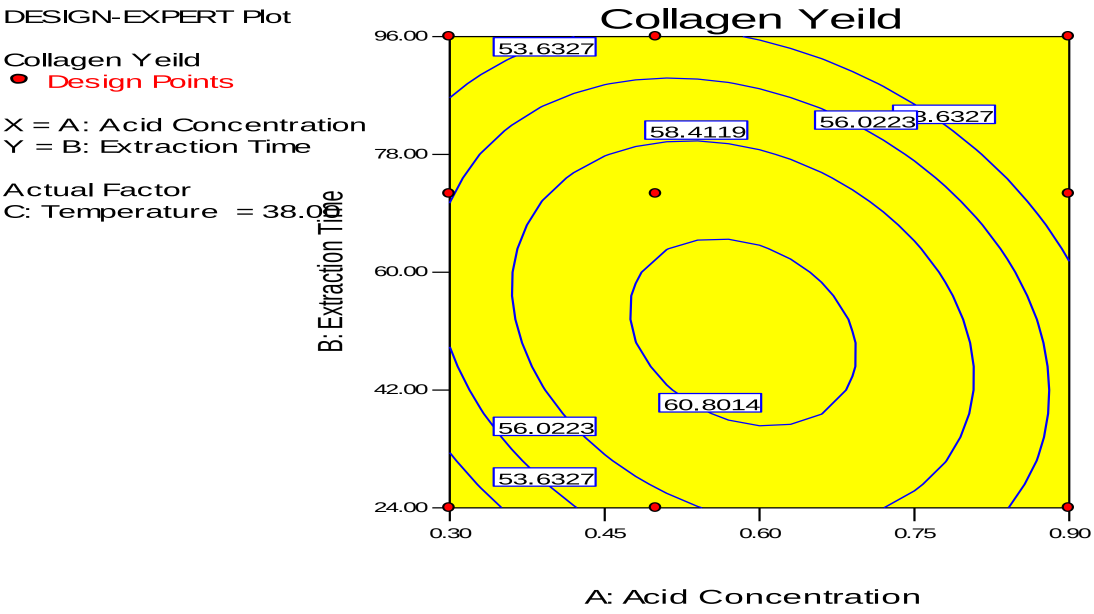

Figure 17: Contour plot of the interaction effect of acid concentration and extraction time versus yield

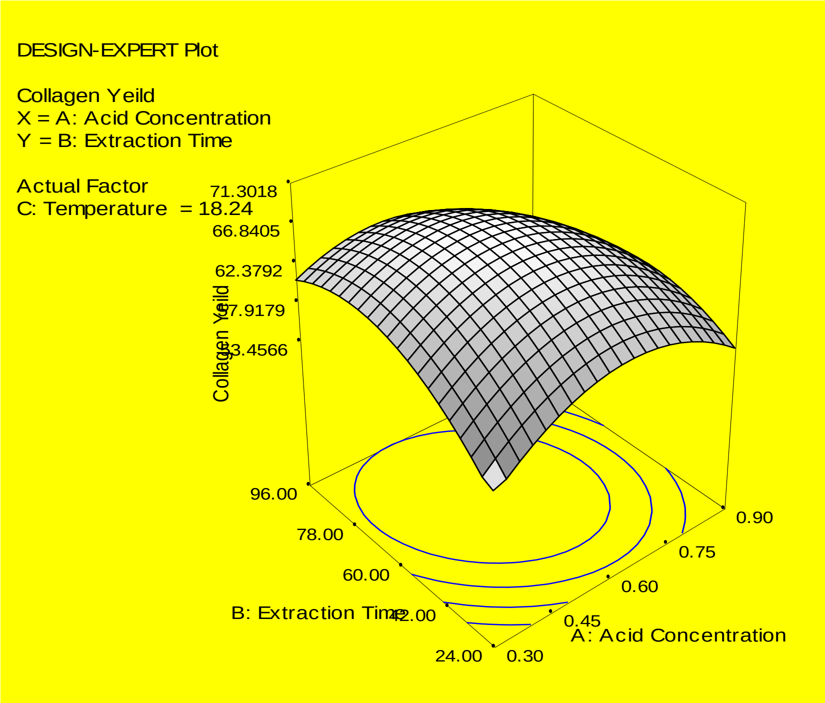

Figure 18: Surface plot of the interaction effect of acid concentration and extraction time versus collagen yield

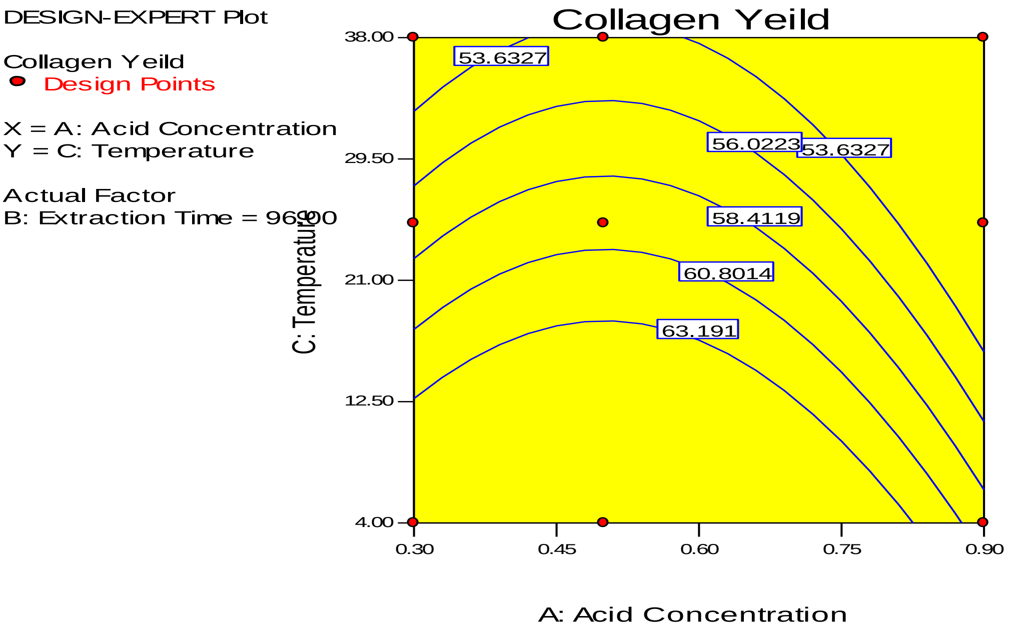

Figure 19: Contour plot of the interaction effect of extraction time and acid concentration versus collagen yield

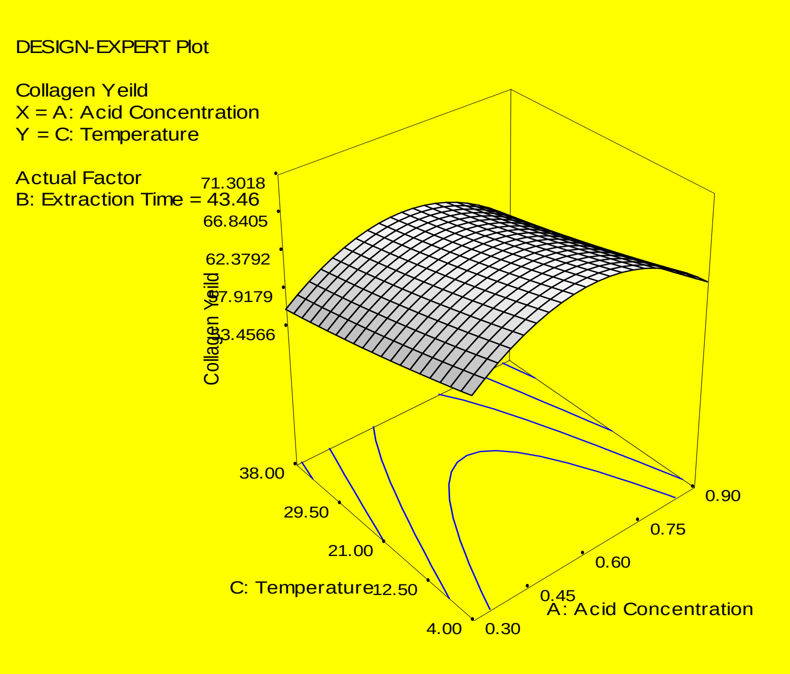

Figure 20: Surface plot of the interaction effects of acid concentration and temperature versus collagen yield

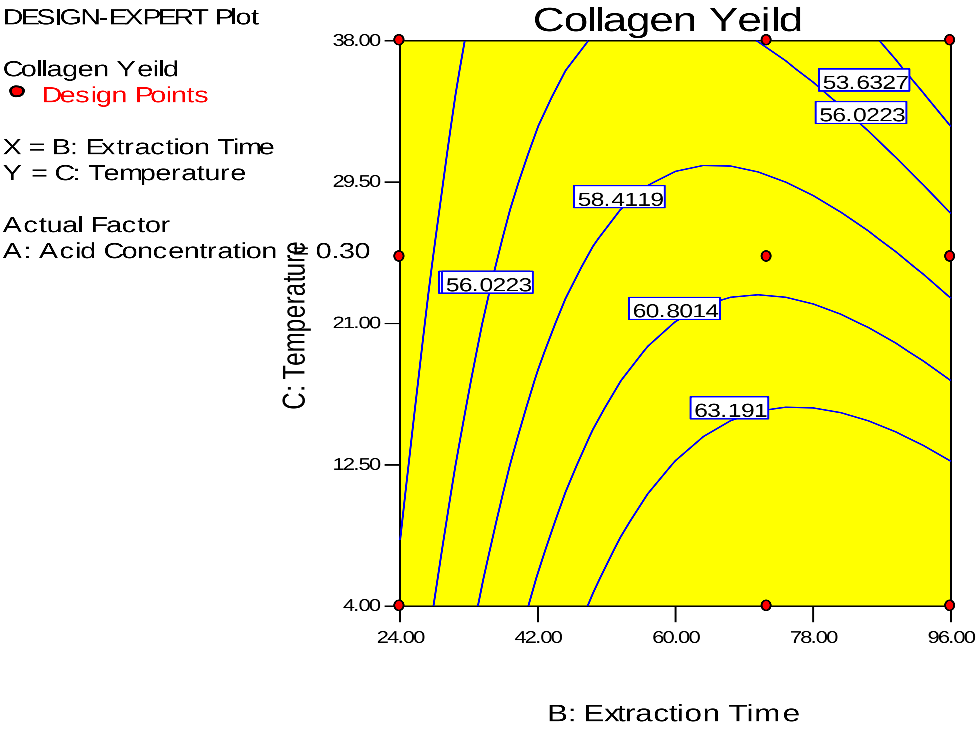

Figure 21: Contour plot of the interaction effects of extraction time and temperature versus collagen yield

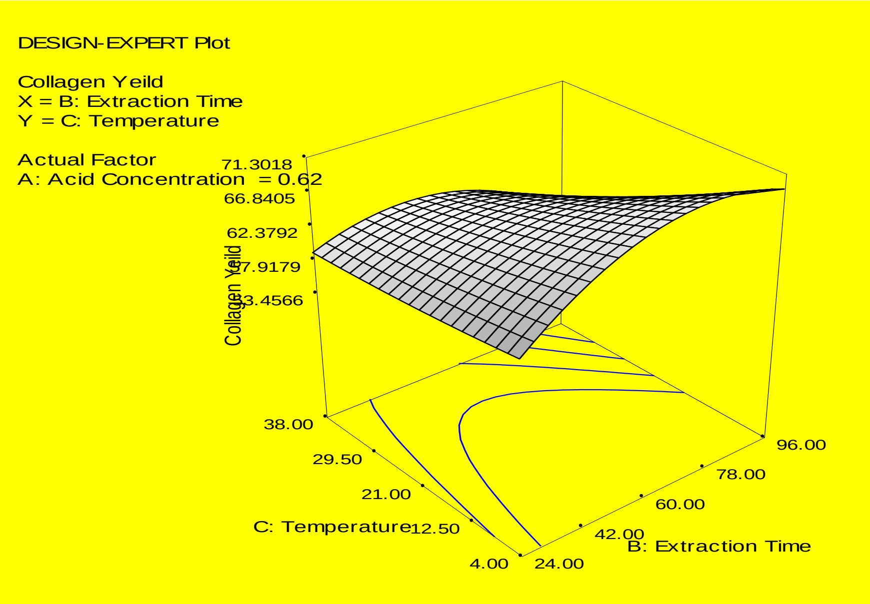

Figure 22: Surface plot of the interaction of extraction time and temperature versus collagen yield

4.12 Yield of Collagen from Fleshing Wastes

The amount of collagen extracted from green and lime fleshing wastes were presented in Table 17. The amounts of collagen extracted from lime/green fleshing wastes were obtained 12. 73% and 14.87% w/w, respectively. The ability of extract collagen using acetic acid from lime and green fleshing were determined to be 84.87% and 96.53%, respectively in depicted Table 10. The difference yield of collagen was conducted a characterization of SDS-PAGE, FTIR and CD analysis where collagen extracted from green fleshing higher ellipticity than collagen that obtained from lime fleshing at 222nm, which is characteristic of triple helical structure, for the same volume of collagen.

Table 17: yielding of collagen from lime and green fleshing wastes

Types of fleshing wastes	Yields of collagen (%)
Liming fleshing waste	84.87 ± 0.5
Green fleshing waste	96.53 ± 1.0

4.13 Characterization of Extracted Collagen from fleshing Wastes

The collagen extract from fleshing wastes using acetic acid was characterized by using CHNS-Elemental Analysis, SDS-PAGE, FTIR, CD spectra and thermal behaviour. The purity and triple helical structure of native collagen were confirmed by the following characterization methods including their data analysis.

4.12.1 CHNS - Elemental Analyser

The results of elemental analysis of elements are presented in Table 18. The nitrogen content of collagen from lime and green fleshing was found to be 14.62% and 15.68%, respectively whereas L-Cystine was 13.03%. The carbon percentage of collagen extracted from lime/ green fleshing were pronounced 44.5% and 43.04%, respectively whereas L-Cystine was 42.18%. The amount of hydrogen in collagen that obtained from lime and green fleshing wastes was given results 6.12% and 7.45%, respectively and L-Cystine (standard) was 5.03%. The percentage of sulphide content in collagen which was extracted from lime fleshing wastes that pronounced 0.26 % whereas L-Cystine was 0.56%. Generally, elemental analysis showed that the extracted collagen from lime fleshing and green fleshing and L-Cystine indicated the protein sample highly purity and any non-proteinous contaminants are negligible.

Table 18: Elemental analysis of collagen extracted from fleshing wastes

Name of Elements	% Amounts		
	Collagen from Lime Fleshing Waste	Collagen from Green Fleshing Waste	L-Cystine (standard)
Nitrogen	14.62±0.9	15.68±0.9	13.03
Carbon	43.04±1.3	44.50±1.8	42.18
Hydrogen	6.12±0.6	7.45±0.6	5.30
Sulphide	0.26±0.1	0.00	0.56

4.12.2 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis

SDS-PAGE analysis conducted to assess the purity of extracted collagen which obtained from delimed and green fleshing wastes is shown in Figure 23. The gel clearly indicated that three bands, namely α, β and ϒ-chains which has a molecular weight of 100, 200 and 220Kda, respectively which are pronounced as type I collagen (Senaratne, et al, 2006) where β, ϒ are dimer and trimmer forms of the α-chain respectively. The collagen that obtained from deliming and green fleshing were confirmed the presence of type I collagen. The oriented pattern of collagen that obtained from deliming and green fleshing showed that the molecular weight of α-chain was around 100kDa and β-chains about 220kDa indicated in SDS-PAGE are covalently cross-linked of double α-chains of two different collagen molecules (Tanaka, et al, 1988). The absence of any other bands in the gel profile showed that sample used contained highly pure type I collagen.

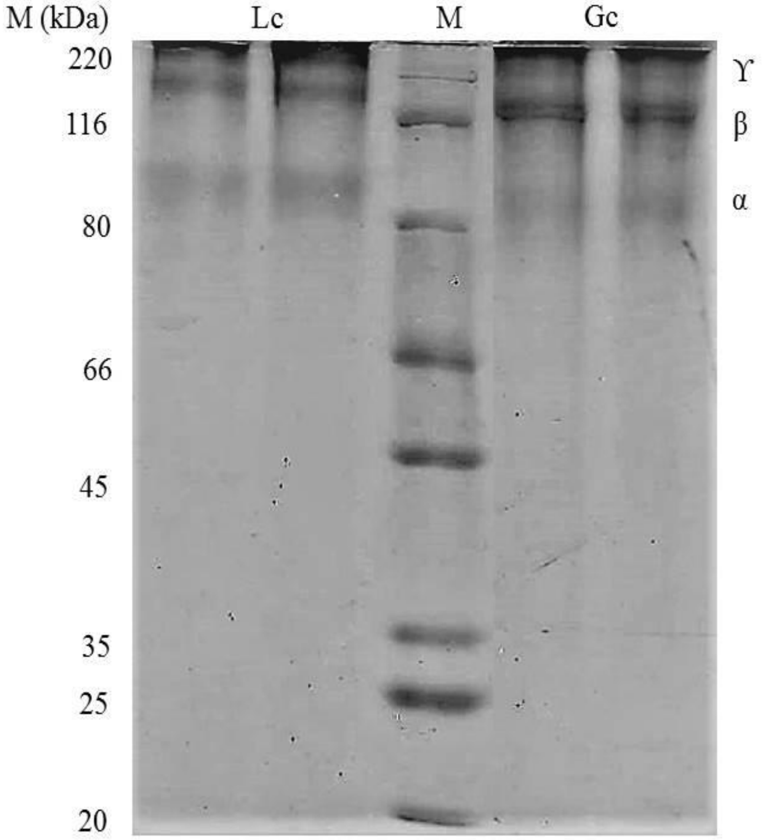

Figure 23: SDS-PAGE analysis of protein that extracted from liming fleshing (Lc) and green fleshing (Gc) with benchmark protein marker (M)

4.12.3 Fourier Transform Infrared Spectroscopy of Collagen (FTIR) Analysis

The triple helical structure of purified collagen was proved by using FTIR analysis. In order to assess the triple helical structure of collagen, FTIR spectrum of collagen gives characteristic Amide A, Amide B, Amide I, Amide II and Amide III peaks. Amide A band is commonly associated with N-H stretching vibration and occurs in the wave number range of 3400-3440cm^{-1} (Doyle, et al, 1975). The collagen extracted from lime fleshing and green fleshing wastes indicated the Amide A peak at 3375cm^{-1}. Amide B peak, representing CH_2

asymmetrical stretch was observed at 2850cm^{-1}. The amide-I band between 1600-1700cm^{-1} is mainly associated with the C=O stretching vibration and is directly related to the backbone conformation which is used to verify the secondary structure of protein (Payne & Veis, 1988). The Amide II band is associated with plane NH bending vibration combined with C-N stretching vibration in the presences of small amount percent of C-C stretching vibrations and normally occurs in between 1550 – 1600cm^{-1}. The amide III band found in the range of 1220-1320cm^{-1} bond indicates a combination of N-H deformation coupled with C-N stretching vibrations. The presence of all characteristic peaks without any major deviations showed that the extracted Type I collagen was in the native form which shown in Figure 24.

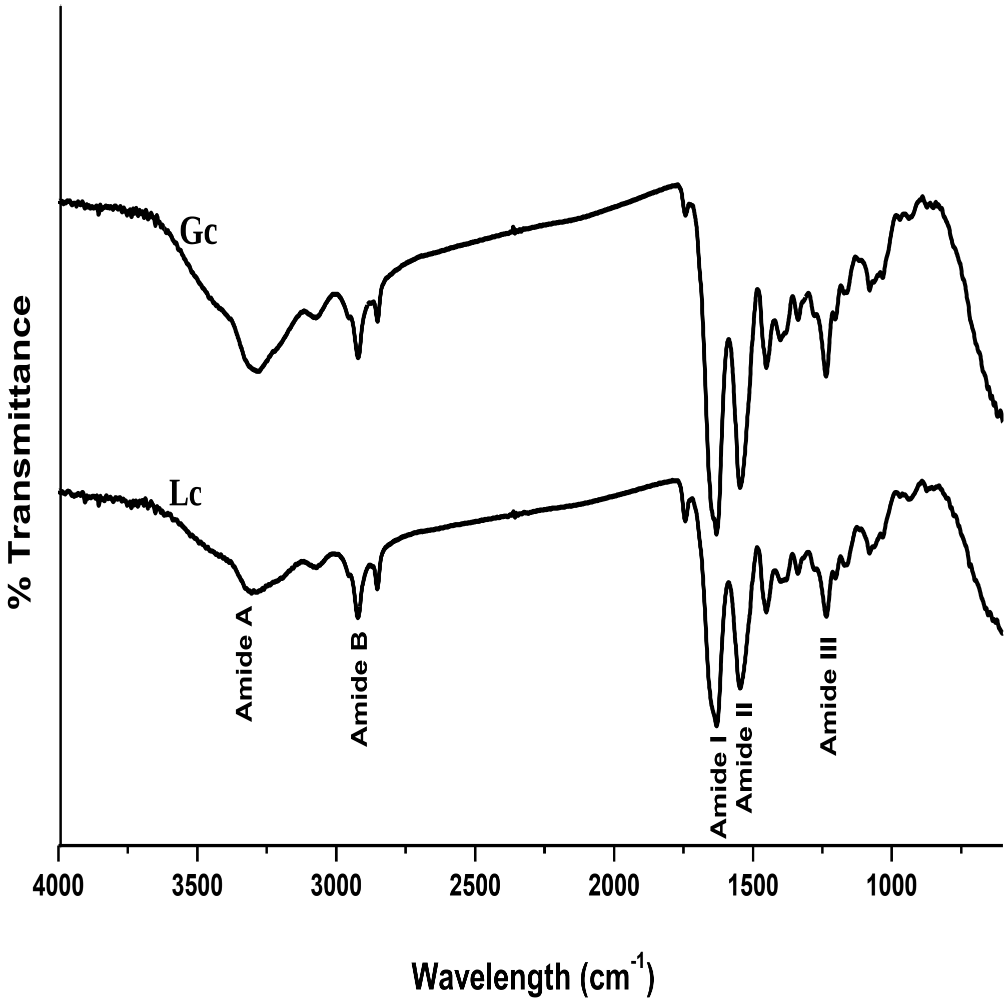

Figure 24: FTIR Spectrum analysis of extracted collagen for from lime fleshing (Lc) and green fleshing (Gc)

4.12.4 Circular Dichroism Spectra (CD) Analysis

CD spectroscopy is a well know technique used for assessing secondary structure of proteins and polypeptides in solution (Greenfield, 1999). Characteristic CD spectrums of native triple helical collagens have been a positive peak at around 222 nm and a negative peak around 198 nm (Heidemann & Roth, 1982). The extracted and purified collagen was subjected to CD spectroscopy between 250 and 190nm, and as expected a positive peak at 222nm and a negative peak at 198nm were seen (Figure 25). This confirmed, along with the data obtained from FTIR, which the extracted collagen is in its native form. Further, the thermal melting temperature of the collagen sample was studied by monitoring the ellipticity at 222nm between 10 and 60°C. The thermal denaturation curve is shown in Figure 26 and the minima of the first derivative of the curve showed that the thermal denaturation temperature of the collagen sample is 40°C.

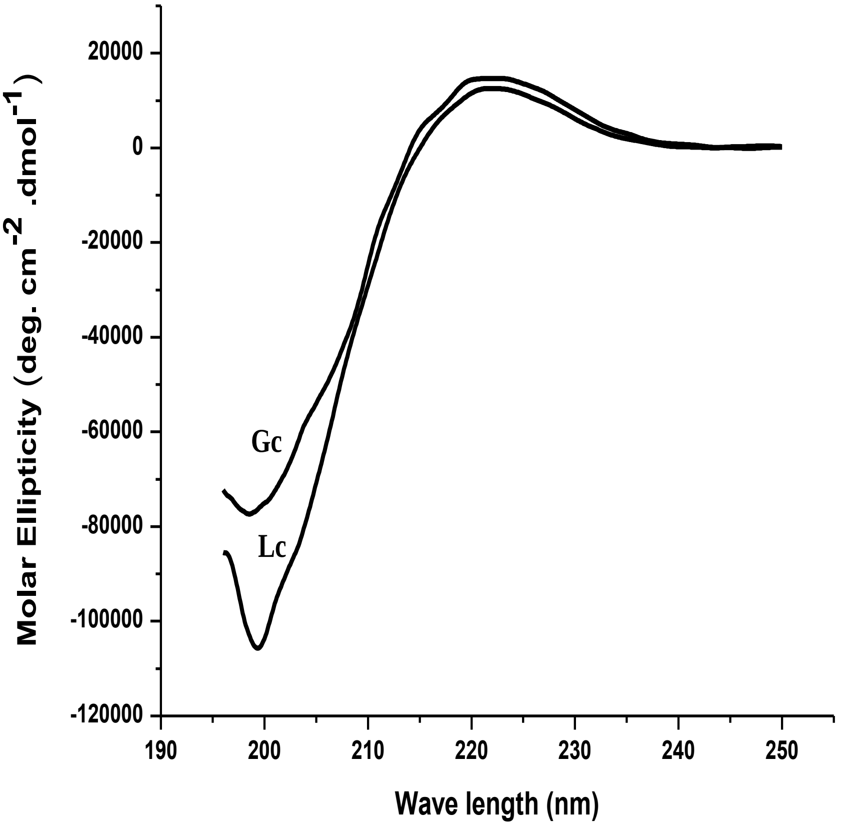

Figure 25: CD spectra analysis of collagen from lime fleshing (Lc) and green fleshing (Gc) wastes with concentration of 0.5mg/L at 25°C

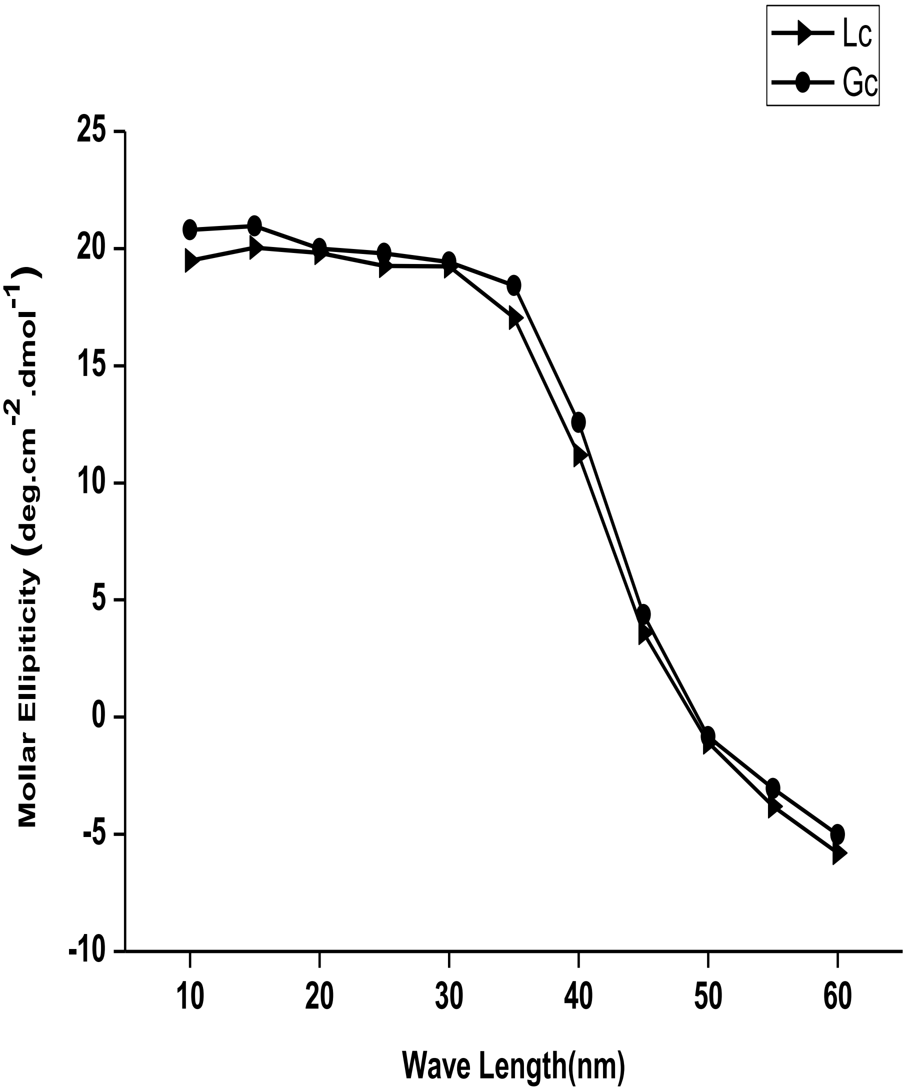

Figure 26: Thermal melting transition of extracted collagen from lime (Lc) and green fleshing (Gc) observed by CD spectra which concentration was 0.5mg/mL.

CHAPTER FIVE

4. CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Beamhouse process generates wastes when it manufactured leather articles. Due to this reason, environment changed their nature by increasing pollution load. 70% of wastes produced from beamhouse process (Kanagaraj, eta al, 2015). Bioprocess is one of the alternative technologies to save the nature of environment. This study focused on enzymatic soaking and dehairing process. Among different concentration of alkali protease feed on soaking process, 0.8% of concentration showed an effective fibre opening of hide and provided high yielding of collagen from conventional process of lime fleshing wastes. In addition to this, the struggles of enzymatic unhairing were solved by 1% of lipase followed by 4% of protease. The efficacy of unhairing was assessed by SEM analysis, water analysis and quality of leather at natural crust level. All the analysis of performance of unhairing was confirmed the standard of their requirement. Fleshing wastes were generated in beamhouse process. The major components of fleshing wastes are proteins and fats. Experiment designed to utilize wastes which generate revenue. Collagen extracted from lime and green fleshing using acid solubilisation which gave 84.78% and 96.53%, respectively. Extracted collagen was confirmed further analysis by SDS-PAGE, FTIR and CD spectra. All analysis pronounced Type I collagen and in native forms. Collagen has many applications in agriculture, cosmetic, medicine, food and pharmacy. Therefore, this study deliver the development of cleaner beamhouse and effective utilization of fleshing wastes which showed road map of generate an income from high value of purified collagen.

5.2 Recommendations

Cleaner beamhouse process is one of alternative solution for reducing pollution which wastes generate from tanneries. This study showed the road map of minimizing of wastes by utilization of chemicals that manufacture by biotechnology. An enzymatic soaking/unhairing need further works in the advantages of cost, time and weather condition. Therefore, enzymatic process should be design a minimum cost and time.

Green fleshing wastes were collected from soaking process which utilized for extraction of bio-product (collagen) that generates revenue instead of wastes. In this study, only collagen extracted from green fleshing. Green fleshing can be used as raw material resource collagen hydrolysate, gelatin and oil. These different product provide variety of application viz. cosmetic, agriculture, syntan utilize for post tanning, tissue engineering, packing material, food and pharmacy application.

Generally, the output of this study will direct further work look as follows.

- In Ethiopia, the application of enzyme in leather industries were not familiar especially fibber opening and unhairing. Hence synthesis the enzyme and apply in Ethiopia leather industries under its weather condition with consideration of cost and time.
- Here the study was extract only collagen from green/lime fleshing wastes. We can further prepare gelatine, collagen hydrolysate, packing materials, gelatin films and collagen sheet for different application.

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Appendixes

Appendix A

Conventional beamhouse process

Process	Chemicals	Concentration (%)	Duration(minute)	Remark
Soaking	Water	300	20	Wash
	Water	300	Left overnight	Total time taken 18 hr. Next day drain out
	Sod. Carbonate	0.25		
Liming	Water	250	Left overnight	Total time taken 14 hr.
	Lime	8		
	Sod. sulphide	2.5		PH (12- 13)
Washing	Water	150	10	Run drum 4 rpm
Re-liming	Water	200	Left for overnight	Run for 15 minute
	Lime	4		
Fleshing	Pelts were fleshed and weighed the pelt			
Deliming	Water	100	40	PH adjust to 8.2-9.0
	Amm. Chloride	2.5		
Washing	Water	100	30	Run the drum & drain out
Pickling	Water	100	3 X 10 + 30	Run the drum and acid was dilute with water (1:10 ratio) and added in three feeds. Check the pH (2.8-3.0)
	Sod. Chloride	10		
	Sulphuric acid	2		

Appendix B

Process for making natural crust for Enzymatic (Table 1& 2) and conventionally beamhouse process

Process	Chemicals	Concentration (%)	Duration (minute)	Remark
Chrome tanning	BSC	8	3 x 10’ + 90’	
Basification	Sod. Formate	1	3 x 10’ + 30’	PH adjust to 3.8-4.0
	Sod. Bicarbonate	1		
Neutralization	Sod. Format	1	3 x 10’ + 30’	P^H adjust to 4.8 -5.0
	Sod. Bicarbonate	1		
Post tanning	Water	100	30’	
	Relugan RE	2		
	Basyntan DI	4		
	Basyntan FB6	3	30’	
	Relugan RE	2		
	Lipodermliq SAF	4	60’	
	Balmol SXE	4		
	Lipodermliq FB16	4		
Fixation	Wattle GS powder	4	30’	
	Formic acid (1:10 dilute with water)	1	3x10’ + 30’	pH (3.9-4.0) Drain
Washing	Water	100	10’	Drain, pile, set, dry, stack, trim and buff

Appendix C

Estimation of Hydroxyproline (HP) Content

Principle

The degraded collagen content in the sample was determined based on the hydroxyproline index according to the method of Wossenr (1961). Hydroxyproline was liberated from the hydrolysed collagen, which was oxidized by Chloramine-T to yield Pyrrole-2-carboxylic acid. The reaction was arrested with perchloric acid and the resulting product was reacted with P-dimethyl amino benzaldehyde acid to give a coloured complex which can be read at 557nm on a spectrophotometer.

Reagents

Hydroxyproline Standard

A stock solution is prepared by dissolving 25mg of vacuum-dried L-hydroxyproline in 250 mL of 0.1N HCl standards are prepared daily by diluting the stock with water to obtain concentrations of 1-5 µg/2mL.

Buffer

Fifty grams of citric acid monohydrate, 12mL of glacial acetic acid, 120g sodium acetate trihydrate, and 34 g sodium hydroxide are made to a final volume of 1 litre in distilled water. The pH is carefully adjusted to 6 and the buffer is stored in refrigerator under toluene.

Chloramine T (Sodium P-Toluene Sulfon Chloroamide)

A 0.05M solution is prepared fresh daily by dissolving 1.41g chloramine T in 20mL water. 30mL of methyl cellosolve and 50mL buffer are added. The solution is kept in a glass-stoppered flask.

Methyl Cellosolve (Ethylene Glycol Mono-methyl Ether)

Preparations free of interfering substances

P-Dimethyl Amino Benzaldehyde

A 20% solution is prepared shortly before use by adding methyl cellosolve to 20g of pp-dimethyl amino benzaldehyde to give a final volume of 100mL. This may be warmed in 60

^{0}C water bath to facilitate solubilisation. If the solutions are deep blue or purple, recrystallization of this reagent by the method of Adams and Coleman may be necessary.

Perchloric Acid

A 3.15M solution is prepared by diluting 27mL of 70% Perchloric acid to 100mL with water.

Procedure

Samples (proteins, tissue, fluids etc.) are hydrolysed by adding HCl to final concentration of 6N. The samples are sealed in small Pyrex test tubes and hydrolysed for 3 hour at 130^{0}C. The tubes are then opened. The contents are decanted into a graduate cylinder or volumetric flask. The tubes are washed thoroughly with water and the washings were combined with the hydrolysate. Several drops of 0.02% methyl red indicator are added, followed by 2.5N NaOH for neutralization. Final adjustments are made with dilute HCl and NaOH until the indicator turns slightly yellow corresponding to pH 6-7.

Reaction Procedure

Samples are prepared as above. 2mL portions containing 1-5µg hydroxyproline are placed in 16x150mm test tubes. A series of standards is prepared containing 0-5µg hydroxyproline in 2 mL. hydroxyproline oxidation is initiated by adding 1ml chloramine T to each tube. The tube contents are mixed by shaking a few times and allowed to stand 20min at room temperature. Chloramine T is destroyed by adding 1ml Perchloric acid to each tube in the same order as before. The contents are mixed allowed to stand for 5 minutes. Finally, 1ml p-dimethyl amino benzaldehyde solution added. The mixture is shaken until on schliren can be seen. The tubes are placed in a 60^{0}C water bath for 20 minutes. Then cooled in tap water for 5 minutes (the occurrence of colour is stable for at least 1 hour). The absorbance of the solutions is determined spectrophotometrically at 557nm).

Notes

- Absorbency is linearly related to the amount of hydroxyproline over the range 0-5 µg
- In the region 5-10µg there is a slight negative deviation of about 2% hence it is recommended that samples be diluted to contain 1-5µg HP per 2mL.
- The mill molar extinction coefficient of the chromogenic is found to be 56.3 at 557nm.
- Samples (purified calfskin gelatin) had an average HP content of 14.34±0.16%.

Appendix D

Extraction of Fat from Fleshing Wastes

Preparation of Sample

After fleshing wastes collected, liming fleshing wastes carried out deliming with 3% of ammonium chloride and ready for extraction. Green fleshing cleaned three times with normal water and ready for extraction of fat.

Extraction Procedure

The fat was extracted using Soxhlet apparatus and by heating at 80^0C. Soxhlet apparatus is the most commonly method that used to extract the amount of oils and fats. The extraction was conducted by petroleum ether solvent as the fat is soluble in this solvent. For this experiment, Soxhlet apparatus was used to fat content from another substance like insoluble matter and protein by applying solvent at 80^0C.

Procedure

- Petroleum ether solvent was poured into bottom flask.
- 50g of the sample was put in the thimble and inserted in the centre of the extractor
- The Soxhlet was heated to a boiling temperature of solvent.
- The extract was collected and all the solvent evaporate
- The experiment was repeated by placing the same amount of the sample into the thimble again.
- The weight of the oil was determined for run hours.

Appendix E

Analysis of Unhairing Efficacy

Scanning Electro-Microscope (SEM)

SEM analysis showed the opening up of fibber and avoid of hair root.

Total Organic Content (TOC) Analyser

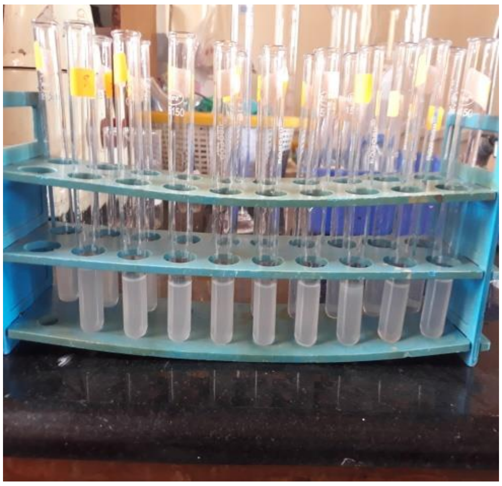
Wastewater Analysis
TOC Analyser gave information about the statues of environmental pollution

Appendix F

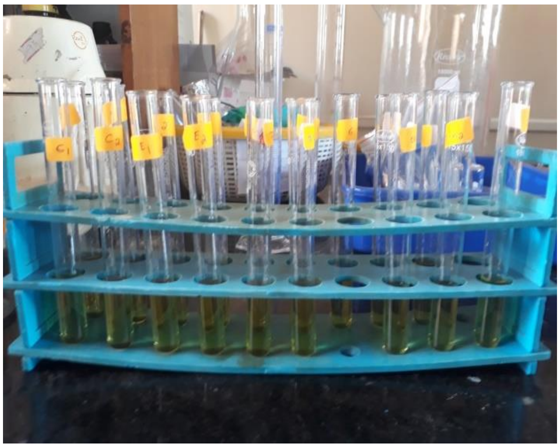
Collagen Content and Characterization Analysis

Estimation of Hydroxyproline Content

3µg hydroxyproline in 2 ml hydroxyproline oxidation is initiated by adding 1ml chloramine T to each tube. The colour of solution becomes colourless. (Initiation the chemical reaction)



Chloramine T is destroyed by adding 1ml Perchloric. Now the colour changes to white cloud. (Stop the chemical reaction)

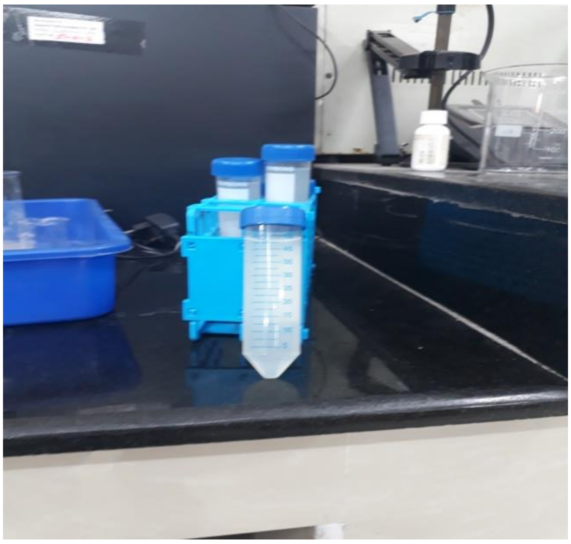


1ml p-dimethyl amino benzaldehyde solution added. The mixture is shaken until on schliren can be seen. The colour of solution was show light green

Appendix G

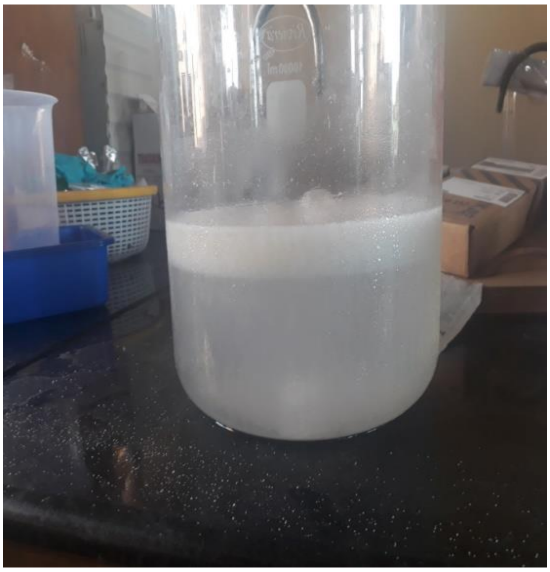
Extraction of Collagen

After added small amount of acetic acid (0.5M) into fleshing samples, the solution mix uniformly. This process is called acid swelling.

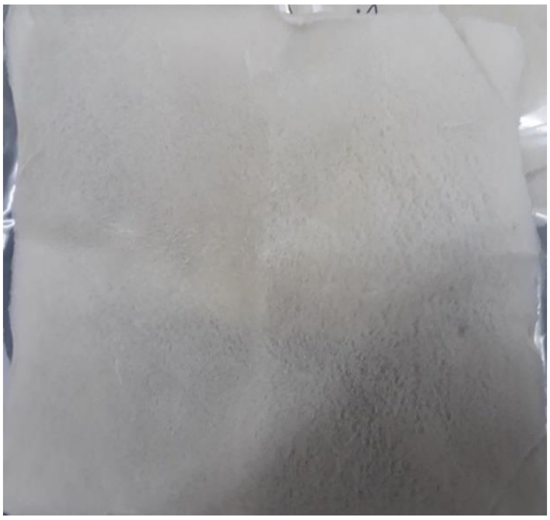


The homogenise fleshing solution with acetic acid was ready for centrifuge process. The centrifuge instrument used to settle the collagen in the form of pellet.

After centrifuge process completed, pellet form of collagen was settle in the bottom of in the centrifuge tube.



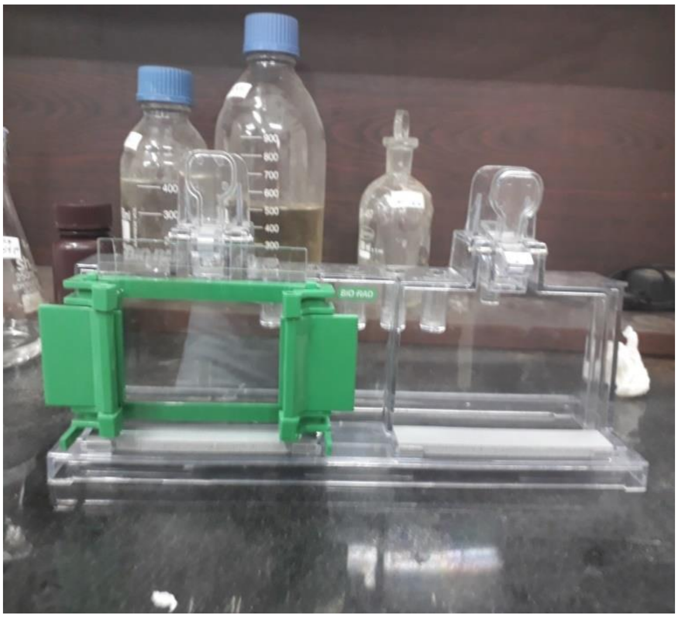
After centrifuge the solution, added 5% (w/v) of salt (NaCl) into this supernatant and kept for 5 hours which separated supernatant and the image is observed as diagram.



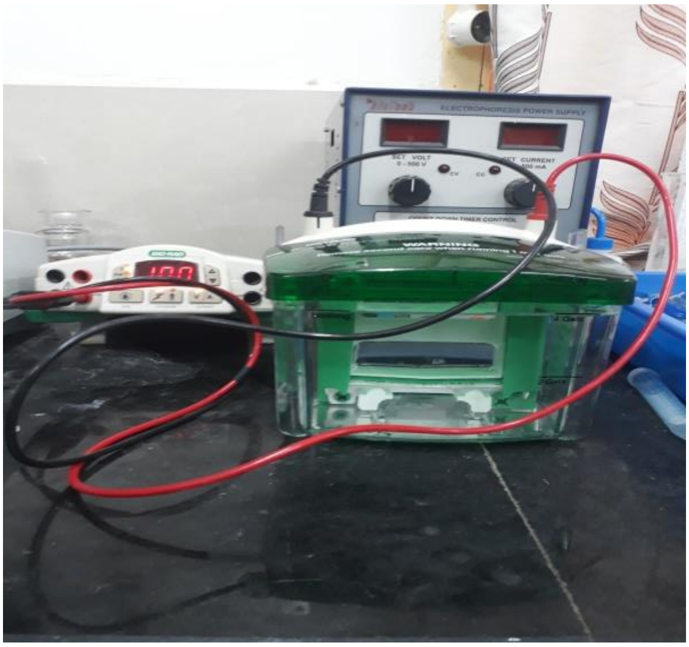
After dialysis the extracted collagen, the collagen lyophilised and it forms this image.

Appendix H

Characterization of collagen



The purity of collagen was checked by SDS-PAGE. This diagram showed that gel film and samples was filling on this stand.



This diagram indicated that the sample run on the gel film till reach bottom.