**ADDIS ABABA UNIVERSITY**

**ADDIS ABABA INSTITUTE OF TECHNOLOGY**

**SCHOOL OF CHEMICAL AND BIO ENGINEERING**

**ENVIRONMENTAL ENGINEERING STREAM**

# FICUS FRUIT ENZYME PREPARATION AND APPLICATION AS ALTERNATIVE TO SULFIDE DEHAIRING

A Thesis Submitted to School of Chemical and Bio Engineering, Addis Ababa Institute of Technology in Partial Fulfillment of the Requirements for the Degree of Masters of Science in Chemical Engineering (Environmental Engineering)

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**Certification**

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TITLE: FICUS FRUIT ENZYME PREPARATION AND APLICATION AS ALTERNATIVE TO SULFIDE DEHAIRING

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APPROVED BY BOARD OF EXAMINERS

This is to certify that we have read this MSc thesis and that in our opinion; it is fully adequate, in scope and quality, as an MSc research proposal for the Degree of Master of Science in school of chemical and bio engineering environmental engineering stream.

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## Declaration

I declare that this thesis entitled “Ficus Fruit Enzyme Preparation and Application as Alternative to Sulfide Dehairing” is my own original work done under the supervision of Professor Zebene Kiflie at School of Chemical and Bio Engineering in Addis Ababa Institute of Technology, Addis Ababa University and I have not previously submitted it entirely or in part for obtaining any qualification at any other university.

Name: Liya Shawel

Signature:____________________

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**Acronyms**

BOD Biochemical Oxygen Demand

BBD Box-Behnken design

COD Chemical Oxygen Demand

TDS Total Dissolved Solid

TSS Total Suspended Solid

ANOVA Analysis of Variance

FS Ficus sycomorous

SEM Scanning electron microscope

UV Ultraviolet

Conc Concentration

EDTA Ethylenediaminetetraacetic acid

TCA Trichloroacetic acid

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**Abstract**

Many tanning industries use many chemicals such as chrome, lime, sodium sulfide and ammonium salts etc. in different processing stages which can be hazardous for human health and the environment. The pre-tanning (liming) stage uses lime and sodium sulfide for dehairing skins and hides. These, however, result in significant increases of the biological oxygen demand (BOD), chemical oxygen demand (COD), suspended solids, etc. the objective of this thesis work is to investigate the potential of protease enzyme as an alternative to lime-sulfide for dehairing application. Protease enzyme was extracted from Ficus Sycomorous fruit using isolation of enzyme from plant source method. The proteolytic activities of the extracted enzyme were characterized by spectrophotometer and showed 18.1892 U/ml. Effects of different operating conditions such as protease enzyme concentration, temperature and time on the dehairing performance were investigated. The enzymatic de-haired skin was analyzed by scanning electron microscope (SEM). Results show that the enzyme was able to dehair 93% fresh goat skin within 17hr at 8 pH and $35^{0}$C. In addition, the generated waste was characterized and showed reduction of 87%BOD, 93%COD, 86.5%TDS and 96.5%TSS. The use of ficin protease enzyme on goat skin resulted in a highly promising hair removal efficiency as alternative to lime-sulfide chemical de-hairing process.

Key words ; ficin, dehair, proteolytic activities, scanning electron microscopic (SEM)

# 1. Introduction

## 1.1. Background of study

Natural contamination has been a major aggravation to mechanical advancement. Chemical and chemical-based businesses are the prime targets of the environmentalists for their campaign against contamination, and leather industry has too not been cleared out of the figuring (Kamini et al., 1999). In any case, leather and the environment can be depicted as two sides of the coin. For case, leather making can be seen as a handle that employments potential squander from the meat industry (i.e. HIDES or SKINS) to form leather through the tanning process. In spite of the leather industry making traceable and unmistakable impacts on socio-economics through business and send out profit, the industry has picked up a negative picture in society owing to the coming about contamination (Thanikaivelan et al., 2004).

Leather could be a tough and adaptable fabric made by changing over creature crude stow away and skins. It is made through a handle known as tanning where the crude cover up and skins are changed over to non-putrescible fabric which stand up to bacterial assault, chemical debasement and stand up to mechanical miss happening. The fabric picks up aqueous steadiness, great breathability, solidness, tall quality among others characteristics (Maina et al., 2019). Calfskin making handle is compartmentalized into four stages of pre-tanning, tanning, post tanning, and wrapping up. Each of the organize contributes to water, arrive, and discuss contamination. Pre-tanning and tanning stages contribute to 80–90% of add up to contamination stack. Dehairing of creature skins/stows away is accomplished by chemical-based strategy called liming. Liming could be a handle, which chooses the last quality of the leather. All things considered, such vital process step includes a major disadvantage of utilizing sparingly water-soluble lime and naturally hurtful sodium sulfide. The proceeded activity of Goodness and SH from the soluble base and sulfide causes breakdown of disulfide bond in cysteine, resulting in disintegration of the fundamentally sound hair protein, keratin. Hair from the crude fabric is either pulped or evacuated intaglio. Pulping of hair increments the biochemical oxygen request (BOD), chemical oxygen request (COD), and TS of the wastewater. This handle closes up in creating loads of hair squanders and lime slime as strong squanders. In expansion to this, the sulfide outflow from the method makes discuss pollution(Ramesh et al., 2018). Additionally, the utilize of lime within the dehairing handle requires its expulsion, as arule by the expansion of ammonium salts, which speaks to high amounts of nitrogen within the stream of wastewater, one of the most contaminants of fluid effluents (Dettmer et al., 2013). For the most part among the several phases of the tanning prepare, the liming/dehairing is dependable for most of the overall environmental affect, because it producesleast83%ofbiochemical oxygen request (BOD), 73 % of chemical oxygen request (COD),60%ofsuspended solids and 76 % of the entire contaminating charge delivered amid the fabricating process of stows away (Dettmer et al., 2012).

Numerous endeavors have been made in arrange to supplant or minimize the utilize of harmful chemicals. One of the foremost critical advances being considered in this prepare is the utilize of proteins that can be utilized in substitution of the chemical dehairing process (Maina et al., 2019). Numerous initiatives have been undertaken to reduce or eliminate the usage of hazardous substances. The employment of enzymes, which could replace the chemical dehairing procedure, is one of the most important technologies being studied in this process (Dettmer et al., 2012). One of the most promising approaches to lessen the harm that leather manufacturing does to the environment is the employment of enzymes. When enzymes are used in this process, less chemicals are used, the hide's structure can be intentionally altered, and leather with a cleaner grain layer is produced. The benefits of enzymatic dehairing are as follows: (i) hair thinning without causing harm to the dermis's fibrous collagen and without solubilizing hair, (ii) lowering or perhaps getting rid of all sodium sulphide, (iii) good quality hair recovery and (iv) improved working condition (Ranjithkumar et al., 2017). Moreover, proteins can be used in hair sparing dehairing where chemicals assault the bond between the hair and the derma and, hence, lead to free hair (Valeika et al., 2009). The main utilized enzyme preparations are proteases. Proteases are enzymes that catalyse the breakdown of peptides and proteins. They are used in a variety of industrial processes and play a vital part in many physiological processes in living things (Liggieri et al., 2009). Plant peroxidases have been isolated, purified, and characterised from a variety of sources, including Eruca vesicaria, tea, Ficus, lettuce, citrus, broccoli, royal palm, soybean, Leucaena leucocephala, papaya, wheat grasses, Solanum melongena, lemon, and so forth. Because they are active throughout a broad range of pH and temperature values, they have particular significance in industry and medicine (Pandey et al., 2017). Therefore the present study aims to produce dehairing protease from Ficus Sycomorus plant through conventional extraction using appropriate solvent and investigate its dehairing efficiency.

### 1.2. Problem statement

Leather making is a process that uses potential waste from the meat industry which is environmentally friendly. Raw hide and skin that is obtained from the meat industry should pass many processing stages in order to make it non putrescible. Workers in tanneries and the environment are highly impacted with sulfide concentration and lime sludge coming out of the dehairing process, basic hydrogen sulfide out of tanning, and non-biodegradable chemicals at various stages of leather making processes. At present tanners in Ethiopia are fully dependent on lime- sulfide based dehairing practice which adversely affects the environment with a poisonous sludge while sodium sulfide is highly toxic and has obnoxious serious health hazard. The dehairing stage is the most polluting process contributing up to 80–90 % of total pollution load in terms of BOD, COD and TDS (total dissolved solids) and TSS (total suspended solids). In this regard, replacing the chemical(s) used in the dehairing process can contribute to the reduction of the pollution load on the environment and tannery workers. This study, therefore aims at producing protease enzyme from Ficus Sycomorus and study its dehairing performance.

### 1.3. Research Objectives

#### 1.3.1. General objective

The general objective of this research is to prepare an eco-friendly protease enzyme from Ficus Sycomorus plant fruit for application in tanneries as alternative to lime-sulfide dehairing.

#### 1.3.2. Specific objectives

- To extract protease enzyme from Ficus Sycomorus fruit
- To test the extract (protease) proteolytic activity
- To investigate the suitability of Ficus Sycomorus fruit for dehairing purpose
- To study the effects of different parameters like time, temperature, and enzymes concentration on the enzymatic dehairing performance.
- To study the effect of enzymatic dehairing on the BOD and COD of the waste and compare with those of conventional method.

### 1.4 Significance of the study

The findings of this research will help to raise awareness about the impacts of (lime-sulfide dehairng) conventional dehairing on the environment and human health. These will also indicate how conventional dehairing being a pollution generator as a solid, liquid and gas waste, we can instead substitute by enzymatic dehairing which is ecofriendly to the environment. The data that will arise from the study will help the other researchers to investigate the material for similar purpose. It will also be able to enhance the use of cost-effective other local plants to be further more studied and applied. Moreover, it will serve as the baseline information on the extraction of plant protease enzyme for substitution of conventional dehairing for other research activities.

### 1.5 Scope and limitation of the study

The study will be delimited to extraction, application of Ficus sycomorous plant protease called ficain dehairing on goat skin and characterization of the final waste.

### 1.6 Expected output/outcome

At the end of this research, the findings will reveal an alternative lime-sulfide free dehairing, enzymatic deharing from plant protease of Ficus sycomorous plant and will generate important data for further research work.

# 2 Literature review

## 2.1 Leather industry and its impact on the environment

Leather industry is early established enterprise converting food industry waste into variety of end items. Those items are appealing, practical, and sustainable. These products include footwear, apparel, fashion accessories, furniture, and automobile accessories. Tanning putrescible animal rawhide and skin involves various production techniques. It rang from cottage industry to heavy industry to produce flexible and durable leather material (Abdrie Seid et al., 2018). In this respect, the leather industry handles waste products from the manufacturing of meat. It might have been clearly differentiated as an ecologically benign industry in this regard. Nonetheless, the leather industry has frequently been linked to excessive pollution. It is because of its stench, organic waste, and high water usage during production. Varying types of waste arise from turning skins into leathers in hundreds of factories worldwide. From archaic to contemporary have an adverse effect on the environment (Ozgunay et al., 2007)(Yorgancioglu et al., 2020). Nearly all of the tannery's operations are wet, requiring large amounts of water. Approximately 90% of water needed is produced as effluent, which degrades receiving water bodies'. And the land's natural life (Chowdhury et al., 2015) according to data from the FAO, during the processing of every tonne of rawhide used in the global leather industry, an additional 30-35 $m^3$ of waste water is disposed of into the environment. If left untreated, tannery waste can have detrimental effects on the air, water, and land. These are reflected throughout the tannery operation process due to chemicals that are used. Including : calcium hydroxide, sodium chloride, sodium sulfide, acids, carbonates and sulphates. Without appropriate treatment, negative effects borne through three basics: gaseous emissions, wastewater, and solid waste. Gaseous emissions are airborne particles and chemicals that come principally during cutting and preparing hides. As well as when workers handle chemical reagents gas emission occur. To eliminate hair, extremely alkaline chemicals are employed in the soaking, liming, and de-liming procedures. Which need large volumes of water. Lastly, because extra material is disposed of, wastewater also contains large quantities of organic waste (*Mitigating Tannery Pollution in Sub Saharan Africa and South Asia Draft Version: 15 March 2022*, 2022).

### 2.1.1 Leather processing

The manufacturing process for leather preparation can be divided into four basic sub-processes: beam house stage, tanning stage, post tanning stage and finishing based on many studies (Maina et al., 2019; Ozgunay et al., 2007). The figure below shows the process of sub-divided stages in leather tannery.

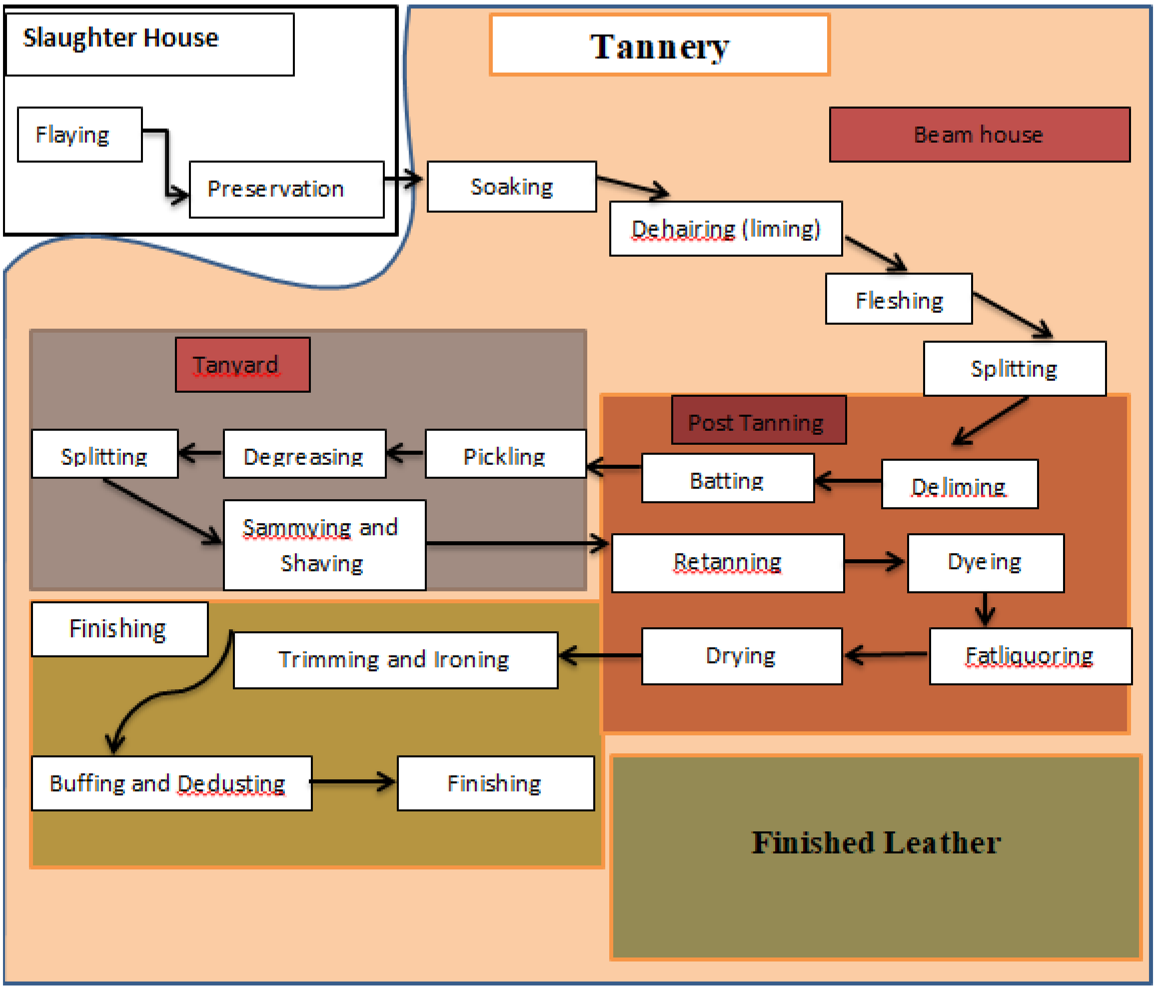


Figure 1 Processes and sub-divided stages in tannery

i. Beam house Processes: After the preserved hides have been trimmed to eliminate any undesired sections. Soaking is to replenish water that has been lost and get rid of dirt, blood, and conservation salt. The wetted hides are treated with a strong alkali solution of lime($Ca(OH)_2$) and sodium-($Na_2S$). It is to ensure hair and wool removal (dehairing procedure) after they have been fleshed. Also to remove the surplus meat and fat sticking to the hide (hypodermis). Hides swells in liming process by immersing in strong alkaline bath to open-up collagen structure. After liming, the hides could undergo a second fleshing procedure to clean the flesh. At this point, the splitting procedure is applied to the skins, sub-dividing them into two or three layers. Deliming is then performed to decrease the pH level in order to remove the lime. And also to make the hide more receptive to the chemicals that used further stages. Through bating operation, hides are exposed to an enzymatic effect for both opening-up the structures and elimination of undesirable proteins from the hide. Following the bating process, a degreasing process is applied. It is to for removing the excess natural fat in hide structure. And also providing a homogeneous distribution of the fat in it.

ii. Tanning Processes: The hides at this stage are first treated with pickle process. It has a solution composed of salt and acids so as to obtain a homogeneous distribution of tanning materials through the cut. After the hides are conditioned as above, the tanning operation is applied with various tanning materials (materials able to form stable bonds with collagen) in order to provide the leather with a stable form and high thermal stability. Tanning materials such as vegetable tannins, mineral tanning materials and syntans (synthetic organic tanning materials) are used in tannage. Because chromium imparts special qualities to leather and saves time during the tanning process, it is the most extensively utilised mineral tanning substance in the leather industry. In the process of making leather, aluminium and vegetable tanning agents are also frequently utilised. The setting out and samming process is used, and shaving is done to get the required thickness of the leather before the leathers are handled with other procedures.

iii. Post-Tanning Processes: in this step the leathers, which are tanned and even to a preferred thickness, pass through re-tannaing process with various retanning agents. It improves the requested characteristics of products. In this process, structural differences within leathers are compensated to obtain uniform structure. Fat-liquoring process is applied using combination of various fat-liquoring agents. It is in order to allow the leather to be more supple and softer. In the dyeing process leathers are dyed to the desired color. Finally, leathers are hung and dried to prepare for finishing process through certain mechanical operations. The unwanted parts are trimmed and removed.

iv. Finishing Processes: After leathers are fat-liquored and dyed in tanning process, they are passed to finishing process. It involves series of coatings on surface to improve resistance, produce appealing and uniform surface. After this process, leathers are trimmed for a final form and sent to confection.

Industries in Ethiopia are few but their influence in terms of pollution is enormous. This is due to the majority of industries lack treatment plants. And also discharges their waste in form of liquid, solid and gas to the environment. Tanning industries are among the industries that produce high concentrations of toxic metals and pollute(Menbere, 2019).

However, the tanning process varies according on the final product, and the kind and volume. The waste generated might also fluctuate greatly. Traditionally, majority of tannery companies process all leather types. Beginning with dehairing and ending with re-tanning procedures (Abdrie Seid et al., 2018). Enormous amount of water and pollutants are discharged during the entire process. Conventional beam house and tanning processes accounts for nearly 90%of total pollution from tannery.

## 2.2 Beam house operations

The processing of hide and skin involves different process and operations. Those are pre-tanning or beam house operations, tanning operations, post tanning and finishing. These operations are for getting the desired features of the leather. The beam house is extremely important in leather manufacturing. It involves a series of unit operations to remove the non-collagen components and to open the collagen fiber-bundles for tanning. Besides, the conventional beam house includes a series of complex unit operations which release many waste as shown in the figure below based on many literature (Beghetto et al., 2013; Covington & Wise, 2020; Liu et al., 2010; Maina et al., 2019).

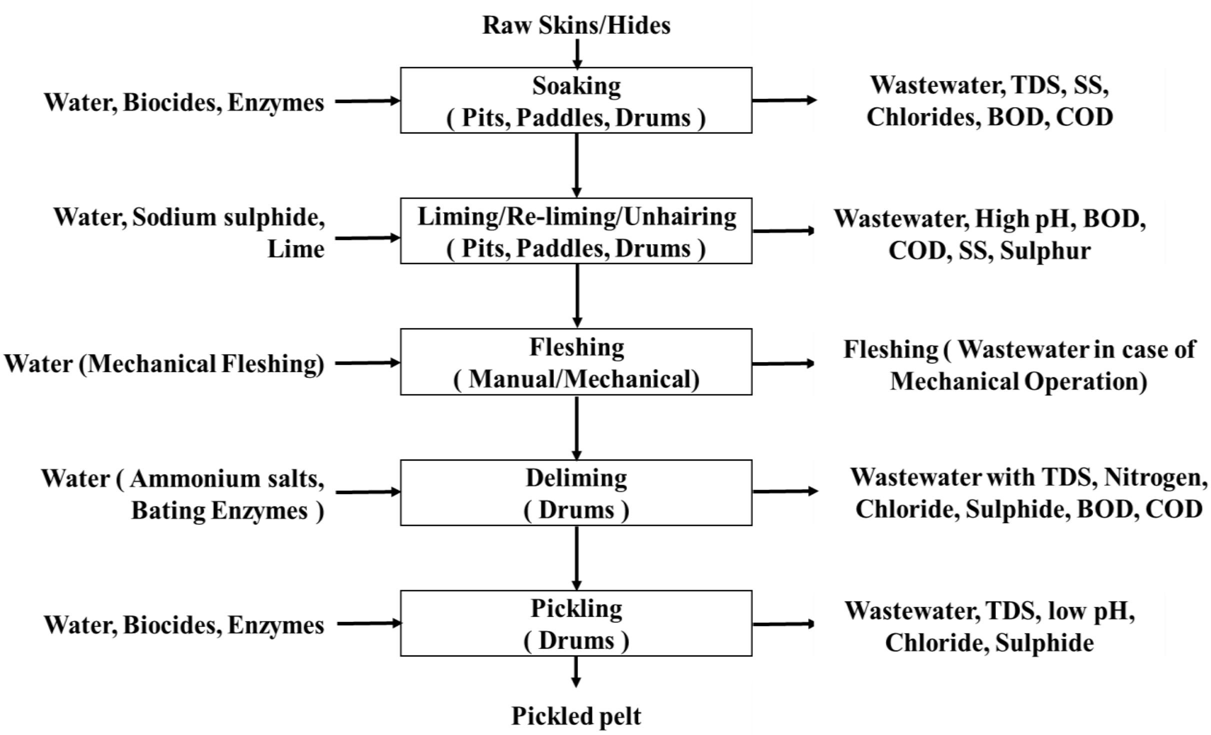


Figure 2 beam house operations

a. Soaking - This is the first step in leather processing. It is an operation which can be carried out in pits, paddles or drum. The purpose of soaking is to remove curing salt from salted skin. Also to rehydrate the skin proteins that cause the fibres to open up, and clear off surface debris such dung, grime, and blood stains. In the process bath, a little residual concentration of sodium chloride is still preferred. Because it facilitates water diffusion along the hierarchical structure of skin fibres for simpler rehydration. Soaking should be done under certain conditions of float, temperature, pH, time and mechanical action. Soaking float is dependent on condition of the skin in range from 200-300 % salted pelt. Green hides are soft enough not requiring soaking, but to remove the blood and dirt. Dried hides need more float for rehydration. Bactericidal agent can also be added to float to prevent bacterial growth during process. Since raw material has a denaturation/ shrinkage temperature of about 65˚C. The temperatures should be limited to 30˚C so as not to destroy the collagen. In conventional batch soaking, salted hides require 6hours or more to remove salt. Also to ensure pelt is completely rehydrated at center and down the hierarchy of structure. Dried skins and hides require 24–48 hours or more.

b. Liming and dehairing - Soaked skins are treated with milk of lime (calcium hydroxide) and sharpening agents sulfides. It aids in the removal of hairs, and other keratinous matter. Liming loosens the collagen fibers and improves the flexibility and fullness of the leather. This process swells up the pelt which contributes to opening-up of the fibre structure. Liming raises pH to alkaline making good environment for the hydrolysis of amide side chains. If the pH is lower, the dehairing chemistry does not work. Its due quilibrium between the non-dehairing hydrosulfide ion and the dehairing sulfide ion is unfavorable. Liming also helps in splitting of fiber structure at the level of fibril bundles. This allows for better penetration of chemicals and more effective reaction. Natural fats are partially saponified, interfibrillar proteins are eliminated, mucoids are degraded.

c. Fleshing - is a mechanical operation done to fresh side of the pelt to remove adhering. Fleshing is done manually for lighter pelts, for heavy pelts a fleshing machine is used. Adequate fleshing allows the penetration of the chemicals in the subsequent processes. Fleshing reduces chemical uptake during liming and assists achieving uniform liming effect to enhance quality.

d. Deliming – removing residual dehairing chemicals like sodium sulfide and lime. During deliming, the pH value of the hides and skins is brought to the required level for the next stage, bating.

e. Bating - This process helps to make finished leather smooth, flat, flexible, soft and stretchy. It involves the addition of proteolytic enzymes. These proteolytic enzymes open the fibrous structure of the pelt to make it softer. Bating also removes the remaining lime in the pelt. Scuds are loosened and other unwanted proteins are removed and these increase degree of stretch. Bating de-swells swollen pelts and prepares pelt for tanning. The process is performed at optimum temperatures for the enzymes 35-40 ˚C.

f. Pickling - the process is primarily conducted to adjust the collagen condition required by tanning reaction. This process lowers the pH by addition of an acid and salt. The low pH ends the bating process and improves the penetration of subsequent tanning agent. And prevents to prevent the rapid combination of the skin substrate with chromium compound. The pickling agents normally used are 5-10% common salt or sodium sulfate, and O.6-1.5% acids. The function of the salt is to prevent acid swelling. The function of the acid is to acidify the collagen, to protonate the carboxyl groups. Where the reactivity is modified, because the chrome tanning reaction only involves ionized carboxyl groups. Acidic treatment minimizes negative charge of carboxyl groups and maximizes positive charge of amino groups. Thus making the pelt positively charged. After this process skin can be shipped or stored for long period without deteriorating.

Nearly 90% of the total pollution from a tannery discharged during the entire tanning process. It accounts for Conventional pre-tanning and tanning processes. Beam house process results in variation in pH. And also causes increase in chemical oxygen demand (COD), total dissolved solids (TDS). Chlorides, sulphates are available in tannery wastewaters. Generally water consumption is highest in the beam house (pre-tanning) areas. But significant amounts of water are also consumed in the post-tanning processes. In the liming stage, protein, hair, skin and emulsified fats are removed from the hides. Then released in the effluent and increase its total solids contents. And also de-liming and bating contain alkaline effluent, sulfides, ammonium and calcium salts (Chowdhury et al., 2015).

### 2.3 Conventional Dehairing operation and its impact on the environment

Dehairing as above mentioned it is the process of removing the hair from the pelt. Once hair shaft detaches from hide surface it is free to float in the bath. And can be separated by filtration for hair saving or chemically dissolved in the bath. On the one hand hair dissolution implies a much higher organic pollution of the wastewater. The mechanism of dehairing is based on chemical attack at sulfur bridge of cystine (CyS-SCy). Sulfur bridge is most abundant (14%) in keratin while it is practically missing in collagen(Dixit et al., 2015).

As conventional dehairing is one of the initial processes of tannery which produces polluting waste. This process in leather making utilizes large amount of lime and sodium sulfide which is hazardous and poses serious waste disposal concerns. Presence of these chemicals in tannery waste is responsible for tremendous pollution. Also cause health hazard to the tannery workers. Lime produces a poisonous sludge while sodium sulfide is highly toxic and has obnoxious odor(Nilegaonkar et al., 2007). The effluent problems arising from lime-sulfide treatment are due to its high alkalinity and suspended solids which contribute to 80–90% of the total pollution load in the waste water, besides generating noxious gases such as hydrogen sulfide as well as solid wastes in the form of lime and sodium sulfide sludge, causing a serious health hazard(Ranjithkumar et al., 2017). Further, the conventional dehairing process involves destruction of the hair. These is leading to increased levels of biological oxygen demand(BOD) , chemical oxygen demand (COD), total dissolved solids (TDS) and total suspended solids (TSS) in the effluent (Ranjithkumar et al., 2017). After dehairing there is deliming process which is to remove the lime and other alkalies. It is done by repeated washing with water and chemicals such as ammonium salts. Weak acids can also be used; boric acid, acetic acid. These chemicals release nitrogenous pollution to environment and also release ammonia gas to air(Maina et al., 2019).

However, 60 to 70% of total pollution load in leather manufacturing is from conventional dehairing. It has big impact on the environment. The conventional dehairing process with sodium sulfide and lime accounts to 84% of biochemical oxygen demand (BOD). And also 75% of COD, 92% of suspended solids (SS) from a tannery(Dixit et al., 2015)(Senthilvelan et al., 2012). Sodium-sulfide not only gives rise to unfavorable consequences on environment also affects efficacy of effluent-treatment. Especially, large amounts of sulfide/calcium hydroxide are used in the dehairing (liming) and reliming. It generates serious pollution to the environment. In Ethiopia also tanneries are using lime sulfide dehairing method as permanent procedure and releasing wastes untreated hair removal of skin, Liming and unhairing produce the effluent stream with the highest COD value(Abdrie Seid et al., 2018; Awulachew, 2021). To overcome environmental impacts and make leather processing ecofriendly, alternative methods were generated to dehair.

### 2.4 Effect of lime sulfide dehairing in Ethiopia

The leather industry sector is one of the fast growing economic sectors in Ethiopia. Currently there are 19 functioning leather tanneries. Hides and skins are the basic raw materials for the leather industry. And Ethiopia is capable of supplying 16 to 18 million hides and skins per annum(Ababa, 2000). Leather industry processes raw hides and produces semi-processed and finished leather for export and local. There are 16medium and large scale footwear manufacturers, 15garments and goods factories, 3gloves factories. There are also 368micro and small scale enterprises producing leather products. The industries are also sources of employment. This is why the sector needs great attention by the government and all concerned stakeholders(T. A et al., 2018).

The current production capacity is based on 15tanneries. It is estimated at 6,000,000 to 8,000 000 kilograms of hides and 20, 000, 000 to 25,000,000 skins. The hides are processed up to wet blues, crust and finished leather. The wet blues and crust hides are produced for export. And remaining low quality are finished for local market inform of garments uppers and linings (Asmare et al., 2021)(Federal Environmental Protection Authority, 2012). Since all skin and hide pass the dehairing stag, the tanning process is different. Kind and amount of waste produced may vary but tanneries process all kind of leathers. Thus starting from dehairing to re-tanning with same processes(Abdrie Seid et al., 2018). The amount of sodium sulfide used is 3% per ton of rawhide(Faki et al., 2019; Nazer et al., 2006). Contaminating load of Sulfide on the final effluent (per ton of skin or hide), applying both a hair-burning and a hair-saving dehairing process is 8.5Kg/t meaning about 40% of sulfide is not absorbed by pelt and discharged to the waste water (Galarza et al., 2016)(Jing et al., n.d.). From this we can conclude that 51,000Kgsulfide from processing hide and 170,000Kgsulfide. From processing skins will be loaded to the effluent of the leather tannery. Many industries in Ethiopia discharge harmful wastes into the rivers and land. In which pollutants are characterized by long persistence compounds (remaining active for a long period of time) and reversing effects is almost impossible(Kebede, 2017; Minas et al., 2017). The discharge of untreated effluent to water bodies causes environmental and health problems on inhabitants. The liquids effluent from leather processing contains, chromium, sulphide organic matter. And also solid waste includes fleshing parts, trimmings, wet blue splits, and shavings, buffing dust. This leads to unwanted odor generated from decomposition of protein solid wastes. Presence of ammonia, hydrogen sulphide, and volatile organic compounds are normally associated with tanning activities (Ayalew & Assefa, 2014; Ryan et al., 2013).

### 2.5 Alternative methods used for the reduction of the amount of lime and sulfide

The conventional liming and dehairing process uses calcium hydroxide and sodium sulfide. To overcome BOD, COD, TDS and TSS caused by dehairing, alternatives have been proposed. It has been a long quest to develop alternative system to do without lime sulfide.

which are associated with far reaching environmental impacts and hazardous effects(Jian et al., 2011).

Enzymatic based leather dehairing using proteolytic has been considered environmentally friendly alternative to chemical process. And also it's gaining increasing importance in dehairing process and partially substitute sodium sulfide. These enzymes catalysis breakdown protein having the ability of enzymes to digests. Those are basal cells in the hair bulb and the cells of the malphigian layer. It is catalyzed more efficient without disturbing the native state of skin(Madhavi et al., 2011). Loosening of hair occurs with subsequent swelling and breakdown of inner root sheath of hair. This aids in reduction of lime and sulfide from the total waste. The origin of the enzymes can be bacterial, animal, plant or fungi. The enzymes can be used in hair saving dehairing where enzymes attack the bond. The bond is between the hair and derma thus lead to lose the hair. Recovering the hair eliminates the discharge load of COD and nitrogen(Maina et al., 2019).

Reuse of the dehairing–liming liquids before discharging them. This is to reduce the concentration of the chemicals. It reduced the environmental impact of the process. 24%, COD was reduced by 50% as well as sulfide which was reduced by 73% when the process water was recycled four times but it is labor intensive and didn't completely reduced sulfide (Maina et al., 2019).

The use of sodium aluminate an alternative to calcium hydroxide or lime was tested. In liming process it resulted to good opening up of fibers and reduces pollution. The pelt dehaired and opened up using sodium aluminate, sodium sulfide and sodium alkali. Due absence of calcium compounds in derma neutralized using 2% ammonia sulphate or 3% boric (Širvaityte et al., 2016).

An oxidative one based on chemical agents (sodium hydroxide and hydrogen peroxide) were used to de-hair in place of toxic sulfide. when the conventional liming is compared with it can be seen that COD and sulfide values has lower and lower than conventional one(Zengin et al., 2010). While wastewaters coming from oxidative treatments, due to oxidative damage, only for lesser value applications(Anzani et al., 2017). However, enzymatic de-hairing is promising approach eliminating toxic sulfide de-hairing process and higher chemical costs (Thanikaivelan et al., 2004).

Enzymatic – oxidative dehairing using protease enzyme in combination with hydrogen peroxide. Alkaline protease used for chemical free enzymatic dehairing process. To reduce the pollution load this causes deleterious effect on the environment. Further, enzyme completely removes hair without causing damage either to keratinous material or collagen matrix. Moreover, it helps in opening up of fiber matrix by removing other globular proteins.

An enzyme-assisted de-hairing process has been developed that uses 85% less sodium sulfide than the conventional lime-sulfide de-hairing process. The loosening of hair was attempted using a commercially available enzyme alkaline protease. Because the process does not require lime for de-hairing, the formation of lime-sludge is completely eliminated, and the complete removal of hair can take place at an unusual pH (pH 8.0) compared with that required for processes that use lime (pH 12.0). Studies to optimize the concentration of enzyme, sodium sulfide and penetrating-aid for depilation were carried out on cowhides using a commercial enzyme formulation. The results indicate that a sodium sulfide concentration of 0.5% and enzyme concentration of 1% is required for complete hair removal. Painting on the grain side of the cow SIDES resulted in complete de-hairing, which was not achieved with other application methods. Strength and bulk properties of the leathers (especially softness) show some improvement as a result of grain softening by the enzymatic action. In addition, it was shown that this de-hairing process has an environmental benefit, reducing COD and total solids loads by 45% and 13%, respectively but still there was no total elimination of sulfide from the waste(Thanikaivelan et al., 2004)(Senthilvelan et al., 2012).

All strategies were developed to decrease the environmental impact of tanneries. It implies replacing sodium sulfide with other chemicals, in order to avoid release of H2S. This chemical is indeed associated with neurological and respiratory problem(Senthilvelan et al., 2012)

## 2.6 Enzymatic de-hairing

Development of environmentally sustainable processes is a challengeable task for the current bio economy. In this direction use of biocatalysts, enzymes, in various process is considered as ecofriendly approach. Therefore, identification of newer sources for such novel enzymes with desired properties is important (Pandey et al., 2017). Enzymes have been pursued as one of the promising alternates to lime and sodium sulfide (Saravanan et al., 2014). It can react with specific substrate depending on the purpose of the process. This changes the paradigm from chemical-based production process into using biological agents in process(Nakagawa et al., n.d.). As an alternative to conventional lime sulfide process, enzymatic unhairing has received much attention(Jian et al., 2011). An important enzyme used in pre-tanning processes belongs to the group of proteolytic enzymes (Kamini et al., 1999). The main utilized enzyme preparations are proteases. Proteolytic enzymes are great commercial importance contributing more than 60%of the world's commercially produced enzymes (Ranjithkumar et al., 2017). Proteases refer to group of enzymes whose catalytic function is hydrolysing peptide bonds of proteins. They are also called proteolytic enzymes or proteinases. Proteases form a large group of enzymes belonging to the class of hydrolases(Mahajan et al., 2015). They are used in enzymatic-dehairing to reduce pollution load to environment and better leather. Their origin can be animal (e.g. from bovine or porcine pancreas), bacterial, fungal or plant (Maina et al., 2019).

The enzymatic dehairing is a very special case of the application of enzymes in beamhouse. Proteolytic enzymes attack the hair roots and the epidermis (Dettmer et al., 2013). Proteoglycans are cementing substances playing important role in stability of fibrils and collagen fibrous structure. It is the disruption of proteoglycans that leads to the detachment of hair from skin. And opening up of fiber structure for the following processes(Jian et al., 2011). Proteases selectively hydrolyze the non-collagenous protein constituents and remove proteins such as albumin and globulin. when applied onto the skin under appropriate conditions(Ranjithkumar et al., 2017). There are three methods of application commonly used in the enzymatic dehairing process. Paint method, Dip method, Spray method. In case of paint method, enzyme solution made into thin paste, adjusted to required pH. Then it is applied on flesh side of hides and skins, piled flesh to flesh. Covered with polythene sheets and kept till dehairing takes place. In dip method enzymatic dehairing, skins are immersed in enzyme at required pH in pit. The spray method is to add extracted enzyme in sprayer and apply to the skin. Dehairing is not the only stage that involves enzyme application. There are also enzymatic application in other beam house and tanning operations until waste treatment as table 2-1 show.

Table 2-1 Enzyme application in different tannery process stages

| Stages | Enzymes involved | Function of enzymes |
|---|---|---|
| Curing | Enzyme are directly not involved | To preserve hides and skins |

| | | |
|---|---|---|
| Soaking | Alkaline and pancreatic proteases | To remove non fibrillar proteins |
| Dehairing | Alkaline and neutral proteases | To improve the waste water quality |
| Degreasing | Lipases and proteases | To remove fats |
| Bating | Trypsin and alkaline proteases | To make soft, supple and pliable |
| Tanning | Enzyme are directly not involved | To influence the quality of tanning |
| Waste processing | Trypsin and proteolytic enzymes | Chrome tanned waste processing |

### 2.7 Protease enzyme

Proteolytic enzymes belong to the hydrolase class of enzymes(EC 3). They are grouped into the subclass of the peptide hydrolases or peptidases (EC 3.4). The proteases can be classified depending on the site of enzyme action. The proteases can be subdivided into exopeptidases or endopeptidases as figure3 depicts. Exopeptidases catalyze hydrolysis of peptide bonds (external peptidic linkages) near N- or C-terminal substrate ends. Endopeptidases cleave internal peptide bonds in the protein. Nature of active sites; catalytic mechanism and presence of amino acid residue(s) at active site. They are cysteine (also known as thiol proteases), serine, aspartic (first called acid proteases), glutamic. Also others like metallo-endopeptidases, threonine, and peptidases with unknown action mechanism(David Troncoso et al., 2022; Mótyánetal.,2013).

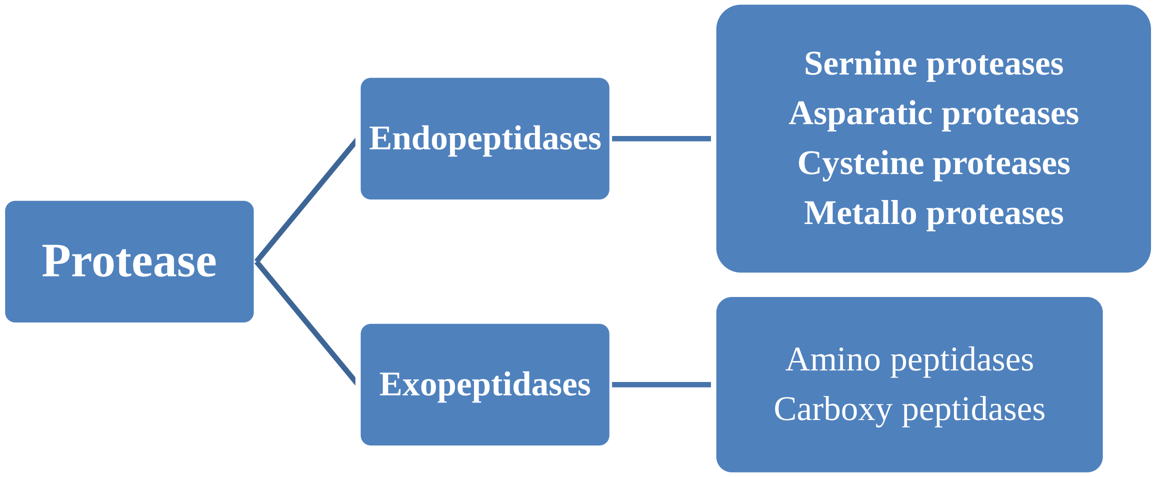


Figure 3 subdivision of protease enzyme

Proteases (also called proteinases or proteolytic enzymes) are able to hydrolyze peptide linkages in proteins. It represents one of the three largest groups of industrial enzymes. And find application in detergents, leather industry, food industry, pharmaceutical industry and bioremediation processes(Shanti Naidu, 2011). From economic perspective, research of new proteases is expanding because they represent 60%of all enzymes (David Troncoso et al., 2022). Industrial applications of plant proteases are of considerable commercial importance due to strong proteolytic activity against a broad range of protein substrates. Most industrial applications based on many literatures(David Troncoso et al., 2022; Feijoo-Siota & Villa, 2011; López-Otín & Bond, 2008; Mahajan et al., 2015; Pandey et al., 2017) of these enzymes are

i) Beer and Alcohol Production - Light and clear beers are preferred by consumers. Treatment with proteolytic enzyme results in beer that remains clear and bright when chilled.
ii) Baking Industry - Proteases are used in baking industry because dough maybe prepared more quickly. If the gluten it contains has been partially hydrolyzed. When high-gluten varieties of wheat are used gluten must extensively degraded for making biscuits. It prevents shrinkage of commercial pie pastry. Protease treatment improves dough relaxing and bread volume, prevents dough shrinkback, and allows faster bakery.

iii) Food Processing - Enzymatic hydrolysis is strongly preferred over chemical methods because it yields hydrolysates. It contains well-defined peptide mixtures and avoids destruction of L-amino acids and toxic formation. Hydrolysis of animal or vegetable food proteins is carried out for different purposes. To improve nutritional characteristics, to retard deterioration, the modification of different functional properties (solubility, foaming, coagulation, and emulsifying capacities), the prevention of undesired interactions, to change flavors and odors, and the removal of toxic or inhibitory factors, among others.

iv) Hydrolysis of Protein-Based Substrates - huge amount of waste rich in protein substrates is wasted. Mostly in the fishing, dairy, food, and bakery industries. Plant proteases have emerged as an affordable technology. It valorize these wastes via hydrolysis of cheese whey, keratinous materials from poultry feathers (keratin, collagen, and gelatin).

v) Leather Industry - Soaking is the first step in leather processing and use of enzymes is recommended. Since it has advantages of reducing the soaking time, initiating the opening of fibers. The enzymes used in soaking target broad-spectrum of reactions not specific thus obtaining solubilization. Conventional leather processing involves consecutive unit operations (soaking, liming, hair removal, deliming, bating, degreasing, and pickling). Where harmful chemicals are used (lime, solvents, sodium sulfide, and ammonium salts). The effluents from leather factories generate serious pollution when they are discharged without correct treatment. Within this context, plant proteases are eco-friendly alternative to replace use of sodium sulfide for dehairing and tanning leather.

vi) Textile Industry – Protease like Papain can be used for processing wool, boiling cocoons, refining silks. As a result, the products will not shrink and will be quite soft. Natural silk and the engulfing gums produced by silk worms are both proteinaceous in nature. Since papain can dissolve sericin but unable to affect silk fiber protein. It can be used for the refinement of the mixture of bombycine and vinegar fiber. In the past, papain has been widely used to 'shrink-proof' wool. A successful method involved the partial hydrolysis of the scale tips. This method also gave wool a silky luster and added to its value.

vii) Cosmetic Medicine and Beauty Products Industry - The cosmetic market is in continuous growth worldwide. Cosmetics are articled to promote cleansing, beautifying, and attractiveness without introducing modifications to body structure. Cosmetic preparations also are used to prevent diseases or protect the body. Such as sunscreen or antidandruff shampoos. Plant proteases like papain used as exfoliates remove dead cell layer (stratum corneum) of skin. And they are commonly used in aging skin, photodamaged skin, acne, and dry skin treatment. Plant protease bromelain is also attractive nutraceutical for cosmetic dental applications, especially for tooth whitening. Also protease of plant show attractive properties for Haircare removing the dirty adsorbed on hair.

Proteases are found from prokaryotes to complex organisms (plants and animals). The type of protease as well its functional properties, directly depend on its particular function. And the organism conditions of the host. Proteases are involved in the physiology of the plants. Physiology is entire cycle life (chloroplast photo-inhibition, defense mechanisms, protomorphic genesis, and seed germination). The most abundant type of protease in plants is cysteine(CPs), serine proteases(SPs) and aspartic proteases(APs).

### 2.8 Cysteine Proteases

Cysteine proteases are present in all living organisms. More than twenty families of cysteine proteases have been described. Many of which (e.g. papain, bromelain, ficain, animal cathepsins) are of industrial importance. They catalyse the hydrolysis of peptide, amide, ester, thiol ester and thiono ester bonds. The Cysteine proteases family can be subdivided into papain, bromelain, ficain, cathepsins(Cotabarren et al., 2007).

i. Papain

Papain is the proteolytic active constituent in plant of tropical papaya fruit having green color. Its name is Carica papaya L. (Caricaceae)(Domsalla & Melzig, 2008). Papain is the most widely studied member of the cysteine proteinase class of enzymes. Best known cysteine protease since 1879(Cotabarren et al., 2007). Papain exhibits endopeptidase, amidase and esterase activities. Papain has function essential of proteolysis widely for peptides of amino acid in all livings. General mechanism of cysteine protease action has been well studied, with papain as model enzyme. Enzymatic activity of papain is exerted by a catalytic formed by Cys and His residues. Which in the pH interval 3.5-8.0 form an ion-pair (Cotabarren et al., 2007). Its decreased activity during storage is due to oxidation of the active site thiol group. This oxidation can be partially reversed by thiol reagents. There are many biological importance of papain, especially industrial applications for different purposes. Which includes food, Pharmaceuticals, Breweries, leather, meat, detergent and others, with help of technological advancement(Errasti et al., 2020). Many studies showed papain has dehairing potential without lime and sulfide at 25C in 24hr (Errasti et al., 2020; Mahajan et al., 2015).

ii. Bromelain

Bromelain was originally given to mixture of proteases found in juice of the stem. And fruit of pineapple (Ananas comosus). Bromelain is still used as collective name for enzymes found in various members of Bromeliaceae-family. The major endopeptidase present in extracts of plant stem is termed ‘stem bromelain’. Whereas major enzyme fraction found in juice of the pineapple fruit is named ‘fruit bromelain’. Some other minor cysteine endopeptidases (ananain, comosain) are also found in the pineapple stem. Its strong proteolytic activity has created a wide interest in numerous applications. Main application is in tenderization, foods, detergents, and the textile industry. It can be used for meat tenderization, grain protein solubilization, beer clarification, baking cookies etc. It has been studied and verifies bromelain acts as enzymatic browing inhibitor in fresh apple. It helps to hydrolyse fish protein to generate fish protein hydrolysate. It also helps to treat acne, wrinkles, and dry skin. Bromelain also envisaged to have extensive applications as active ingredient in tooth-whitening and skin products(Arshad et al., 2014). It has been also used for skin dehairing having 4.7U/ml in 24hr(Mohan et al., 2016).

iii. Cathepsins

Cathepsins are important group of enzymes that are responsible for number of physiological processes. It include cellular protein degradation and they are rapidly inactivated at neutral pH(Cotabarren et al., 2007).

iv. Ficain (ficin)

Ficain(EC 3.4.22.3; synonym: ficin) is name for cysteine protease isolated from different Fig tree species. Ficain requires cysteine or other reducing agents for activation. Most common enzymes present in fruit are Protease. Fruits enzyme that breakdown proteins have their own uses. Ficain is proteolytic enzyme extracted from fig tree, which belongs to a class of proteinases. It is a specific enzyme which can hydrolyze the chemical bond in natural protein. It has good stability, and its structure and hydrolysis mechanism(Chatterjee & Sharma, 2018). The enzyme has broad specificity with the acceptance of hydrophobic amino acid residues(Mahajan et al., 2015). The optimum pH range is from 5.0 to 8.0. It is used in Brewing industry. The application of proteolytic enzymes to chillproof beer was patented by Leo Wallerstein in 1911. Ficin are used in brewing industry in order to obtain good colloidal properties low temperatures. Thus eliminating cloud formation(Feijoo-Siota & Villa, 2011).

### 2.9 Ficus sycomorous

Ficus sycomorus, is commonly known as ‘Shola’ or ‘Bamba’ (Amharic). The plant is also known by several English names such as wild, strangler, Sycamore fig. Bush fig, and common cluster fig is also its other name. It is a fig species that has been cultivated since ancient times. Ficus sycomorus belongs to Family Moraceae, comprising about 40genera and over 1,400species of trees. Shrubs, vine and herbs, often with milky latex juices. Ficus sycomorus is a large, semi deciduous spreading tree, up to 21m. The bole can be up to 100cm in diameter and is occasionally buttressed. Bark on young stems is pale green with a soft powdery covering. Grey green , fairly smooth, with scattered grey scales and pale brown patches on older stems. Leaves are broadly ovate or elliptic. The flowers are unisexual, cyclic and greenish. Ficus sycomorus is 2–3cm in diameter and found in leaf axils up to 10 cm. It has leafless branches on old wood as in single or paired form. The seeds are numerous, round and very tiny.

Ficus sycomorus is native to South West Africa, Ethiopia, Kenya, Egypt, Middle East and Israel. And in its native habitat, usually found in rich soils along rivers and mixed woodlands. The plant is sometimes cultivated, mainly in the east and south Mediterranean Regions (Crete, Israel, Syria, Yemen). It is one of oldest cultivated fruit plants in Egypt and Ethiopia. Often it depicted in old Egyptian mural and tomb paintings. It grows well in the area mean annual temperature range of 0- 40°C (K. A & Y, 2015).

Ficin can be extracted from latex, leaf and fruit ficus specieses for different purpose. It can be used for preparation of an alcoholic beverage, Meat tenderization: myofibrillar proteins hydrolysis. Milk clotting, Bioactive peptides production, Synthetic fibers hydrolysis, Biomedicine, hemostatic, Brew industry are other applications.

Many uses of enzymes in dehairing processes has been studied in previous researches. The application and use of ficin enzyme have been found seldom up to my knowledge. Furthermore, previous studies indicate enzymatic dehairing processes are costly when compared to the conventional processes. Since finding alternative sources that are abundant in local area was found to be significant. This study will contribute to showing the possibility of using alternative sustainable dehairing material namely that is cost-effective and widely available. The study will contribute as a standing point for future researches that will focus on optimization and standardization of enzymatic dehairing through the use of the ficus sycomorous fruit enzyme extraction.

# 3 Materials and Methods

## 3.1 Materials

The raw materials used in this study were Ficus sycomorous fruit and wet-salted goat skins. Chemicals used for enzyme extraction and enzymatic quantification were NaCl, EDTA, Cysteine Casein, Sodium hydroxide, Trichloroacetic acid(TCA), Skimmed milk Agar, Tyrosine, Casine and Ninhydrine while NaOH and Phosphate Buffer (monobasic dihydrogen phosphate and dibasic monohydrogen phosphate) were used for pH adjustment. The instruments used were Spectrophotometer for measuring absorbance in order to get the proteolytic activity of extracted enzyme, Centrifuge for separating particles from the fluid, soaking pits/Drums for soaking the raw goat skin for the removal of dirt and blood, Incubator for temperature regulation and Auto clave to sterilize equipment and instruments. Other materials used were test tubes, petridish, graduated measuring cylinders, vacuum filter micropipette, refrigerator, soaking pits/drums, pH adjuster and consumables are filter paper and aluminum foil.

## 3.2 Methods

Within this chapter the methodology to carry out this study are collection and washing ficus sycomorous fruit to remove dirt, extraction of ficain protease, preparations of various reagents, qualitatively and quantitatively testing extracted protease,

### 3.2.1 Collection and Enzyme isolation from Ficus sycomorus fruit

Ficus sycomorus fig tree fruit were collected from Gurd-shola Addis Ababa Ethiopia and washed to remove the dirt. The collected fruits were homogenized mechanically using blender due to the case of plant tissues. Cells are covered with strong cell walls. Mechanical homogenization seems to be one of best methods for disruption. It isolate the desired natural products from the raw materials(Toldrá & Nollet, 2013). And with 0.9% NaCl containing 5mM EDTA and 5mM cysteine soaked for 3hours. Then filtered using vacuum filer. The filtrate was centrifuged at 6000rpm for 10 minutes(Daussant et al., 1992; Ranjini et al., 2020). To the supernatant, 0.2% tannic acid was added. Then incubated at $4^{o}C$ for 2hours and centrifuged at 6000rpm for 10minutes to remove residual solvents. To the supernatant, phosphate buffer containing 5mMEDTA and 5mMcysteine added and centrifuged at 6000rpm for 10minutes(Meza-Espinoza et al., 2018). Adding cysteine while extracting the protease enzyme help to keep the enzyme active. It also reduce the chance of a significant loss in activity by increasing stability. While EDTA as reducing agents used to improve the cysteine proteolytic activity. Also protect the enzyme from oxidation occurring during extraction. It chelates metal ions that could lead to inactivation of enzyme by attacking sulfhydryl group (David Troncoso et al., 2022).

### 3.2.2 Protein detection test of the extracted enzyme

To identify the extract of Ficus sycomorous fruit as protein, Ninhydrin test was carried out. According on the amount of amino acids available, this test provides colour responses. This chemical test determines the presence of primary or secondary amines, amino acids, and ammonia. Using the procedure that Chaiwut et al. (2007) outline (Arshad et al., 2014). The test entails adding ninhydrin reagent to test sample in presence of an amino group. It causes the production of a deep blue colour known as Ruhemann's purple. The color change occurs when ninhydrine bind to free amino groups in the protein. This is helpful tool for detecting presence of proteins in sample and to distinguish between protein and non-protein substances.

One milliliter of ninhydrin solution was added to two milliliters of sample to conduct the ninhydrin test in a test tube. After that, the tube spent five minutes in a 91°C water bath. After that, the tube was placed in a cold water bath to reach room temperature.

The second qualitative test was done using skimmed milk agar prepared by suspending 51.5 grams of skimmed milk agar powder in 1000ml of distilled water which was then heated to boiling to dissolve the medium completely. Hence it was sterilized by autoclaving at15lbs pressure ($121^0$C) for 15 minutes. Mix well and pour in to sterile Petri plates. After 10 min of standing, the skimmed milk/casein plate was solidified and 0.1ml of the enzyme was poured to the hole. Then incubated $37^0$C for 18hr. After 18hr of incubation the clear zone can be observed implying availability of protease due to hydrolysis of the skimmed milk agar plate.

### 3.2.3 Protease assay

This assay carried out to determine enzyme activity using casein as substrate. It based on Sigma's Non-specific Protease Activity Assay(Cupp-enyard & Aldrich, 2008). A substrate is casein. Tyrosine is released together with other amino acids after casein digestion by protease testing. Peptide fragments are another. A blue-colored chromophore is largely produced by the reaction of folin and Ciocalteus Phenol with free tyrosine. It is measurable and expressed on a spectrophotometer as an absorbance value. Greater chromophores are produced, more tyrosine is liberated from casein, and protease activity is increased. The absorbance values produced by the protease activity are contrasted with a reference curve. This was produced by reacting F-Creagent with known amounts of tyrosine to correlate absorbance changes. With amount of tyrosine in micromoles. From standard curve activity of protease samples can be determined in terms of enzyme units. It is the amount in micromoles of tyrosine equivalents released from casein per minute.

Then the absorbance of the test sample from the spectrophotometer should be identified. To calculate umole tyrosine equivalents released. Inserting absorbance value for one of the test samples into the slope equation. And solving will result in micromoles of tyrosine liberated during this particular proteolytic reaction as showed in Equation 1.

$$\text{Activity of enzyme in units}\frac{\text{per}}{\text{ml}} = \frac{umoleTER * TVA}{VE * TA * VC}$$

Equation 1

- TER- Tyrosine equivalents released
- TVA- Total volume (in milliliters) of assay
- VE- Volume of Enzyme of enzyme used
- TA- Time of assay as per the Unit definition
- VC- Volume used in Colorimetric Determination
- The volumes are in milliliters and time in minutes

### 3.2.4 Preparation of skin samples for dehairing experiment.

Fresh just after flaying goat skins was soaked in water for 3hr to remove the dung, dirt and other unwanted materials. Thus, soaked goat skin cut into pieces 5cm by 5cm $25cm^2$ for the dehairing experiments and grouped into different sets to study effects of enzyme concentration, temperature and time duration on dehairing.

### 3.2.5 Adjusting the pH based on proteolytic activity

Optimum pH of Ficus sycomorous was found in range of pH 5 to 8 as referred from (Cotabarren et al., 2007) and 5 - 7.0 from Pandey et al., 2017. So the wide range is taken from 5 to 8 and using spectrophotometer the proteolytic activity has been tested at pH 5, 6, 7 and 8. Then the higher proteolytic activity pH was used.

### 3.2.6 Experimental Design

In this study experimental design and data analysis was done by using design expert software. The experimental data analysis was provided by using Box-Behnken design(BBD). It is one type of response surface methodology. The BBD was chosen among the various experimental RSM models because it is an independent. And rotatable quadratic model without embedded factorial points. Furthermore, BBD outperforms CCD and the three-level full factorial design when comparing the various RSM tool design methodologies. Furthermore, the BBD is typically quite profitable and successful. Compared to other experimental design methodologies, it is because fewer experiments are needed. Response functions at the intermediate level can also be computed using it. Then calculate the system's performance at each test point within the examined range. Consequently, BBD is used more often than other experimental design techniques to maximise research on industrial wastewater treatment. Additionally, the Box-Behnken design achieves its highest efficiency in an experiment with three layers and three components. Additionally, there have been many less tests done on this, which is a benefit over the other RSM design (Ferreira et al., 2007). Therefore, response surface approach was utilised to investigate the influence of three parameters for enzymatic dehairing. The response variable was the dehairing of hair removal efficiency, while the operational parameters were temperature, concentration, and time.

### 3.2.7 Effect of temperature on dehairing process

The effect of temperature for dehairing performance was investigated in the temperature range of 35 to 55°C (Cotabarren et al., 2007; and Pandey et al., 2017). Temperature affects the rate of an enzyme reaction by increasing the thermal energy of the substrate molecules. This increases the proportion of molecules with sufficient energy to overcome the activation barrier and hence increases the rate of the reaction. In addition, the thermal energy of the component molecules of the enzyme is increased, which leads to an increased rate of denaturation of the enzyme protein due to the disruption of the noncovalent interactions holding the structure together.

### 3.2.8 Effect of enzyme concentration for dehairing process

In this study, on samples of soaked goat skin flesh side Crude enzyme solution of varying concentrations was applied. Such as solution of enzyme was diluted and 70, 85 percent and direct extracted enzyme without dilution is applied on experimental samples.

### 3.2.9 Effect of time duration for dehairing process

The dehairing experiment was carried out to determine time duration required for complete removal of hair. Experimental samples were treated with crude enzyme and diluted enzyme for time duration range of 16 to 18 hr (Badgujar & Mahajan, 2013; Senthilvelan et al., 2012; Sundararajan et al., 2011) and assessed for hair removal and pelt color.

Box-Behnken experimental factorial design matrix was used for the studying combined effect of temperature, enzyme concentration and time on protease activity ANOVA.

The factors and levels are given in Table3-1. The factors, i.e., Temperature, enzyme concentration and time were designated. According to BBD the total number of experiments can be calculated as: N = k2+k + Cp, where k is a number of factors, and Cp is a central replication point(Ferreira et al., 2007).

Table 3-1 Experimental levels of selected variables for Box-Behnken design

| Variables | Unit | -1 (low) 0 | 0 (medium) | +1 (high) |
|---|---|---|---|---|
| Temperature | $^0C$ | 35 | 45 | 55 |
| Concentration | Percent | 70 | 85 | 100 |
| Time | Hr | 16 | 17 | 18 |

### 3.2.10 Analysis of waste water

Environmental parameters were studied by measuring the pollution parameters of wastewater. After de-hairing process the experimental waste sample was collected and analyzed. Pollution parameter such as Biochemical oxygen demand of the waste water BOD, chemical oxygen demand COD, total dissolved solids TDS and total suspended solids TSS of the waste water were measure. BOD test was measured as per the standard of 5 day incubation (Azide modification). Chemical oxygen demand, total dissolved solids and total suspended solids of the waste water were measured as per standard APHA 5220-B, APHA 2540-D and APHA 2540-C respectively.

# 4 Result and Discussion

## 4.1 Enzyme isolation of ficus sycomorus fig fruit

Washed fruits homogenized using blender in order to facilitate the isolation of wanted material from raw source as showed on figure 4 and 5, then ficin cysteine protease isolated from the fruit after soaking, filtration and centrifugation.

Figure 4Washed and dried FS fruit

Figure 5 FS fruit homogenization by blender

Figure 6 Filtered and centrifuged FS fruit

### 4.2 Protein identification test

After the extraction of ficain protease enzyme, by the ratio 1 ml of Ninhydrin solution was added to 2 ml of enzyme sample. Then the sample color change and this confirmed the presence of protein in the extract as figure 7 show.

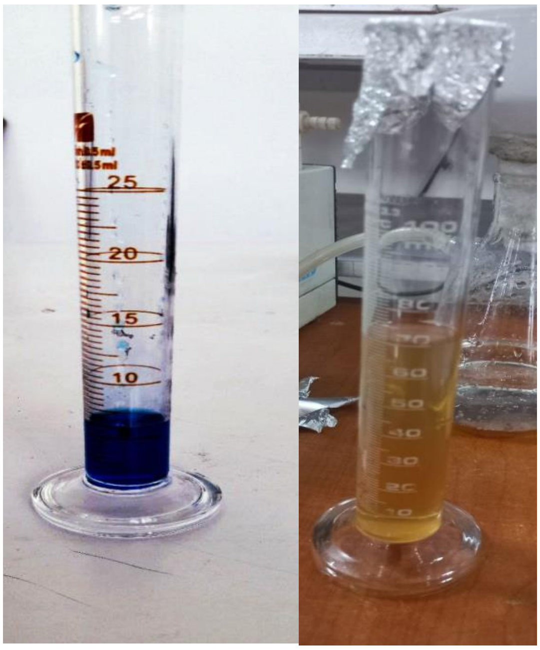

Figure 7 Ninhydrin test color change of FS fruit

The second qualitative test was using skimmed milk agar for the presence of protease. The diameter observed was 10 squares which is equal to 50mm under fluorescent light. And when compared to result of papain it is 12 squares which is equal to 60mm and the control or distilled water was 0 and the result is comparably good.

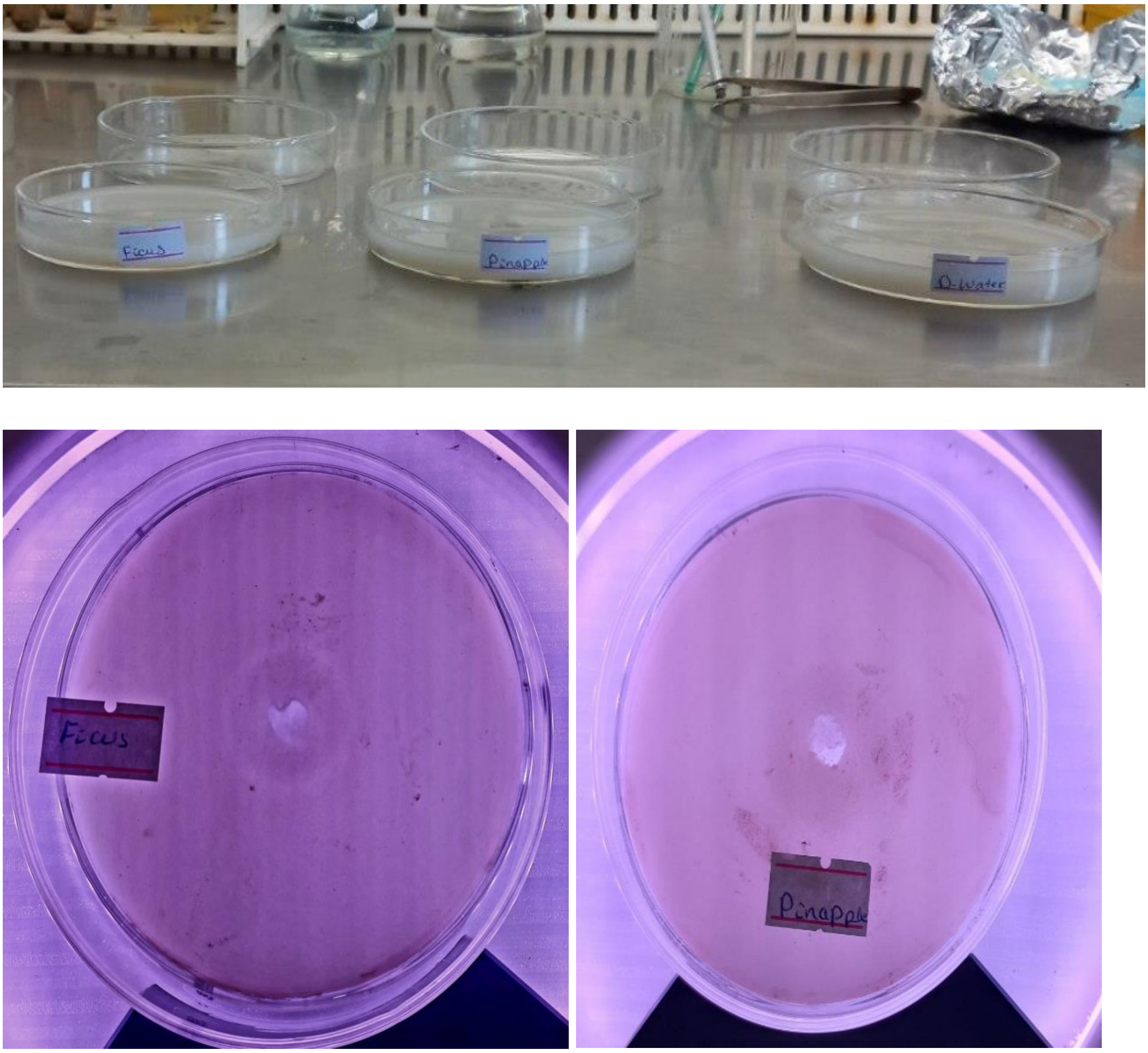


Figure 8 Skimmed milk agar tests for FS and Pineapple

### 4.3 Proteolytic assay

The activity of isolated ficin enzyme from ficus sycomorous fruit was studied by UV–VIS spectrophotometer using casein as a substrate. The activity of ficin enzyme calculated from concentration equation.

The equation obtained from the standard curve having $r^2$ of 98.1 is $C = 30.93 * A + 11.48$ Equation 2

The absorbance measurement of ficin from UV–VIS spectrophotometer was 1.520 having Concentration =57.875. The proteolytic activity found to be 18.1892 U/ml.

This proteolytic activity result is similar to the proteolytic activity obtained from a Bromelia karatas fruit 16.57U/ml and Bacterial Extracellular Alkaline Protease having 16.5u/ml proteolytic activity used to dehair goat skin research(Meza-Espinoza et al., 2018; S.Y et al., 2017). When we also see ficin plant protease used for other application, ficus sycomorous fruit enzyme also has a convenient and promising activity. On a research made on ficin enzyme from Ficus septica Burm F stem latex it was reported that the proteolytic activity is 18.91U/ml and (Wahyuni et al., 2017).

### 4.4 Adjusting pH

PH determination was done by two steps. The first method was by spectrophotometer, in which four enzyme solutions was prepared having different pH at 5,6,7,8 by phosphate buffer. As the pH of the enzyme increases the proteolytic activity increases until it reaches 8, then after the proteolytic activity starts to decrease as listed on table 4-1.

Table 4-1 Absorbance and proteolytic activity for different pH values

| pH | Absorbance | Proteolytic activity in U/ml |
|---|---|---|
| 5 | 0.564 | 8.26 |
| 6 | 0.89 | 11.14 |
| 7 | 1.2 | 14 |
| 8 | 1.520 | 18.1892 |
| 9 | 1.3 | 15 |

Ficin enzyme has very low activity at very acidic pH levels (pH 3–6). That is because functional groups in the active site are disrupted by the excessive H+ ions. At pH 7–9 the enzyme activity is relatively maximum because the enzyme reaches expected degree of ionization. At pH 10–12, enzyme activity is decreased due to an excess of OH- ions(Wahyuni et al., 2017). Also collagen which is the most important component of skin and hide, in the leather making prefer a pH of low alkalinity, when pH exceeds to high alkaline, in fact, a rapid hydrolytic damage of collagen takes place and must absolutely be avoided. And the alkaline pH allow hydrolysis of amide sidechains, peptide bonds and swelling of the skin which is called opening-up, this opening up leads the collagen easily loses the hair due to the enzyme penetration to the hair root(Beghetto et al., 2013).

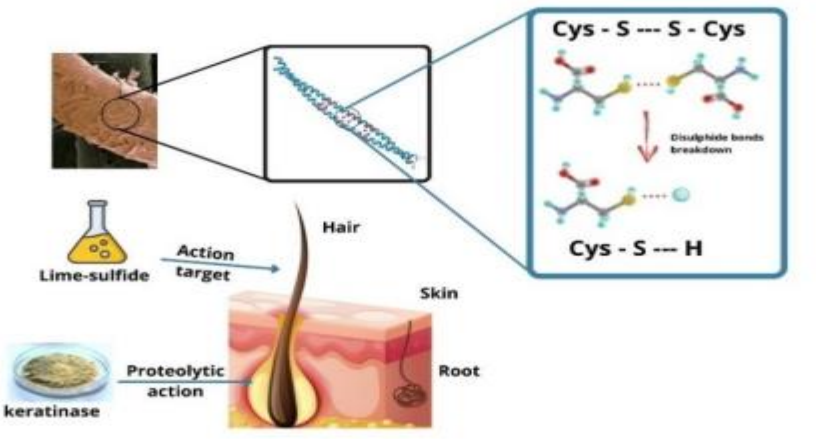


Figure 9 Proteolytic action on hair root

### 4.5 Statistical analysis and Analysis of variance (ANOVA)

The result of response was calculated based on the SEM (scanning electro-microscope) captured picture from sample of the runs. The pores were seen by scanning electro-microscope and were counted from the sample inserted. Numbers of pores were changed to percent based on samples obtained from conventional liming to know how many pores should be available per area. Goat skin per $5.2mm^2$(2.65mm*1.96mm) has 65 pores and $3mm^2$(1.99mm*1.47mm) has 38 pores. Areas of the samples were taken from the SEM picture word pad field of view. This test is done due to lack of information on Ethiopian goat skin pore per area while others countries like India is available but pores differ due to temperature. As temperature increase the pores on the goat skin increases. Also from the run samples run 2 proves that the area of $1.1mm^2$ has 14pores which is relatively equal as $5.2mm^2$ area pores.

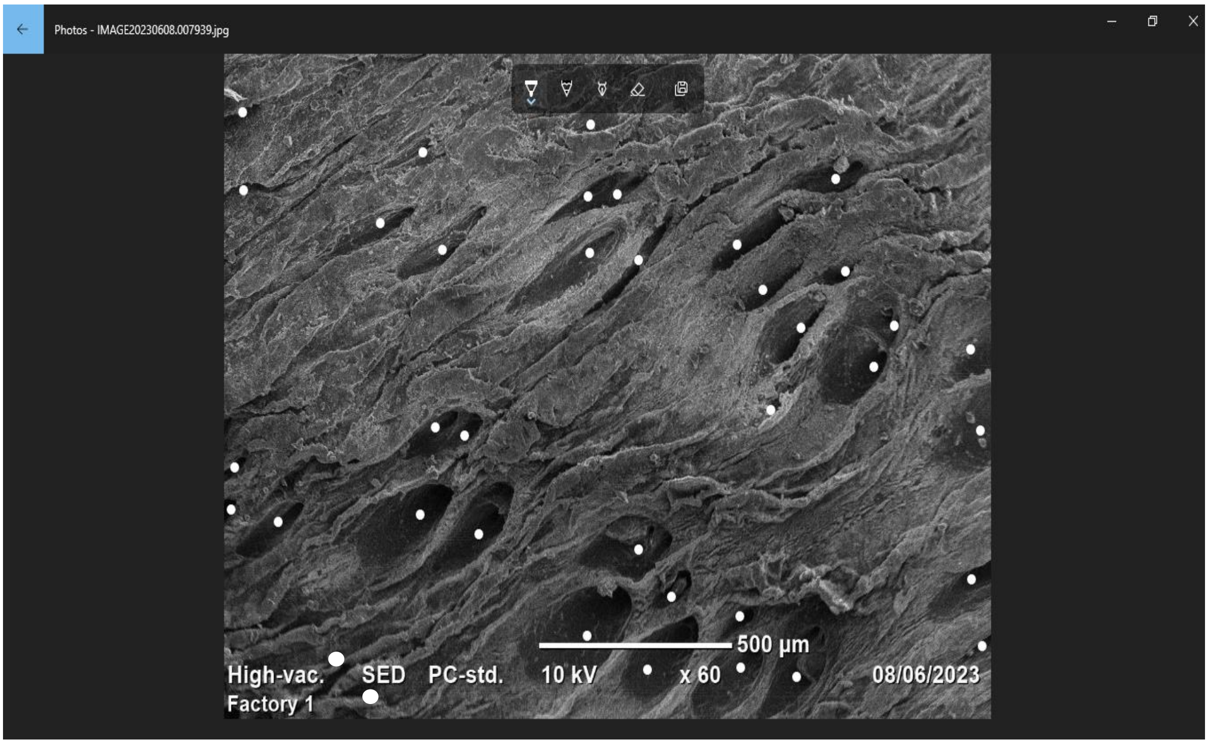


IMAGE20230608.007939 - Notepad

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Ln 1, Col 1 | 100% | Windows (CRLF) | UTF-8

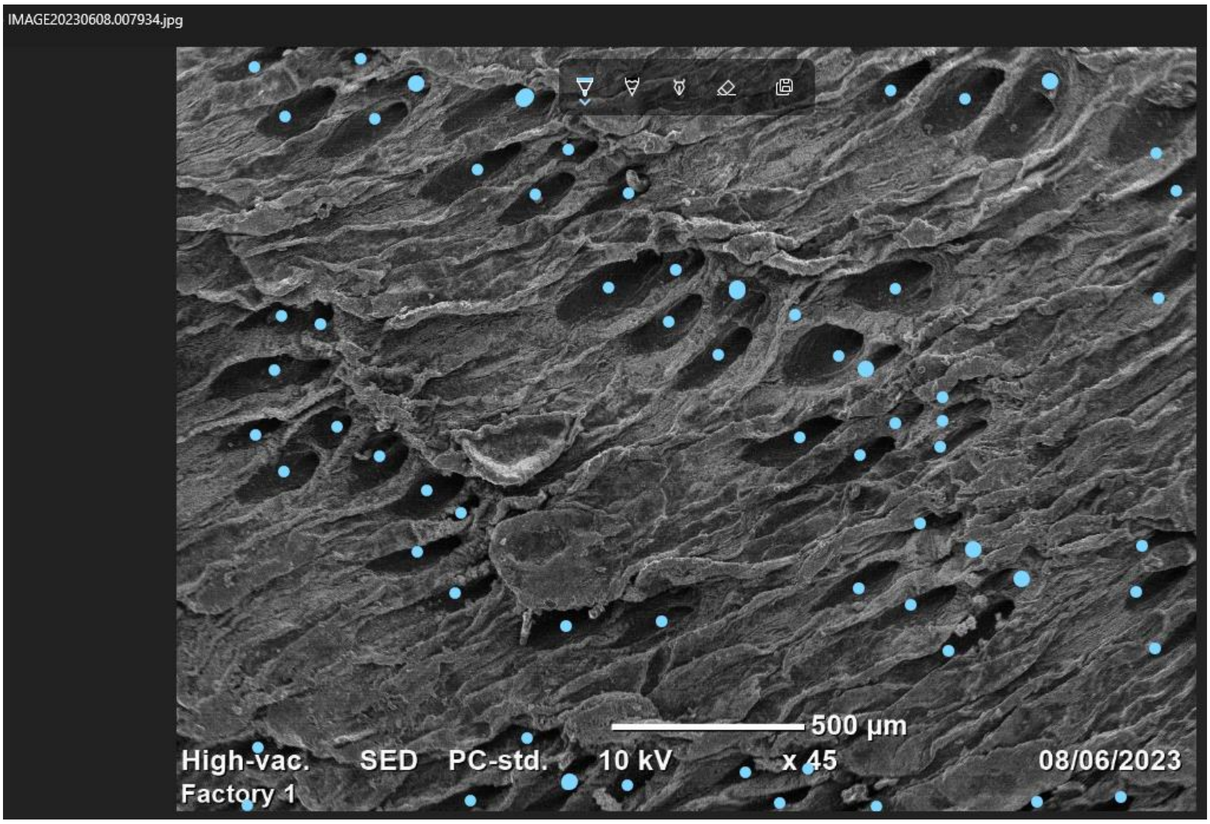


IMAGE20230608.007934 - Notepad

File Edit Format View Help

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Ln 1, Col 1 | 100% | Windows (CRLF) | UTF-8

Figure 10 conventionally dehaired sample SEM results

The calculation of changing dehaired pore number per area to percentage is as followed. Areas of the captured pictures were obtained from the SEM picture Note pad field of view for each picture.

Run 1

Number of total dehaired skin pores per 14.4mm$^2$ was expected to have 180 pores if 5.2 mm$^2$ conventionally dehaired goat skin has 65 pores as above mentioned.

```
IMAGE20220928.005917 - Notepad
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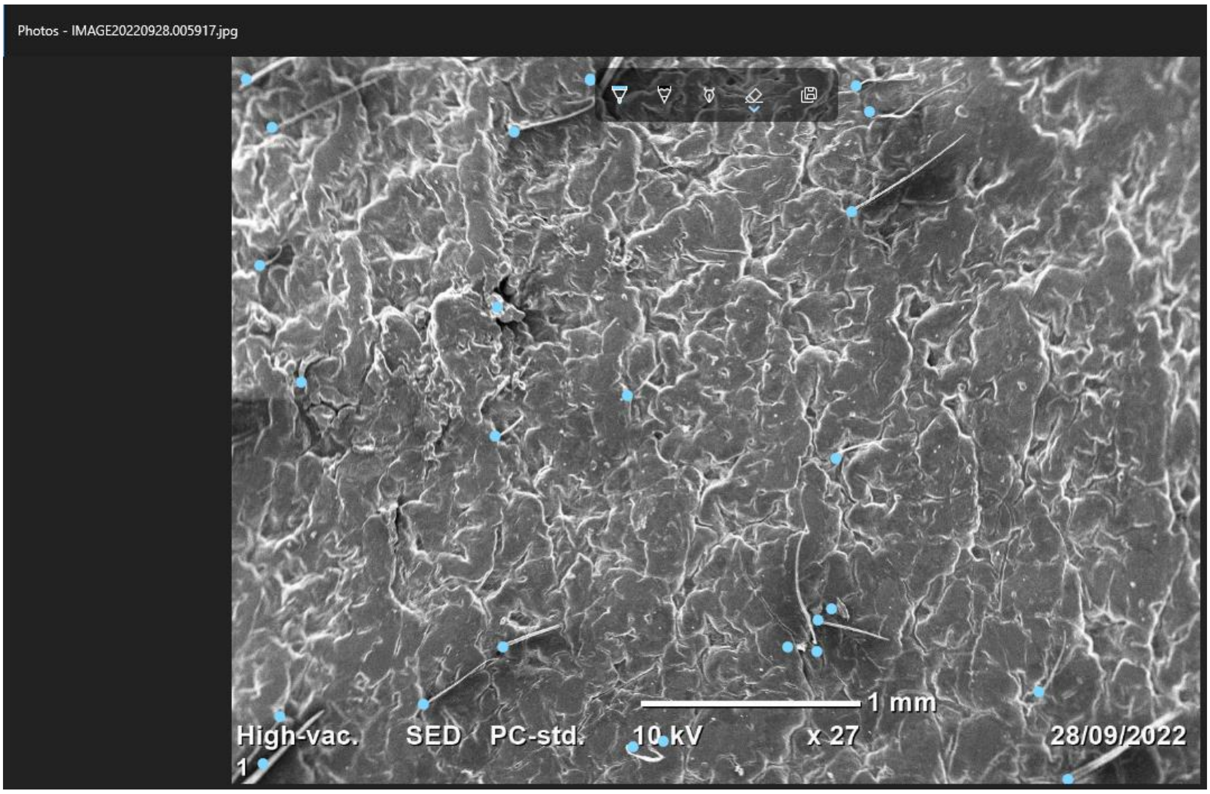



Figure 11Run 1 SEM result

Run 1 has 25 not dehaired pores

Total pores – not dehaired pores= dehaired pores, then dehaired pores changed to

180-25=155

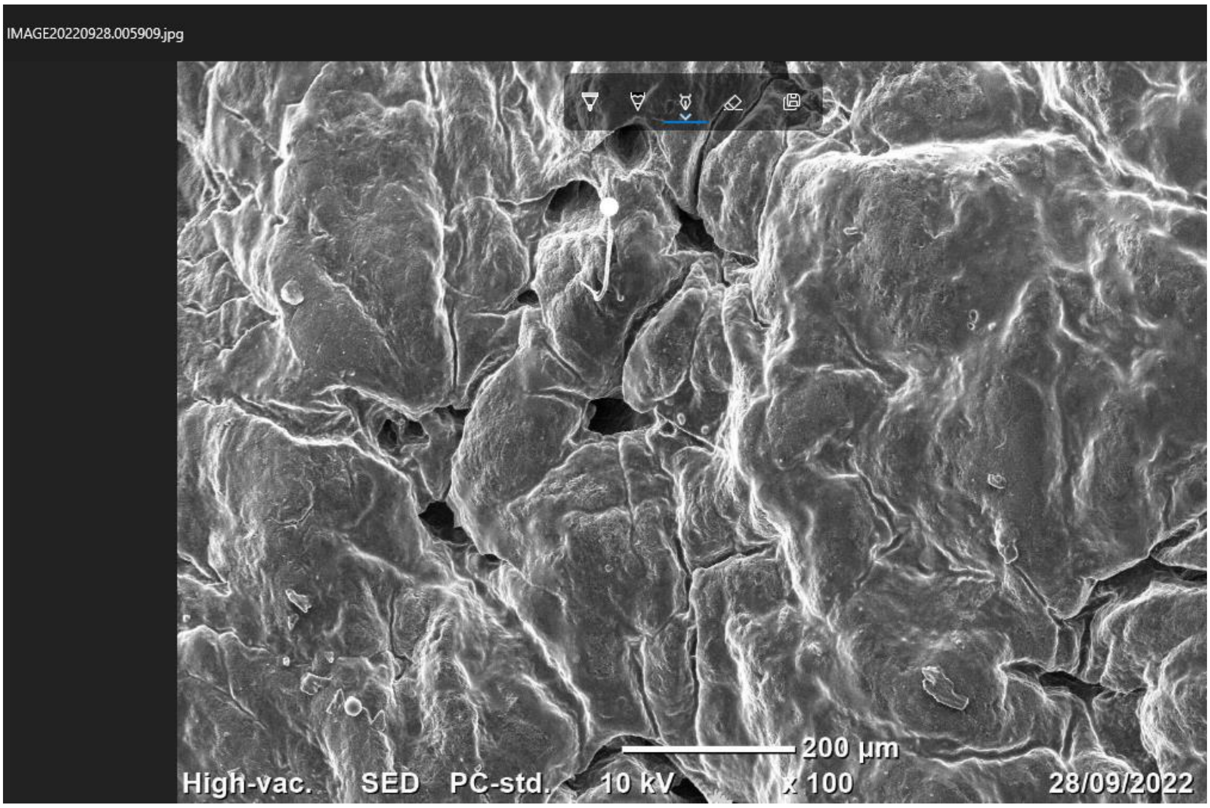
If 180pores=100%

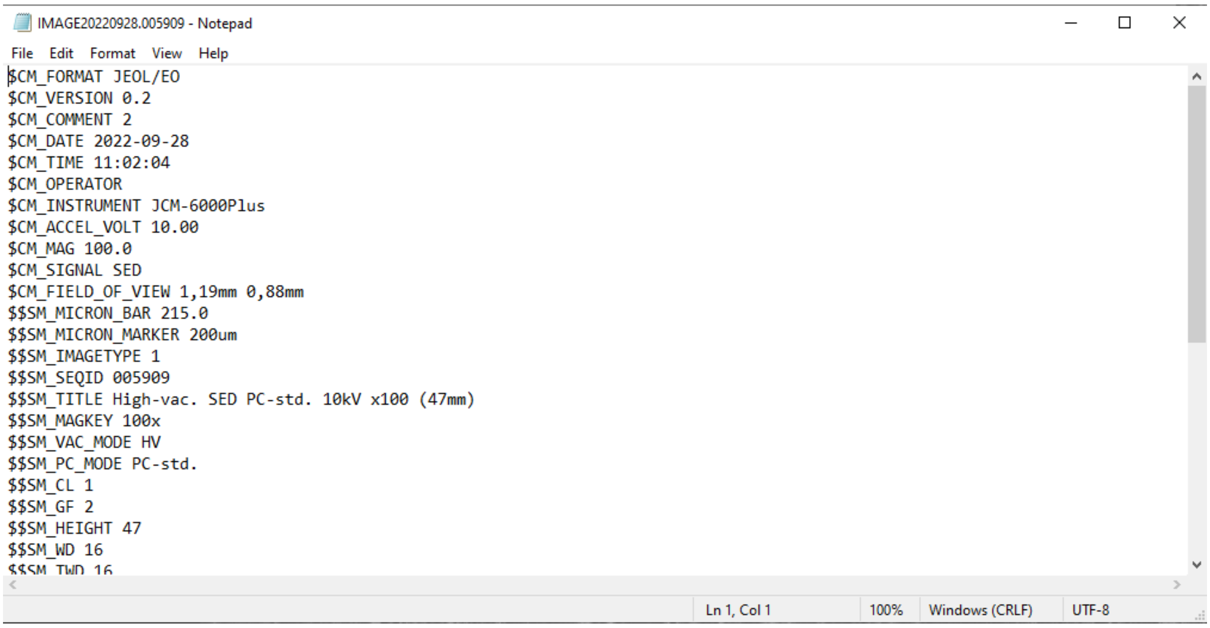
155 pores=?

15500/180=86%

Run 2

Number of total dehaired pores per 1.1mm$^2$ is expected to have 14 pores and considered as 100%.





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```

Figure 12 Run 2 SEM result

Run 2 has only one pore which is not dehaired 14-1=13

14=100%

13= ?

1300/14=93%

Run 3

Number of total dehaired pores per 5.4mm$^2$ is expected to has 68 pores and considered as 100%.

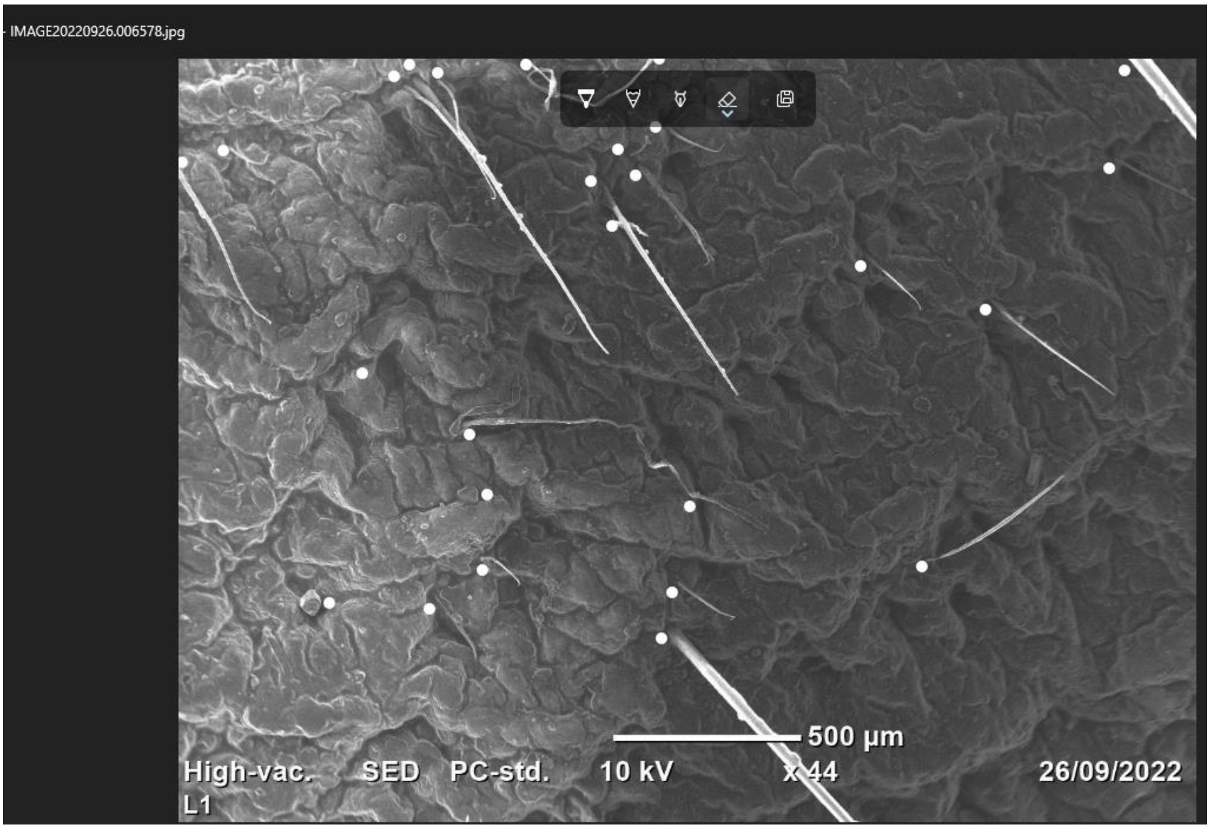



IMAGE20220926.006578 - Notepad

File Edit Format View Help

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$$SM_WD 16
$$SM_TWD 16
```

Ln 1, Col 1 | 100% | Windows (CRLF) | UTF-8

Figure 13 Run 3 SEM result

Run 3 has 24 not dehaired pores among 68 pores, 68-24=44

68=100%

44=?

4400/68=65%

Run 4

It has 2.71mm*2.01mm=5.4mm$^2$ area with 68 pores as 68 total pore is 100%.

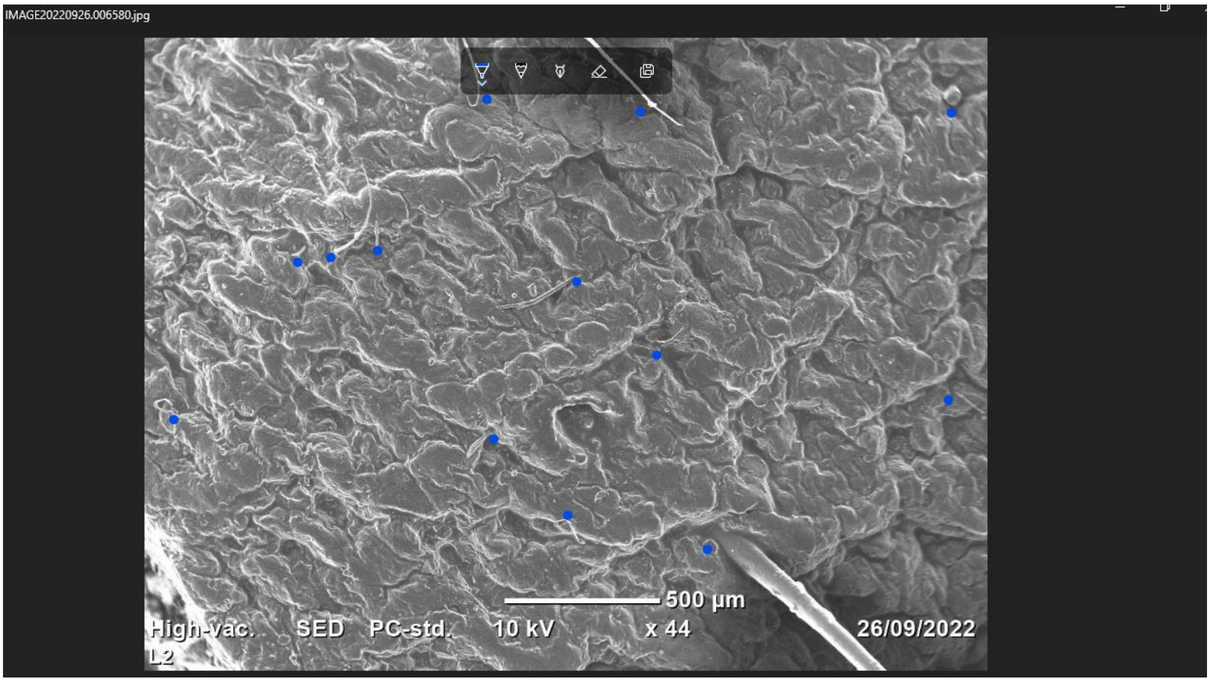


IMAGE20220926.006580 - Notepad

File Edit Format View Help

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```

Ln 1, Col 1 | 100% | Windows (CRLF) | UTF-8

Figure 14 Run 4 SEM result

There is 13 not dehaired pores among 68 total pores, 68-13=55

68=100%

55=?

5500/68=80.8%

Run 5

It has 2.71mm*2.01=5.4mm$^2$ area.

Total pore is expected to be 68pores among that 14pores are not dehaired. 68-14=54

68=100%

54=?

5400/68=79%


IMAGE20220926.006579 - Notepad

File Edit Format View Help

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```

Ln 1, Col 1 | 100% | Windows (CRLF) | UTF-8

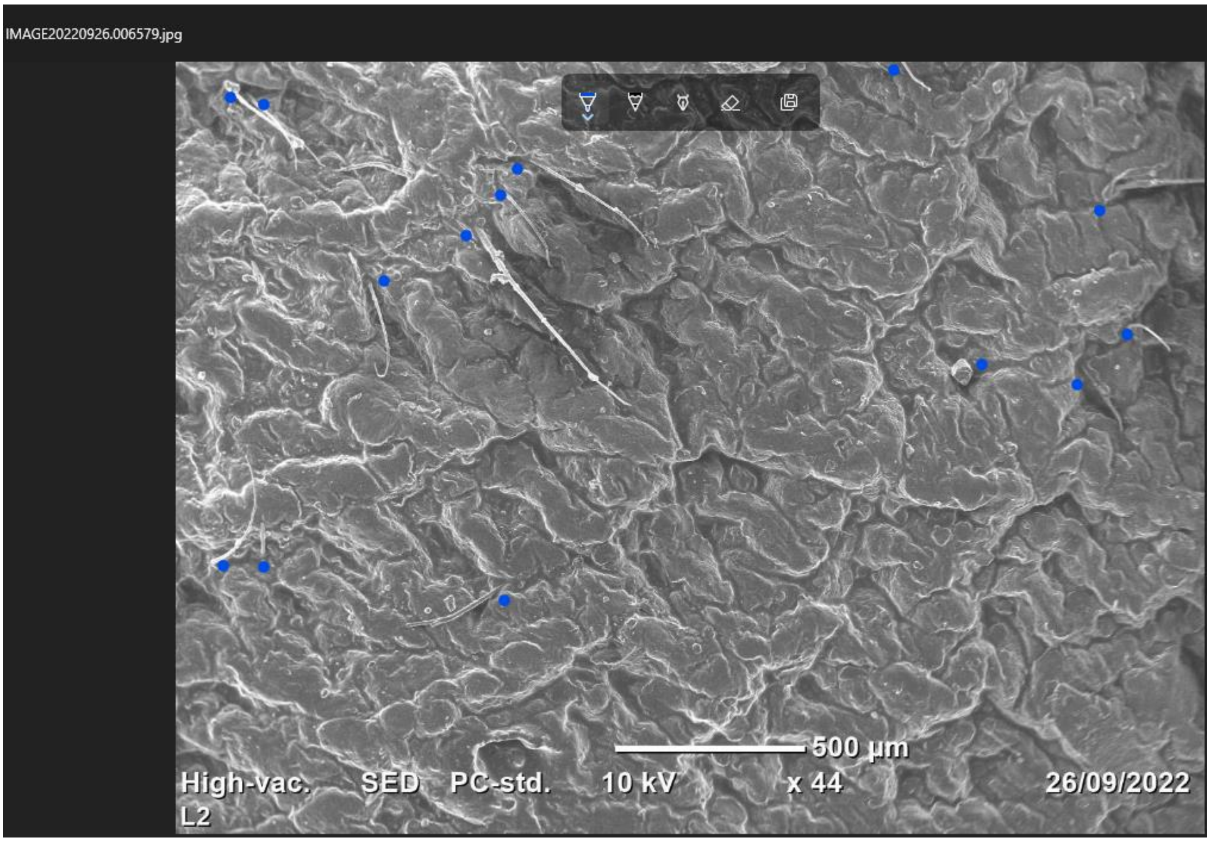


Figure 15 Run 5 SEM result

Run 6

It has 2.71mm*2.01mm=5.4mm$^2$ and expected to have 68 pores.

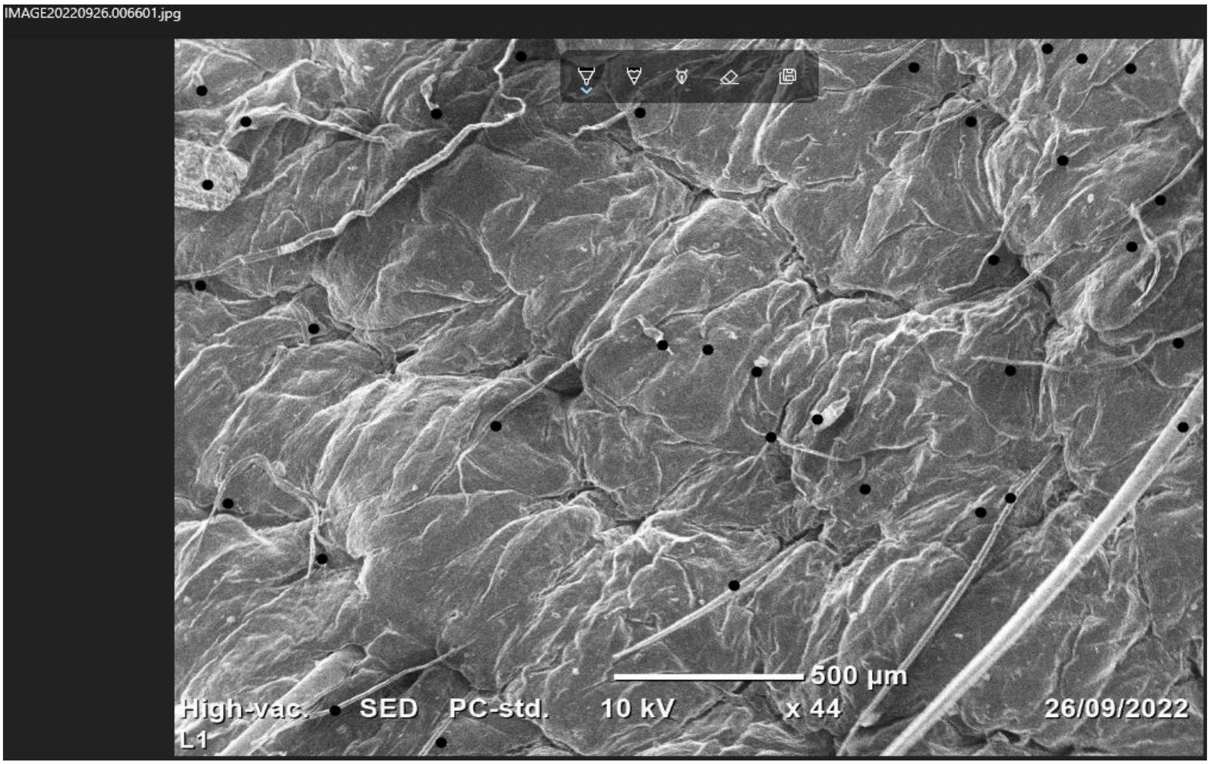

IMAGE20220926.006601 - Notepad

File Edit Format View Help


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```


Ln 1, Col 1 | 100% | Windows (CRLF) | UTF-8

Figure 16 Run 6 SEM result

68 pores= 100%

34 pores=?

3400/68=50%

Run 7

Has 14.4mm$^2$ area with 180 pores among that 60 were not dehaired.

180-60=120

180pores=100%

120pores=?

12000/180=66.7%

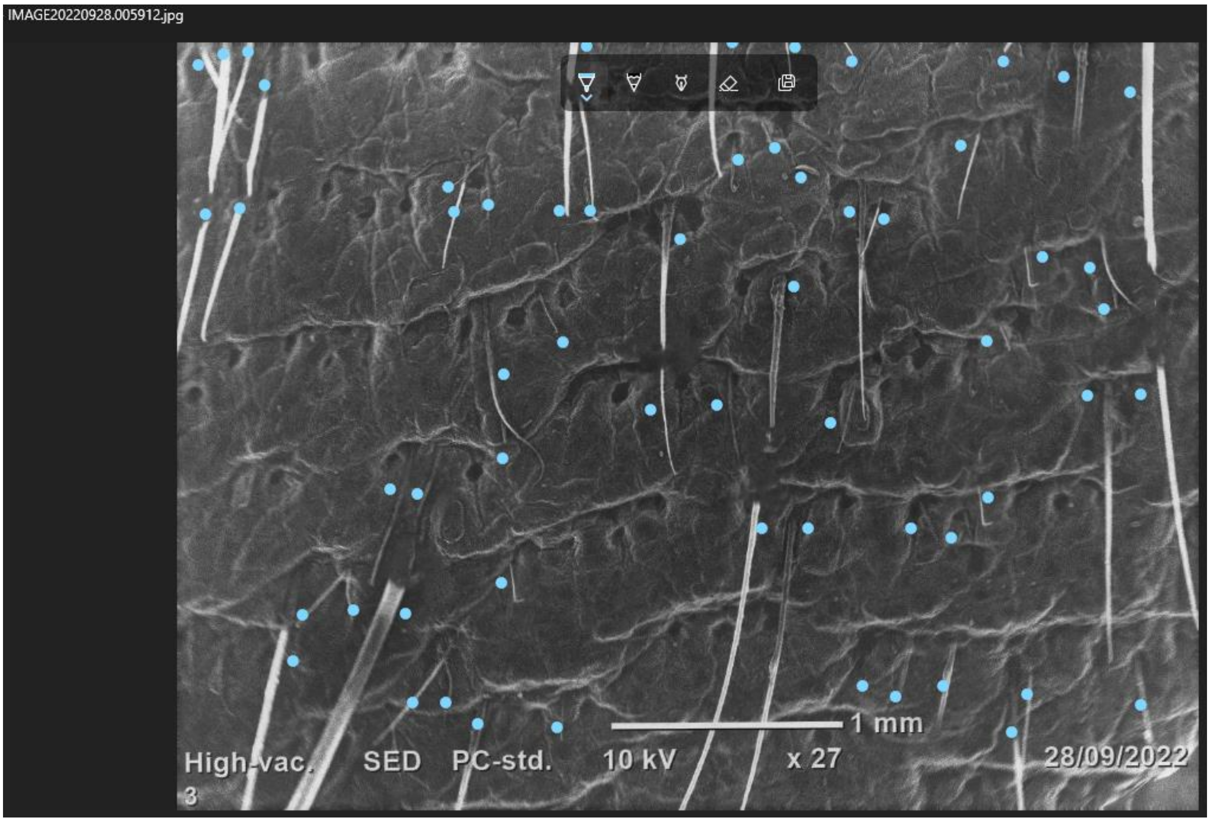


IMAGE20220928.005912 - Notepad

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Ln 1, Col 1 | 100% | Windows (CRLF) | UTF-8

Figure 17 Run 7 SEM result

Run 8

It has area of $1.1mm^2$ and only 4 dehaired pores. It was expected to have total 14 pores.

14pores=100%

4pores=?

400/14=28.5%

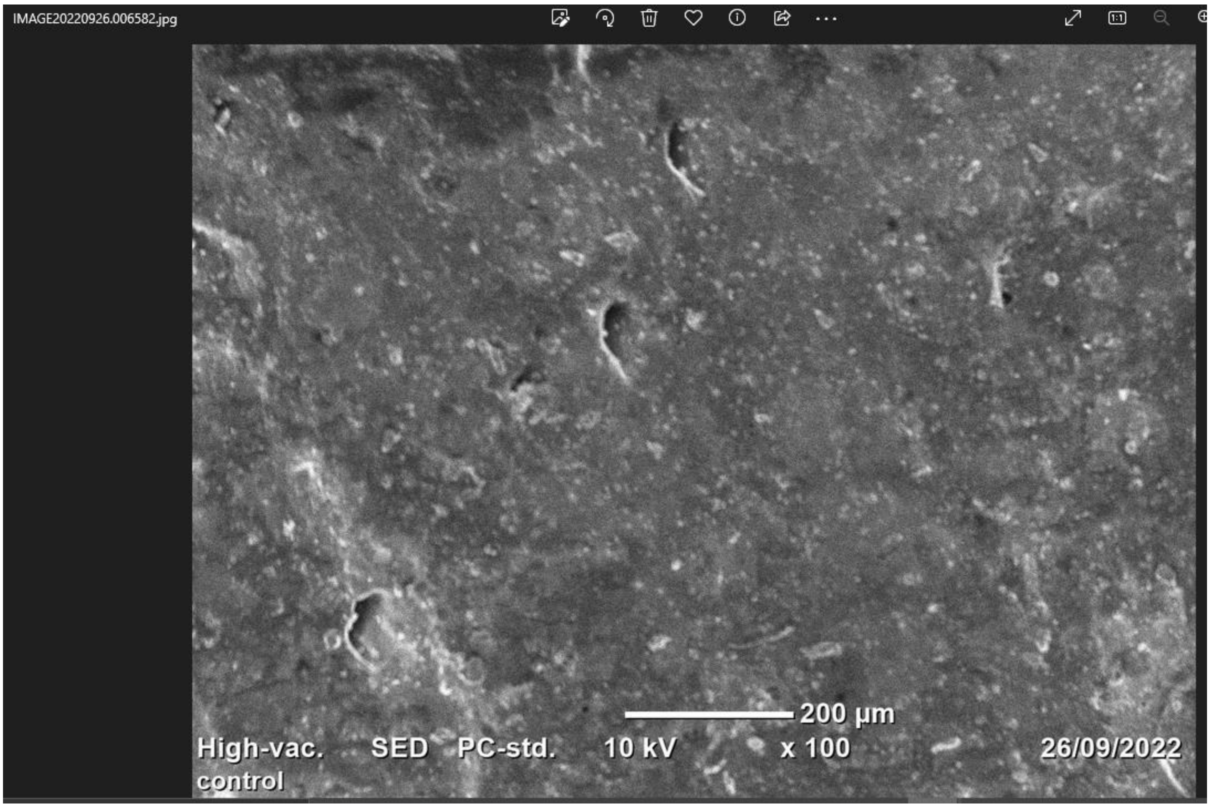

IMAGE20220926.006582 - Notepad

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```

Ln 1, Col 1 | 100% | Windows (CRLF) | UTF-8

Figure 18 Run 8 SEM result

Run 9

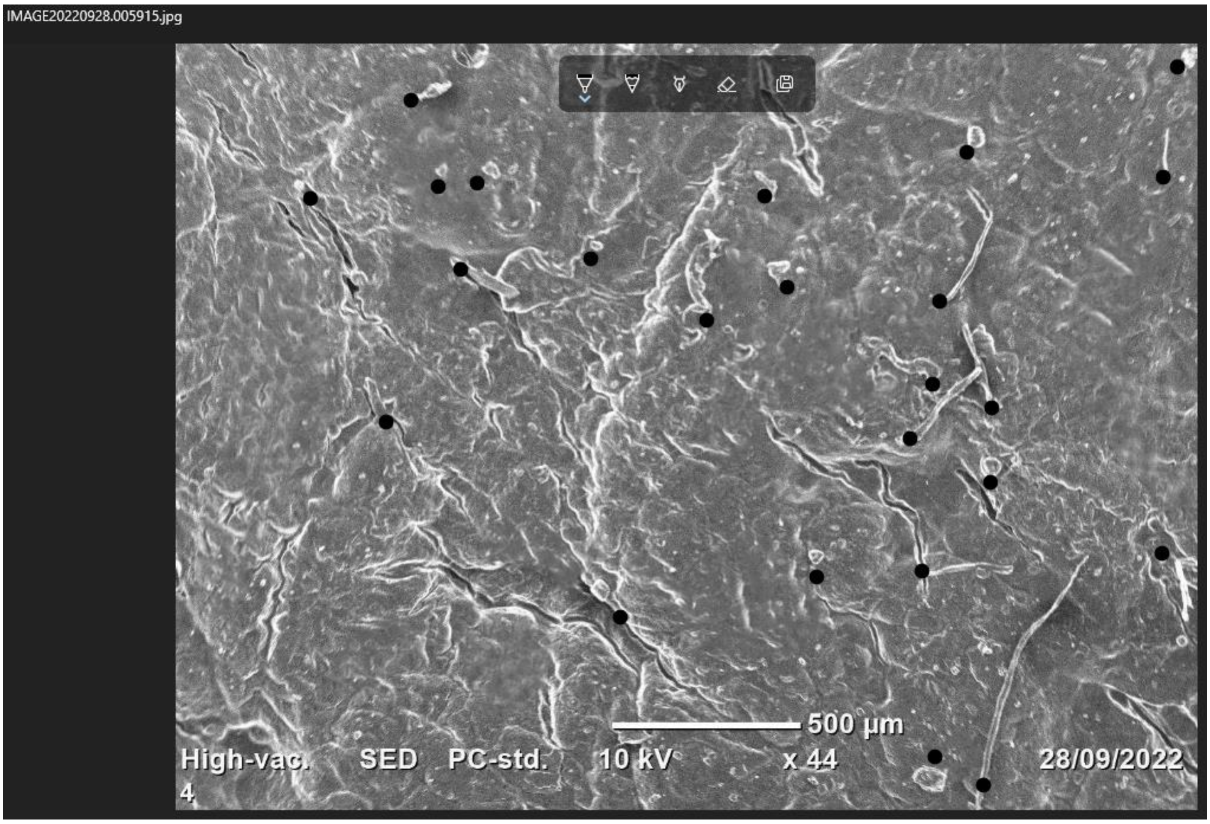
Has 5.4mm$^2$ area expecting total 68pores and 24pores were not dehaired.

68pore-24pore=44pore

If 68pore=100%

44pore=?

4400/68=64.7%





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$$SM_TWD 18
```



Figure 19 Run 9 SEM result

Run 10

The sample of run 10 has 2.71mm*2.01mm=5.4mm$^2$ area having 23 not dehaired pores.

68pores=100%

45pores=?

4500/68=66.17%

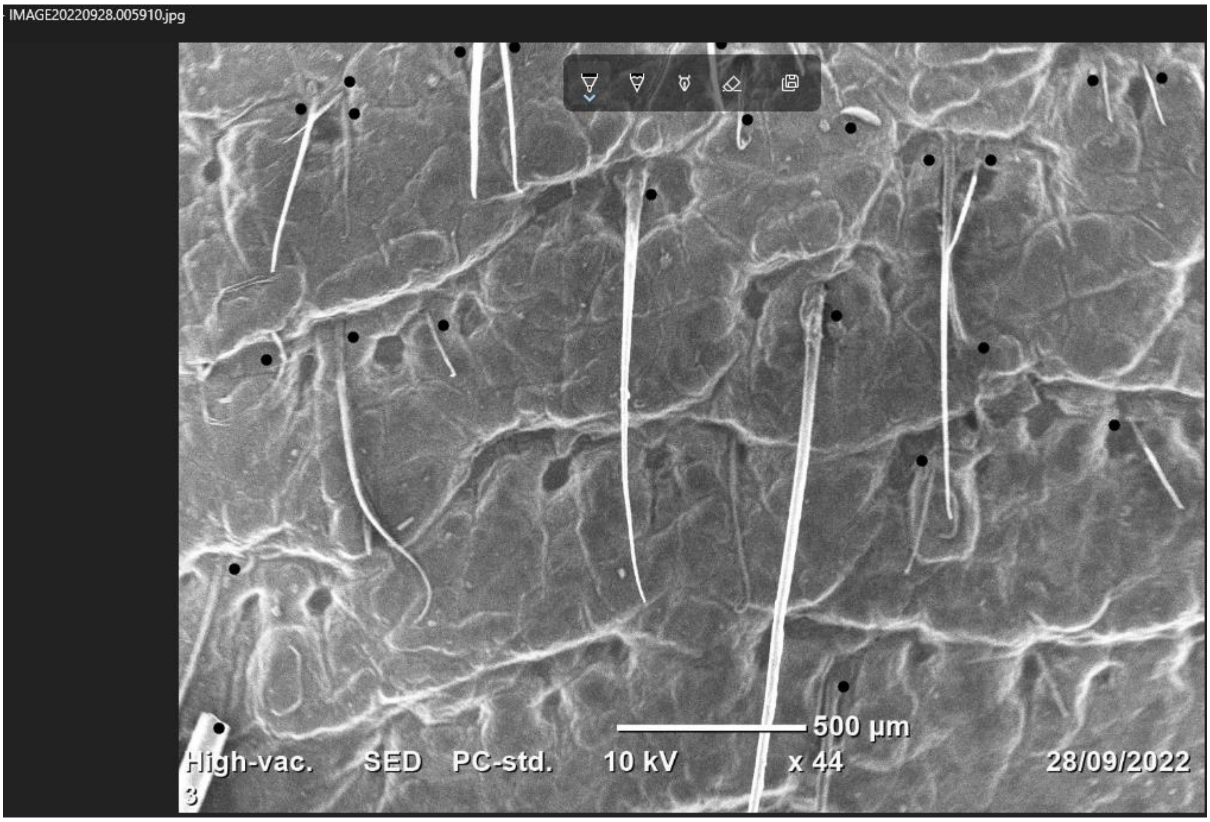

IMAGE20220928.005910 - Notepad

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```

Ln 1, Col 1 100% Windows (CRLF) UTF-8

Figure 20 Run 10 SEM result

Run 11

It has 1mm$^2$ area expecting 13 pores among those 7pores were not dehaired.

13pores-7pores=6pores

If 13pores=100%

6pores=?

600/13=46.2%

IMAGE20220928.005907 - Notepad

File Edit Format View Help

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Ln 1, Col 1 100% Windows (CRLF) UTF-8

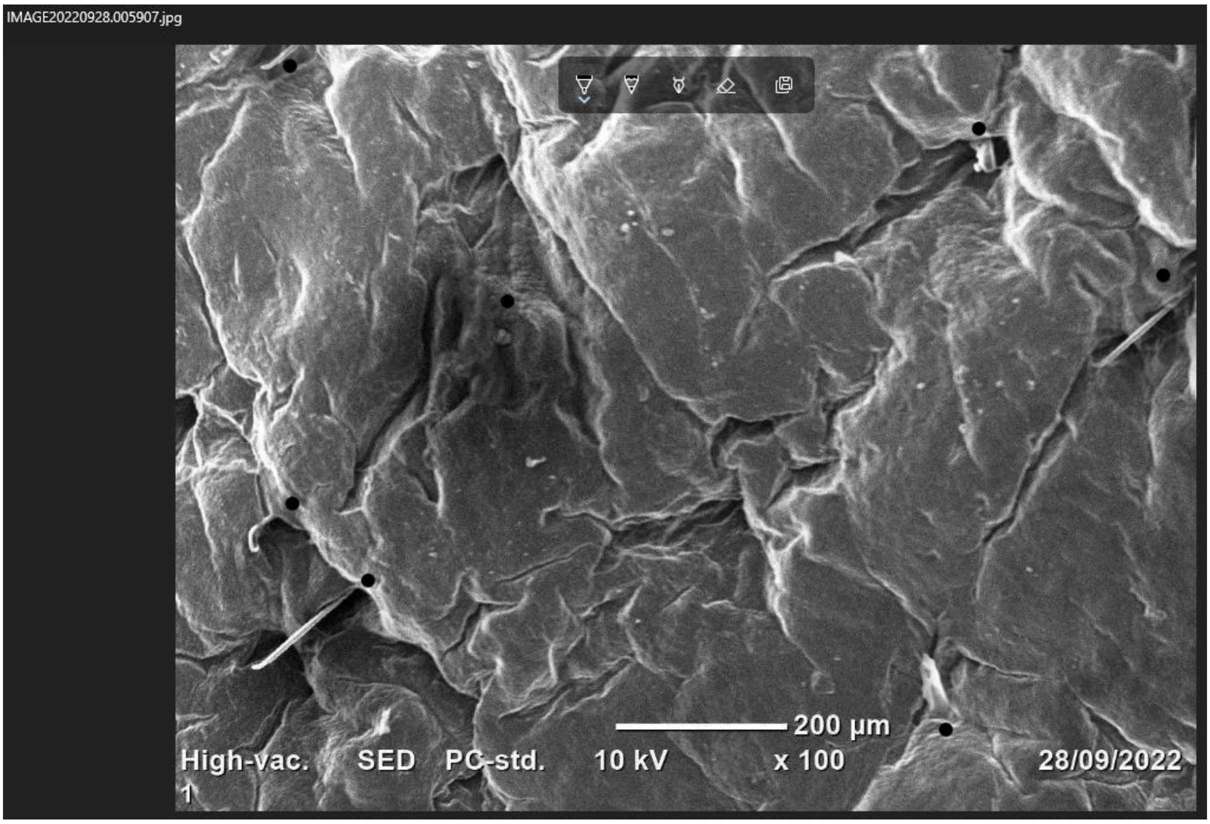


Figure 21 Run 11 SEM result

Run 12

It has 14.4mm$^2$ area expecting 180pores and has 63 not dehaired pores.

180-65=117pores

If 180pore=100%

117 pores=?

11700/180=65%


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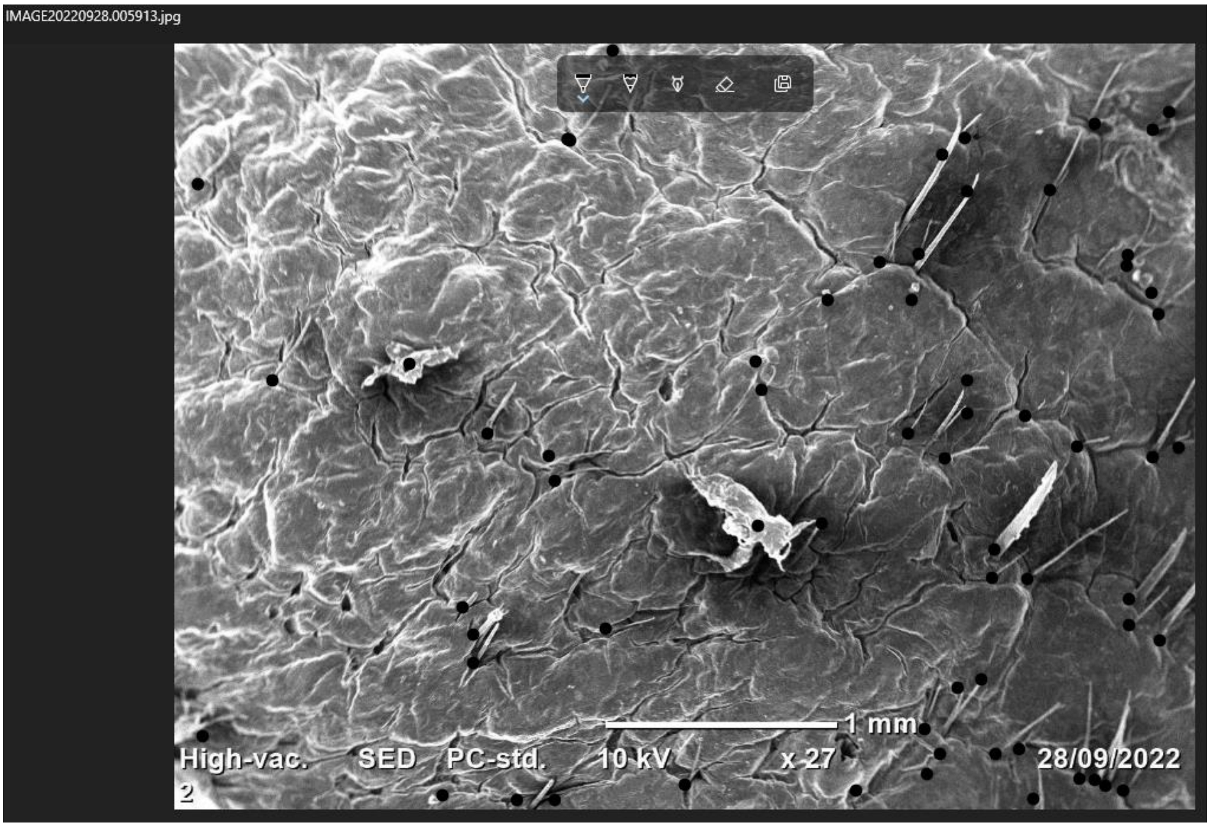



Figure 22 Run 12 SEM result

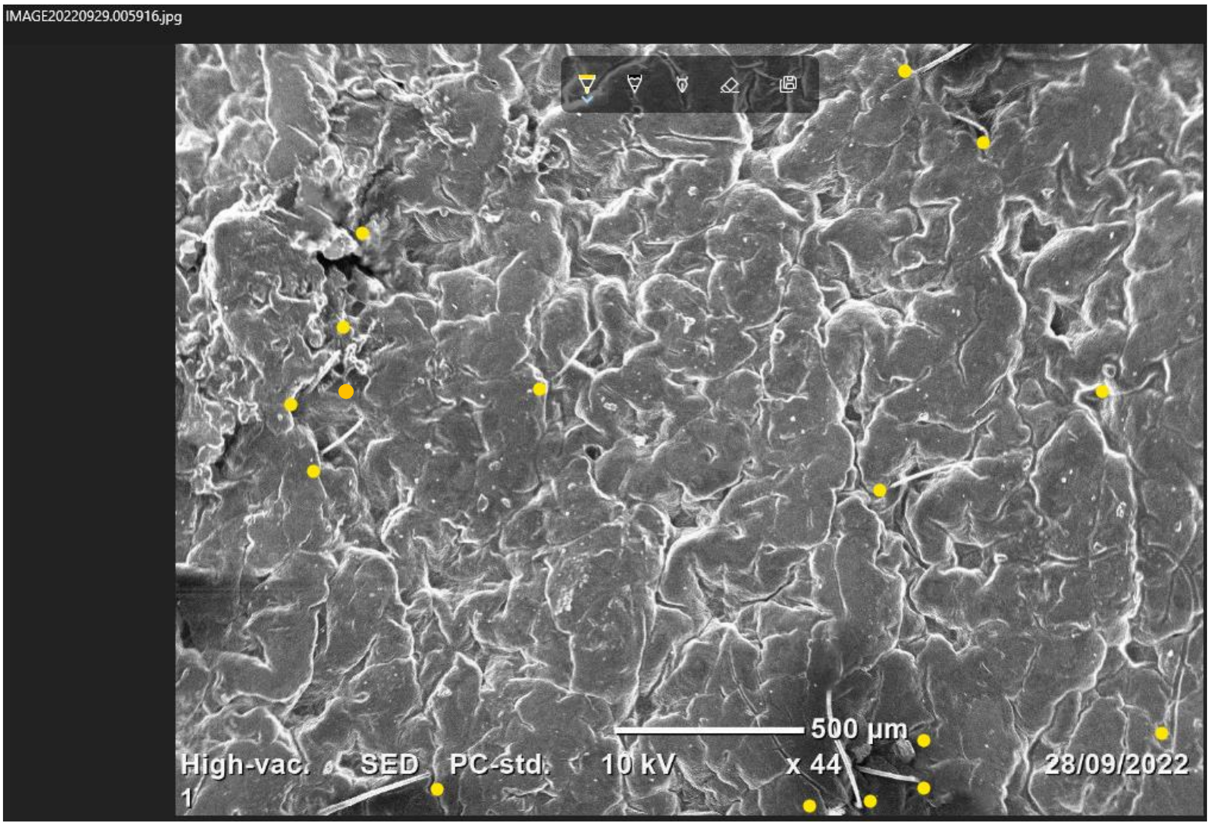
Run 13

It has 2.71mm*2.01mm= 5.4mm$^2$ area, expected to have 68 pores and among those 15pores were not dehaired. 68-16=52

68pores=100%

52=?

5300/68=76%

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```

Figure 23 Run 13 SEM result

Run 14

It has 2.71mm* 2.01mm=5.4mm$^2$ area expected to have 68 pores. Among 68 pores 31pores were not dehaired. 68-31=37pores

68pores=100%

37pores=?

3700/68=54%

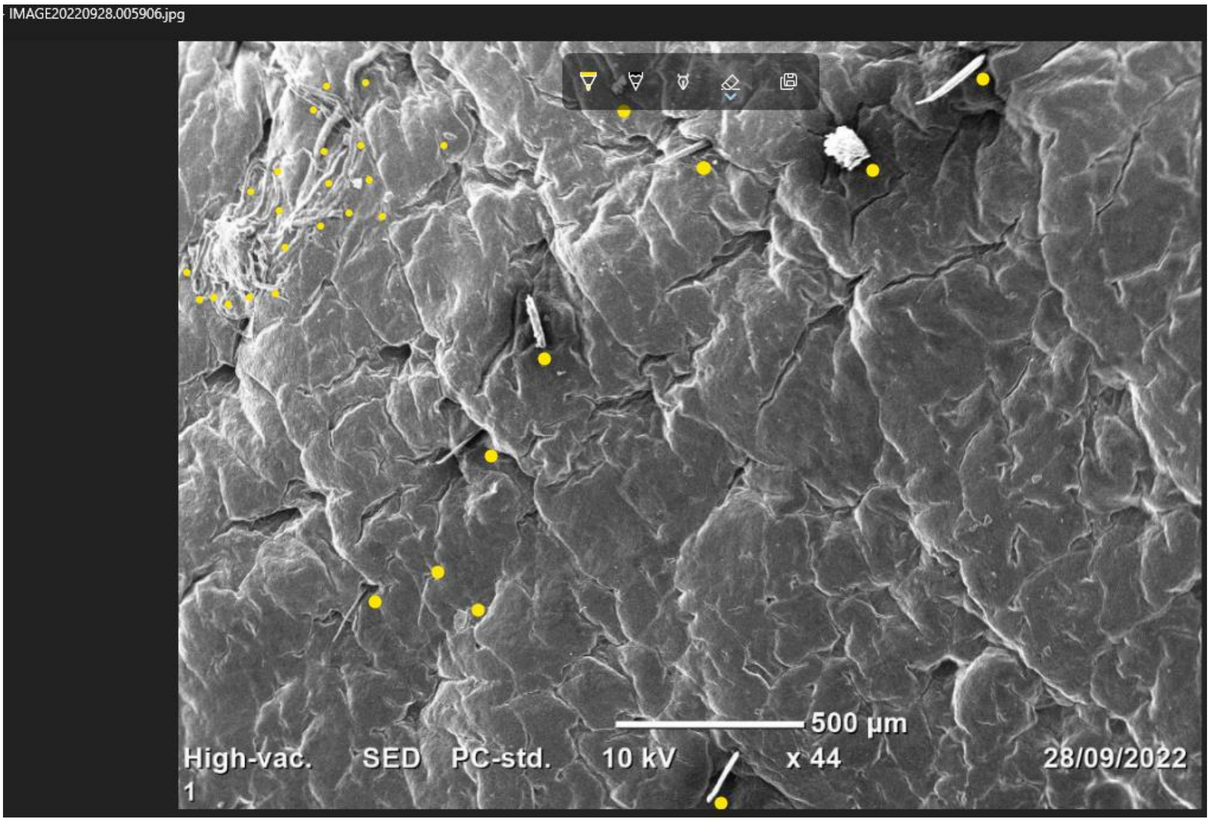



IMAGE20220928.005906 - Notepad

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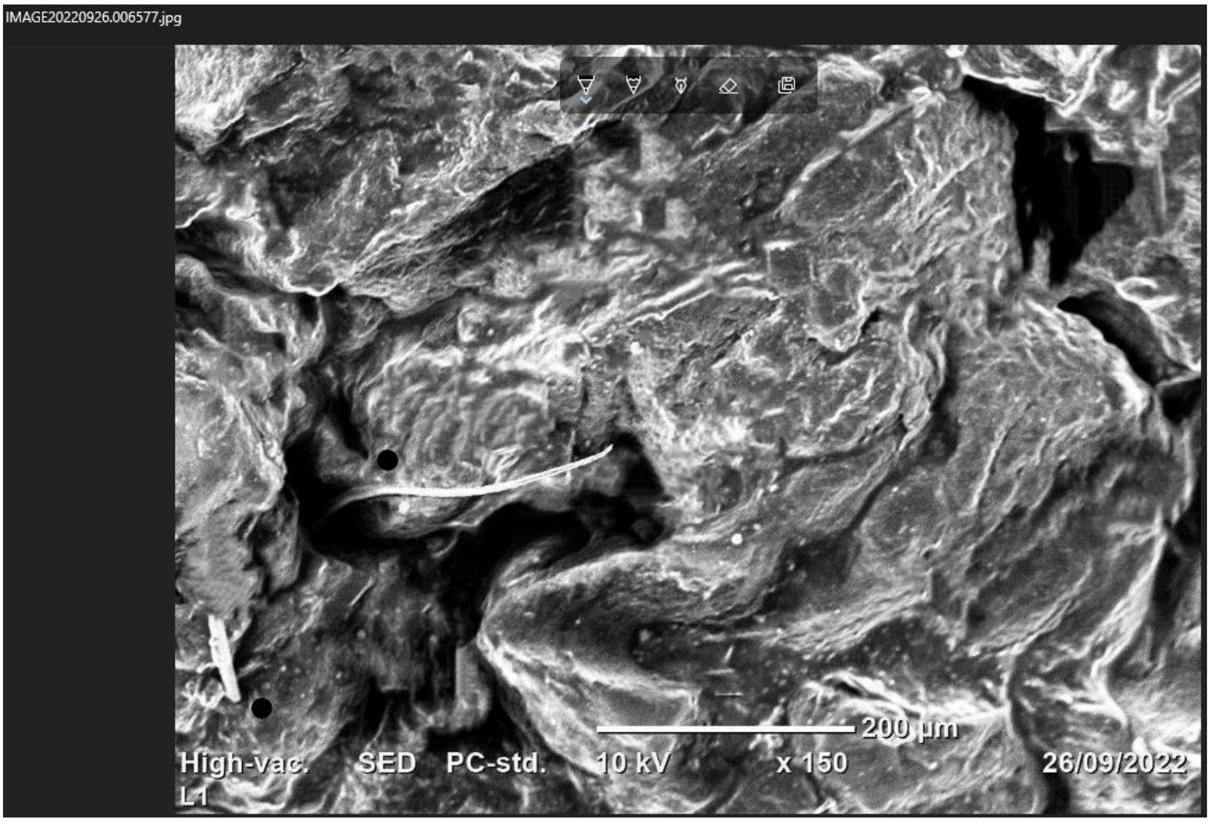
Ln 1, Col 1 100% Windows (CRLF) UTF-8

Figure 24 Run 14 SEM result

Run 15

Has 0.795mm*0.588mm=0.5mm$^2$ expecting 6pores and 2 pores were having hair that is not dehaired. If 6pore is 100% then 4pore that is dehaired will be how much percent?

400/6=67%





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$$SM_MAGKEY 150x
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$$SM_PC_MODE PC-std.
$$SM_CL 1
$$SM_GF 2
$$SM_HEIGHT 46
$$SM_WD 17
$$SM_TWD 18
```



Figure 25 Run 15 SEM result

Run 16

It has 2.65mm*1.96mm=5.2mm$^2$ area expecting 65 pores. Among those pores 28 pores were not dehaired, so 65-28=37

65pores=100%

37=?

3700/65=57%

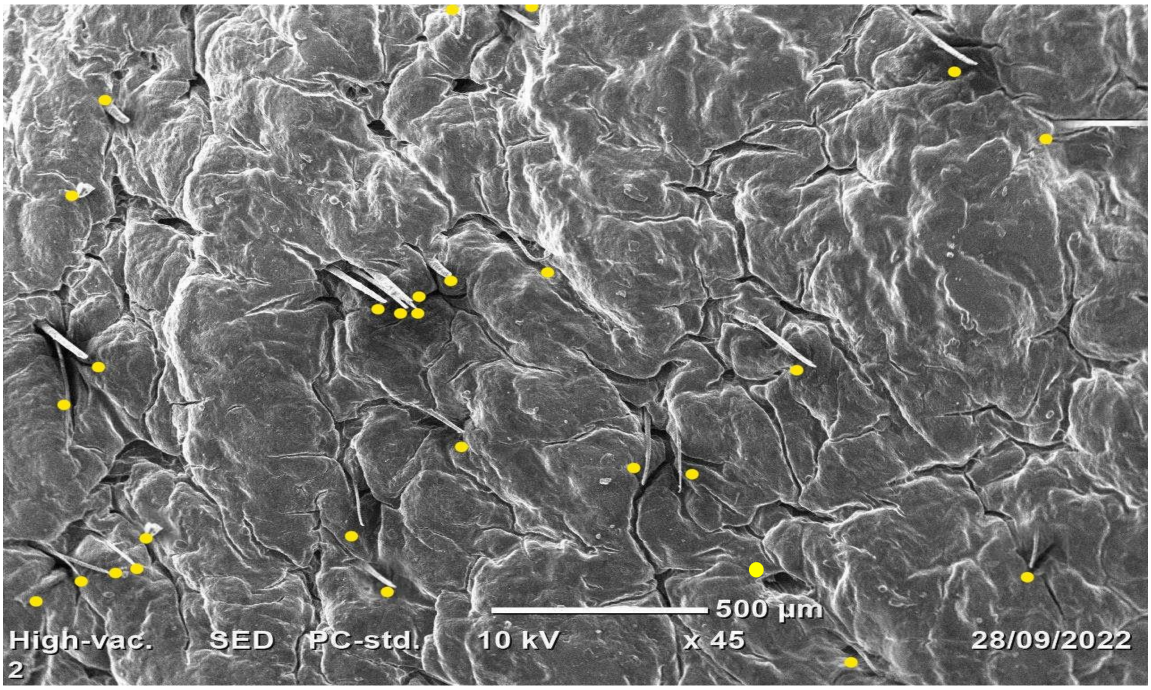



IMAGE20220928.005908 - Notepad

File Edit Format View Help

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$CM_TIME 10:59:55
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$CM_INSTRUMENT JCM-6000Plus
$CM_ACCEL_VOLT 10.00
$CM_MAG 45.0
$CM_SIGNAL SED
$CM_FIELD_OF_VIEW 2,65mm 1,96mm
$$SM_MICRON_BAR 242.0
$$SM_MICRON_MARKER 500um
$$SM_IMAGETYPE 1
$$SM_SEQID 005908
$$SM_TITLE High-vac. SED PC-std. 10kV x45 (47mm)
$$SM_MAGKEY 45x
$$SM_VAC_MODE HV
$$SM_PC_MODE PC-std.
$$SM_CL 1
$$SM_GF 2
$$SM_HEIGHT 47
$$SM_WD 16
$$SM_TWD 16
```

Ln 1, Col 1 100% Windows (CRLF) UTF-8

Figure 26 Run 16 SEM result

Run 17

Has 1mm$^2$ area expecting 13pores and 8 hairs were available.

13-8=5pore

If 13pore=100%

5pore =?

500/13=39%

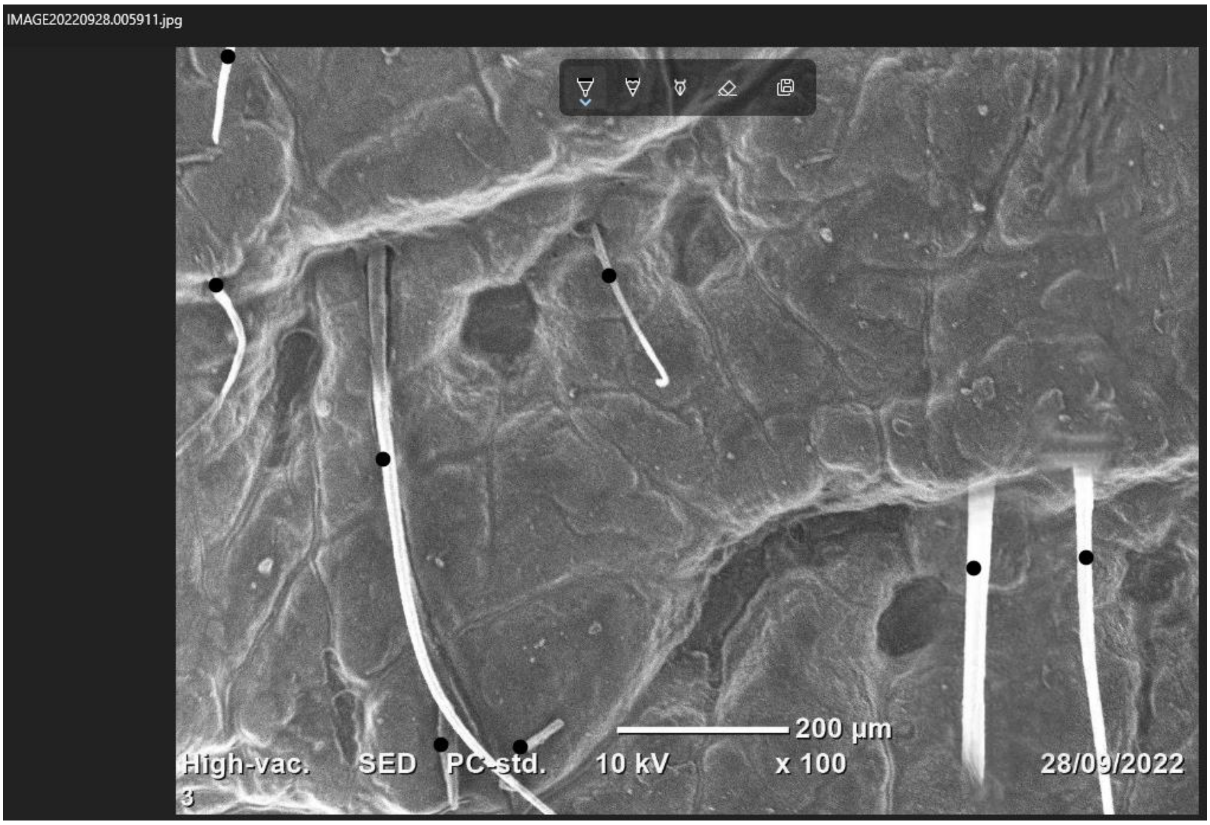


IMAGE20220928.005911 - Notepad
File Edit Format View Help

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$CM_VERSION 0.2
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$CM_TIME 11:21:11
$CM_OPERATOR
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$CM_ACCEL_VOLT 10.00
$CM_MAG 100.0
$CM_SIGNAL SED
$CM_FIELD_OF_VIEW 1,19mm 0,88mm
$$SM_MICRON_BAR 215.0
$$SM_MICRON_MARKER 200um
$$SM_IMAGETYPE 1
$$SM_SEQID 005911
$$SM_TITLE High-vac. SED PC-std. 10kV x100 (47mm)
$$SM_MAGKEY 100x
$$SM_VAC_MODE HV
$$SM_PC_MODE PC-std.
$$SM_CL 1
$$SM_GF 2
$$SM_HEIGHT 47
$$SM_WD 16
$$SM_TWD 16

Ln 1, Col 1 100% Windows (CRLF) UTF-8

Figure 27 Run 17 SEM result

Analysis of variance (ANOVA) helps us to identify the significance of a given parameters and their interaction effect by evaluating their P and F value. When the P- value is small and F-value is large the effect is considered as significant.

Table 4-2 Model performance indicators for hair removal during for dehairing.

| **Source** | **Sum of Squares** | **DF** | **Mean Square** | **F Value** | **Prob > F** | |
|---|---|---|---|---|---|---|
| Model | *4529.72* | *9* | *503.30* | *217.62* | *< 0.0001* | *significant* |
| *A* | *1493.31* | *1* | *1493.31* | *645.68* | *< 0.0001* | |
| *B* | *66.13* | *1* | *66.13* | *28.59* | *0.0011* | |
| *C* | *2899.41* | *1* | *2899.41* | *1253.65* | *< 0.0001* | |
| $A^2$ | *6.01* | *1* | *6.01* | *2.60* | *0.1511* | |
| $B^2$ | *0.97* | *1* | *0.97* | *0.42* | *0.5375* | |
| $C^2$ | *62.23* | *1* | *62.23* | *26.91* | *0.0013* | |
| *AB* | *0.16* | *1* | *0.16* | *0.069* | *0.8001* | |
| *AC* | *0.063* | *1* | *0.063* | *0.027* | *0.8741* | |
| *BC* | *0.010* | *1* | *0.010* | *4.324E-003* | *0.9494* | |
| Residual | *16.19* | *7* | *2.31* | | | |
| *Lack of Fit* | *12.02* | *3* | *4.01* | *3.84* | *0.1132* | *not significant* |
| *Pure Error* | *4.17* | *4* | *1.04* | | | |
| Cor Total | *4545.91* | *16* | | | | |

The Model F-value of 320.12 implies the model is significant. There is only a 0.01% 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 A, B, C and C2 are significant model terms. The lack of fit F-value of 3.84 is not significant relative to the pure error; There is a 11.32% chance that a "Lack of Fit F-value" this large could occur dueto noise. Non-significant lack of fit is good. Lack of fit compares the variation between the actual data and predicted value, to the variation between replicates. This indicates that the model equation can be used to predict values and the experimental results are statistically acceptable.

### 4.5.1 Fit summary and Fit statistics

Table 4-3 Summary of model fitness.

| **Source** | **Std. Dev.** | **R-Squared** | **Adjusted R-Squared** | **Predicted R-Squared** | Remark |
|---|---|---|---|---|---|
| Linear | 2.59 | 0.9808 | 0.9764 | 0.9657 | |
| 2FI | 2.95 | 0.9809 | 0.9694 | 0.9258 | |
| Quadratic | 1.52 | 0.9964 | 0.9919 | 0.9563 | Suggested |
| Cubic | 1.02 | 0.9991 | 0.9963 | | Aliased |

The experimental data was checked for model adequacy to see if the fitted model would produce inaccurate or deceptive results. The experimental data were fitted using four-degree polynomial models, including linear, interactive (2FI), cubic, and quadratic models. The statistical summary for each model is shown in Table 4-3, that was the output by Design Expert. A quadratic model was suggested, nevertheless it has lower $R^2$ and adjusted $R^2$ values than a cubic model. This is because the cubic model is aliased, which means that the effects of each variable that results dissimilar signals become not distinguishable. For the remaining relation like a linear relationship, the R2 and Adj-R 2 values are lower from the quadratic. Therefore, it is clear that the relationship is not adequate for the experimental data. Thus, the quadratic model was selected to fit the experimental data for the selected condition.

The coefficient of determination (R2 ) is well-defined as the ratio of the explained variation to the total variation and is a measure of the degree of fit (Ferreira et al., 2007), the "Pred R-Squared" of 0.9563is in reasonable agreement with the "Adj R-Squared" of 0.9919. This means that the response model evaluated in this study can describe the reaction very well, with R2 of 0.9964 and an Adj R-Squared of 0.9919 indicating a better model prediction.. In addition, the model is very significant, as it is evident from its F-value (F model = 217.62) and very low probability value (p = 0.0001) as shown from above Table 4-2.

Table 4-4 Fit of statistics for hair removal

| | | | |
|---|---|---|---|
| Std. Dev. | 1.52 | R-Squared | 0.9964 |
| Mean | 63.77 | Adj R-Squared | 0.9919 |
| C.V. | 2.38 | Pred R-Squared | 0.9563 |
| PRESS | 198.80 | Adeq Precision | 56.071 |

The statistic known as standard deviation (Std.Dev) quantifies how dispersed a set of data is in relation to its mean. The ratio of the standard deviation to the mean is known as the coefficient of variation (C.V.%). Generally speaking, the coefficient of variation calculates the ratio of the standard deviation to the mean, whereas the standard deviation indicates the distance between the average value and the mean. The degree of dispersion around the mean increases with increasing coefficient of variation. A smaller coefficient of variation is preferable since it would indicate that the distribution of data values is closer to the mean. The Adjusted R2 of 0.9919 and the Predicted R2 of 0.9563 are reasonably in agreement; that is, the difference is less than 0.2. Adeq Precision calculates the ratio of signal to noise. Ideally, the ratio should be higher than 4. The ratio of 56.071 in this instance denotes a sufficient signal. The design space may be navigated using this concept.

### 4.6 Determination of Second-Order Polynomial Equations and validation of model

The finding of the experiment, which was based on the Box-Behnken experimental design, was fitted to the input variables using an empirical relationship defined by a second-order polynomial equation with interaction terms. Based on the responses, the software recommended that a quadratic model simulating the process be used to better describe the dehairing process as shown in equation 3.

Hair removal = +74.22274+1.54918* Enzyme conc-14.82033* Time+1.40047*Temprature -5.30889E-003* Enzyme conc2+0.48050 * Time 2-0.038445*Temprature 2+0.013333 *Enzyme conc * Time +8.33333E-004* Enzyme conc * Temprature +5.00000E-003 * Time * Temprature …Equation 3

As BBD was selected to investigate the interactions between the independent factors and find their optimal points. Table 4-5 of supplementary material depicts the details of the experimental design matrix of BBD-RSM and the experimental data (actual and predicted) for Dehairing processes.

Table4-5 Experimental and predicted response under different condition

| Std | Run | Block | Factor 1 A:Enzyme con% | Factor 2 B:Time Hr | Factor 3 C:Temprature $^{0}C$ | Response 1 Hair removal % | Pridicted value |
|---|---|---|---|---|---|---|---|
| 10 | 1 | Block 1 | 85.00 | 18.00 | 35.00 | 86 | 84.41 |
| 6 | 2 | Block 1 | 100.00 | 17.00 | 35.00 | 93 | 93.45 |
| 5 | 3 | Block 1 | 70.00 | 17.00 | 35.00 | 65 | 66.37 |
| 4 | 4 | Block 1 | 100.00 | 18.00 | 45.00 | 80.8 | 81.94 |
| 9 | 5 | Block 1 | 85.00 | 16.00 | 35.00 | 79 | 78.76 |
| 1 | 6 | Block 1 | 70.00 | 16.00 | 45.00 | 50 | 48.86 |
| 16 | 7 | Block 1 | 85.00 | 17.00 | 45.00 | 66.7 | 65.91 |
| 7 | 8 | Block 1 | 70.00 | 17.00 | 55.00 | 28.5 | 28.05 |
| 13 | 9 | Block 1 | 85.00 | 17.00 | 45.00 | 64.7 | 65.91 |
| 15 | 10 | Block 1 | 85.00 | 17.00 | 45.00 | 66.17 | 65.91 |
| 12 | 11 | Block 1 | 85.00 | 18.00 | 55.00 | 46.2 | 46.44 |
| 14 | 12 | Block 1 | 85.00 | 17.00 | 45.00 | 65 | 65.91 |
| 2 | 13 | Block 1 | 100.00 | 16.00 | 45.00 | 76 | 75.79 |
| 3 | 14 | Block 1 | 70.00 | 18.00 | 45.00 | 54 | 54.21 |
| 17 | 15 | Block 1 | 85.00 | 17.00 | 45.00 | 67 | 65.91 |
| 8 | 16 | Block 1 | 100.00 | 17.00 | 55.00 | 57 | 55.62 |
| 11 | 17 | Block 1 | 85.00 | 16.00 | 55.00 | 39 | 40.59 |

### 4.7 Diagnostics plots and Model graph of response on the Experimental Variables

An accuracy check is important to obtain an adequate and exact model as shown in Fig. [28,29 and 30]. Additionally, by contrasting the actual (experimental) and predicted values for removal efficiency, the model's accuracy was tested. The values of the actual and anticipated removal efficiencies for a specific circumstance are displayed in Table 4-5. Secondly, it was possible to obtain a normal plot of residuals between the normal probability (%) and the internally studentized residuals. This allows for the measurement of the standard deviations separating the experimental and predicted values, as well as the checking of the residuals to see how well the model adheres to the ANOVA assumptions(Wahyuni et al., 2017).

The normal probability plot of the residuals given in Figure 28 indicates the residuals are generally along a straight line, and this implies the residuals have a normal distribution in the given probability range. The residuals are the difference between the actual and predicted values of experimental runs by the design expert. Thus, one can generalize that there is no problem with the normality in the data. Hence, there are no severe outliers to be found. The points in the plots for this experimental data fit in to the straight line in the figure, demonstrating that the quadratic polynomial model satisfies the analysis of variance (ANOVA) assumptions that the error distribution is estimated normally.

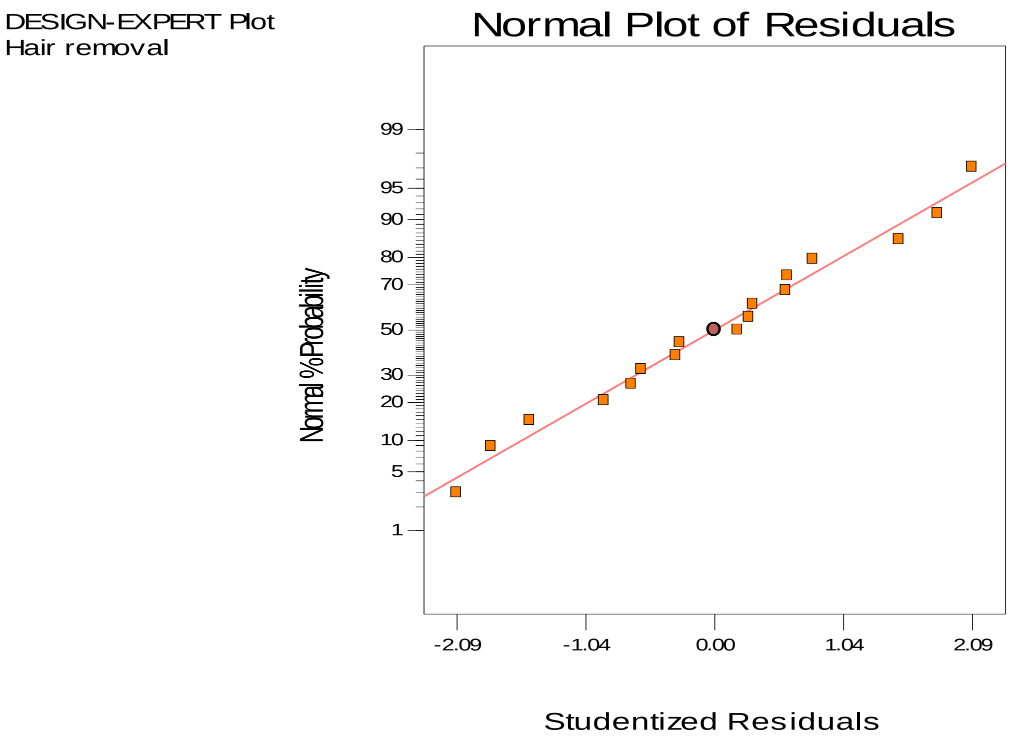


Figure 28 Normal plot of residuals experimental value for dehairing

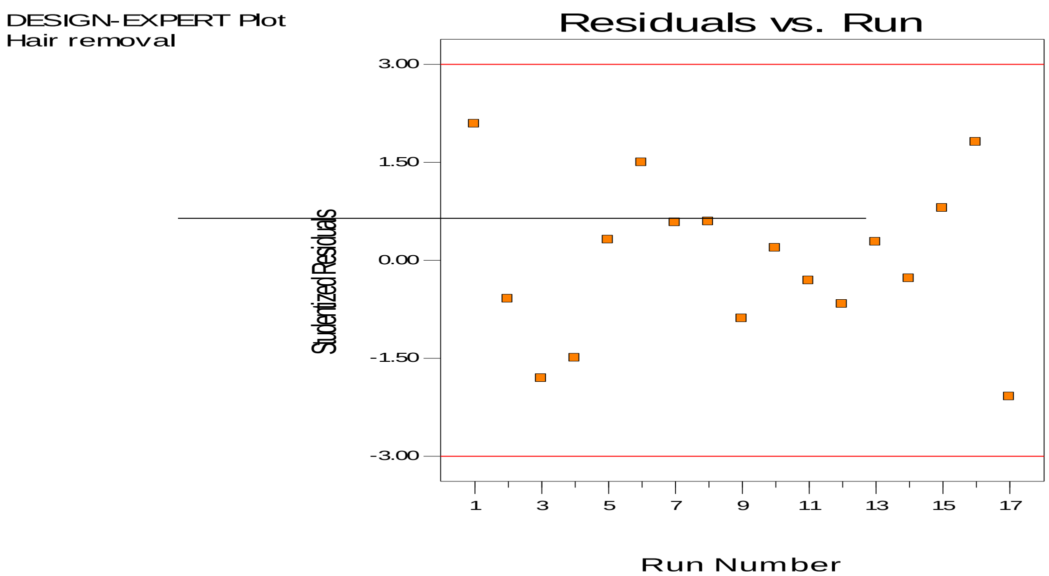


Figure 29 Residual vs experimental value for Dehairing

Figure 29 shows the plot of residuals versus the experimental run order. It checks for lurking variables that may have influenced the response during the experiment. The two bounder lines of graph are drowned at 3 and -3 of external residuals whereas the values of residual verses run are at the center line in the boundary and no outline found.

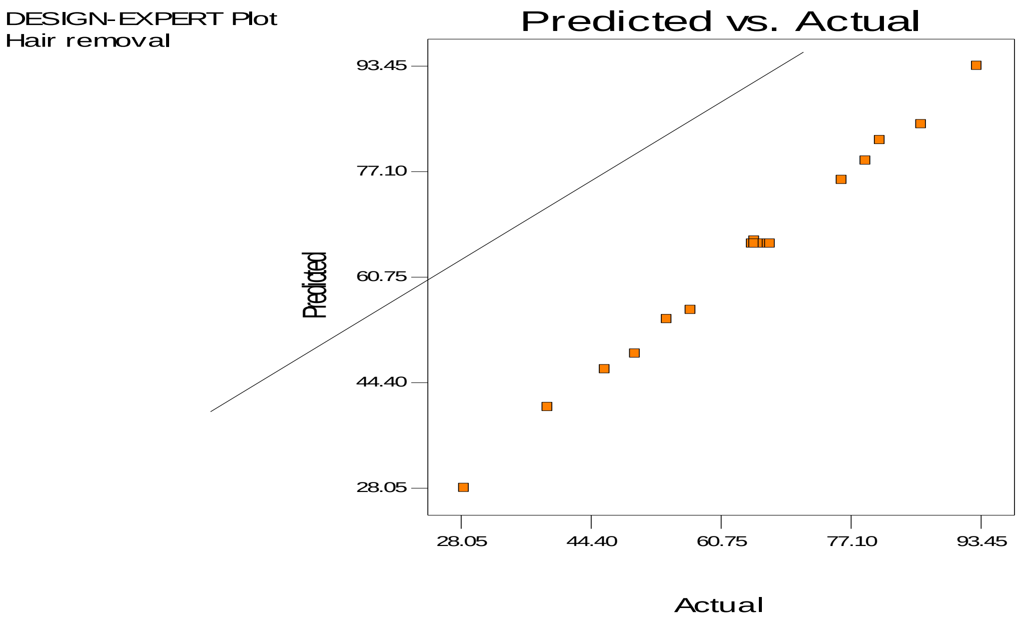


Figure 30 Predicted vs actual value for dehairing

Another crucial diagnostics plot that should be taken into account is the plot of predicted against actual (Figure 30), which is a graph of the predicted response values versus the actual response values. The goal is to identify a value or set of values that the model is unable to predict with reasonable accuracy. Looking at the graph, we can see that the predicted and actual values follow a straight line, indicating an acceptable fit between the laboratory result and the prediction and demonstrating the strong predictive capacity of the constructed model.

### 4.8 Effect of Variables (Parameters) on Enzymatic dehairing

In the present work, the extraction of ficus sycomorous fruit enzyme and its dehairing performance was observed through by scanning electro-microscope. The effects of each variable and their interaction on de-hairing performance were studied. Enzyme concentration, time and temperature play a key role in the enzyme de-hairing performance efficiency and the results are depicted as surface plots in Figures.

### 4.8.1 Effect of temperature on enzyme activity to dehair

The Figure below shows the effect of Temperature (35, 45 and $55^0$C) on enzymatic dehairing at a time. It was shown that this element had effect on the dehairing efficiency meaning that when temperature is lower the enzymatic dehairing efficiency is high. And when time increases the enzymatic dehairing efficiency increases.

At high temperature, ficin decreased its activity, possibly because of it enzyme incurred damage resulting in loss of catalytic activity as stated on literature (Wahyuni et al., 2017). Protease activity increases along with increasing temperatures until the optimum temperature is reached, after which a further increase in temperature will cause decreased protease activity. At lower temperatures than the optimum temperature, the enzyme activity is also low. That is because lower activation energy is available. Increased temperature also affects the conformational changes of the substrate so that the substrate actively experience barriers to entering the active site of the enzyme and causes a fall in the activity of the enzyme. In this study also the higher enzymatic dehairing was achived at temperature $35^0$C, this is similar to studies Latex peptidases of Calotropis procera for dehairing of leather as an alternative to environmentally toxic sodium sulfide treatment(Lopéz et al., 2017), Dehairing of animal hides and skins by alkaline proteases of Aspergillus oryzae for efficient processing to leather products in Tanzania in literature(Never et al., 2019). and also on application of Carica papaya (Cp) and Vasconcellea quercifolia (Vq) obtained from latex on the dehairing process(David Troncoso et al., 2022). As temperature increase the efficiency to dehair reduced which show opposite relation, and the lowest dehairing efficiency was achived at 55 $^0$C. And at high temperature above optimum (like $65^0$C) not only the structure of the enzyme molecule composed of amino acids containing hydrogen bonds in the polypeptide chain will be stretchable and so broken but also the rawhide shrinkage temperature will be reached(Chahine, 2000; Maina et al., 2019).

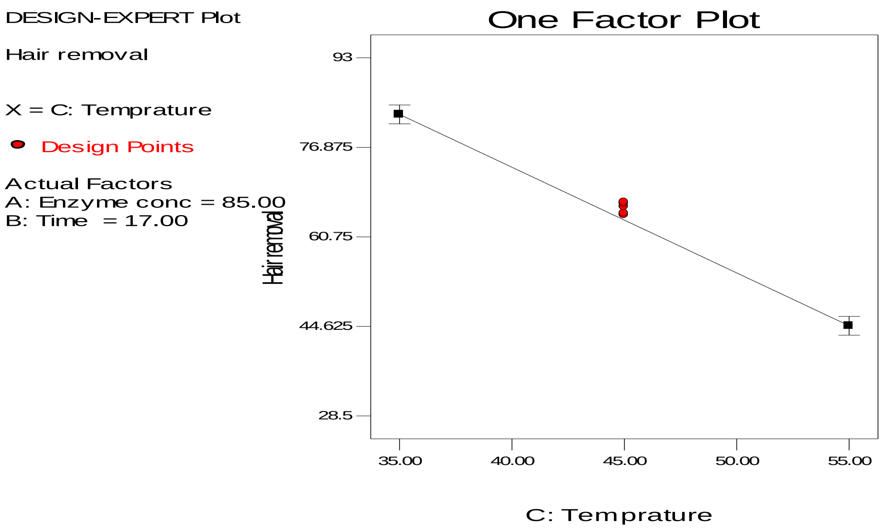


Figure 31 Effect of temperature on dehairing

### 4.8.2 Effect of Time on dehairing performance

The Figure below shows the effect of time on enzymatic dehairing. As we can understand from the graph this element had positive effect on the dehairing efficiency, results showing that when time is lower the enzymatic dehairing efficiency is low and when time is increasing the enzymatic dehairing efficiency increases. This implies enzyme gets efficient time to breakdown non-collagenous skin constituents and removes non-fibrillary proteins(Badgujar & Mahajan, 2013). Within the range 16 to 18hr there was significant change on the dehairing performance or hair removal from the raw skin. Also while checking time intervals the pores opened up and hairs on the raw skin of goat started to be dehaired after 12hr incubation but it required force instead of gentle scalding. Then checked at 14hr the very long hairs were easily removed but many fine hairs that can be seen and feel by touching were not dehaired. At a time from 16 to 18hr there was significance change to the hair removal which is similar to study made on Characterization of Thermo- and Detergent Stable Antigenic Glycosylated Cysteine Protease of Euphorbia nivulia Buch.-Ham. and Evaluation of Its Ecofriendly Applications(Badgujar & Mahajan, 2013).

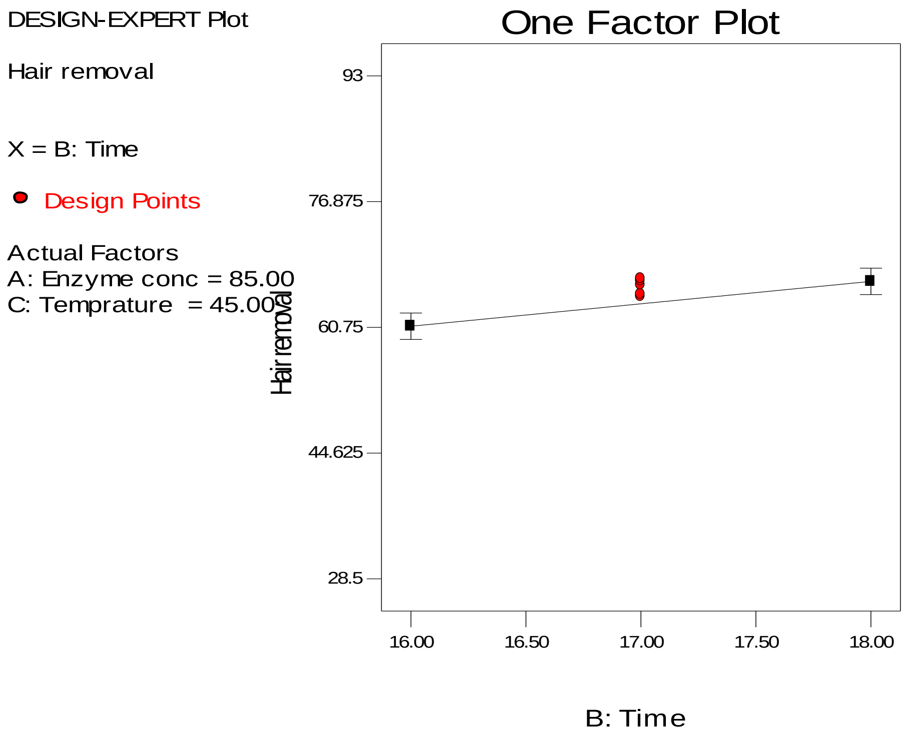


Figure 32 Effect of time on dehairing

### 4.8.3 Effect of enzymatic concentration on dehairing performance

The effect of enzymatic concentration on dehairing performance was tested in range of 100, 85 and 70 percent. As the Figure below shows the effect of current on removal percent has been noticed that the hair removal or dehairing efficiency increased with increase in enzymatic concentration, due to the fact that by increasing the enzymatic concentration, it means a full concentration has great efficiency to dehair or remove the hair from the skin but with lowest temperature and highest time. As shown in the figure full concentration with highest temperature and lower time has low (39) percent of hair removal while with lowest temperature and at high incubation time 94 percent hair removal achieved.

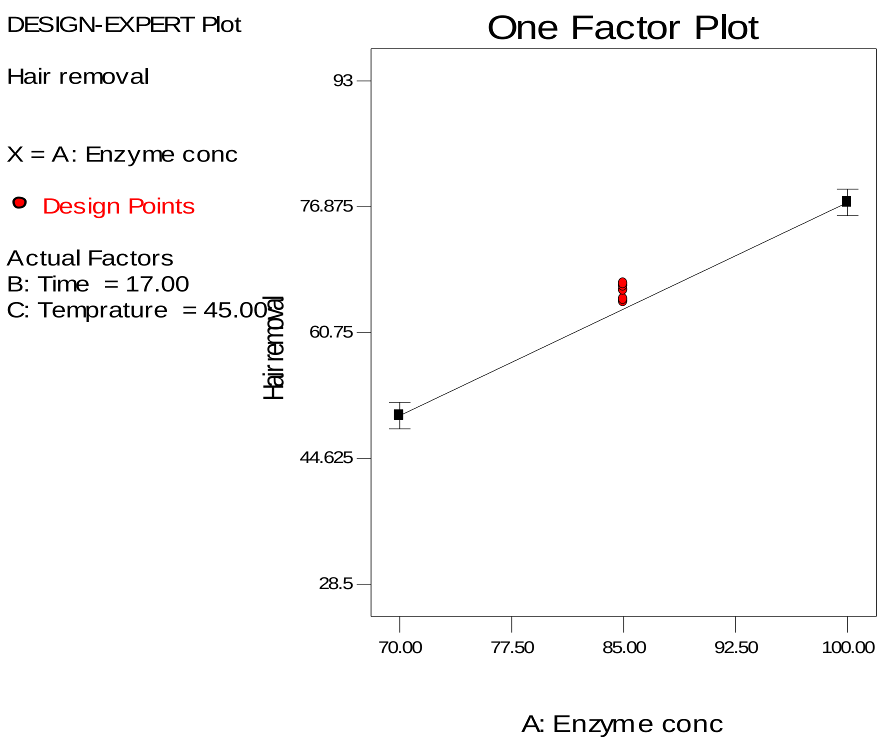


Figure 33 Effect of enzyme concentration on dehairing

### 4.8.4 Interaction effect of parameters on the dehairing performance

An interaction effect is a combination effect of two or more individual factors that can possibly affect the response in the same or different way. Interaction effects should always be under consideration if and only if the P-value of the combined factors found to be less than 0.05 or 5% (i.e., the probability of the model) Figure (34) shows the interaction effect between enzyme concentration and time seen from table of ANOVA the effect has a P-value greater than 0.05 i.e., 1.0000 the results obtained indicates that the interaction effect is not significant. The graph shows that full concentration with 17hr has 93 percent hair removal at $35^0$C also full concentration with 16hr has 80.8 percent hair removal at $45^0$C. Therefore the combination effect of time and enzyme concentration is positive as both increase the deharing or hair removal efficiency increases as Figure 34 shows. Temperature has great effect on the dehairing or hair removal due to the cysteine protease, it is dependent on temperature. As both graph of temperature with time and temperature with enzyme concentration shows in fig 35 and 36 the hair removal increase as the temperature decreases. This implies that the temperature negatively affect the hair removal process due to the cysteine protease has effective removal efficiency at temperature around 35. As studies also shown Euphorbia nivulia Buch.-Ham Cysteine protease could remove hairs cow hides after 18 hr incubation of hide at 30°C easily with no observable damage on the collagen. Therefore, the dehaired skin exhibits clean hair pore and clear grain structure(Badgujar & Mahajan, 2013). Also compared to earlier reports protease from Bacillus cereus and Aspergillus tamarii dehaired the goat skin in 21 and 24 h, respectively(Senthilvelan et al., 2012) indicating its potential application in leather industry for ecofriendly economizing the process. Also the skin treated by ficus sycomoros enzyme was clean and has clear pores as seen on scanning microscope.

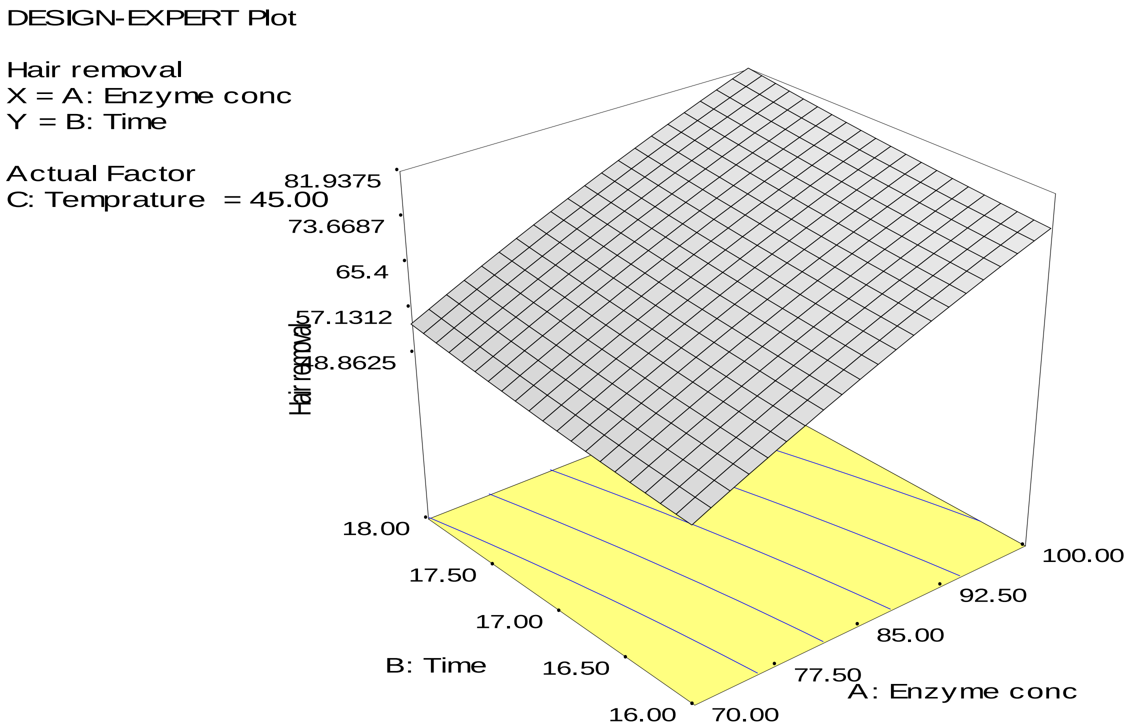


Figure 34 enzyme concentration and time effect on dehairing

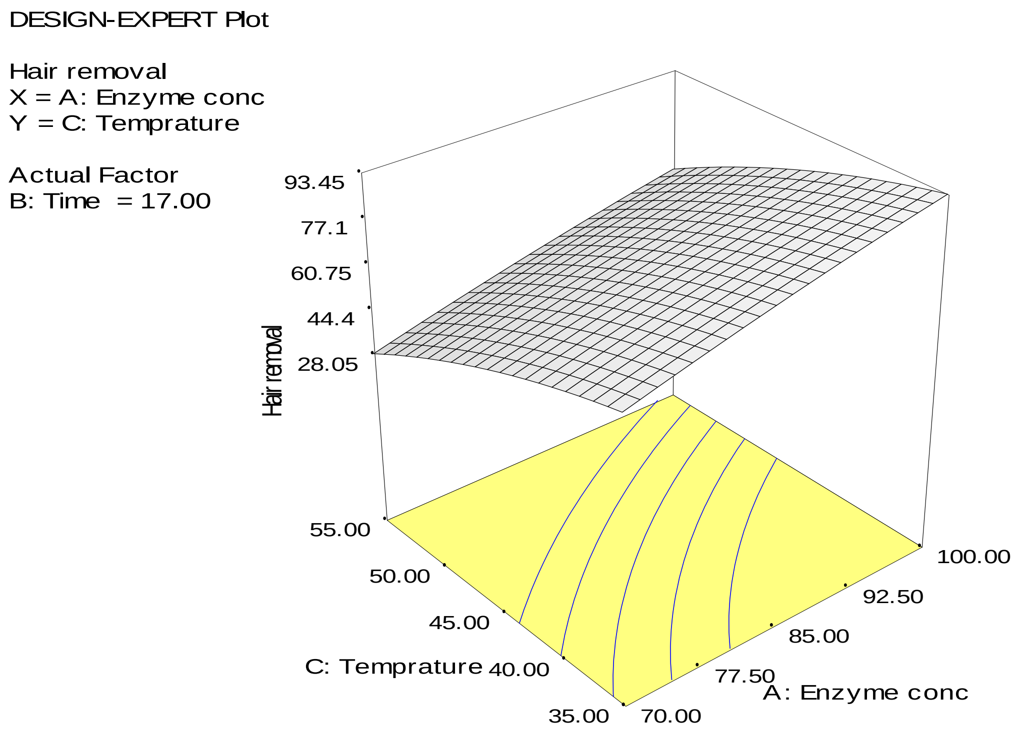


Figure 35 Effect of enzyme concentration and time

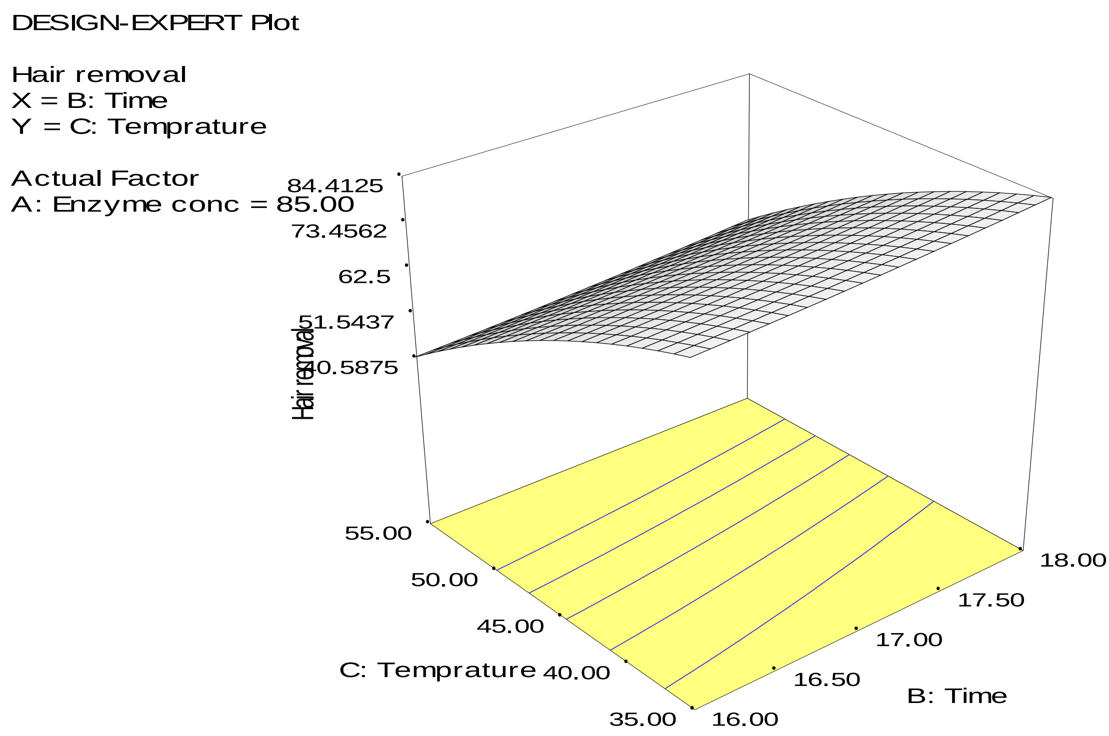


Figure 36 Effect of time and temperature

## 4.9 BOD and COD results

The final waste was characterized for BOD, COD, TDS, and TSS tests the result was as followed

Table 4-6 waste result

| Tests | Results |
|---|---|
| BOD | 210.0 |
| COD | 932.2 |
| TDS | 2105 |
| TSS | 582 |

As shown on the table above the results in mg/L was relatively seen and compared with effluent from modjo and batu tannery in Ethiopia. In batu and modjo tannery the characteristic of the effluent in liming stage is presented as follow from literature (Abdrie Seid et al., 2018)

| Table 1. (Batu Tannery ) | | | | | | |
|---|---|---|---|---|---|---|
| Parameter | Soaking | Liming | Deliming | Pickling | tanning | Re-tanning |
| PH | 8.37±0.988 | 12.00±0.707 | 8.63±0.989 | 3.25±0.212 | 4.09±0.141 | 4.11±0.127 |
| Total alkalinity | 9157±5503.5 | 15172±4282.2 | 11150.6±7140.8 | - | - | - |
| BOD5 | 1700±141.42 | 1710±56.66 | 1625±66.47 | 190±36.77 | 277.5±88.39 | 280.5±77.075 |
| COD | 11640±1484.92 | 18578±1827.15 | 7485.1±1808.07 | 2707±687.34 | 1716±619.43 | 4487.1±1121.61 |
| Total solid | 36160.5±9772.95 | 21961.35±1695.4 | 25002.1±11543.58 | 23588±12215.97 | 13553.5±16899 | 6272.95±8345.2 |
| Total dissolved Solid | 27067.5±9853.5 | 15157±1636.24 | 19199.95±11596.48 | 23130±12204.6 | 13148.5±16897.7 | 6100.95±8342.37 |
| Suspended Solid | 9093±80.61 | 6804.35±59.18 | 5802.6±52.89 | 458±11.313 | 405±1.41 | 172±2.82 |
| Chloride | 31127.37±849.05 | 5581.2±72.14 | 3862.12±140.89 | 41568.9±1423.37 | 2719.7±364.202 | 2666.15±436.49 |
| Sulfide | 0.035±0.0014 | 2.267±0.583 | 1.365±0.275 | 0.905±0.487 | 0.35±0.125 | 0.280±0.226 |
| Chromium | - | - | - | - | .006 | 1.22 |

| Table 2. (Modjo Tannery ) | | | | | | |
|---|---|---|---|---|---|---|
| Parameter | Soaking | Liming | Deliming | Pickling | tanning | Re-tanning |
| PH | 8.23±0.68 | 12.64±0.00 | 8.52±1.01 | 3.93±0.38 | 3.96±0.212 | 3.98±0.113 |
| Total alkalinity | 3463.1±1220.61 | 10187.15±3392.18 | 3684.5±1321.61 | - | - | - |
| BOD5 | 3161.25±147.4 | 4275.1±568.66 | 3232.1±626.64 | 786±124.451 | 870.5±149.2 | 847±88.4 |
| COD | 11695±1704.13 | 13535±2354.67 | 6401.5±1808.072 | 2707±510.34 | 1716±456.43 | 4487.1±8021.61 |
| Total solid | 19090.33±4679.8 | 19267.36±4787.2 | 18130.75±3896.6 | 18329.25±3987.13 | 17751.69±3662.77 | 5864.5±1461.03 |
| Total dissolved Solid | 23171±4625.15 | 23380.4±4575.05 | 20971.8±3268.14 | 25508.5±5324.54 | 17963.2±3662.8 | 7288±1562.17 |
| Suspended Solid | 7118.9±699.54 | 6987±572.6 | 5559±468.97 | 8670.5±1120.69 | 768.5±198.3 | 1926.5±322.76 |

As we can see from the table BOD for Batu and Modjo is 1710±56.66 and 4275.1±568.66 respectively and according to the BOD result for the waste from enzymatic dehairing is 210mg/L showing that the enzymatic dehairing has less impact of effect on the dehairing BOD having 87% reduction. For COD, TDS and TSS result as LIDI (leather industry development institute) has tested and approved the test result was COD 932.2 while Batu and Modjo has 18578±1827.15 and 13535±2354.67 showing 93% reduction, TDS 2105.0 while Batu and Modjo were having 21961.35±1695.4 and 23380.4±4575.05 showing 86.5% reduction, TSS 582 while Batu and Modjo were having 15157±1636.24 and 19267.36±4787.2showing 96.5% reduction(Abdrie Seid et al., 2018). This shows the COD, TDS and TSS test results also had improved. When we see the improvement in BOD and COD test there was a minimum 93.2% reduction for all BOD, COD, TDS and TSS experimental tests.

Finally when we also compare with other countries, Bangladesh has BOD 700±7.5, COD 4800±4.5, TDS 12500±5.5 and TSS 23300±5.5 here also enzymatic deharing has showed improvement by reduction of 70% for BOD, 80% for COD, 83.2% for TDS and 95% for TSS.

| Company Name: |
|---|
| በኢትዮጵያ የኮንስትራክሽን ዲዛይንና ሱፐርቪዥን ሥራዎች ኮርፖሬሽን |
| Ethiopian Construction Design & Supervision Works Corporation |
| Tel: +251-11-872-3643/+251-11-872-3478/+251-11-869-5125/+251-11-872-1236 |
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| Title: | | Document No: | Issue No. 1 | Page No. |
|---|---|---|---|---|
| Water Quality Test Report | | OF/ECDSWC/0941 | | 1 of 2 |

Lab No.:- 294/2015

Client/Project: - Liya Shawel Tesfaye

Client ID:- -

Location:- -

Date Reported :- 3/10/2022

Client Ref: - Lab./02/Wq/131/22

Date Collected: - 22/9/2022

Source of Sample:- Waste

Date Received:- 22/9/2022

| No | Tests | Test Results | Unit | Test Method | Ethiopian Environmental Standard (mg/L) |
|---|---|---|---|---|---|
| 1 | $BOD_5$ | 210.00 | mg/L | 5 Day incubation(Azide Modification) | 80.0 |

**REMARK**:-The test result can be compared with the Ethiopian Environmental Standard maximum allowable concentration (mg/L) presented on the right column. The water sample is collected and submitted to the laboratory by the client.

Reported by ______ Checked by ______ Approved by ______

Lab Expert Senior Water Quality Expert Water Quality S/P Manager

Among the major services rendered by the Water Quality Laboratory Testing S/Process of Ethiopian Construction Design & Supervision Works Corporation are: Testing all the Physico-Chemical and Bacteriological analysis of potable or drinking water/ Bottled drinking water/, waste water, water for construction and irrigation uses and sampling of water and waste water.

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| LIDI | LEATHER INDUSTRY DEVELOPMENT INSTITUTE<br>TESTING & RESEARCH LABORATORY<br>DIRECTORATE | |
|---|---|---|
| Title | **Test report** | Page : 1 of 1 |

**Test date(s):** 28/09/2022 to 06/10/2022
**Lab. Design .Code No:** WW-17371
**Type of Sample:** Waste Water
**Sample Identification:** _________
**Sampling Condition:** ____________
**Sample Storage and Preservation:** APHA 1060 C
**Environmental test Condition:** Temp. 20.0 ($^0$C) & RH 53.3(%)

**Report No:** WW-17371/22
**Test order No:** ________
Sampling Date & Place: Customer
Sampling location : Secondary outlet
Sample receiving date: 22/09/2022
**Sampled by :** Customer
**Report date:** 06/10/2022

Name of Customer: **Addis Ababa Institute of Technology**
**School of Chemical and Bio engineering**
Address of customer: **Tel : Office: 011-123-24-17**
**Fax No: 011-12394-80**
**E-MAIL: info@cheng.aait.edu.et**
**P.O. Box :**
**ADDIS ABABA, ETHIOPA**

ORIGINAL

**Customer Code:** ____________

| S/No | Type of Test | Test Result | Unit | Test Method | Uncertainty | Standard Required | Remark |
|---|---|---|---|---|---|---|---|
| 1. | Chemical Oxygen Demand (COD) | 932.2 | $mgO_2/l$ | APHA 5220-B | ± 0.02 | 500.0 | |
| 2. | Total Suspended Solids | 582.0 | mg/l | APHA 2540 –D | - | 50.0 | |
| 3. | Total Dissolved Solids | 2105.0 | mg/l | APHA 2540 –C | -- | -- | |

**Remark: 1.** The S.no. 2 & 3 test parameters are not accredited
**2.** The Standard requirement is only for secondary out let & its from Ethiopian Environmental Protection Authority (EPA)
**3.** Samples independently collected delivered to the Laboratory by customer. Sample location described by customer

Tested By: Kidist A. ______ Signature
Ass Researcher II

Checked By: Maereg H. ______ Signature
Researcher I
(Team Leader)

Authorized By: ______ Signature

Aster Mekasha Goshu
Directorate, Research and Testing Laboratory Director

**1. This test report is for technical information of the client only. Not for advertisement, promotion, publicity litigation or legal purpose.**
**2. The test result relates only to the item tested.**
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# 5 Conclusion and Recommendation

## 5.1 Conclusion

Conventional dehairing by using lime sulfide in the current trends of Ethiopian tannery has a serious environmental problem due to the emission of hazardous waste chemicals. The use of protease enzyme as alternative to dehairing of goat skin is studied to totally eliminate lime sulfide and reduce environmental pollution while reducing environmental pollution and hazards. As alternative, Ficin enzyme from Ficus sycomoros was better to substitute such problems due to its characteristics of removing hair. After successful production of Ficin enzyme the enzyme was applied on goat skin to see of dehairing ability and effect. The activity of this ficin enzyme having 18U/ml was better at its temperature $35^0$c and pH 8 and 17hr. Under the investigation of the efficacy of the protease products of the employed recovering and partial purification methods, use of the protease resulted in about 93% dehairing efficiency. Such a higher level of hair removal efficiency is a good indication for the protease products of each method in competing with the conventional chemical dehairing process without taking other technical and economic factors into account. This thesis studies about there is no bad effect of using protease enzyme for the operations of leather processing.

## 5.2 Recommendation

The following suggestions are made for future works, taking into account the extremely promising findings obtained in this thesis;

- The potential of ficus sycomorous should be investigated more than this. In these study ficin enzyme is used only for dehairing process but further investigation can be done on other processing areas such as batting.
- Study is needed to work on the factors affecting the ficin enzyme on dehairing additional to temperature, enzyme concentration and time like applying agitation mechanical action in the drum.
- To improve the dehairing efficiency and reduce time taken for dehairing, in combination with other alternative deharing methods such as lime sulfide should be studied.
- This study was done on goat skin samples but further investigation can be done on other types of hide and skin.
- Accepting that using Ficus sycomorous fruit enzyme, as an alternative to conventional dehairing, it has great reduction on environmental pollution but economic feasibility and practical application for mass production needs further analysis.

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**Annex 1 Ninhydrin Test Procedures**

Procedure - We begin with a 2% solution of ninhydrin which we prepare by dissolving 0.2g of ninhydrin per 10ml of a carrier solvent such as acetone or ethanol.

Next, we prepare a 1% solution of the test compound using distilled water. To this, we add a few drops of our ninhydrin solution.

Next, we place our test tube in a warm water bath for a few minutes.

If the solution develops a deep blue or purplish colour, we have a positive ninhydrin test. Look for the development of blue or violet color.

**But in the case of proline and hydroxyproline, yellow color will develop instead of blue color. Similarly, asparagine will give brown color.

**Annex 2 Protease assay**

Protocol

1. A 50 mM Potassium Phosphate Buffer, pH 7.5. Prepare using 11.4 mg/ml of potassium phospate dibasic, trihydrate in purified water and adjusting pH with 1M HCl. This solution is placed at 37°C prior to use.
2. A 0.65% weight/volume casein solution, prepared by mixing 6.5 mg/ml of casein in the 50 mM potassium phosphate buffer. Gradually increased the solution temperature with gentle stirring to 80-85 °C for about 10 minutes until a homogenous dispersion is achieved. It is very important not to boil the solution. The pH is then adjusted if necessary with NaOH and HCl.
3. A 110 mM Trichloroacetic acid solution, prepared by diluting a 6.1N stock 1:55 with purified water. Trichloroacetic acid is a strong acid and should be handled with care.
4. 0.5 mM Folin & Ciolcaltea's, or Folin's Phenol Reagent, which is the solution that will react with tyrosine to generate a measurable color change that will be directly related to the activity of proteases. Folin's Phenol Reagent is an acid and should be handled with care.
5. A 500 mM Sodium Carbonate solution, prepared using 53 mg/ml of anyhydrous sodium carbonate in purified water. An enzyme diluent solution, which consists of 10 mM Sodium Acetate Buffer with 5mM Calcium Acetate, pH 7.5, at 37°C. This solution is what we use to dissolve solid protease samples or dilute enzyme solutions.

6. 1.1 mM L-tyrosine Standard stock solution. Prepared using 0.2 mg/ml L-tyrosine in purified water and heated gently until the tyrosine dissolves. As with the casein, do not boil this solution. Allow the L-tyrosine standard to cool to room temperature. This solution will be diluted further to make our standard curve.
7. Protease solution. Immediately before use, dissolve protease in enzyme diluent solution prepared in step 6.

Setting up the Protease Assay and Standard Curves

1. To begin this assay, find suitable vials that will hold about 15 ml. For each enzyme that will be tested, 4 vials are needed. One vial will be used as a blank, and three others will be used to assay activity of three dilutions of the protease. Three dilutions are useful when checking final calculations against each other.
    a. To each set of four vials, add 5ml of our 0.65% casein solution. Let them equilibrate in a water bath at 37°C for about 5 minutes.
    b. Add varying volumes of enzyme solution that will be tested to three of the test sample vials, but not the blank. Mix by swirling and incubate for 37°C for exactly ten minutes. The protease activity and consequential liberation of tyrosine during this incubation time is what will be measured and compared between test samples.
2. After this 10 minute incubation, add the 5ml of the TCA reagent to each tube to stop the reaction. Then, add an appropriate volume of enzyme solution to each tube, even the blank, so that the final volume of enzyme solution in each tube is 1 ml. This is done to account for the absorbance value of the enzyme itself and to ensure that the final volume in each tube is equal. Incubate the solutions at 37°C for 30 minutes. During this 30 minute incubation, you may want to set up your tyrosine standard dilutions. Use 6 dram vials (dram vials can be substituted with polypropylene tubes) that can easily hold 8 mls. To the six vials, add the 1.1 mM tyrosine standard stock solutions with the following volumes in mls: 0.10, 0.20, 0.40, 0.50. Don't add any tyrosine standard to the blank. Lower standards may be needed for impure test samples that will yield little color change. Once the tyrosine standard solution has been added, add an appropriate volume of purified water to each of the standards to bring the volume to 2 mls.

3. After the 30 minute incubation, filter each of the test solutions and the blank using a 0.45 um polyether sulfone syringe filter. Filtration is required to remove any insolubles from the samples.
    a. Add the filtration 2 mls of the test samples and blank filtrate to 4 dram vials that can hold at least 8 mls. The same type of vial in which the standards were prepared can be used.
    b. To all of the vials containing the standards and standard blank, add 5mls of sodium carbonate. For best results, add 1 ml of Folin's reagent immediately afterwards.
    c. Add sodium carbonate to regulate any pH drop created by the addition of the Folin's reagent.
    d. Add sodium carbonate to the test samples and test blank. These solutions become cloudy after the addition of sodium carbonate. Add the Folin's reagent, which will react primarily with free tyrosine.
    e. Mix the dram vials by swirling and incubate at 37°C for 30 minutes.

Table 0-1 Tyrosine standard

| | Sta.1 | Sta.2 | Sta.3 | Sta.4 | Blank |
|---|---|---|---|---|---|
| Standard soln. | 0.10ml | 0.20 ml | 0.40 ml | 0.50 ml | - |
| Distilled water | 1.9ml | 1.80 ml | 1.6 ml | 1.5 ml | 2ml |
| sodium carbonate | 5 ml | 5 ml | 5 ml | 5 ml | 5ml |
| Folin's reagent | 1 ml | 1 ml | 1 ml | 1 ml | 1ml |

Table 0-2 Test samples preparation

| | test1 | test2 | test3 | Blank |
|---|---|---|---|---|
| Casein | 5ml | 5ml | 5ml | 5ml |
| Enzyme | 0.5ml | 0.7ml | 1.0ml | - |
| TCA | 5ml | 5ml | 5ml | 5ml |
| Enzyme solution | 0.5ml | 0.3ml | - | 1ml |
| 2ml of each of test filtrate | | | | |
| sodium carbonate | 5ml | 5ml | 5ml | 5ml |
| Folin's reagent | 1ml | 1ml | 1ml | 1ml |

After this incubation notice that the standards have a gradation of color correlating with the amount of tyrosine added; the highest concentrations of tyrosine appearing darkest. Also notice appreciable color change in our test samples. 2ml of these solutions are filtered using a 0.45 um syringe filter into suitable cuvettes. After the assay is performed, you can proceed to the spectrophotometer to record the absorbance values. Absorbance will be measured at 660nm in spectrophotometer. Once all of the data has been collected, the standard curve can be created. In order to generate the curve, difference in absorbance between the standard and standard blank must be calculated. This is the absorbance value attributable to the amount of tyrosine in the standard solutions. After this simple calculation, plot the change in absorbance of our standards on the Y axis, versus the amount in micromoles for each of our 4 standards on the X axis finally we have got Tyrosine standard curve.

Figure 37conducting protease assay

Pictures before and after dehairing

Figure 38 Pictures before and after dehairing

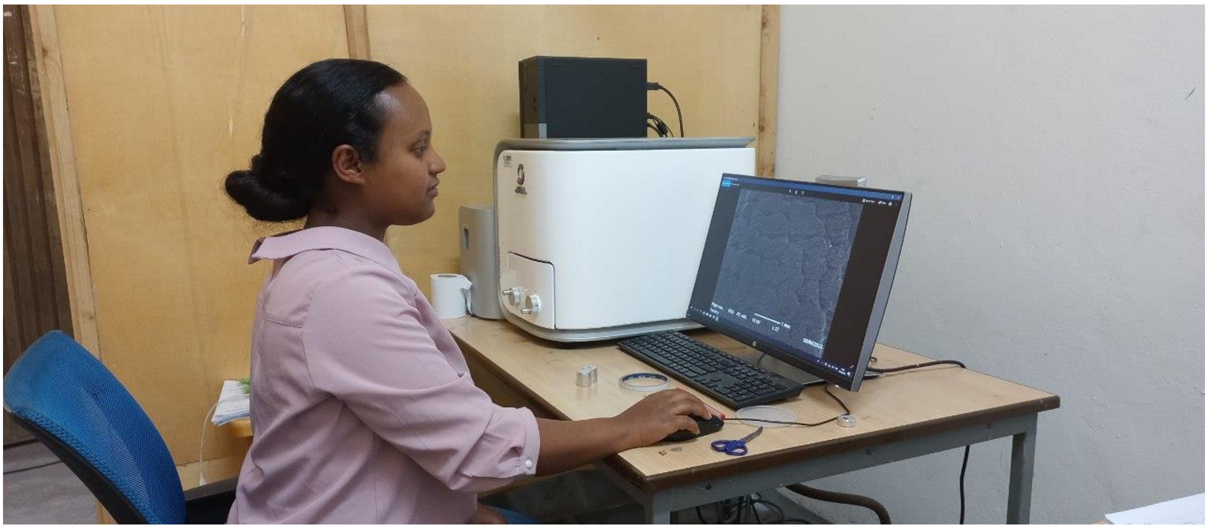

Figure 39 taking samples SEM pictures