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OPTIMIZATION OF SERICIN REMOVAL FROM TUSSER SILK BY AUTOCLAVING

A thesis submitted in partial fulfillment

of the requirements for the degree of

Bachelor of Technology

In

Biotechnology

By

Sonali Nanda

108BT020

Under the guidance of

Dr B. P. Nayak

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National Institute of Technology, Rourkela

2012

CERTIFICATE

This is to certify that the project entitled "**Optimization of sericin removal from Tusser silk by autoclaving**" submitted by Sonali Nanda in partial fulfillment of the requirement for the award of Bachelor of Technology in Bio Technology at National Institute of Technology Rourkela, is an authentic work carried out by her under my guidance and supervision.

To the best of my knowledge, the matter embodied in the thesis has not been submitted to any other institute/university for the award of any degree or diploma.

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CONTENTS

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A project of this nature could not have been attempted without inspiration from others work. I would like to thank all the authors whose names are mentioned in the reference section.

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(Sonali Nanda)

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ABSTRACT

Silk is an important biomaterial for tissue engineering purposes due to its improved mechanical and biological properties. The natural fiber has two major components – the structural centre protein chain called fibroin and the outer glue like covering fiber called sericin. It has been well documented that sericin has the immunogenic properties in animal and human physiological system and so the silk is used only after the sericin is removed thus improving the mechanical and biological properties of silk. Removing sericin from silk is called degumming i.e. “removing the gum”. It should be done carefully in a way that the fibroin is not damaged. Sericin is removed by using a base such as anhydrous sodium carbonate as well as autoclaving by using water under pressure. By varying one parameter and keeping the other two fixed we find out the optimum temperature, time, concentration as well as pressure to degum silk such that the sericin is removed optimally and the structural integrity of silk is not lost. By SEM analysis the morphological characteristics of degummed silk was studied.70^0C, 0.02M Na_2CO_3 and 35 minutes for degumming using Na_2CO_3 and 15psi, 121^0C, 15 minutes were found to be the optimum parameters for degumming.

Key words: Silk, Sericin, Fibroin, Degumming, Autoclaving, SEM

CHAPTER 1

INTRODUCTION

INTRODUCTION

Silk degumming is basically a method of cleavage of peptide bonds of sericin by hydrolytic or enzymatic action and subsequent removal of sericin from silk fiber. Neutral, acidic or alkaline environment is used for hydrolysis of sericin. Boiling – off in alkaline soap is the most popular method [1] .Degumming is done to improve the colour and texture of silk.

SILK: 18 kinds of amino acids such as alanine, sericin, glycine are present in silk [2]. Silk contains (22-25%) sericin and (62.5-67%) fibroin protein [3]. Fibroin is basically the structural centre of silk and sericin is a sticky material surrounding it. Sericin's molecular formula is $C_{30}H_{40}N_{10}O_{16.}$ Sericin is amorphous and acts as a gum binder to maintain the structural integrity of cocoon [3,6]. Sericin hides the luster and whiteness of silk [4]. Sericin is more water soluble than fibroin [3]. Silk has wide application in the field of tissue engineering and scaffold designing [2]. Sericin is a protein created by *Antheraea mylitta* (Tusser silkworm) in the production of silk. [5]. Special property of Sericin is its opalescence resulting from light scattering effect. Sericin has high affinity for keratin due to which it binds effectively with skin and hair to form a multi functional protective film. Sericin gives a stiff feeling to silk thereby hiding its luster and whiteness [3]. It prevents the penetration of dyes and other solution into silk [7]. The structure of fibroin is basically in the form of β-pleated sheets. Its composition repetition is Gly-Ser-Gly-Ala-Gly-Ala. It is mainly responsible for the whiteness and luster of silk. Silk is used because it has good mechanical properties also it is quite biocompatible in nature. Silk offers a broad range of mechanical and functional properties for bio medical applications [13]. The silk fibroin has very good biocompatibility for cells *in-vitro* too, as well as it maintains cell function [9]. As compared to synthetic polymer silk fibroin is produced at lower temperature [10].

CHAPTER 2

LITERATURE

REVIEW

REVIEW OF LITERATURE:

Degumming is done to improve the characteristics and properties of silk. Silk is an excellent bio material, [l] we need it for many purposes starting from wound healing to scaffold design. Sericin which hides the luster and whiteness of silk is removed in the process of degumming. Cell degumming is basically a method of cleavage of peptide bonds of sericin by hydrolytic or enzyme action and subsequent removal of sericin from silk fibre as proposed by M.L.Guljarani [1]. Acidic, alkaline and neutral environment are mainly applied for the degumming process to hydrolyze sericin. Boiling -off in alkaline soap is the most popular method. Degumming results in decrease in stress at the proportional limit (yield strength) which is exhibited by change in shape of force verses displacement curves. Immersion in water at 25^0c (room temperature) or heating in presence of oxygen for 30 minutes at 100^0c are both quantitatively same as 30 minutes degumming treatment in boiling water if effect of tensile property of silk is taken into account [11]. Sericin is removed as it has some impurities like waxes, fats, mineral salts and pigments. Sericin and Fibroin (components of silk filament) have same amino acid residues. Care should be taken so as fibroin is not damaged [12].Removing sericin thus subsequently removing impurities likes waxes and fats which makes dyeing and printing process easier. Silk fiber becomes highly absorbent for dyes and chemicals. The luster of fibroin is revealed which improves the appearance of silk. Following degumming methods were purposed by Attard et.al. Degumming with water under pressure at 115^0c, Degumming with soap at 98^0c, Degumming with synthetic detergent at 98^0c, Degumming with acids, Degumming with enzymes. SEM is usually used to access the morphological characteristics of the silk fiber.

The degree of fiber damage is accessed by determining the viscosity of degumming solution, tensile properties or amino acid sequence of fiber. The gravinimetric method is the most common and simple method to study the effectiveness of degumming .It measures the weight loss of the sample but it dosen't reveal any residual sericin thereby we cannot know if there is damage to fibroin or not. By measuring tensile strength damage to fiber is assessed [12] .In staining method we evaluate if any sericin residue is present on the sample or not but this method is basically qualitative not quantitative. The viscosity of degumming solution and amino acid sequence of the fiber is difficult to monitor [12].

Fibroin is a single protein insoluble in hot water. Silk contains (22-25%) sericin and (62.5-67%) fibroin. Sericin is amorphous and acts as a gum binder to maintain structural integrity of cocoon [3]. Fungal protease degumming method was proposed by Gulrajani. This method is energy saving, time consuming and requires low temperature. It also dosen't employ any soap, alkali or acids. The silk produced by this method has a good luster.

Mulberry silk fabric degummed by five methods (acid, alkali, soap, enzyme) was found to have low stress mechanical properties because of non uniform treatment was proposed by chopra et al [13].

Muga, Tusser, Eri craem silk had less amount of sericin but it took more time than mulberry silk was proposed by Vaishali etal. So, sericin is strongly embedded in wild silk as compared to mulberry [14].

Upon reacting soaps (alkali) with sericin a progressive degradation of sericin takes place in solution was proposed by Roy.H.Waters etal [15].

The grams of sericin combining with one gram equivalent of soap or alkali when equivalence is attained is expressed by the equation.

$B=-1000LOG_{10}R-2180$

B is the binding weight of sericin.

R is the initial ratio of gram equivalence of total soap or alkali per grams of total sericin initially present.

The physio mechanical properties like (tenacity, modulus of elasticity, intrinsic viscosity) are taken into account for characterization of degumming sample [16].

CHAPTER 3

MATERIALS AND METHODS

3.1 MATERIALS

Tusser silk cocoons (*Antheraea mylitta)* were collected from RMB, Chaibasa of the West Singhbhum (Jharkhand) while chemicals such as sodium carbonate was purchased from HiMedia. Autoclave, SEM, weighing balance and water-bath were used for the purpose of our study.

3.2 METHODS

3.2.1 Preparation of sample

The pupa from Tusser silk cocoons were removed then dried Tusser cocoons were cut into small pieces for our study.

3.2.2 Degumming by using anhydrous Na_2CO_3

5 grams of cocoons were weighed using weighing balance. They are washed properly then dissolved in a certain concentration of Na_2CO_3 then it is heated at a certain temperature for a certain period of time in water bath. After this the cocoons are taken washed repeatedly with distilled water till the turbidity of the water is removed. The yellowish liquid which is Sericin is separated from cocoons. The cocoons are found to be clumped to each other so they are washed properly so that maximum amount of sericin is removed. Then the cocoons are stretched in aluminium foil it is kept in a hot air oven and dried overnight for 24 hours. Then dried fibre is taken out after 24 hours for analysis. Drying is done to remove all the water.

3.2.2.1 Varying time keeping temperature and concentration fixed.

Here the temperature is fixed at 70^0c and concentration is fixed at 0.02 molar and time is varied from 20-60 minutes.

3.2.2.2 Varying temperature keeping time and concentration fixed.

Here temperature is varied between 50-70^{0}C keeping time fixed at 35 minutes and concentration at 0.02M.

3.2.2.3 Varying concentration keeping temperature and time fixed.

Here the concentration of sodium carbonate is varied between 0.01-0.05M keeping time at 35 minutes and temperature at 70^{0}C.

3.2.3 Degumming by autoclaving method.

5 grams of cocoons are weighed in a weighing balance then it is dissolved in 100ml of water taken in a beaker. Cocoons dissolved in water are kept in autoclave at a certain set temperature and pressure for a certain time. Then the cocoons are taken out from the autoclave and the sticky liquid i.e. sericin is drained out and the cocoons are washed properly with distilled water till the turbidity of the water goes and maximum amount of sericin is separated out. Then the cocoons are kept in an aluminium foil stretched from all sides and kept overnight in a hot air oven at 37^{0}C for drying. Then the dried sample is taken out for analysis.

3.2.3.1 Degumming by varying pressure keeping temperature and time constant

Here the pressure is varied between 11-15 psi and time and temperature are kept constant at 15 minutes and 121^{0}C respectively.

3.2.3.2 Degumming by varying temperature keeping pressure and time constant

Here the temperature is varied between 111-121^{0}C keeping pressure and time fixed at 15 psi and 15 minutes respectively.

3.2.3.3 Degumming by varying time keeping temperature and pressure constant

Here time is varied between 5-15 minutes keeping temperature and pressure constant at 121^0C and 15 psi respectively.

3.2.4 SEM analysis

The samples degummed at constant concentration i.e. 0.02M and constant time of 35 minutes and varied temperature between 50-70^0c and autoclaved sample at 15psi, 121^0C and 15 minutes were analyzed using JEOL JSM-6480LV SEM at 15KV, room temperature and resolutions at 100X. These 6 samples were air dried over night and affixed with the help of carbon tape to SEM holders and 20 nm layer of platinum is used for vacuum coating.

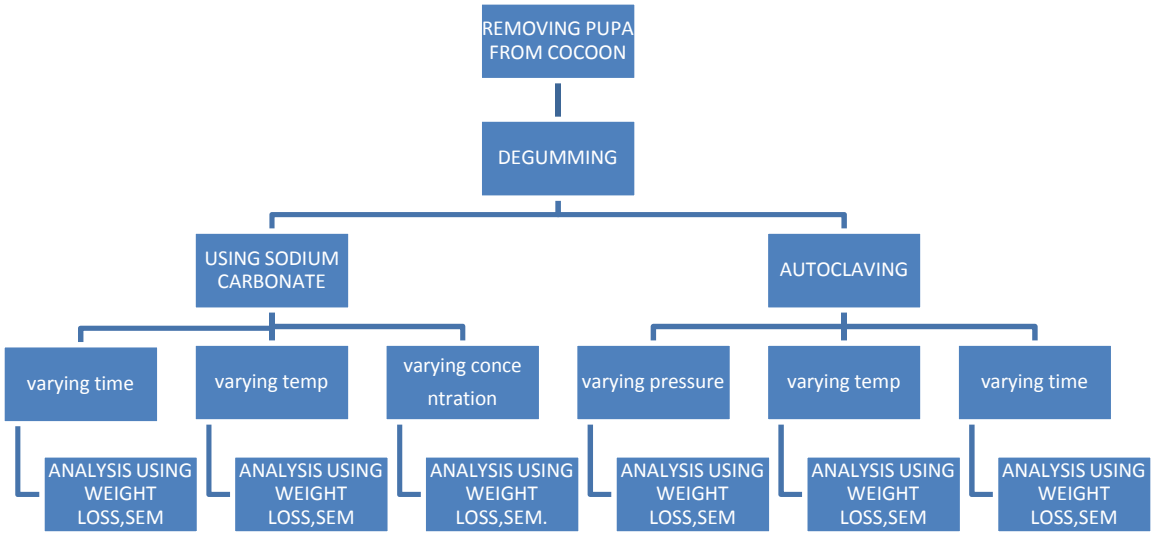


FIG 1: FLOW CHART SHOWING DEGUMMING METHODOLOGY.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 DEGUMMING WEIGHT LOSS ANALYSIS

The samples are dried overnight for 24 hours then its weight is measured with weighing balance. There was a decrease in weight of the sample before degumming and after degumming.

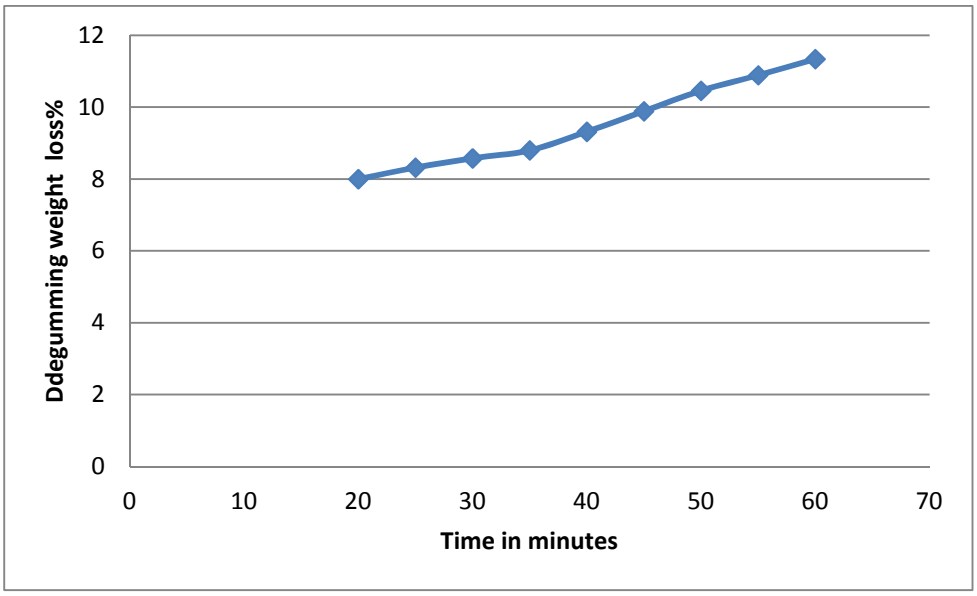
4.1.1 Degumming weight loss in case of varying time in Na_2CO_3 method

The weight loss increases with increase in time showing that more amount of sericin is removed as the time progesses this may be due to the hydrolysis of interlinking bonds between sericin and fibroin as time progresses moreover more amount of sericin is hydrolyzed by salt as the time increases from 20-60 minutes. Weight loss is maximum at 60 minutes but the fibers are also burned if the temperature is proceeded beyond 35 minutes. So, 35 minutes is the optimum temperature.

Table 1: Degumming weight loss in case of varying time

Time (in minutes)	Temperature (in ^{0}C)	Concentration (in moles/liter)	Weight before degumming (in grams)	Weight after degumming (in grams)	**%** Degumming weight loss
20	70	0.02	5	4.4	8
25	70	0.02	5	4.584	8.32
30	70	0.02	5	4.571	8.58
35	70	0.02	5	4.5595	8.81
40	70	0.02	5	4.534	9.32
45	70	0.02	5	4.5055	9.89
50	70	0.02	5	4.477	10.46
55	70	0.02	5	4.4555	10.89
60	70	0.02	5	4.433	11.34

Graph 1: Degumming weight loss analysis at variable time



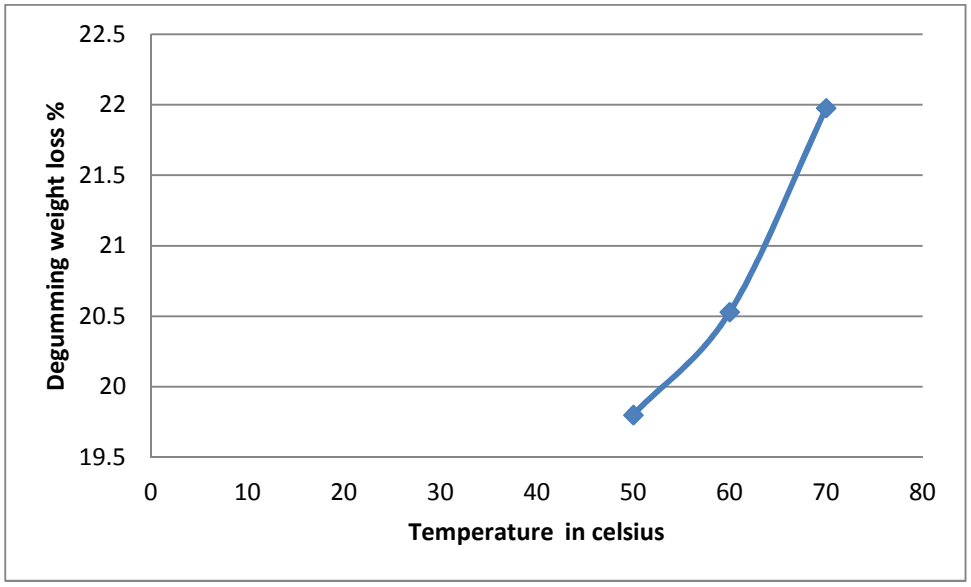
4.1.2 Degumming weight loss in case of varying temperature in Na_2CO_3 method

Weight loss increases as temperature increases. Weight loss is maximum at 70^0c without burning of fiber. This may be due to increase in activation energy due to breaking of interlinking bonds. It also increases the solubility of partially hydrolyzed sericin fractions.

Table2: Degumming weight loss in case of varying temperature

Temperature (in ^{0}C)	Concentration (in moles/liter)	Time (in minutes)	Weight before degumming (in grams)	Weight after degumming (in grams)	% Degumming weight loss
50	0.02	35	5	4.01	19.8
60	0.02	35	5	3.9735	20.53
70	0.02	35	5	3.9012	21.976

Graph 2: Degumming weight loss in case of variable temperature.



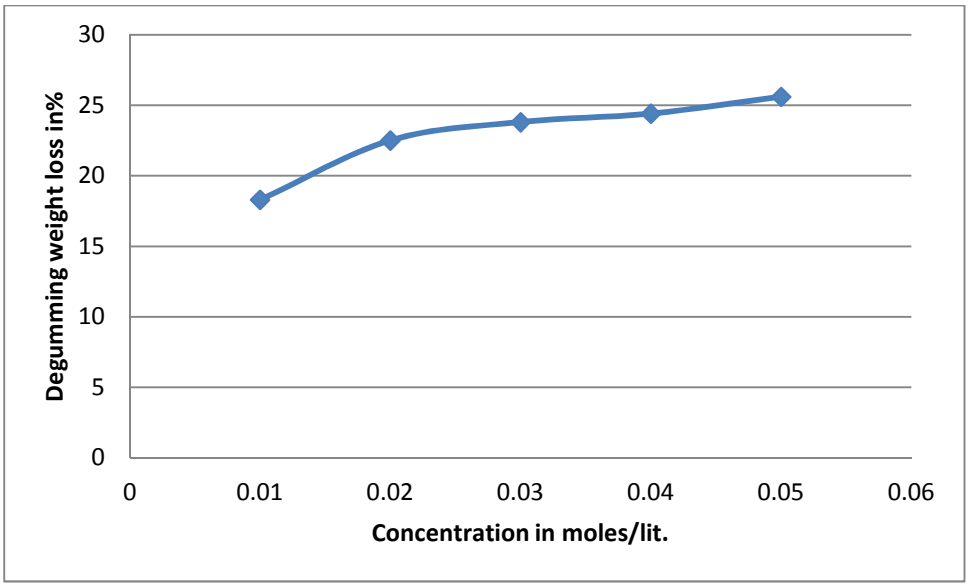
4.1.3 Degumming Weight loss in case of varying concentration in Na_2CO_3

Weight loss increases as concentration increases and amount of silk fiber decreases. Maximum weight loss is achieved at 0.04M. Concentration is taken minimum to maintain the structural integrity of silk and protect it from harsh chemicals which is turn prevents hydrolytic degradation of silk. Also the fiber burns after the concentration increases from 0.02M.

Table 3: Degumming weight loss in case of varying concentration

Concentration (in moles/liter)	Temperature (in ^{0}C)	Time (in minutes)	Weight before degumming (in grams)	Weight after degumming (in grams)	% Degumming weight loss
0.01	70	35	5	4.085	18.3
0.02	70	35	5	3.875	22.5
0.03	70	35	5	3.81	23.8
0.04	70	35	5	3.78	24.4
0.05	70	35	5	3.72	25.6

Graph 3:Degumming weight loss in case of varying concentration



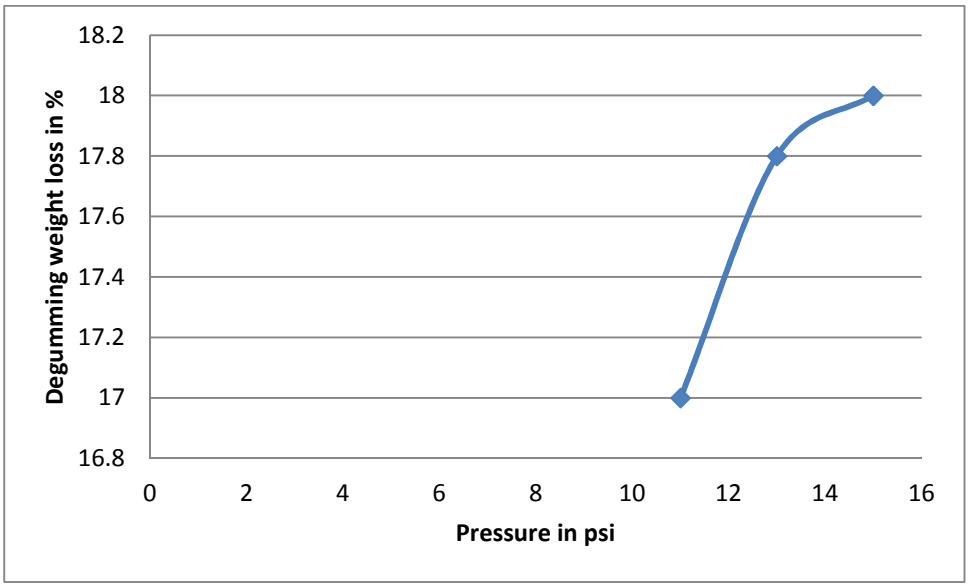
4.1.4 Degumming weight loss in case of varying pressure in autoclaving method:

Weight loss increases with increase in pressure. With increase in pressure interlinking bond between sericin and fibroin decreases. At 15 Psi optimum amount of sericin is removed and from SEM analysis we see that the sample at 15 psi, 121^{0}C, 15 minutes.

Table 4: Degumming weight loss in case of variable pressure

Pressure (in psi)	Time (in minutes)	Temperature (in ^{0}C)	Weight before degumming (in grams).	Weight after degumming (in grams)	% Degummimg weight loss
11	15	121	5	4.15	17
13	15	121	5	4.11	17.8
15	15	121	5	4.1	18

Graph 4: Degumming weight loss in case of variable pressure



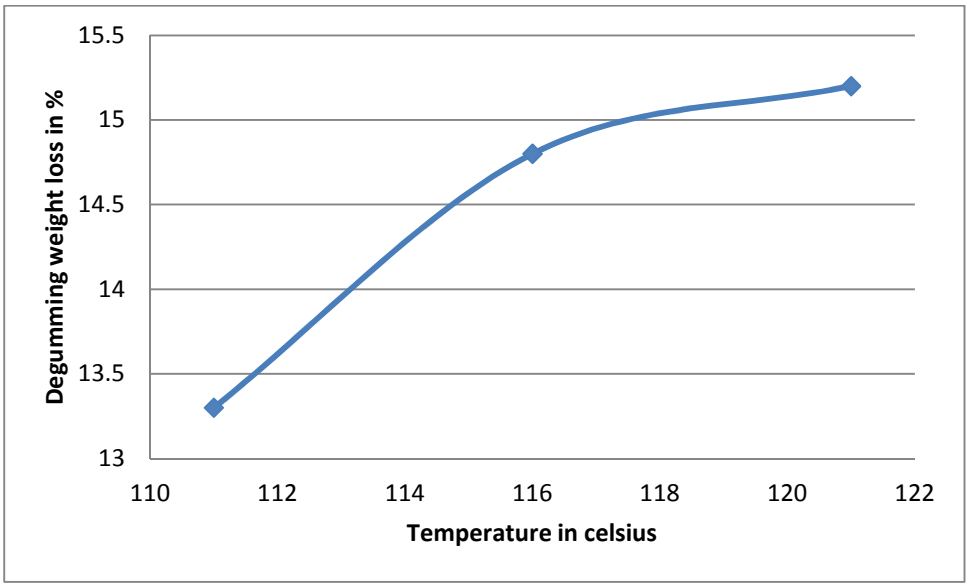
4.1.5 Degumming weight loss in case of varying temperature in autoclaving method

Weight loss increases as temperature increases due to hydrolytic degradation of sericin in solution. Maximum amount of sericin is removed at 121^{0}C without burning of fiber.Also we get smooth images in SEM analysis.

Table 5: Degumming weight loss in case of variable temperature

Time (in minutes)	Pressure (in psi)	Temp (in ^{0}C)	Weight before degumming (in grams)	Weight after degumming (in grams)	% Degumming weight loss
15	15	111	5	4.335	13.3
15	15	116	5	4.26	14.8
15	15	121	5	4.24	15.2

Graph 5: Degumming weight loss in case of variable temperature



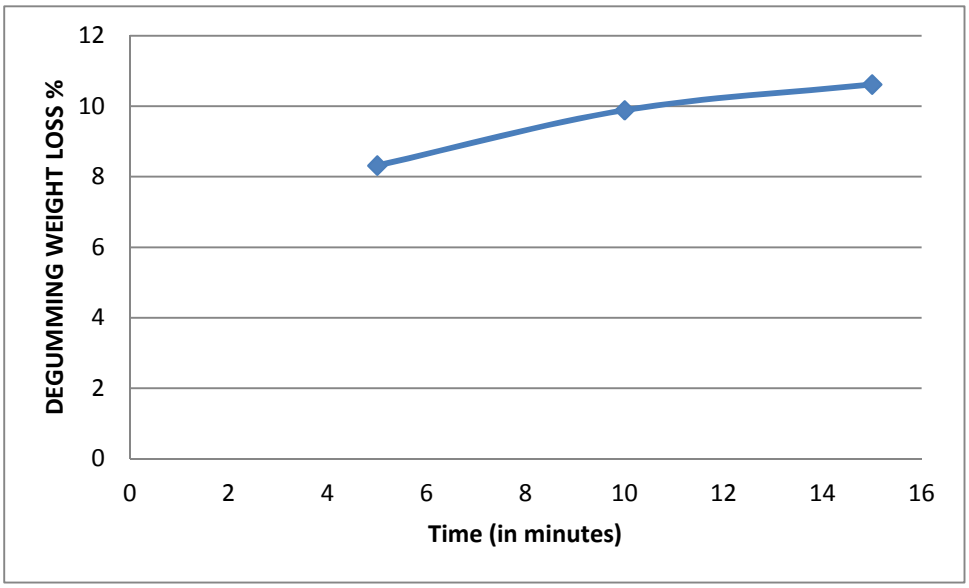
4.1.6 Degumming Weight loss by varying time in autoclaving

The weight loss % increases with time because as the time is increased sericin is exposed to hydrolysis of salt more and more. At 15 minutes maximum amount of sericin is removed without burning of fiber.

Table 6: Degumming weight loss in case of variable time

Time (in minutes)	Pressure (in psi)	Temperature (in ^{0}C)	Weight before degumming (in grams)	Wt after degumming (in grams)	% Degumming weight loss
5	15	121	5	4.584	8.32
10	15	121	5	4.5055	9.89
15	15	121	5	4.469	10.62

Graph 6: Degumming weight loss in case of variable time



4.2 SEM analysis

SEM images of the samples are obtained and it is compared to optimize the parameters.

4.2.1 SEM images

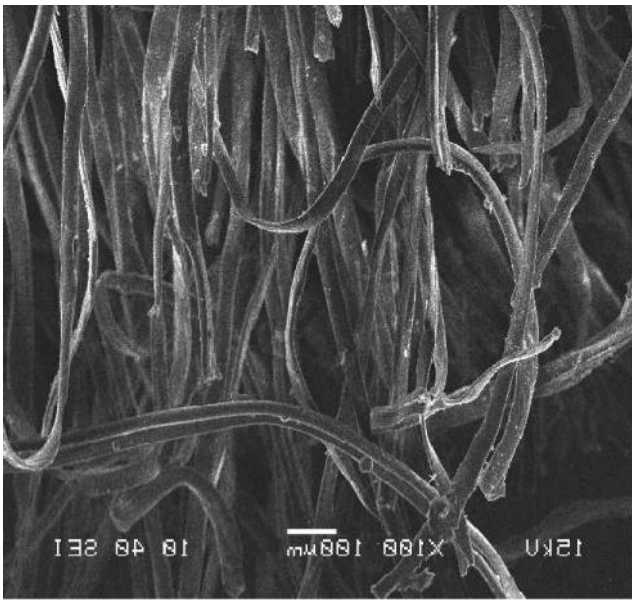


FIG 2: SEM images of degummed silk using autoclaving method at 121^0C, 15 psi, 15 minutes.

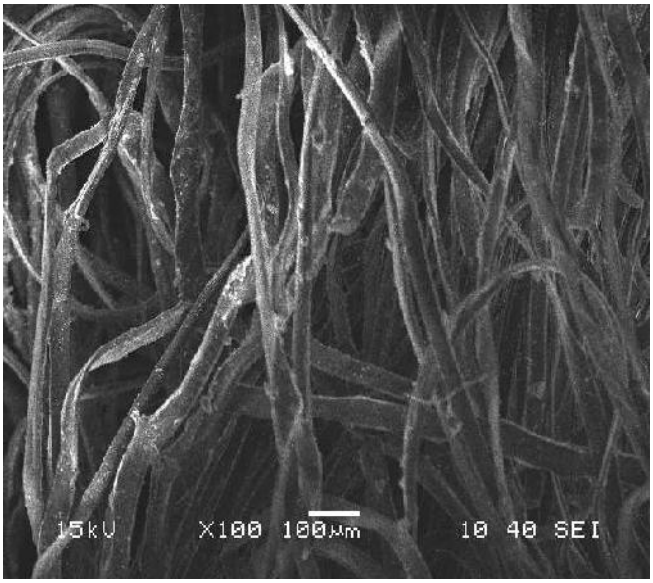


FIG 3: SEM images of degummed silk using autoclaving method at 121^0C, 15 psi, 10 minutes.

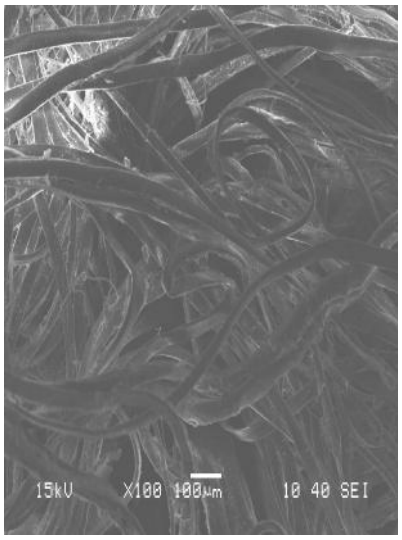


FIG 4: SEM images of sample at 70^0C,0.02M Na_2CO_3, 35 minutes

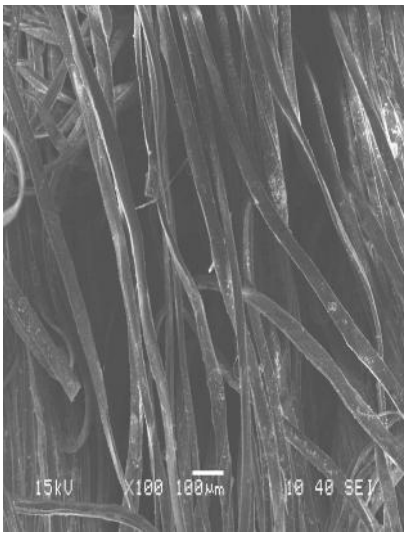


FIG 5:SEM images of sample at 60^0C,0.02M Na_2CO_3 35 minutes

FIG 6: SEM images of sample at 50^0C,0.02M Na_2CO_3,35 minutes

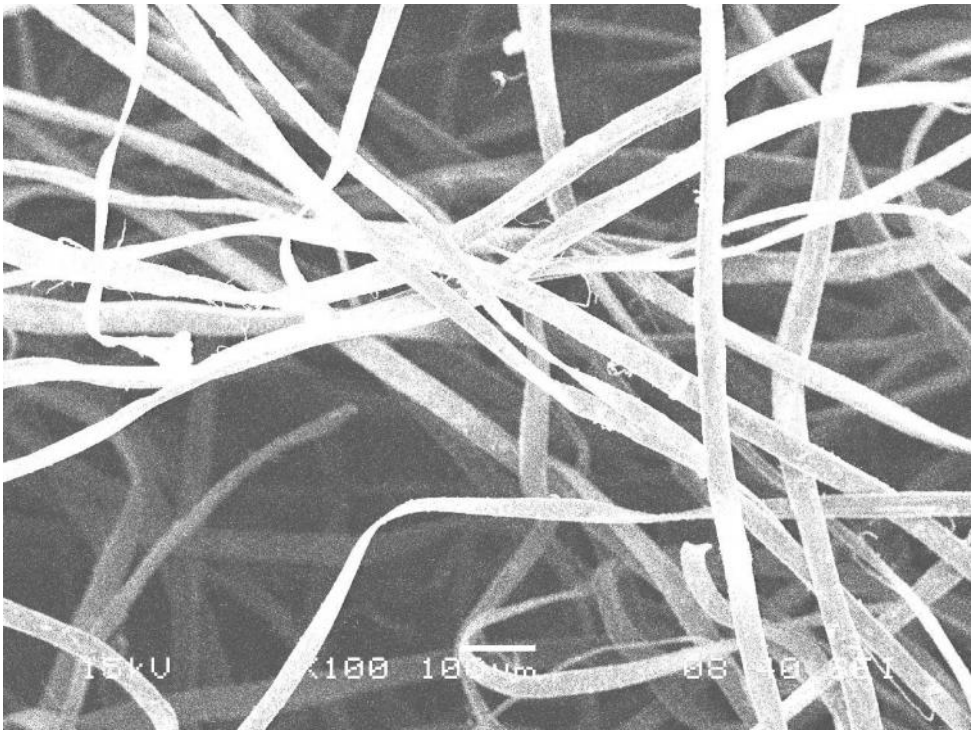


Fig 7: SEM images of undegummed silk

4.2.2 Interpretations from SEM images

We see that as the temperature is increased the whitish portion in the image decreases clearly indicating that Sericin is removed also fiber becomes more smooth with increase in temperature. In case of autoclaving the whitish portion decreases as the time period increases clearly indicating that the amount of sericin in the fiber decreases with increase in time. Also degumming using Na_2CO_3 is a better method than autoclaving as the amount of white portion is less in using Na_2CO_3 than autoclaving .Also the amount of white portion is less in degummed silk as compared to undegummed silk as clearly seen from the figure .This happens due to the reason that individual filaments which are glued together are separated after sericin removal. WE also conclude that the dull and lusterless appearance of silk gives way to shiny and luster fiber after degumming.

Sericin is basically the whitish stuff (observed in SEM analysis) which is removed after degumming. Sericin is mainly present in the interlocking in silk fibers. Also as we increase the degumming temperature clear and distinct longitudinal strains of fiber are obtained.

CHAPTER 5

CONCLUSION

CONCLUSION

We conclude that with increase in degumming time, temperature and concentration, more amount of sericin is removed but care should be taken to ensure that the structural integrity of silk is not lost. From SEM analysis we see that smooth and soft fibers are obtained at 70^0C, 0.02M, 35 minutes sample. Also for autoclaving somewhat optimum fiber is obtained at 15 psi, 121^0C, 15 minutes. Removal of sericin improves the luster of silk thereby improving the mechanical properties. A through understanding of Degumming process will play a vital role is using silk fiber for 3-D scaffold designing and Tissue engineering applications.

CHAPTER 6
FUTURE WORK

FUTURE WORK

We need to further optimize the autoclaving method by degumming. We have to vary the temperature, pressure and time to a greater extent such that we obtain more optimum parameters. We also need to analyze the autoclaved degummed silk sample using universal testing machine to analyze its mechanical property as autoclaved degummed silk is free from any chemicals so there might not be any alternation in its chemical structure. Also by FTIR analysis of autoclaved degummed silk and comparing it with undegummed silk we can know the changes in functional groups after degumming.

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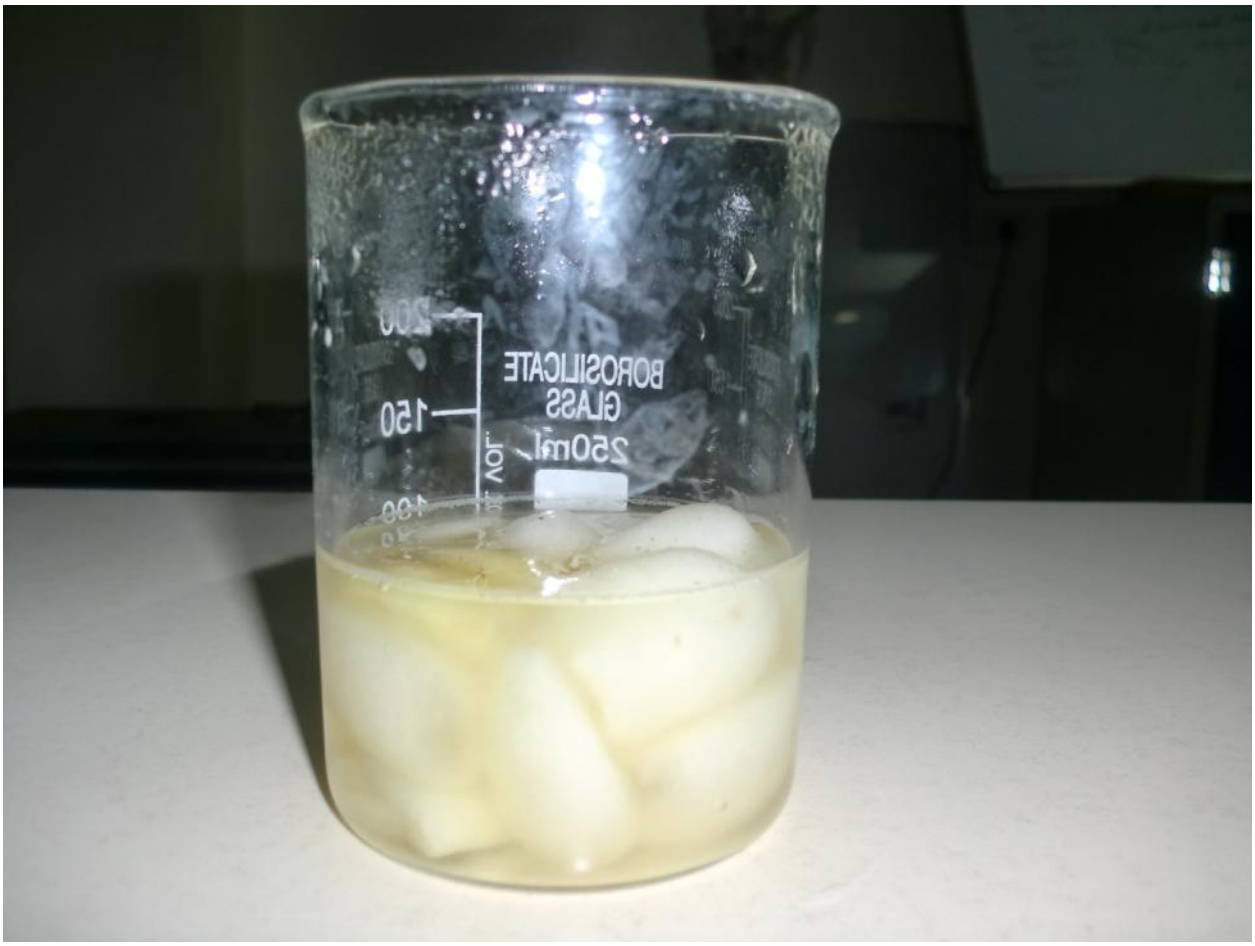
APPENDIX-1: OBSERVATIONS AFTER DEGUMMING



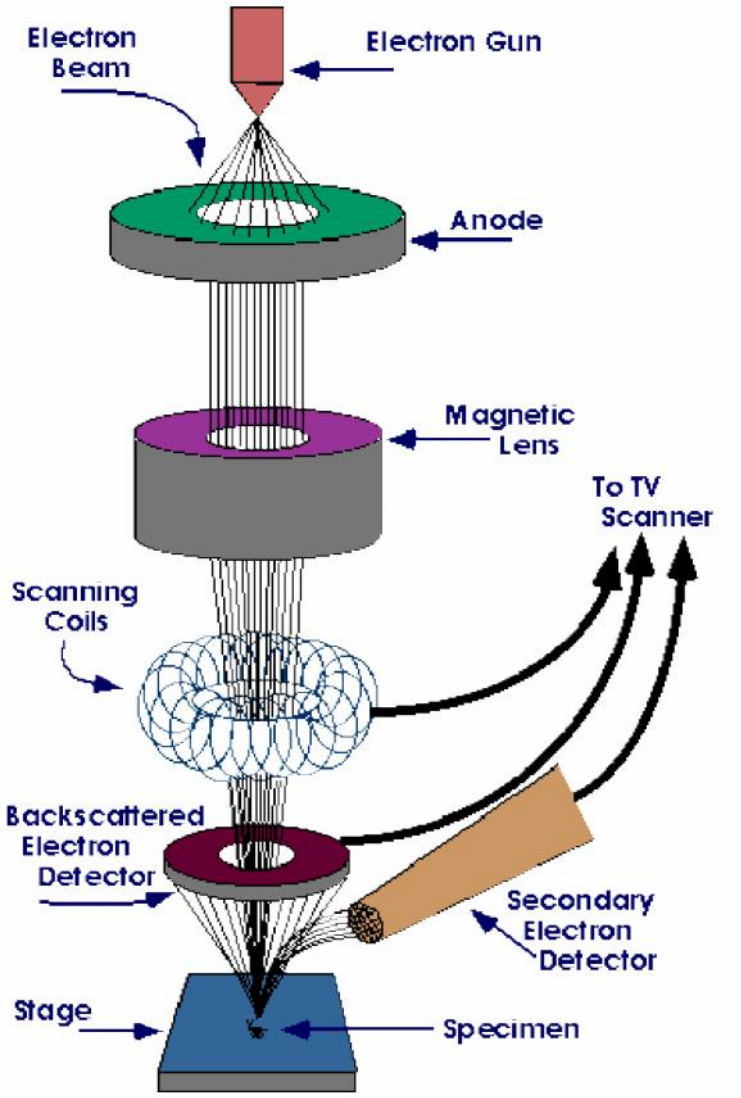
Cocoons after degumming are clumped together. Yellowish liquid is Sericin which is sticky to feel. A thick viscous yellowish liquid which is sticky to feel formed at the bottom after taking it out from hot water bath which is sericin only.

The gel like structure is sericin and thread like structure is fibroin. Cocoons are clumped because they are cross linked.

APPENDIX-2: SEM analysis

Diagram of a scanning electron microscope (SEM)

Principle: A beam of electrons are focused on the sample. The interaction of electrons with the atoms produces signal which gives information about the sample's composition and topography. Various types of signals are produced by SEM such as secondary electrons,back scattered electrons, charecteristics X-rays, light(cathodoluminiscence), specimen current, transmitted electrons.



Schematic view of SEM.

APPENDIX 3: IMAGES OF FIBER AFTER DRYING

Fibers obtained after air drying at 37^0C for 24 hours.