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RESEARCH ARTICLE

Degumming of Silk Using Microbial Protease

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Abstract

Silk, the elegant and luxury fabric has enchanting man for various decades. Degumming of silk i.e. breaking the peptide linkage of amino acid in sericin structure into a small water soluble group is the most crucial step in preparation of silk fibers. Conventional methods used for degumming like treating with alkali and acid damages the silk fibres, and also these methods are not eco-friendly. Enzymes are gradually being used to replace the harsh chemicals in degumming process. Enzymatic degumming involves proteolytic degradation of sericin using specific proteases which does not damage silk. As microbes are the good source of these proteolytic enzymes, the present study was planned to use the microbial protease for degumming of silk. To economize the production of Proteolytic enzyme, the fermentation conditions were optimized. The efficiency of the enzyme was studied in terms of weight loss, whiteness index, dyed and assessed for color value, texture, feel and luster. Eco-friendly degumming of silk yarn was best done at a temperature of 50°C and pH 9 for 2 hours.

Keywords: *Bacillus subtilis*, Degumming, *Penicillium citrinum*, Proteolytic enzyme, Silk

Abbreviations: NA – Nutrient Agar, PDB-Potato Dextrose Broth, PDA – Potato Dextrose Agar, *P. citrinum- Penicillium citrinum, B. subtilis-Bacillus subtilis*

Introduction

Silk, the Queen of Textiles is a splendid gift of nature to the mankind known for its elegance, refinement, beauty and luxury. The story of silk is fascinating, romantic and adventurous too. In India no religious ritual is completed without the use of silk cloth. Textile is a vast field ever growing with the improvement in the field of science and technology. Though vast varieties of fabrics are available in the market, silk continues to be the queen of fabrics (Krishnaveni and Rajkumar, 2007). Silk is a product of long and tedious process starting from production of silk filament by silk worms, spinning of the silk filament from the cultivated or wild cocoons, weaving of the silk fabric and giving the final treatments to get the desired kind of product. Natural silk is a continuous protein-filament spun by the silk worm (Ibrahim *et al.,* 2007). Degumming is the process of cleavage of peptide bonds of sericin either by hydrolytic or enzymatic methods and its subsequent removal from silk fibroin (Trotman, 1984). The fibroin filaments of cocoon silk are naturally gummed with the protein sericin and also small amounts of non proteinaceous impurities like dust, minerals, pigments and waxy matter. Sericin acts as an adhesive for the twin fibroin filaments and conceals the unique luster of fibroin. Thus, there is a need to remove sericin (degum) that covers the fibre surface in order to obtain a luster, soft handle and the other desired properties of silk for further processing (Ministry of Textiles, 1990). The removal of sericin from the raw silk is a preliminary and important step in silk processing to obtain an ideal fiber for textile industry, and is known as "Degumming." In the traditional methods of degumming the raw silk, fibers are treated with alkali and soap at 95°C–100°C or are boiled at elevated temperature and/or pressure for 1–2 h. Disadvantages associated with these methods are uneven degumming, strength loss of fibers and high resource consumption with respect to water and energy as well as high output of effluents with polluting substances (Gulrajani *et al.,* 2000a; Freddi *et al.,* 2003).

The conventional method for the degumming of silk under alkaline conditions at a pH of 10 to 11 near boil has a long history. The principle behind silk degumming process is increasing the silk gum solubility by breaking the peptide linkage of sericin structure into small molecule such as amino acid and its oligomer with hydrolysis reaction (Sonthisombat and Speakman, 2004). Silk degumming can be performed by numerous methods such as using alkaline and synthetic detergent. However, alkaline conditions are harmful to silk fiber because silk has poor resistance to alkaline conditions. In the present scenario the proteolytic

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enzyme can be used to solve this problem but has some disadvantages like specific conditions to function and high costs (Joonlaiad, 1990). Proteases cover the 60% of total enzyme market and amongst the most valuable commercial enzyme. Alkaline proteases hold a great potential for application in the detergent and leather industries and there is an ever increasing trend to develop environment friendly technologies. Plants, animals and microbes are the main sources for protease production (Fufeungsombut *et al.,* 2009).

Enzymatic degumming is gaining lot of attention in recent years, as it is a milder process with negligible input of hazardous chemicals and recovery of valuable by products such as sericin is also possible. However, the use of enzymes in the silk industry is relatively unexplored and it has generated a lot of interest only in the last twenty years (Gulrajani 1992; Chopra and Gulrajani 1994; Gulrajani *et al.,* 2000b; Freddi *et al.,* 2003; Arami *et al.,* 2007; Fan *et al.,* 2010).

Animal, plant, and microbial proteases are reported to be used for degumming of silk. Microbial proteases used for degumming are mainly from *Bacillus* species, though few fungal proteases are also used (Gulrajani *et al.,* 1996, 2000b; Freddi *et al.,* 2003; Anghileri *et al.,* 2007; Arami *et al.,* 2007)

Enzymatic application in Textile wet processing is extremely expensive and availability of enzymes is also scarce, more so in the handloom sectors like silk. In the present study an attempt was made to explore alternative microbial sources for enzymes which could be cost effective both in the manufacturing and application. It is possible that incorporation of natural sources in the degumming recipes could foster microbial fermentation in the handloom sector. The study was to be able to optimize the fermentation conditions so, that the enzyme is activated at a lower temperature.

Materials and Methods

Selection of Yarn

Raw Mulberry silk yarn of 21-25 denier was obtained from Central Silk Board, Varanasi. It was in the form of hanks and creamish in color.

Procurement of Protease Producing Microbe

Microbial cultures were procured from Division of Plant Pathology, IARI i.e. *Penicillium citrinum, Bacillus subtilis, Aspergillus niger* and *Aspergillus flavus* that were non pathogenic to human and can produce protease in suitable medium.

Conventional Methods of Degumming

Different reagents were used for degumming of silk yarns by conventional methods like soap 10g/l and sodium carbonate 2g/l pH-9.5; soap, sodium carbonate, EDTA, pH-10.3; ezee, pH-10; citric acid and Non ionic detergent pH-6 ; soap and sodium silicate at pH-6

Enzymatic Method of Degumming

The commercially produced trypsin enzyme was used for degumming (Table 1) and its degumming efficiency was compared with the chemically treated degummed sample.

Table 1. Enzymatic Method of Degumming

Trypsin	*1, 3 and 5 %*
Sodium carbonate	*1%*
Sodium phosphate	*1%*
Non ionic wetting agent	*1%*
MLR	*1:20*
Time	*1 hr*
Temperature	*50°C*

Cultivation of Fungi and Bacteria for Protease Production

Fungi and Bacteria were inoculated respectively cultured on PDA and NA slants and were incubated for 3-4 days at 28±2°C for fungi and 1-3 days at 37±2°C for bacteria.

Preparation of Culture Media

Different broth media were prepared namely Minimal medium, PDB, NB and casein (1%), malt extract 1%, polypeptone 1% and sodium carbonate 1% at pH-10

Preparation of Casein Agar Plates

Casein agar plates (casein-1% and agar -2%) were commonly used for the initial screening of proteolytic activity. The clear zone of casein agar hydrolysis was an indication of protease production; the isolates were selected on the basis of the size of the zone of clearance. Those producing larger size zone of clearance were selected for further study.

Procedure for Degumming

The cultured broths were centrifuged and the cell-free supernatants were used for the degumming that was carried out in the orbital shaker at 50°C for 2 hours at pH-9. The medium used for degumming contains
Culture filtrate-50ml

Non-ionic wetting agent -1g/l

Sodium bicarbonate- 2g/l

As the zone of proteolysis on casein agar plate was maximum with the Minimal medium broth, it was used for further study. Maximum protease activity was detected with *Penicillium citrinum* and *Bacillus subtilis*, it was decided to carry further work with these strains.

Use of Various Nitrogen and Carbon sources

To cut down the production cost various natural as well as cheap sources were explored as nutrients for microbial growth to maximize protease production. Six different nitrogen sources- soya bean, gelatin, whey protein, peptone, casein and almond cake ; and Seven different carbon sources- bread crumbs, potato peel water, potato peel , rotten banana, peanuts cake, wheat bran and rice water were used (Table 2 and 3)

Use of Combined Nitrogen and Carbon Sources

Both nitrogen and carbon sources were added as supplements to minimal medium to check their effect on protease production (Table 4). For *Penicillium citrinum* rotten banana and almond cake, bread crumbs and almond cake and rice water and almond cake. *Bacillus subtilis* rotten banana and almond cake and rotten banana and soya bean was used. The inoculated media were then incubated for 7 days in incubator at 28±2°C (*Penicillium*) and at 37±2°C (*Bacillus*). Both were grown in static condition.

Table 2. Natural Nitrogen Sources Used in Minimal Medium Broth

Microbes	Minimal Medium	Nitrogen Sources (1%)	pH	Temperature (°C)	Conditions
Penicillium citrinum	*Glucose – 0.5%* *$MgSO_4.7H_2O$ – 0.5%* *K H_2PO_4 – 0.5%* *$FeSO_4.7H_2O$ -0.001%* *Nitrogen source - 1%*	*Soya bean*	*6*	*28 ± 2*	*Static*
		Gelatin	*6*	*28 ± 2*	*Static*
		Whey protein	*6*	*28 ± 2*	*Static*
		Peptone	*6*	*28 ± 2*	*Static*
		Casein	*6*	*28 ± 2*	*Static*
		Almond cake	*6*	*28 ± 2*	*Static*
Bacillus subtilis	*Glucose – 0.5%* *$MgSO_4.7H_2O$ – 0.5%* *K H_2PO_4 – 0.5%* *$FeSO_4.7H_2O$ –0.001%* *Nitrogen source - 1%*	*Soya bean*	*7.5*	*37 ± 2*	*Static*
		Gelatin	*7.5*	*37 ± 2*	*Static*
		Whey protein	*7.5*	*37 ± 2*	*Static*
		Peptone	*7.5*	*37 ± 2*	*Static*
		Casein	*7.5*	*37 ± 2*	*Static*
		Almond cake	*7.5*	*37 ± 2*	*Static*

Table 3. Natural Carbon Sources Used in Minimal Medium Broth

Microbes	Minimal Medium	Carbon Sources (1%)	pH	Temperature (°C)	Conditions
Penicillium citrinum	*Peptone – 0.5%* *$MgSO_4.7H_2O$ – 0.5%* *K H_2PO_4 – 0.5%* *$FeSO_4.7H_2O$ -0.001%* *Carbon source – 1%*	*Bread crumbs*	*6*	*28 ± 2*	*Static*
		Wheat bran	*6*	*28 ± 2*	*Static*
		Rice water	*6*	*28 ± 2*	*Static*
		Peanut cake	*6*	*28 ± 2*	*Static*
		Rotten banana	*6*	*28 ± 2*	*Static*
		Potato peel	*6*	*28 ± 2*	*Static*
		Potato peel water	*6*	*28 ± 2*	*Static*
Bacillus subtilis	*Peptone – 0.5%* *$MgSO_4.7H_2O$ – 0.5%* *K H_2PO_4 – 0.5%* *$FeSO_4.7H_2O$ -0.001%* *Carbon source – 1%*	*Bread crumbs*	*7.5*	*37 ± 2*	*Static*
		Wheat bran	*7.5*	*37 ± 2*	*Static*
		Rice water	*7.5*	*37 ± 2*	*Static*

Table 4. Natural Carbon and Nitrogen Sources

S.No	Microbes	Minimal Medium	Carbon + Nitrogen Sources (1% each)	pH	Temperature (°C)	Conditions
1.	***Penicillium citrinum***	*$MgSO_4.7H_2O$–0.5%* *K H_2PO_4 – 0.5%* *$FeSO_4.7H_2O$-0.001%* *Carbon sources – 1%* *Nitrogen sources – 1%*	*Rotten banana + almond cake*	*6*	*28 ± 2*	*Static*
			Bread crumbs + almond cake	*6*	*28 ± 2*	*Static*
			Rice water + almond cake	*6*	*28 ± 2*	*Static*
2.	***Bacillus subtilis***	*$MgSO_4.7H_2O$–0.5%* *K H_2PO_4 – 0.5%* *$FeSO_4.7H_2O$-0.001%*	*Rotten banana + almond cake*	*7.5*	*37 ± 2*	*Static*
			Rotten banana + soya bean	*7.5*	*37°C± 2°C*	*Static*

Weight Loss

Weight of yarn – 0.5grams

Weight loss was calculated by

$$\text{Wt loss \%} = \frac{(Initial\ Weight - Final\ Weight)}{Initial\ Weight} \times 100$$

Dyeing of Sample

Samples of silk fibers obtained after degumming processes were dyed using acid Magenta (C.I Number- A. Red 186)

Results and Discussion

Conventional Method of Degumming

Out of the five chemical methods tested, the one using soap and sodium carbonate was found to be most effective degumming agent resulting in 24% weight loss. The least degumming of silk yarn i.e. 12% weight loss was observed when treatment was done with ezee (Fig. 1)

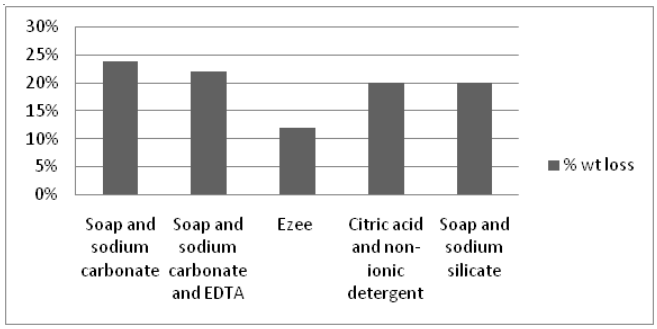

Fig. 1. Percentage Weight Loss in Mulberry Silk Yarn After Conventional Degumming

On comparing whiteness index and K/S value of Mulberry silk yarn degummed with with various chemical methods, maximum whiteness index of 82.85 was found to be of yarns degummed with soap and sodium carbonate, indicating it to be an effective degumming method (Table 5). The K/S value of soap and sodium carbonate degummed sample was 38.82 indicating a good colour intensity when compared to raw silk yarn sample (13.23).

Enzymatic Degumming

Procuring of enzymes was difficult as availability of enzymes was scarce as well as these were extremely expensive. Only trypsin was sourced and degumming was carried out by using it at various concentrations (0.5%. 1%, 3% and 5%).

Percentage weight loss of samples found to vary from 2% with 0.5% of enzyme to 28% with 5% enzyme concentration (Fig. 2). When compared with control sample, developed using the same silk yarn by treatment with soap and sodium carbonate, degumming with trypsin gave better weight loss (28%).

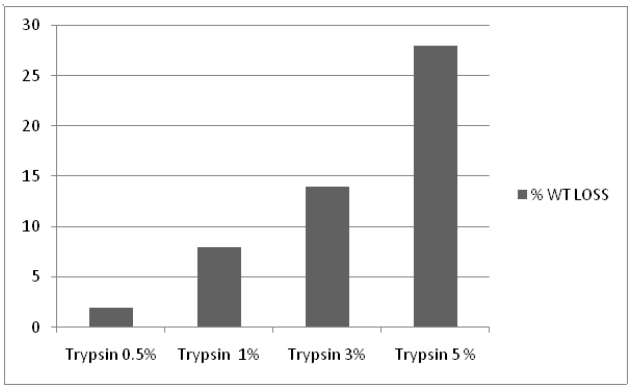

Fig. 2. Percentage Weight Loss in Mulberry Silk Yarn After Enzymatic Degumming)

Table 5. Whiteness Index, K/S and L*, a*, b* Values of Mulberry Silk Yarn After Chemical Degumming)

Treatments	Whiteness Index	K/S	L*	a*	b*
Raw sample	*30.016*	*13.50*	*43.332*	*56.86*	*-8.634*
Soap – 10 g/l *Sodium carbonate – 2 g/l* *pH – 9.5*	*82.859*	*38.72*	*40.95*	*60.70*	*-8.77*
Soap – 15% *Sodium carbonate – 1.5%* *EDTA – 0.05%* *pH – 10.3*	*72.769*	*33.56*	*37.851*	*56.38*	*-12.35*
Ezee – 1% *pH – 10*	*57.182*	*18.20*	*45.06*	*60.22*	*-6.17*
Citric acid – 25% *Non-ionic detergent – 0.2%* *pH – 6*	*64.898*	*32.5*	*46.23*	*66.21*	*-13.8*
Soap – 4 g/l *Sodium silicate – 2.5 %* *pH – 6*	*59.686*	*30.06*	*43.19*	*64.71*	*-13.55*

Table 6. Whiteness Index, K/S and L*, a*, b* Values of Mulberry Silk Yarn After Enzymatic Degumming

Enzyme	Whiteness Index	K/S	L*	a*	b*
Trypsin 0.5%	*80.838*	*28.64*	*36.35*	*62.76*	*-6.15*
Trypsin 1%	*81.772*	*35.39*	*34.63*	*62.32*	*-7.25*
Trypsin 3%	*81.98*	*44.05*	*24.11*	*44.02*	*-5.00*
Trypsin 5 %	*85.69*	*45.30*	*2.40*	*38.43*	*3.49*

Degumming using with trypsin gave better weight loss (28%) and whiteness index (85.69%). Using Trypsin at 5% concentration gave highest K/S value i.e. 45.30 leading to good color intensity as control sample (38.72) (Table 6). Indicating more dye absorption occur in trypsin degummed sample.

Optimization of Broth and Fermentation Condition for Degumming

Four protease producing microbes from Division of Plant Pathology, IARI i.e. *Penicillium citrinum, Aspergillus niger, Aspergillus flavus and Bacillus subtilis* were cultured on different media to observe their protease production efficiency.

After 7 days of incubation, the cultures were filtered out and tested for protease production on casein agar plates. The production of zone of clearance around the wells containing these culture filtrates indicated extracellular protease production and hence can be easily distinguished from the non – proteolytic ones. Zone of clearance was observed in all the casein agar plates though maximum size was observed with the culture filtrate obtained from using Minimal medium.

On comparing protease activity of different microbes, it was found that *Bacillus subtilis* and *Penicillium citrinum* produced a larger zone of clearance while *Aspergillus niger* and *Aspergillus flavus* produces small zones. So, for further study isolates producing larger zone and proteolytic i.e. *Bacillus subtilis* and *Penicillium citrinum* were selected.

Supplementation of Media with Nitrogen and Carbon Sources

Better weight loss was observed when degumming was done with *Bacillus subtilis* and *Penicillium citrinum* grown on minimal medium supplemented with nitrogen sources almond cake, peptone and soya bean supplemented culture filtrates gave weight loss of 28% as compared to control of 24% (Fig. 3). Peptone appeared to be better nitrogen source for *Penicillium citrinum*, whereas soya bean and almond cake were better for *Bacillus subtilis* protease production. It was also reported that protease production can be increased by using appropriate nitrogen sources in media. Complex nitrogeneous compounds supported better protease production (Qureshi *et a.l,* 2011).

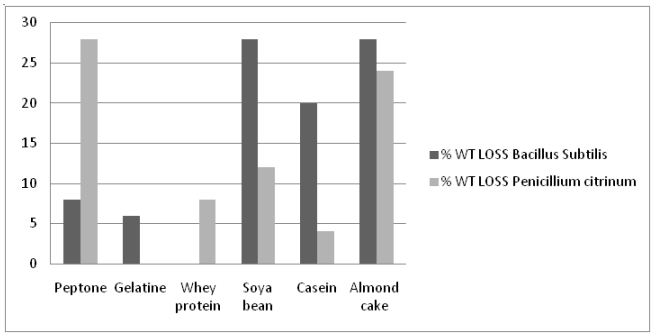

Fig. 3. Percentage Weight Loss in Mulberry Silk Yarn Using Natural Nitrogen Sources in Minimal Medium

From the color value obtained, it was found that *Penicillium citrinum* has higher K/S value than control, hence, *Penicillium citrinum* degummed sample has higher color depth than control . *Bacillus subtilis* also showed equally good K/S value than all other samples.

The luster, texture and feel of *Bacillus subtilis* (soya bean and almond cake as nitrogen source) and *P.citrinum* (peptone and almond cake) was excellent while control shows excellent luster and very good texture and feel. The texture and feel of casein, whey protein, gelatin is very harsh and poor. It may be due to incomplete removal of sericin.

From the results, it was clear that almond cake for both the microbes and soya bean for *Bacillus subtilis* and peptone for *Penicillium citrinum* were best nitrogen sources for protease production. The maximum weight loss was achieved by using these sources. The maximum protease production was achieved after 7 days of incubation period. It was found that almond cake was the best nitrogen source as compared to others.

Degumming with *Bacil*lus subtilis and Penicillium citrinum in medium containing rotten banana as carbon source gave better weight loss of 28% and 26 %respectively as compared to control of 24% (Fig. 4). With rice water *Penicillium citrinum* produced weight loss just similar to control i.e 24 %. All other carbon sources were found to have insignificant effect on protease production.

Table 7. Weight Loss in Mulberry Silk Yarn Using Both Nitrogen and Carbon Sources)

Sources / Microbes	Nitrogen + Carbon	7 Days Weight (gm)	% weight Loss
Bacillus subtilis(Bs)	*Rotten banana + almond cake (RbA1)*	*0.35*	**30**
	Rotten banana + soya bean (RbB)	*0.37*	*26*
Penicillium citrinum(Pc)	*Rotten banana + almond cake (RbA2)*	*0.41*	*18*
	Rice water + almond cake (RbRw)	*0.37*	**26**
	Bread crumbs + almond cake (Rbbc)	*0.39*	*22*

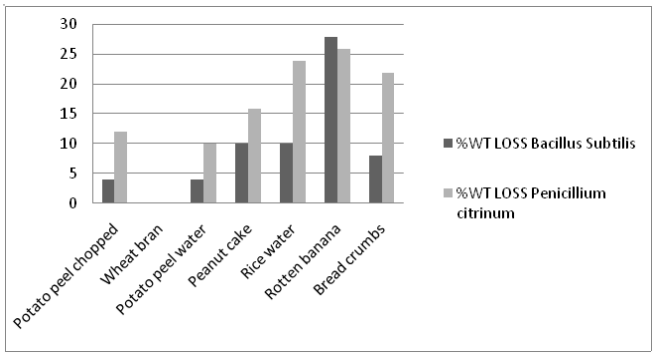

Fig. 4. Percentage Weight Loss in Mulberry Silk Yarn Using Natural Carbon Sources in Minimal Medium)

Bacillus subtilis treated degummed samples showed highest whiteness index of i.e. **87.08** followed by *Penicillium citrinum* i.e **83.09** with rotten bananas supplement**.** Peanut cake, bread crumbs and rice water also shows good whiteness index. It was clear that minimal medium supplemented with potato peel water, potato peel chopped, wheat bran did not show appreciable degumming results and thus were not taken for further study. Rotten banana was a good source of carbon in minimal medium that produce highest weight loss followed by rice water and bread crumbs. Use of agro waste or other waste material was reported for protease production. The utilization of agro industrial waste not only fulfills the requirement as a substrate for the production of several value added products but also reduces pollution (Freddi *et al.,* 2003)

Use of Combined Natural Carbon and Nitrogen Sources

The *B. subtilis* culture in minimal medium supplemented with Almond cake and Rotten banana gave maximum weight loss of 30% and whiteness index of 87.276 followed by that with degummed Rotten banana and Soya bean (26% and 85.20) (Table 7). Rice water and Almond cake supplemented culture of *P. citrinum* gave weight loss of 26% and whiteness index 84.01 respectively

Use of combination of Almond cake and Rotten banana culture gave highest K/S value i.e.39.55 than control degummed samples i.e. 36.82. From this, it was clear that the absorption of dye in this sample is maximum. Other samples also had good K/S values. Samples were also visually examined and found to have darker shade on almond and Rotten banana degummed sample than others. The luster, texture and feel of *Bacillus subtilis* and *Penicillium citrinum* (Rotten banana along with almond cake/ soya bean and rice water with almond cake) was excellent while control showed excellent luster and very good texture and feel.

Conclusion

The results of the study revealed that microbes could be exploited for producing proteolytic enzymes, the characteristics of which depend on the conditions of growth like culture medium, pH, time and temperature. These enzymes can be used for degumming the silk fibers to make the process more eco-friendly. Enzymatically degummed samples were found to be superior in terms of whiteness, dye uptake, luster, feel, softness and percent weight loss as compared to conventionally degummed samples. This is because proteolytic enzymes work under milder conditions.

(Duran, 2000) reported that proteolytic enzymes have become an integral part of silk finishing process. This is also known as enzymatic degumming. It involves degradation of sericin using an enzyme, which does not attack fibroin. This is the reason for proteolytic degummed samples to appear whiter, brighter, shiner and softer than the soap degummed samples.

After optimizing all fermentation conditions like media, nitrogen and carbon sources, time, temperature and pH, final degummed samples were prepared with *Bacillus* and *Penicillium* cultures, which gave maximum weight loss and also reliability of repeatability of degumming was good throughout the study. The best degumming process include was temperature - 50°C, pH - 9 and time - 2 hrs with 1g/l non- ionic wetting agent and 2g/l sodium carbonate. Enzymatic degumming is a milder process regarding temperature, being carried out at temperature 50° C; whereas soap degumming requires higher

temperature of 100 °C which in combination with alkaline treatment caused undesirable changes in texture, feel and luster.

Samples were also visually examined and found to have brighter, whiter and shiny in appearance. The texture, feel and luster of *Bacillus subtilis* and *Penicillium citrinum* degummed sample were excellent. The dye uptake of all these samples was also excellent because enzyme act on the surface bringing about hydrolysis of chains and thereby increasing the number of amino groups for absorption of dye.

From the result, it can be inferred that proteolytic enzymes extracted in lab under different conditions were the best as degumming agents followed by Trypsin and then soaps. So these natural enzymes could be used for silk degumming effectively in industries as they have the added advantages of being eco -friendly.

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