

New Technique to Produce Large Amount of Flat Silk by Biospinning

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Abstract

Beyond the production of silk thread, there are several studies showing that the silk is a great biomaterial for surgical sutures and grafts. This paper shows a new technique to produce silk thread changing the natural cycle of silk production, which is the production of cocoons. This new method has the purpose of producing a silk fabric free of impurities, through flat surfaces. Six different surfaces were tested: Glass, Formica Surface, Steel and Zinc Sheets, Cotton tissue and Burlap Bag. The first five surfaces had not presented enough larvae alive for statistical analysis, because there were several damages in silkworms larvae that resulted in mortality and low silk production. On the other hand, the burlap bag surface presented good results for web construction by biospinning and its use was indicated for silk industry focused on biomaterials. The present study suggested the potential of naturally biospun web, using *Bombyx mori*, to develop a new technique to produce silk thread matrices that will have several applications at the industry and production of biomedical materials.

Keywords

Silkworm, *Bombyx mori* L., Silk Thread, Biomaterial

1. Introduction

Silk is widely known in the textile industry for their mechanical properties and brightness, produced by spiders

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and different insect classes, they produce cocoons, eggs and traps protection [1]. Silk has been also the focus of new researches for tissue engineering and biomedicine. *Bombyx mori*, an insect that belongs to the Lepidoptera order, produces a silk cocoon and because of their easy handling in laboratory, it is widely used in genomic and proteomic studies, being a reference organism in biological studies [2].

Silkworm large-scale production is held by silk farmers, employed mainly by family farming, developed in small lands. This activity is the result of integration between industry and farmers that plant and cultivate mulberry (*Morus* sp.) that feed silkworm larvae, on the other hand, the industry promotes the breeding and production of eggs until the silkworm larvae reach the field [3]. Brazil has a favorable climate for mulberry plantation, as well as for silkworm production, thus sericulture is a good alternative crop for the country, especially for small farmers [4].

The silk thread is secreted by *B. mori* larvae and can measure up to 1500 meters, basically made of two fibroin proteins, which consists in a 70% to 80% hydrophobic protein, and sericin, approximately 20% to 30% hydrophilic coat protein [5]. Structurally, the silk fiber is composed of a 325 kDa and 25 kDa chain, which are connected by sulfide bridges, bound by a sericin chain. The silk fibroin has tripeptide glycine sequence (43%) and alanine (30%), which are interspersed with amino acids, such as serine or tyrosine, and they show a high complex organization [6].

Silk fibroin is a natural polymer with high potential for application as biomaterial due to its elasticity, flexibility, tensile strength, versatility, easy permeability and absorption, enzyme immobilization, substrate for cell growth, minimal inflammatory response, thermal stability, oxygenation, hemostatic property, biocompatibility and biodegradability [5] [7]-[9]. When silk thread is used in surgeries, occasional rejection has been reported and it is attributed by contamination with sericin [10]. In two years, the silk is completely absorbed and is found no trace of the same material [11].

Biomaterials devices used to repair tissue injuries has been utilized for centuries, however most of biomaterials are inadequate and can cause severe inflammatory reactions in patients. The biomaterials industry is constantly looking for materials with appropriate physical and chemical properties that cause the least possible intolerance by the body. The fibroin has several of these properties, since biomaterials of different configurations can be made, depending on requirements: gels, films, 3D porous matrices, microspheres and microfibers [12], supports adhesion of stem cells proliferation and differentiation *in vitro* and promoting tissue repair *in vivo* [10].

Biospinning is a mechanism of natural silk fibers production exploited for decades aiming to produce a silk thread better for be used in biomaterials [13]. The alternative method of non-cocoon silk utilization is the new orientation in which modern sericulture industry develops to [14].

The aim of this study was induce *B. mori* silkworms to spin a silk web without the formation of cocoon, creating a new technique that allows a continuous development of insect life cycle. This technique used a flat base surface, where the silkworms were placed and spinning a tissue for future potential application, which facilitated the extraction of fibroin or used the web as a biomaterial. In this study, we tested different surfaces for flat silk production.

2. Materials and Methods

2.1. Cleaning Methodology

All materials and surfaces used were cleaned and disinfected with 1% calcium hypochlorite. The rearing house was cleaned with 3% formalin in lime water (1 kg of hydrated lime/100 liters of water), and 1% hypochlorite.

2.2. Biological Material

In this work we used commercial silkworm hybrids from Seda Y Fibras SA. The eggs were previously spraying with 3% formalin. The hatched eggs have received a standard treatment, heated in hydrochloric acid at birth, and then 20,000 larvae were placed in each box.

2.3. Silkworm Rearing

During the rearing, four repetitions were performed. In all repetitions, silkworm on first and second age, were fed with chopped mulberry leaves. Larvae on third to the fifth age were fed with mulberry branches, as in the regular sericulture. Corporal disinfectant was not used on any larvae age; only hydrated lime was used in the

base surface. The mulberry leaves were collected manually everyday to feed the larvae, chemical herbicides or chemical fertilizers were not used in these treatments.

2.4. Larvae

It was important to establish a synchronization of silkworm larvae, so that the web could be produced at the same time. For this, commercial phytoecdysteroid was used to increase productivity in sericulture, which is an ecdysteroid derived from plant source, used as a mechanism of defense. This ecdysteroid is structurally closer to the original and natural insect hormone and could be used for synchronizing the maturation activity [15] [16]. 3% - 5% phytoecdysteroid β -ecdysone was used for synchronization of silkworm maturation and was administered in the fifth instar at the time of initiation of spinning behavior [17]. After that, 80% of uniform mature larvae were observed in 24 hours. The larvae were separated when the first thread was spun and placed in contact with the tissue surface.

2.5. Web Spinning

The larvae were placed on a flat surface and the web was naturally spun, without formation of cocoon. Some tests were made with six different surfaces for conducting the web as sheet steel, zinc sheet, glass, formica, cotton tissue and burlap bag. All these webs obtained for these different techniques were analyzed. So, six different surfaces were tested for silkworm spinning.

During the spinning the web was manually cleaned, carefully to not remain any organic waste, fecal matter, mulberry leaves, dust and dead larvae between the threads. Once the larvae stopped the spinning, they were collected before the transformation to pupae, to prevent fat residues in the web. Then the tissue was cleaned with a common vacuum cleaner to remove dust and dried in a stove for 3 hours at 60°C. Finally the tissue should be free of impurities and organic waste, as well as chemical residues that could have fallen during the house cleaning or the silk gland by oral incorporation.

3. Results and Discussion

3.1. Surface Analysis

The 3% - 5% phytoecdysteroid β -ecdysone hormone was used and in this study promoted the larvae synchronization. Just when, 80% of uniform mature larvae were observed in 24 hours. The larvae were separated when the first thread was spun and placed in contact with the tissue surface.

Glass and Formica Surface. Formica Surface (**Figure 1(A)**) has a format of a tray with edges where larvae spun the cocoon. Also, as a glass surface, was observed that the larvae escaped around the edges and fell in the floor. Both materials showed low absorption and a poor contact surface for the silk fiber to be spun. These surfaces were not used in these paper results, but were considered for future research. *Steel and Zinc Sheets.* These surfaces (**Figure 1(B)**) showed some problems as the other two surfaces described previously, where larvae escaped and fell in the floor. However, these sheets did not offer adherence surface required that the larvae could walk or spin the silk thread. Both were discarded and were not considered for future research. *Cotton tissue.* In this technique, the cotton tissue was stretched over a wood support (**Figure 1(C)**). Also occurred that some larvae fell into the floor, however the problem with this surface was that it had high adherence which made the cleaning and hygiene of the tissue, so this technique has been discarded. *Burlap Bag.* This material was placed over an extended breeding box (**Figure 1(D)**), with some inclination, which made some larvae escape and fall in the floor, but in low quantity. The larvae had adherence which permitted 2000 larvae during 2 - 3 days spinning a tissue with 1800 cm \times 1000 cm. This surface showed adherence, absorbent, easy cleaning, easy disinfection and it is inexpensive. The silk tissue was spun in this surface and the final product overcame the expectations. This was the surface and the final silk web chosen as the better and more advantageous for analysis corresponding production and use in the silk industry.

3.2. Burlap Bag Tissue

The result with burlap bag showed an average of 1370 grams of raw silk with a devoid standard (DS) 210, 1220 grams of dry web with a DS of 187% and 49.75% loss with a DS of 13.84, that increased by 20,000 larvae in

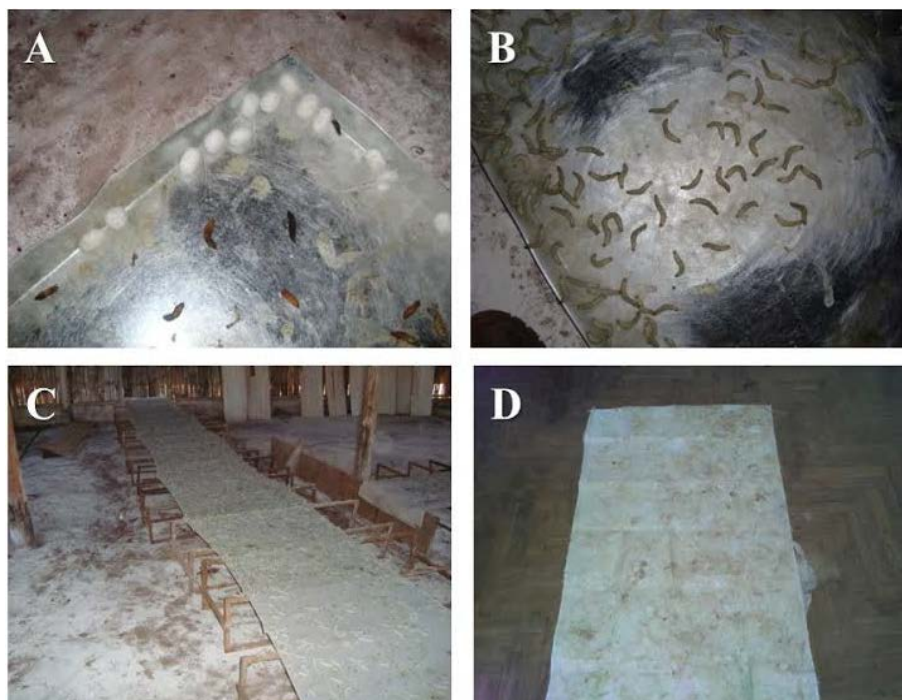


Figure 1. (A) Formica Surface. The larvae spun cocoons on the edges; (B) Steel or Zinc. Sheets surface. Non-absorbent surface which the larvae presented difficult to walk and spun silk fiber; (C) Cotton Tissue. The cotton stretched over wood; (D) Burlap Bag Surface. The final silk tissue finished on surface.

each brood box or repetition. The average production of a raw silk per larvae was 0.14 grams. The value obtained was lower than in some studies available in the literature relates to cocoon weight [18]-[22].

Bacterial infection was observed in the ending of larval cycle, even on a period that the larvae walking around and some felt into the ground. The stress caused by changing the natural cycle makes the silkworm waste more energy, together with the other symptoms described, the spinning cocoon until finally were weaved the tissue and the others could be lowered the immunological defenses of the silkworms becoming susceptible to diseases [23] [24]. This larval behavior can explain the loss estimated at 49.75% for disease, which was equivalent to a variation of silk released larvae. However, it is common that the larvae stop the feeding before making the cocoon, preparing for ecdysis, as a wander to locate a suitable place for cocoon formation, which causes a low stress [25] [26].

The results showed satisfactory relation of time and production of silk fiber, considering the natural spinning of this tissue, and the only addition of this technique was a phytoecdysteroid (β -ecdysone) to ensure the same time of silk spinning and pupation. The phytoecdysteroids are a class of chemicals that plants synthesize for defense against phytophagous, such as insects. These compounds are mimics of hormones used by arthropods in the molting process known as ecdysis. In sericulture, the β -ecdysone is used to increase productivity and reported this administration at a particular time also increase cocoon weight, shell weight and shell percentage, with accepted results about uniform maturation in *B. mori* [15]-[17].

During the experiment, the temperature suddenly dropped below 10°C causing loss in the four replicates. These results agree with the temperature parameters chosen for this methodology of production that could not be less than 20°C, because the pupae were not with the cocoon protection. This technique also allowed to the pupae complete their life cycle, after collected over the tissue, what not occurred in the usual sericulture method of cocoon production.

3.3. Silk Production without Formation of Cocoon

The cocoon is an intricate structure potentially serving numerous simultaneous functions, providing protection to the pupa against biodegradation and dehydration [27]. The presence of the cocoon provides the local envi-

ronment of the pupa ensuring optimal conditions for successful pupation, besides accelerate pupal development by raising the body temperature of the developing pupae. Silk cocoons already possess anti-bacterial and anti-fungal properties [28] [29]. However, this protection occurs after the cocoon spinning and to the technique success the temperature control above 20°C, showed significant result creating a good environment to the larvae, before and during the silk spinning.

Recent analyses of the fibers and structural properties of the cocoon showed multiple layers composed it [30]. The fibers arranged randomly in the plane of the cocoon walls, are bound by sericin to form a non-woven porous structure. This form a graded layers shows different morphologies of non-woven and in these multiple layers there are relatively weak bonds by sericin between them [31].

This heterogeneous structure consisting of two distinct types of silk fibers, large-diameter “outer” fibers, with ~26 nm diameter, and smaller diameter inner fibers with ~16 nm diameter, forming a tightly woven cocoon wall [32] [33]. The structure formed by silkworm cocoon can occur in this silk tissue technique without formation of cocoon, many methods use in the biomaterial industry showed mats weave randomly with silk fibers capable of similar structures founded and described in cocoon wall.

The synchronous maturation is important for large scale silk production and cocoon spinning process could be synchronized. The phytoecdysteroid do not have any effect on the properties of silk, but these administration is need based in technologies of sericulture for physiologically manage the silkworm crop.

3.4. Silk Tissue as a Biomaterial

The biomaterial design is an important element of tissue engineering, this product provides a structural ways to cell attachment and must be biocompatible and elicit little to no host immune response [10] [34] [35]. Silk represent a biocompatible, degradable, mechanically superior family that offer uncountable properties [36].

A wide range of techniques support the silk biomaterial industry, some of them possess similarities with the technique showed in this paper. Eletrospinning is an attractive one, to produce fibers of the desired diameter for tissue engineering applications, but requiring instrumental set-up. Eletrospinning fibers can be produced by a method from aqueous silk fibroin solution mixed with polyethyleneoxid established a fiber morphology with less than 0.8 nm in diameter [37]. However, this method needs high expensive equipment and chemicals solution that degree the quality of silk fiber.

Non-woven technique were prepared by native silk fibers partial solubilization, the silk fibroin mats are a biomaterial used to cell culture as a guide to cells migration, adhesion and differentiation [10] [36] [38].

This technique creates a silk fibroin mats with 10 - 30 nm in diameter and this similar thickness was found in cocoon wall [33] [34] [39].

The similar structure formed in a non-woven mats and a multilayer cocoon could be related and found in this technique for producing silk without the formation of silkworm cocoons. This fabric could have properties to attempt to replicate its characteristics in non-woven fibers used in everything from textiles to scaffolding for skin grafts.

Both of these methods can be used in the industry of silk biomaterials however its use caused damaged in the silk fiber, it has been reported that artificial silk fibers have an inferior mechanical properties compared to naturally spun silk, giving an edge to this natural method over others [40]-[44].

One of the advantages of using this silk fiber instead of cocoons is the easily extraction of fibroin and improve its use, because there were less organic residuals, such as pupae oil, remaining products of the metamorphosis, exuvia etc, that can increase the cost of the extraction. Unlike the cocoon, that contain the exuvia and raise the cost by the oil residues on it, then in this case it is necessary the chemical extraction of fibroin, using products with higher cost. This was the main reason why this studies was conducted, in other words, to reduce the cost of fibroin extraction and increase the amount of fibroin extracted.

The evaluation of silk quality is based on the raw silk percent, which is the amount of silk produced after unwinding the cocoon, the wire length can also be evaluated, among other characteristics. In the present study these characteristics were not evaluated due the fact that the cocoon material is not intentioned to be spinning, thus this analysis will not be relevant in this work. In future studies there is the intention to evaluate protocols that prove the easily fibroin extraction in this silk fiber, comparing to the silk from the cocoon and the silk yarn, also the amount of fibroin obtained is this different protocols. In this work we aimed to produce a new technic aimed the silk production focused on the extraction of a pure and clean fibroin, therefore its higher production.

3.5. Biospinning

Another new technique related was observed in the wild Saturniid silkworm *Antheraea mylitta* denominated “biospinning” [13]. This was the only technique present in the literature, until now, using a natural method to biofabricate a silk fiber matrices that can be used for future applications as a biomaterial. This method consists in *A. mylitta* larvae spinning fibers and fabricating matrices with linear or mixed orientations on coverslip surfaces measuring 20 × 25 mm. In the randomly aligned only one silkworm were placed on Teflon-coated glass plates and spun layer by layer on it, while in the technique of *B. mori* silk tissue, many larvae produced a largest tissue in a cheaper surface.

The silk tissue of *B. mori* also could be denominated biospinning and showed some advantages, *B. mori* has higher economic importance, has a consolidated rearing, the mulberry silk is the most studied, searched and used in the world. The present technique has suggested the potential of naturally biospun tissue, by mulberry silkworm *B. mori*, for develop a new technique for production of silk fibrous matrices and various industry and biomedical applications in the future. It is worth emphasizing that methodology can be applied commercially on a large-scale.

4. Conclusion

This new technique demonstrated a new perspective to the sericulture based in producing a silk web without formation of silkworm cocoon. This method, produced a new and alternative route using silk fibers directly from silkworms, woven the tissue, with natural spinning process without any chemical processing, or impurities, or interrupt the life cycle of the insect, could be used in the textile industry and biomaterial engineering application. In accordance with the results obtained, it was found that the five surfaces (Glass and Formica Surface, Steel and Zinc Sheets and Cotton tissue) did not show larvae alive in sufficient number for statistical analysis. Therefore, these treatments have inflicted severe damages in the silkworm larvae and resulting in mortality and low silk production. On the other hand, the burlap bag surface presented good results for web construction by biospinning and its use was indicated for silk industry focused on biomaterials.

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