UTAH STATE UNIVERSITY

SPIDER SILK MASP1 AND MASP2 PROTEINS AS CARBON FIBER PRECURSORS
DE-EE0006857
DOE-USU-0006857 FINAL TECHNICAL REPORT

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ACKNOWLEDGMENT
The information, data, or work presented herein was funded in part by the Office of Energy Efficiency and Renewable Energy (EERE), U.S. Department of Energy, under Award Number DE-EE0006857.

ABSTRACT/EXECUTIVE SUMMARY

The objective of this project is to develop an unconventional non-petroleum based carbon fiber precursor which has the potential to be produced in high yield and quantities. Methods will be developed to produce pilot-scale quantities of fibers from spider silk proteins with mechanical properties at least 75% that of the natural dragline silk fibers in tensile strength and elongations of less than 5%. The precursor fibers will be converted to carbon fibers, with a goal of >250Ksi strength and 1-2% elongation. Cost analysis will be performed and the process optimized.

Task 1: Subtask 1. Protein production: We exceeded the go/ no go milestone of 1.0g/L of one of the spider silk protein (MSp2) purified last FY and have now increased from 5L to 500L fermentations. We have made a series of changes to the purification protocol from the initial report last FY. These led to a reduction in the time needed for the purification and reduced the purification costs by nearly 90%.

Subtask 2. Fiber spinning: The major focus has been to produce more material to send 24 fiber thread to ONRL. We are still developing the methodology to successfully spin 24 fiber yarns. This involves both the spinning dope solutions as well as the methods to keep the fibers from fusing during the post spin stretch.

The second area of focus has been to standardize the spin dopes for making the fibers. We now know that the conductivity (indicative of salt remaining with the protein after purification) is an important factor in successful spinning as is the pH. We now know that we need to be below 600 uS conductivity and that the most effective pH is protein dependent.

Subtask 3. Silkworm silk: We have found the transgenic silkworms made using gene replacement at the fibroin light chain instead of heavy chain as we did previously have a higher tensile strength. See figures below showing the curve for the top end of the cocoon fibers. This tensile strength is the same as the average for spider dragline silk.

Task 2. Carbonization: The major accomplishment in the latter part of the work is that the ONRL group has successfully heated the spider silk protein fibers all the way up to 1700°C and produced a very competitive carbon fiber based on mechanical properties. Several important factors were discovered during these initial trials: 1) the ramp speed for increasing the temperature is critical; 2) maintaining tension on the fiber during the heating process because as it is heated it tends to expand; and 3) narrow temperature window in which stretching the fiber during heating leads to much better final materials.

Task 3. Techno-Economic Methods: The techno-economic analysis was expanded to determine the relative cost of production with the bacterial production system compared to the transgenic alfalfa and goat production systems.

The comparisons show two important things. For all systems the key factor in the final price is the amount of spider silk protein produced for whatever measure of volume or weight is used. Second alfalfa can be the cheapest but is subject to the possible regulatory control unless the US develops a more comprehensive approach to GMOs. The silkworm analysis was not completed due to a variety of confounding factors. The primary one was that if the production were shifted overseas then the cost would likely be nearly equivalent to current silk prices of $5-15/kg. However if concerns about the location of production is important then it would need to be done in the US and initial costs would be much higher but if the later scenario is utilized then the cost would be lowered but it was not possible to calculate exact costs.

Accomplishments

- We exceeded the go/no go milestone of 1.0g/L of one of the spider silk protein (MSp2) purified which has now been scaled to 500L of fermentation.
- The spider silk protein fibers have been successfully heated all the way up to 1700°C and still maintained a useable carbon fiber with excellent tensile strength.
- Making the transgenic silkworms using gene replacement at the fibroin light chain instead of the heavy chain generates fibers a higher tensile strength, at the average of spider dragline silk.
- A techno-economic model has been created that describes the relative percent costs of all the inputs to spider silk protein production via fermentation with the latest modifications to the process.

Future Directions (BUT NOT FUNDING AVAILABLE)

- Increase spider silk protein production via fermentation.
- Improve further the tensile strength of the transgenic silkworm silk.
- Generate methods to increase the tensile strength of the thermally stabilized the spider silk derived carbon fibers.

Technology Assessment

- Target: Increase spider silk protein production to 2g/L recovered protein.
- Gap: Bacterial production and purification technology both have to be improved.
- Target: Increase the tensile strength of carbon fibers by stabilizing spider silk fibers by crosslinking.
- Gap: The best crosslinking method need to be identified.

Introduction

We propose to develop more efficient carbon fibers by using unconventional, non-petroleum precursors that are high strength, low cost and high yield. To our knowledge, the use of spider silk as a carbon fiber precursor is entirely novel. Specifically, in this two-year project, we will assess the feasibility of spider silk fiber as a model for engineering biomimetic polymer precursors to replace petroleum-based polyacrylonitrile (PAN) precursors. Utilization of this unconventional precursor has several potential impacts. From a material science perspective, spider silk has many desirable properties including tensile strength as high as Kevlar and elongation equivalent to nylon, which would impart new properties to carbon fiber. From a sustainability perspective, silks are proteins and, thus, are renewable resources. Finally, from the perspective of national competitiveness and security, unlike PAN precursors, spider silk can be US sourced. Techno-economic modeling will be performed to understand the commercial feasibility of the production processes being investigated with data feedback used to highlight

key areas for research focus to improve the economics of the processes.
The technical target for this feasibility project is to produce carbon fibers with uniform mechanical properties of at least 250Ksi (1.72 GPa) tensile strength, 25Msi (172 GPa) tensile modulus and 1% ultimate tensile strain. Spider silk protein will be produced via E. coli fermentation at a pilot-scale level of multiple 500L fermenters. Recent advances in fermentation and protein expression indicate that this production level at the pilot scale will produce in excess of 1kg per fermentation, resulting in ~330,000m of silk fiber.
Project Objectives: *The three major goals of this project are: 1) develop methods to produce large (pilot-scale) quantities of fibers from spider silk proteins with mechanical properties at least 75% that of the natural dragline silk fibers in tensile strength and elongations of less than 5%; 2) develop procedures to convert these fibers into carbon fibers with mechanical properties of 250Ksi and elongations of 1-2%; and 3) conduct techno-economic assessment of key process cost points to determine where to minimize costs and the most cost effective alternative method.*

Approach

Task 1: Spider silk protein based fiber production

Task Summary: There are two separate approaches to produce sufficient spider silk protein based fibers (Project Objective 1): 1) spin fibers from *E.coli* produced protein and 2) create transgenic silkworms producing cocoon silk fibers with mechanical properties similar to spider silk (see Fig. 1 for flowchart). This task will be needed to achieve our major deliverable.

Task Details: Initial production methods for spider silk fibers had two deficits: 1) lack of production capacity and 2) inferior mechanical properties. We have improved bacterial production to meet the Go/No Go in FY15. We have continued to improve the production process. The transgenic silkworm system is in theory infinitely expandable and at a low cost, but the fibers produced lack the needed mechanical properties. To overcome this we have directly replaced the silkworm light chain protein gene with a spider silk protein gene.

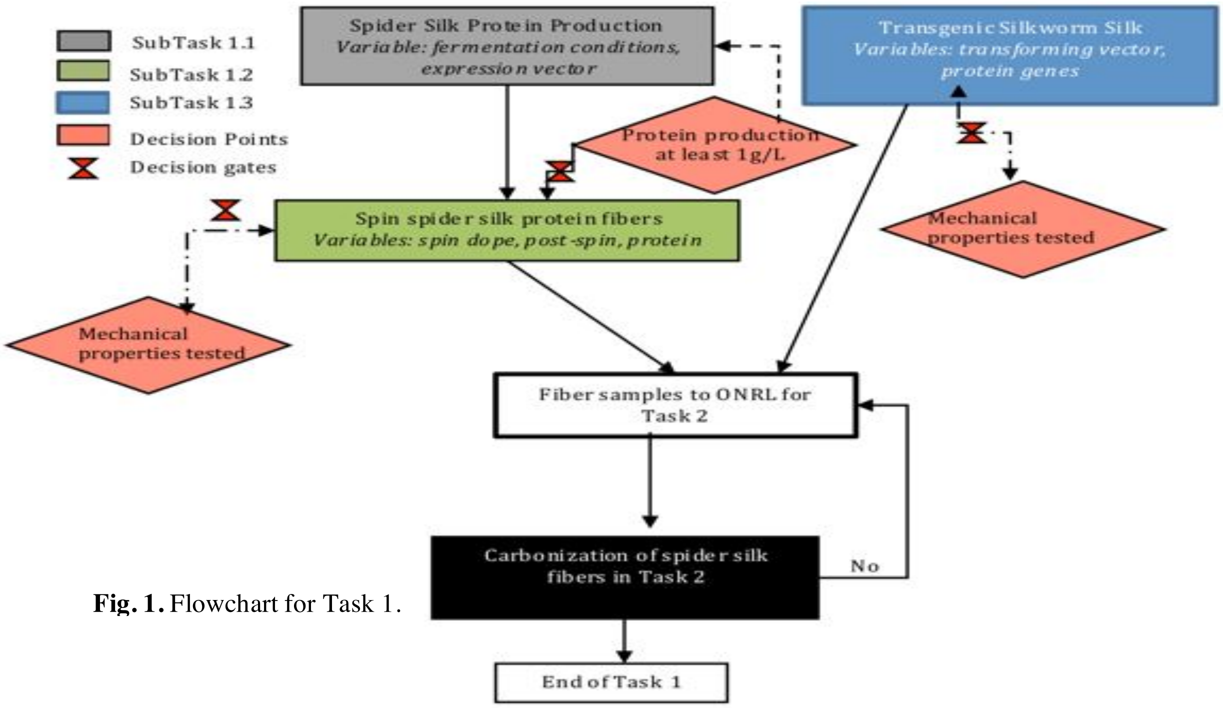

Fig. 1. Flowchart for Task 1.

<u>Task 1:</u>

<u>Milestones 1.1.1, 1.2.1, 1.3.1, and the first go/no go all Completed.</u>

<u>FY accomplishments:</u>

We have made a number changes to the initial protocols we developed as described in the Q3 report. This had led to a substantially more efficient and less costly process. Specifically, we finally have the flow through homogenizer operating as it was designed which allows us to lyse up to 100L/h of fermentation liquid which can then be directly passed to the flow-through centrifuge. With this system we have been able to add two more key steps that greatly decrease the time and cost of the purification. The first is that we have previously shown that the spider silk proteins are highly stable even at temperatures exceeding 90°C. So while still in the fermentation tank we heat the tank to 80°C and then pass the fluid through the homogenizer which increases the temperature to about 85°C. In order to remove as much of the insoluble material as possible we are now adding 0.2% polyethylenimine (PEI) after the homogenization step and cooling it to near room temperature before centrifugation. These steps have led to a decrease in the suspended solids from over 2% to less than 0.1% making all the downstream steps more efficient.

We now immediately add AmSO4 to the supernatant from the centrifuge to precipitate the spider silk protein and a small amount of bacterial proteins. This suspension is then passed through the flow through centrifuge and the precipitate collected containing the spider silk protein. Based on the previously described heat/pressure method to solubilize the spider silk proteins we use a Paar vessel to use larger volumes (>300ml) to resolubilize the spider silk proteins which due to the high temperature prevents the remaining bacterial proteins from solubilizing. This solution is cooled and the AmSO4 precipitation is repeated to generate a very high purity spider silk protein preparation.

The precise recoveries and purity of the final proteins are still be assessed as this complete process has only evolved to its current form in the past couple of weeks. In addition, we are developing a rapid assay using the Waters UPLC to determine both concentration and purity of the spider silk proteins which will eliminate two time consuming and costly steps in analysis.

The transgenic silkworm silk was improved substantially in tensile strength by replacing the silkworm light chain gene with a spider silk protein gene. This led to tensile strengths equivalent to those of the natural dragline spider silk.

Task 2: Develop the spider silk fiber conversion technology to obtain low-cost carbon fiber for automotive and commodity applications

The overall technical objective of the proposed research is to demonstrate continuous mode conversion of spider silk protein fiber precursors to obtain carbonized fibers that are applicable in automotive industries. The specific objectives for this work include: (1) develop modified spider silk protein fiber that can be rendered infusible, (2) demonstrate accelerated stabilization using thermal in air or thermochemical methods with a residence time comparable to traditional polyacrylonitrile fiber residence time, and (3) obtain carbonized fiber from the stabilized precursors by applying optimal conversion parameters. In Task 2 (Fig. 3), we will conduct a systematic approach to optimize the conversion parameters with an overarching goal to produce renewable source spider silk-based, low-cost carbon fiber for automotive application. The final project deliverable will be a continuously processed spider silk-based carbon fiber with satisfactory properties.

Subtask 2.1: Development of Chemical/Physical Pretreatment

We explored two methods for protein crosslinking. The first is a visible light induced photo crosslinking process that can be initiated during the fiber spinning process. The other is chemical crosslinking utilizing specific amino acids in the protein. There are several alternatives for this which are still under investigation but nothing was used in the fiber work.

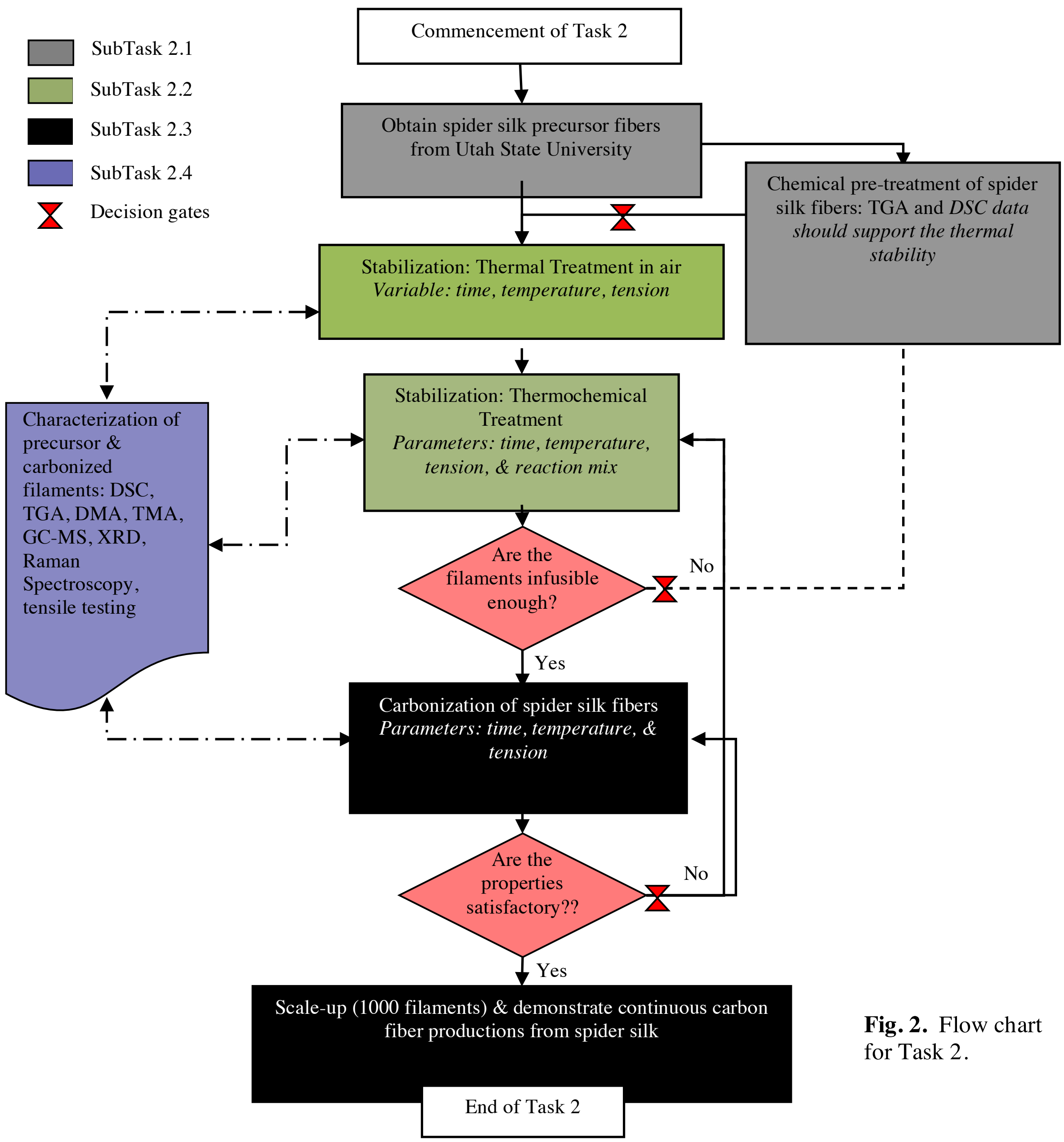

Fig. 2. Flow chart for Task 2.

FY accomplishments:

Several methods for crosslinking are still under study.

Milestone 2.2.1: Conversion to Carbon Fiber

The major accomplishment for is that the ONRL group successfully carbonized the spider silk protein fibers up to 1700°C and still maintained a carbon fiber with excellent mechanical properties. Several important factors were discovered during these initial trials: 1) the ramp speed for increasing the temperature is critical; 2) maintaining tension on the fiber is key during the heating process because as it is heated it tends to expand; and 3) narrow temperature window in which stretching the fiber during heating leads to much better final material's properties.

They have followed up with analyses of the fibers at various final heating temperatures and found that the higher temperature leads to better fiber mechanical properties as shown in the table below. It should be noted that we have achieved the minimum for the second Go/No Go of greater than 50ksi for the peak stress and in fact have exceeded that and exceeded the modulus values as well (1-2 Msi).

Milestone 2.4.1: Micro-structure analysis

The group at ONRL has done several microstructural analyses as seen in the figures below. These include SEM images showing improved fiber shape and uniformity. The Raman spectra show the D and G bands have smaller peak widths which indicates they are better ordered in relationship to the fiber axis.

Task 3: Techno-economic analyses

Milestone 3.2.1, Milestone 3.2.2, Milestone 3.3.1. All Completed.

Task Summary: The scope of the modeling work is focused on the critical evaluation of the proposed process on the metrics of economics and environmental impact. A critical component of the modeling work is the integration of experimental data for validation and data feedback to experimental systems. The scope is divided into two performance periods, the first of which focuses on the development of engineering process models and integration with economic evaluation. The second performance period will continue to refine economic modeling while leveraging engineering process models for life cycle assessment. The results from the first performance period will be an economic assessment of the proposed process and data feedback highlighting areas for improvement. The results from the second performance period will include the updating of economic models based on experimental results as well as quantifying the environmental impact of the production and use of the proposed technology.

FY accomplishments:

The techno-economic analysis was expanded to determine the relative cost of production of the bacterial production system compared to the transgenic alfalfa and goat production systems.

Results and Discussion

Task 1:

Subtask 1. Protein production:

We have made a number changes to the initial protocols we developed as described in the FY15 report. This had led to a substantially more efficient and less costly process. Specifically, we finally have the flow through homogenizer operating as it was designed which allows us to lyse up to 100L/h of fermentation liquid which can then be directly passed to the flow-through centrifuge. With this system we have been able to add two more key steps that greatly decrease the time and cost of the purification. The first is that we have previously shown that the spider silk proteins are highly stable even at temperatures exceeding 90°C. So while still in the fermentation tank we heat the tank to 80°C and then pass the fluid through the homogenizer which increases the temperature to about 85°C. In order to remove as much of the insoluble material as possible we are now adding 0.2% polyethylenimine (PEI) after the homogenization step and cooling it to near room temperature before centrifugation. These steps have led to a decrease in the suspended solids from over 2% to less than 0.1% making all the downstream steps more efficient.

We now immediately add AmSO4 to the supernatant from the centrifuge to precipitate the spider silk protein and a small amount of bacterial proteins. This suspension is then passed through the flow through centrifuge and the precipitate collected containing the spider silk protein. Based on the previously described heat/pressure method to solubilize the spider silk proteins we use a Paar vessel to use larger volumes (>300ml) to resolubilize the spider silk proteins which due to the high temperature prevents the remaining bacterial proteins from solubilizing. This solution is cooled and the AmSO4 precipitation is repeated to generate a very high purity spider silk protein preparation.

The precise recoveries and purity of the final proteins are still be assessed as this complete process has only evolved to its current form in the past couple of weeks. In addition we are developing a rapid assay using the Waters UPLC to determine both concentration and purity of the spider silk proteins which will eliminate two time consuming and costly steps in analysis.

<u>Subtask 2. Fiber spinning:</u>

The major focus is to produce more material to send 24 fiber thread to ONRL. We are still developing the methodology to successfully spin 24 fiber yarns. This involves both the spinning dope solutions as well as the methods to keep the fibers from fusing during the post spin stretch.

As in the previous quarter the second area of focus has been to standardize the spin dopes for making the fibers. We now know that the conductivity (indicative of salt remaining with the protein after purification) is an important factor in successful spinning as is the pH. So we have purchased micro pH and conductivity probes to allow us to very accurately determine these parameters and their effects on spinning success. We now know that we need to be below 600 uS conductivity and that the most effective pH is protein dependent.

<u>Subtask 3. Silkworm silk:</u>

We have found the transgenic silkworms made using gene replacement at the fibroin light chain instead of heavy chain as we did previously have a higher tensile strength. See Figure 3 below showing the curve for the top end of the cocoon fibers. This tensile strength for the transgenic fiber is the same as the average for spider dragline silk.

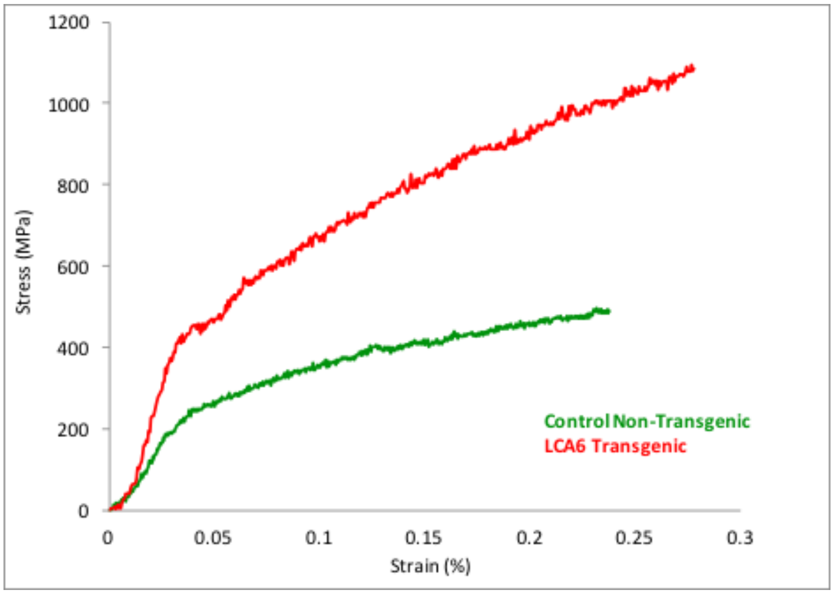

Figure 3. Stress-strain curve for silkworm silk fibers.

Task 2. Carbonization:

Milestone 2.1.1 Pretreatment: Synthetic Spider Silk Protein Modifications due to Cross-linking

USU and ONRL are continuing work on chemical crosslinking methods in addition to our photocrosslinking. Studies are not completed yet for results.

Milestone 2.2.1: Conversion to Carbon Fiber

The major accomplishment was that the ONRL group successfully (Fig. 4). Several important factors were discovered during these initial trials: 1) the ramp speed for increasing the temperature is critical; 2) maintaining tension on the fiber during the heating process because as it is heated it tends to expand; and 3) narrow temperature window in which stretching the fiber during heating leads to much better final materials.

They have followed up with analyses of the fibers at various final heating temperatures and found that the higher temperature leads to better fiber mechanical properties as shown in the Table 1 below. Note the tensile strength in particular is higher at 1700°C than at the lower temperatures and that the diameter is only slightly smaller indicating very little loss of mass in going from 1300-1700°C. The initial modulus and the ultimate strain are relatively unchanged with increasing temperature. As noted previously the fibers are still pliable and not brittle even after 1700°C. It should be noted that we have achieved the minimum for the second Go/No Go of great than 50ksi for the peak stress and in fact have exceeded that of most carbon fiber (50-70ksi) and exceeding the modulus values as well (1-2 Msi).

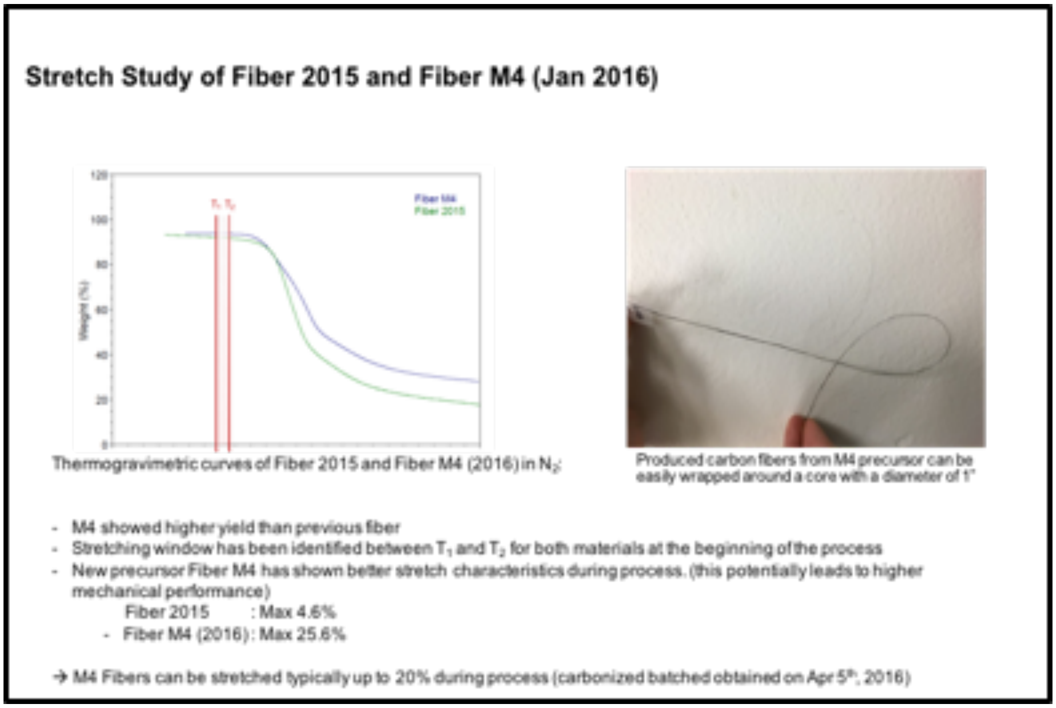

Figure 4. Initial carbon fiber studies from spider silk protein fibers.

Sample ID	Max. temp. of treatment [°C]	Equivalent diameter [µm]	peak stress [ksi]	Modulus [Msi]	Strain at break [%]
Bundle 1	1300	29.37 (1.58)	99.7 (41.0)	7.5 (0.9)	1.28 (0.42)
Bundle 2	1300	29.34 (1.51)	77.3 (41.1)	7.3 (1.1)	1.06 (0.67)
Bundle 3	1500	28.52 (1.35)	69.2 (43.6)	8.9 (1.4)	0.78 (0.50)
Bundle 4	1700	26.08 (3.11)	101.9 (61.9)	7.4 (2.0)	1.32 (0.67)

Table 1: Mechanical test on the fused material that has been produced during FY2016. Those test that have been performed before the end of the fiscal year. For these tests it is assumed that each bundle contains 8 filaments. The diameter of one individual filament is evaluated/measured for each bundle (around 10µm at 1300°C and 9µm at 1700°C). The “equivalent diameter” shown in this chart is a calculation for a “virtual large filament” that would have the same surface of cross-section compare to the bundles of 8 filaments that are tested.

Milestone 2.4.1: Micro-structure analysis

The group at ONRL has done several microstructural analyses as seen in the figures below. These include SEM images showing improved fiber shape and uniformity (Fig. 5).

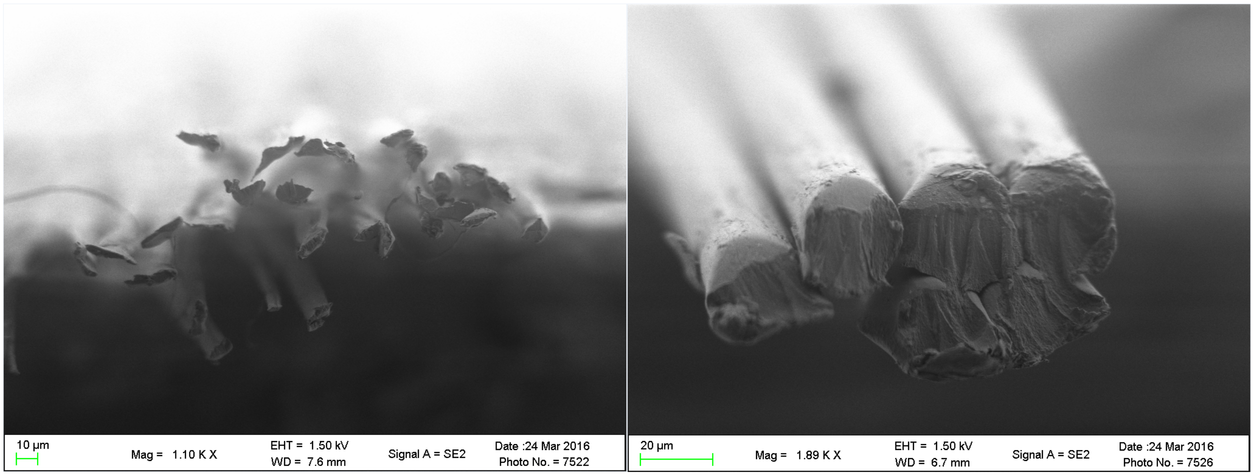

Figure 5: SEM pictures emphasizing the improvement of the production of the precursor between FY2015 (left) and FY2016 (right). On the left, a precursor made of transgenic silkworm/spider silk blend (Apr. 2015). The filaments are not fused but the shape of their cross-section is irregular. The longest dimension is mostly in the 15µm - 22µm range. On the right, a precursor made of spider silk proteins (Jan.2016): its cross-section is almost perfectly circular with a diameter around 28µm. However, the filaments are fused in bundles (usually 8 filaments, which is the maximum).

The Raman spectra show the D and G bands have smaller peak widths which indicates they are better ordered in relationship to the fiber axis (Figure 6). Finally Figure 7 shows that the micro-Raman when overlayed on the visual fiber cross sections demonstrates the same conclusion as the bulk Raman in Figure 3. The D/G peak ratio is lower at the higher temperatures indicating higher order and especially at the highest temperature very uniform ratio compared to the lower temperatures.

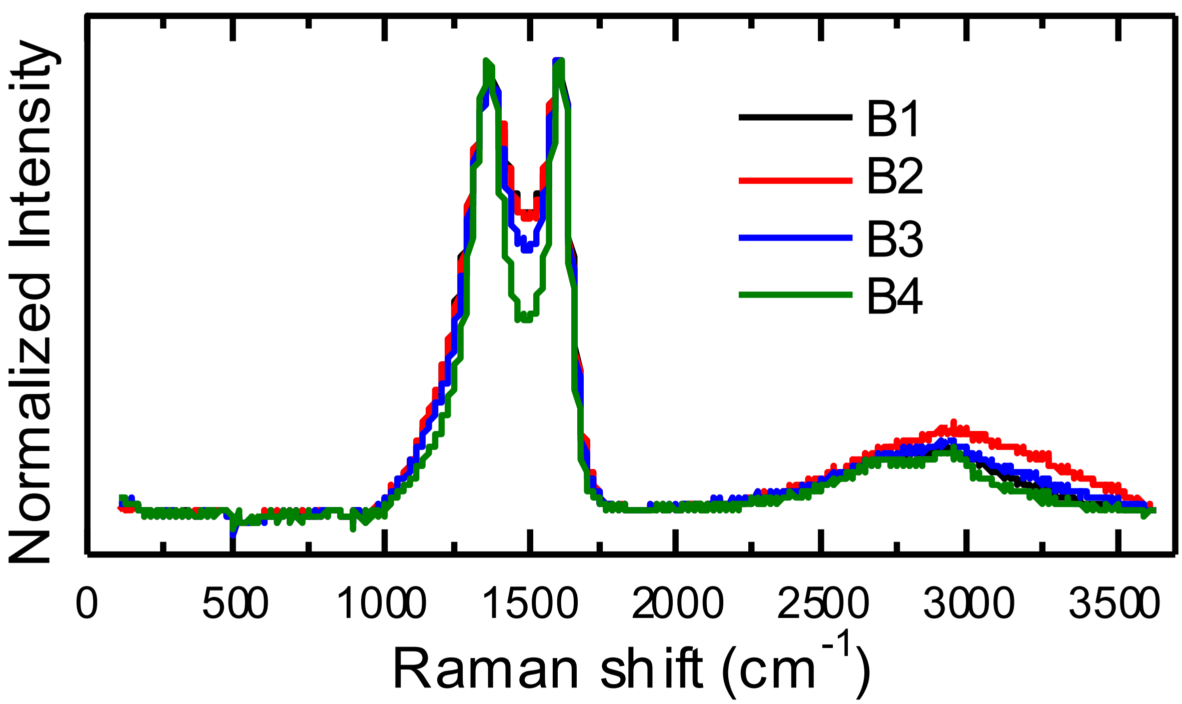

Figure 6: Average Raman spectroscopy of the cross-section of the carbonized fiber which mechanical properties where displayed in

Table 1. The sample B4 shows much narrower D and G bands that the other batches. This means the ordering is better at higher temperatures.

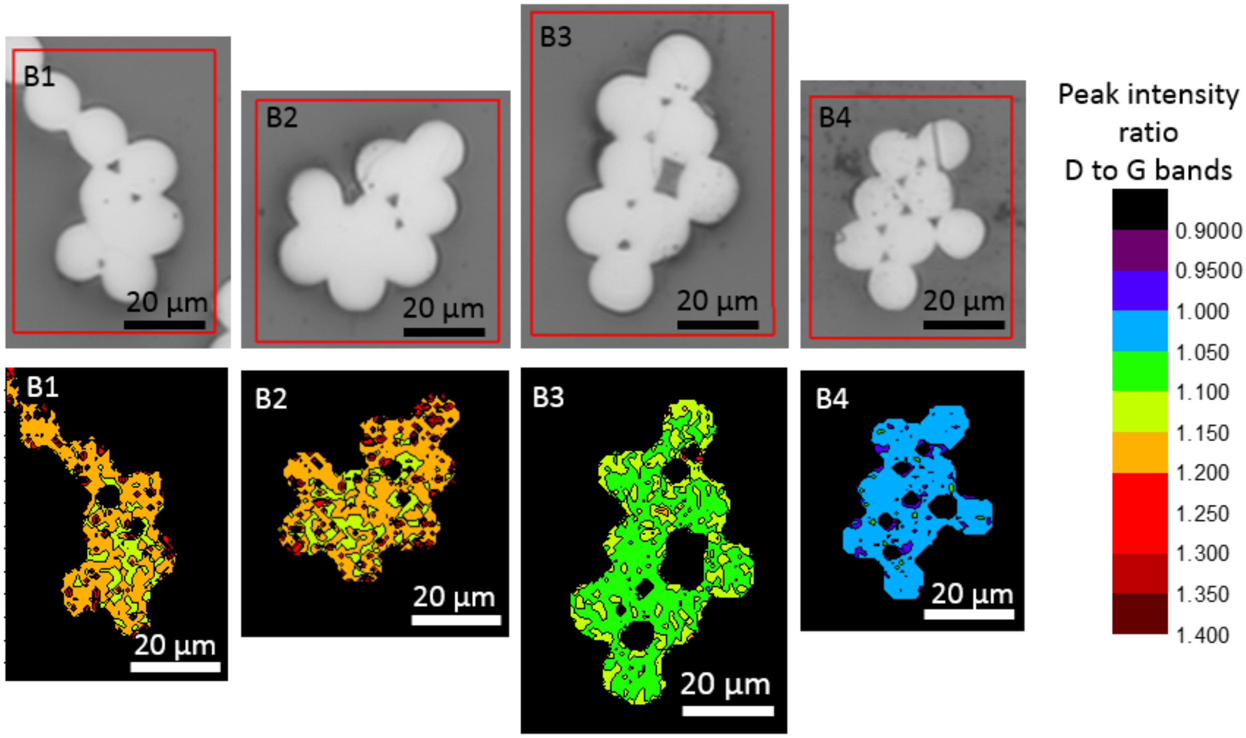

Figure 7: Raman spectroscopy and optical images of the cross section of the bundles B1 to B4 presented in Table 1. The map of D/G shows that this ratio is decreasing with the increased temperature of thermal treatment. Ordering is better at higher temperature (lower D/G ratio).

Task 3.

The techno-economic analysis was expanded to determine the relative cost of production with the bacterial production system (Fig. 5) compared to the transgenic alfalfa and goat production systems (Fig. 9).

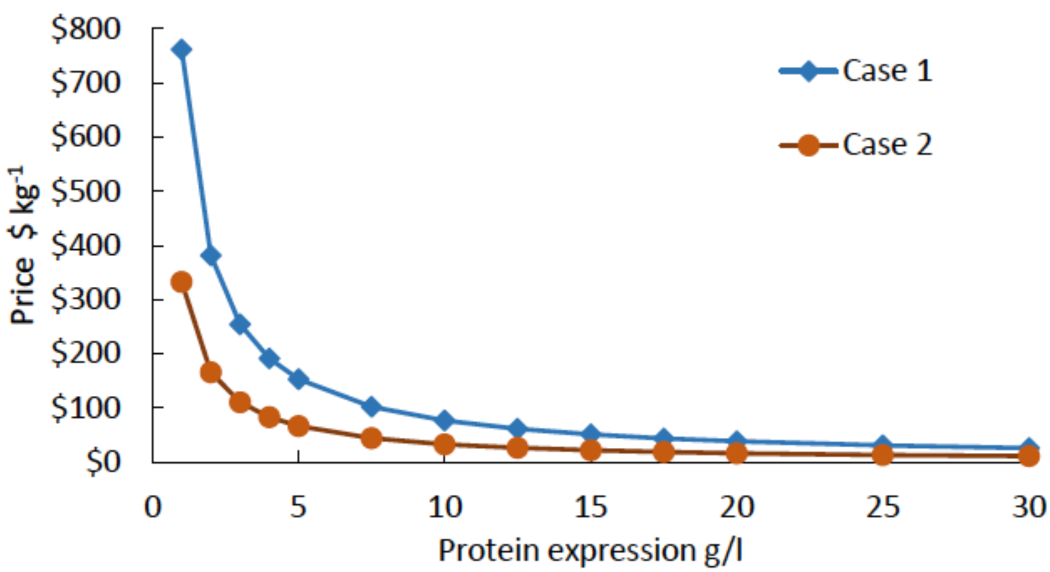

Figure 8: Product sale price as a function of protein expression in E. coli for the initial purification scenario, Case 1 (initial costs) and the new optimized purification scenario, Case 2.

The comparisons show two important things. For all systems the key factor in the final price is the amount of spider silk protein produced for whatever measure of volume or weight is used. Second alfalfa can be the cheapest but is subject to the possible regulatory control unless the US develops a more comprehensive approach to GMOs. The silkworm analysis was not completed due to a variety of confounding factors. The primary one was that if the production were shifted overseas then the cost would likely be nearly equivalent to current silk prices of $5-15/kg. However, if concerns about the location of production is important then it would need to be done in the US and initial costs would be much higher but if the later scenario is utilized then the cost would be lowered but it was not possible to calculate exact costs.

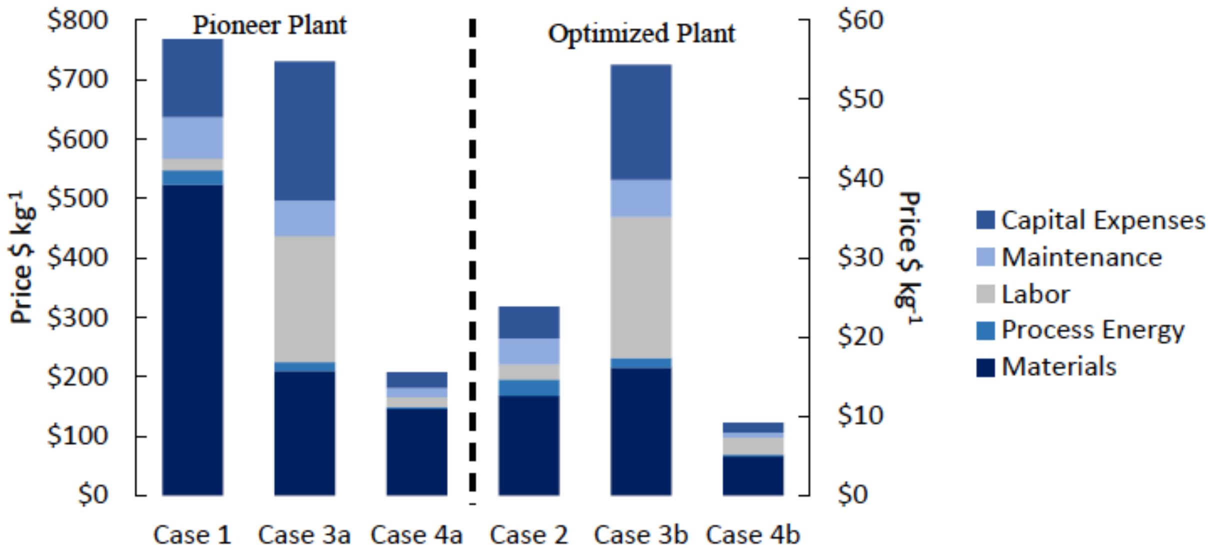

Figure 9: Synthetic spider silk sale price estimates from various facilities and levels of optimization. Cases 1 and 2 are from Figure 5 at low protein production from *E.coli*. Cases 3a and 3b are the initial and optimized goat milk production and Cases 4a and 4b are for alfalfa production initial and optimized.

Technology Transfer Path

We currently are negotiating a joint research effort with an automobile parts designer in CA.

Conclusion

Task 1: Subtask 1. Protein production: We achieved the Go/No Go milestone of 1.0g/L of purified spider silk protein (MSp2) last FY and have now increased from 5L to 500L fermentations. We have made changes to the purification protocol from the initial report last FY. These led to a reduction in the purification time needed and reduced the purification costs by nearly 90%.

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Task 2. Carbonization: The major accomplishment this quarter is that ONRL group has successfully heated the spider silk protein fibers all the way up to 1700°C and still maintained a useable fiber. Several important factors were discovered during these initial trials: 1) the ramp speed for increasing the temperature is critical; 2) maintaining tension on the fiber during the heating process because as it is heated it tends to expand; and 3) narrow temperature window in which stretching the fiber during heating leads to much better final materials. We are now testing different spider silk proteins and crosslinking methods to achieve higher tensile strengths of the carbonized fibers.

Task 3. Techno-Economic Methods: The techno-economic analysis was expanded to determine the relative cost of production with the bacterial production system compared to the transgenic alfalfa and goat production systems. The comparisons show two important things. For all systems the most important factor in the final price is the amount of spider silk protein produced. Second alfalfa can be the cheapest but is maybe subject regulatory issues. The silkworm analysis was not completed due to a variety of confounding factors including IP issues if the production is shifted from the US.

PRESENTATIONS/PUBLICATIONS/PATENTS

(2015) Cameron G. Copeland, Brianne E. Bell, Chad D. Christensen, and Randolph V. Lewis, Development of a Process for the Spinning of Synthetic Spider Silk ACS Biomaterials Science and Engineering 1 (7): 577–584 **DOI:** 10.1021/acsbiomaterials.5b00092

Two manuscripts in preparation and one submitted.