Revisiting the elongation potential of the Davidonis culture (Ideation Idea #8) and staining analysis of biopolymers (Ideation Idea #1)

EXP22000631

WEDNESDAY, 9/21/2022

Instruction:

1. Complete Table 1 before submitting this report for review.
2. Keep entries in a folder dedicated to the assay discipline. i.e. All transformation assays should go into the transformation folder
3. Save entries in folders under a broader theme rather than by OKRs, i.e: Cloning projects should be in a general biomol folder, elongation assays in Fiber development e.t.c.

Table 1: Complete this table before requesting for Review

	A	B
1	Quarter	Q3
2	OKR # and title	OKR1: Create an in-vivo technology for .....
3	KR # and title	KR4: Seed Train and Scaleup
4	Name of the Responsible Scientist	Sergey Savinov
5	Sprint team members	Angelina Cooper
6	Project Title/Experiment Title	Make 100kg of invitro Cotton
7	Start Date	9-12-22
8	End Date	9-19-22
9	Report Due	8-xx-22
10	Submitted On	8-xx-22
11	Progress towards OKR completion (%)	10
12	Relevance to Fiber Development (use the Scale below)	4
13	Reviewed and Approved by	Prince Zogli

DEFINITION OF DONE: DONE MEANS YOU HAVE THE DATA AT HAND AND MADE CONCLUSIONS

Relevance to Fiber Development Scale

5: Very relevant

4: Relevant

3: Somewhat relevant
2: Maybe relevant
1: Not relevant

Scientific Summary:
The 1st generation cotton ovule culture (FJA-Davidonis) has displayed suppressed sensitivity to pro-elongation treatments. On the other hand, this cell line is fully compatible with suspension culturing scale-up and low CapEx bioreactor, providing a venue for obtaining large amounts of fiber-like cellular material.

Purpose: Evaluate solid-media elongation conditions (MSA-G5) on FJA-Davidonis cell line cultured on solid (MSA-KD) media

1.Hypothesis being tested or purpose

- Despite relative reluctance of the FJA-Davidonis cell line to respond to previous pro-elongation treatments, the optimized conditions favoring elongation of HS cells
  Monitoring the time-dependent effect of GA3 on the elongation/aggregation status of HS cells
  are expected to induce at least some cellular elongation

2. Material and Methods

Materials

1. Sterile 100-mm plates
2. Sterilized twitters and spatulas
3. 1x PBS
4. 2 mL Eppendorf tubes
5. Microscopy slides and covers
6. 12-well plates
7. MSA-KD media (per L):
   - MS salts and organics: 4.33 g
   - Glucose: 30 g
   - myo-inositol: 100 mg
   - pH adjustments to 5.8 with 1N NaOH
   - Autoclaving (20 min)
   - Gamborg B5 vitamins: 1000 uL (100 x filter sterilized)
   - Kinetin (filter sterilized 1 mg/mL stock): 538 uL
   - 2,4-D (filter sterilized 1 mg/mL stock): 99.5 uL
   - Dispense 30 mL per 100-mm plate
8. MSA-G5 media (per L):
   - MS salts and organics: 4.33 g
   - Glucose: 30 g
   - myo-inositol: 100 mg
   - Ascorbic acid: 100 mg
   - pH adjustments to 5.8 with 1N NaOH
   - Autoclaving (20 min)

- Gamborg B5 vitamins: 1000 uL (100 x filter sterilized)
- GA3 (1 mg/mL stock in water): 1732 uL
- Dispense 30 mL per 100-mm plate

Biological Materials (List all biological materials used for the project)

Table 1 (Biological Materials)

	A	B	C	D	E	F
1	**Genotype**	**Cell line (name or description)**	**Cell type**	**Media type**	**Media recipee (link or notable details)**	Age of the Cell line
2	FJA		Davidonis	MSA-KD	see above	17 weeks
3						

Protocol:

1. Aliquots of FJA-Davidonis culture was transferred onto "native" (MSA-KD) and "pro-elongation" (MSA-G5) media
2. The plates were sealed with air-permeable tape and incubated in dark at 34 ˚C for 8–10 days
3. Biomasses of each aliquot were measured and doubling times were calculated
4. The aliquoted of each sample were subject to Congo Red staining:
    a. Samples (ca. 50 mg) were combined with 495 uL 1 x PBS and 5 uL and 1% Congo Red and gently agitated to disrupt clumping
    b. The suspensions were incubated at 30˚ C for 15 min
    c. The liquid was withdrawn by pressing the pipette tip to the bottom of the tube
    d. The cells were washed (2x 500 uL) with 1x PBS and resuspended in 500 uL 1x PBS
5. The samples were subjected to microscopic analysis using a polarizing microscope to provide manually collected cell lengths:
    a. A pipetteman with a cut pipette tip was used to transfer a small amount of stained cells onto a microscopy slide. A covers was applied.
    b. Images were taken at 10x magnification with a 100 um scale indicator in bright light.
    c. Manual non-linear length measurements were performed on > 100 random non-overlapping cells, whose length could be discerned unequivocally
6. Automated length measurements were made:
    a. Samples were prepared by dispensing ca. 50 mg of biomass of each sample in 2.0 mL of 1x PBS in duplicate
    b. Each well was subjected to imaging using a confocal microscope and automated length measurements
7. Staining analysis was performed to report on biopolymer content of "naive" sample, elongated sample and mature cotton fiber, using the following stains:

Table1

	A	B	C
1	Stain	Biopolymer Specificity	Comment
2	Safranin	Lignin	Stains lignin regardless of cellulose
3	Toluidine Blue	Lignin + Pectin + Cellulose	Acidophilic metachromatic
4	Astra Blue	Cellulose	Stains cellulose only in the absence of lignin

   a. Samples (ca. 50 mg) were combined with 495 uL 1 x PBS and 5 uL and 100x stocks (0.1% w/v in 1x PBS) of each stain (Safranin, Toluidine Blue and Astra Blue) and gently agitated to disrupt clumping
   b. The liquid was withdrawn by pressing the pipette tip to the bottom of the tube
   c. The cells were washed (2x 500 uL) with 1x PBS and resuspended in 500 mL 1x PBS
   d. A pipetteman with a cut pipette tip was used to transfer a small amount of stained cells onto a microscopy slide. A covers was applied.
   e. Images were taken at 10x magnification in bright light.
8. Automated length measurements were made:
   a. Samples were prepared by dispensing ca. 50 mg of biomass of each sample in 2 mL of 1x PBS in duplicate
   b. Each well was subjected to imaging using a confocal microscope and automated length measurements
9. The "naive" and elongated samples were characterized by the kinetic implementation of Resazurin assay in a separate experiment (see Development and implementation of kinetic Resazurin vitality assay (Ideation Idea #2))

3. Results (Please include conclusions from both positive and negative data, do not omit anything). Tables, figures, and relevant observations. Also provide link(s) to raw data as needed.

Part 1: Data outputs/Deliverables: List all key data outputs required to achieved Objectives

- Biomasses of culture aliquotes
- Calculated doubling times
- Images of Congo Red-stained cells
- Manually measured cell lengths
- Automatically measured cell lengths
- Staining images (Safranin, Toluidine Blue, Astra Blue)

Part 2: Provide the key result(s) as listed in part 1

Raw biomasses and Specific Growth Rates:

	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	AA
y	Initiation	Propagation	Age	Conditions	Period, days	Initial Mass, g	Final mass, g	Specific Growth Rate, 1/days	Period, days	Initial Mass, g	Final mass, g	Specific Growth Rate, 1/days	Period, days	Initial Mass, g	Final mass, g	Specific Growth Rate, 1/days	Period, days	Initial Mass, g	Final mass, g	Specific Growth Rate, 1/days	Period, days	Initial Mass, g	Final mass, g	Specific Growth Rate, 1/days	Mean SGR, 1/days	StDev
	KD	KD	2 years	MSA-K2.5-D0.45	10	0.46	4.44	0.23	10	0.46	4.28	0.22													0.225	0.003
	KD	KD	2 years	MSA-G5	9	0.06	2.00	0.39	9	0.14	2.42	0.32	9	0.22	2.77	0.28	9	0.10	0.73	0.22	8	0.14	2.42	0.36	0.31	0.066

- Specific growth rate is calculated as:

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- Doubling Time can then be calculated as:

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3.3.3. Doubling Time

Doubling time (*dt*) is the time required for the concentration of biomass of a population of cells to double. One of the greatest contrasts between the growth of cultured plant cells and microorganisms refers to their respective growth rates. Although the pattern of growth may be the same, plant cells have dou-

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bling times or division rates measured in days, while this parameter in many microorganisms is in the order of minutes to hours. One of the fastest (and quite exceptional) doubling time recorded for a plant cell culture is 15 h for tobacco cells *(2)*. The doubling time (dt) can be calculated according to the following equation *(6)*:

$$dt = \frac{\ln 2}{\mu}$$

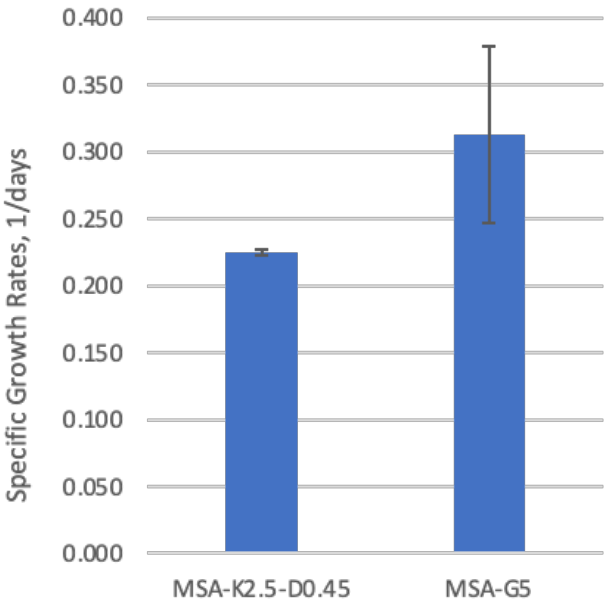
Where μ represents the specific growth rate.

from Plant Cell Culture Protocols (https://link.springer.com/book/10.1007/978-1-61779-818-4#bibliographic-information)

Specific Growth Rates Chart:

- The FJA-Davidonis cells increase biomass significantly faster on pro-elongation media (MSA-G5) than on propagation media (MSA-KD)

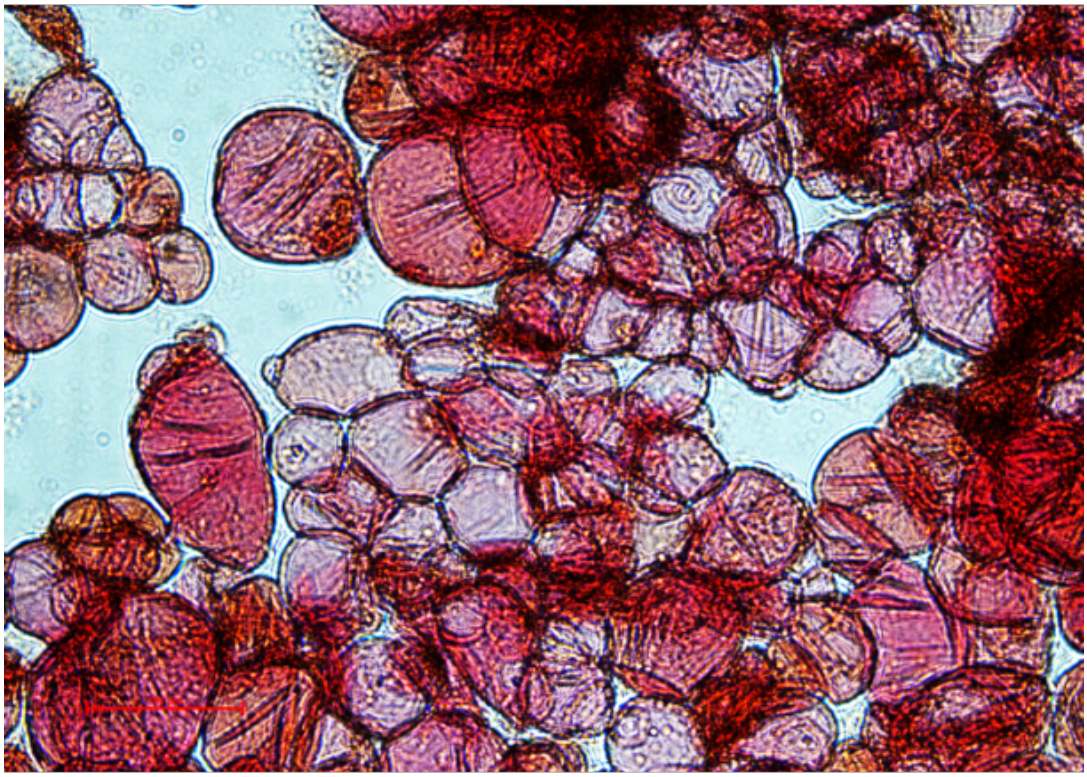
image.png



Microscopic morphologies:

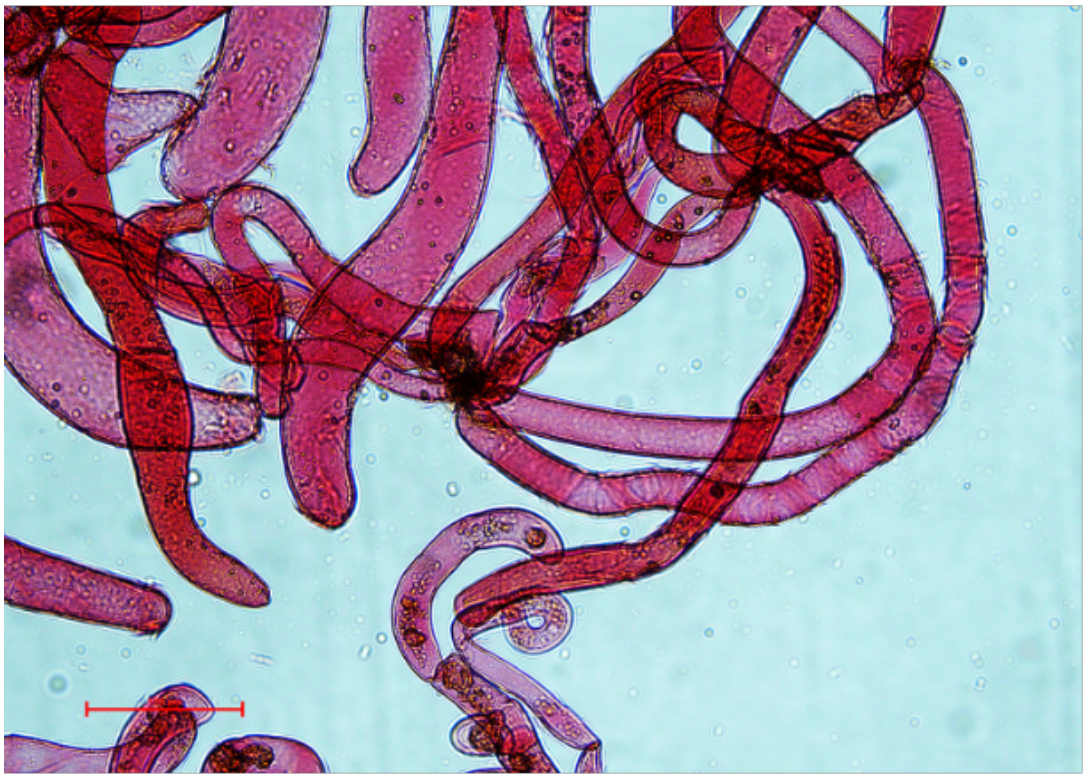
- "Naive" cells: mostly spherical cells

image.png



- Elongated cells:

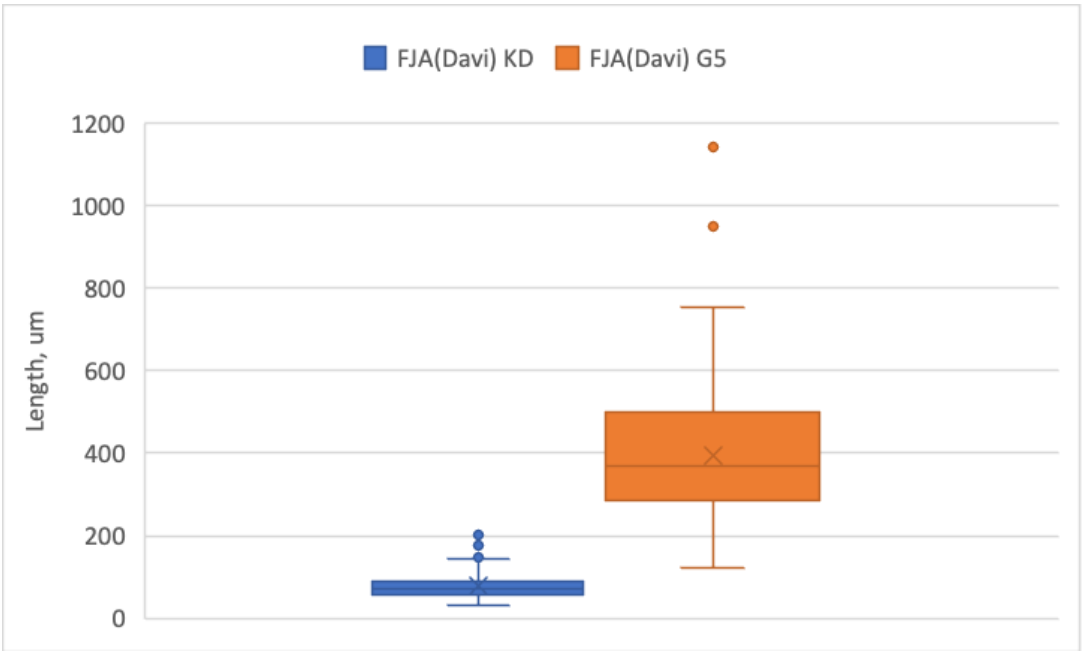
image.png



Manually measured cell lengths:
https://galy.egnyte.com/dl/ZASO8Bf2Jg

Manual Lengths Box Chart:

image.png



Manual Lengths Statistics:

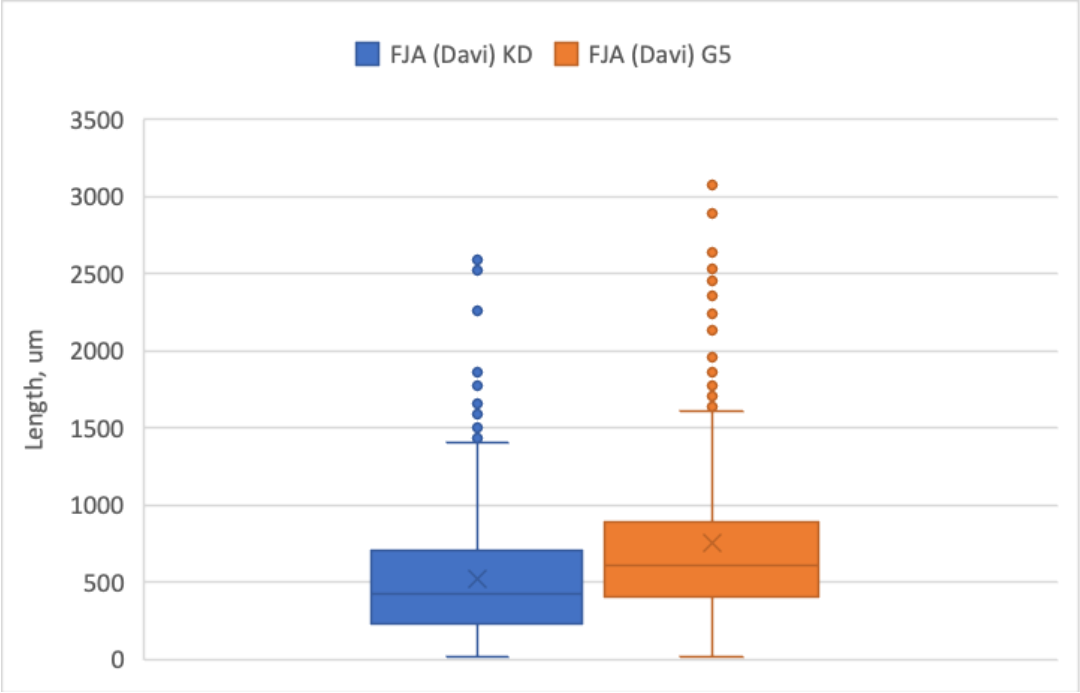
Table3

	A	B	C	D	E
1	Treatment	Mean Length, µm	St.Dev, µm	Minimum, µm	Maximum, µm
2	FJA-KD, KD	393.88	153.45	121.54	1142.14
3	FJA-KD, G5	77.63	32.3	23.79	220.57

Automatically Measured Cell Lengths:
https://galy.egnyte.com/dl/8HNhnWmRJi

Automated Lengths Box Chart:

image.png



Automated and Manual Lengths Statistics:

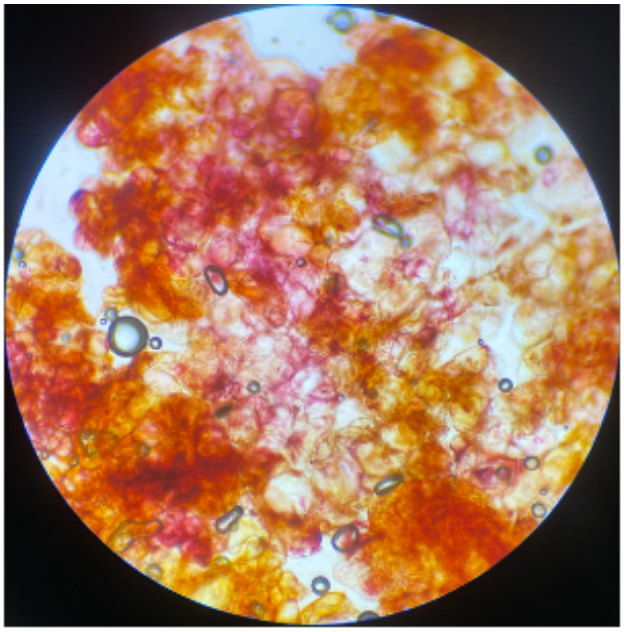
Table4

	A	B	C	D	E	F	G
1	Measurement	Treatment	Mean Length, µm	St.Dev, µm	Minimum, µm	Maximum, µm	Length increase, fold
2	Automated	FJA(Davi), KD	522.78	417.37	12.88	2598.57	1.20
3		FJA(Davi), G5	754.72	531.20	12.88	3119.64	
4	Manual	FJA(Davi), KD	77.63	32.3	23.79	220.57	5.18
5		FJA(Davi), G5	393.88	153.45	121.54	1142.14	

Staining images:

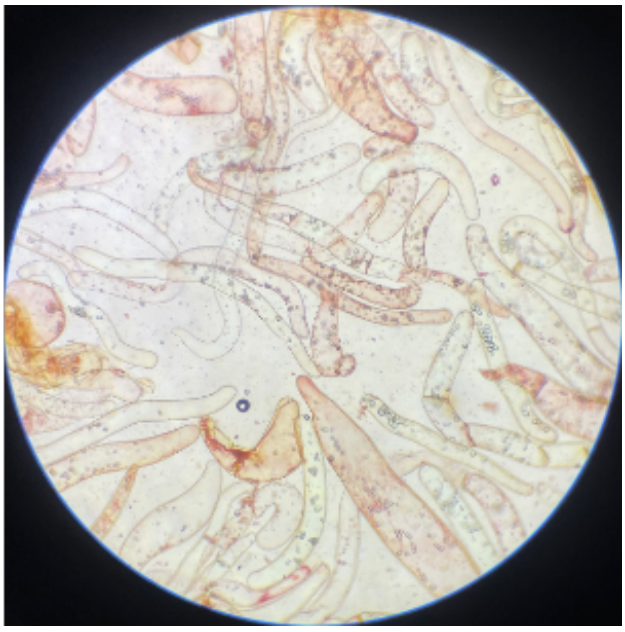
1. Safranin (lignin indicator - red for lignin):
    - "Naive" cells (positive for lignin):

image.png



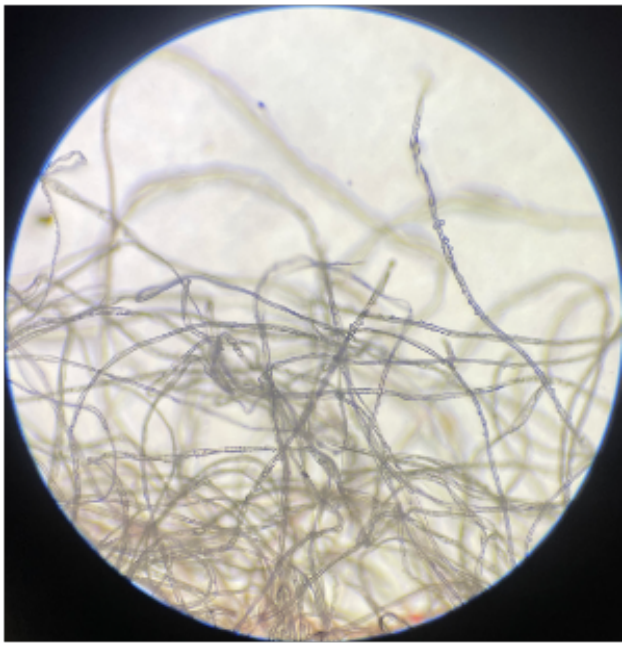
- Elongated cells (negative for lignin):

image.png



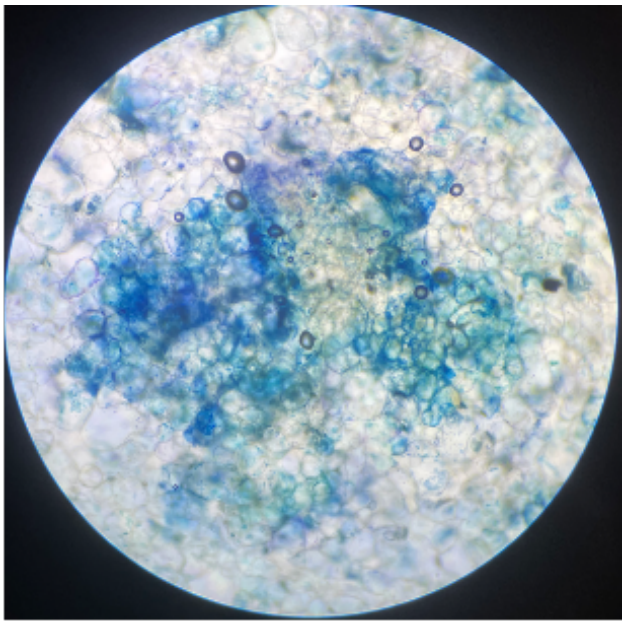
- Mature fiber (negative for lignin):

image.png



2. Toluidine Blue (acidophilic metachromatic indicator - green for lignin, pink/purple for pectin, blue for cellulose):
    - "Naive" cells (positive for lignin):

image.png



    - Elongated cells (partially positive for pectin):

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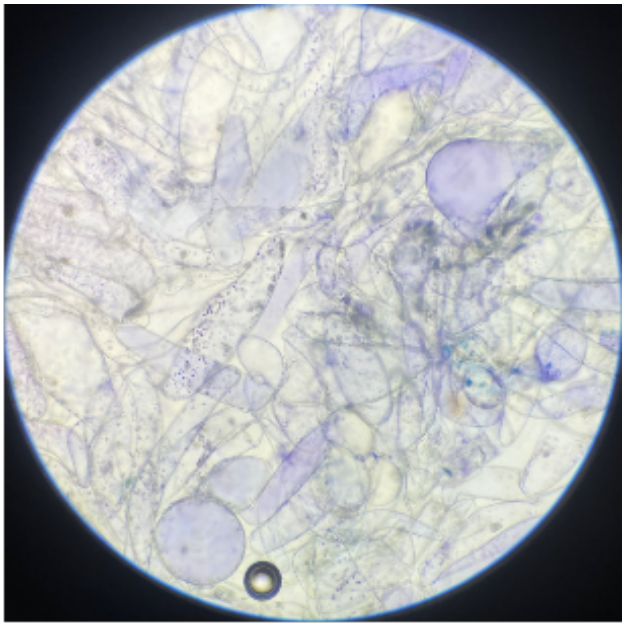

- Mature fiber (positive for cellulose):

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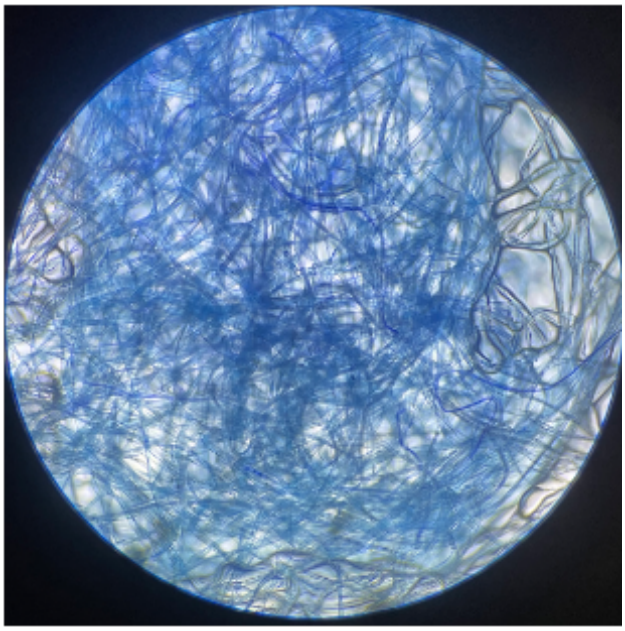
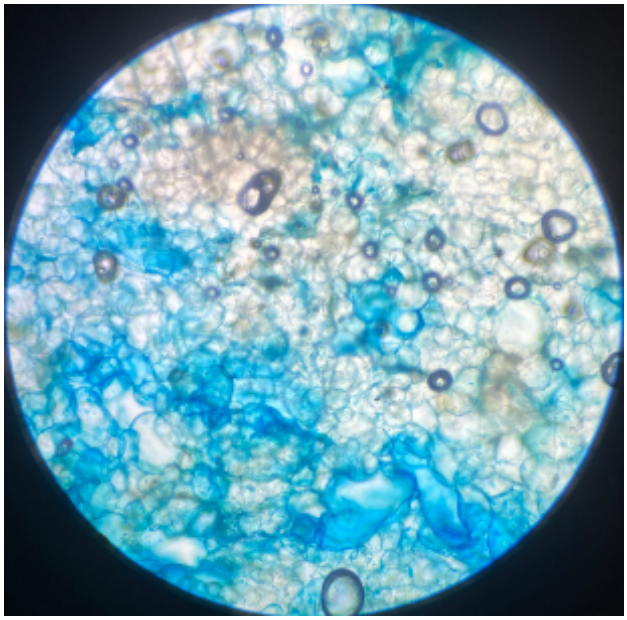


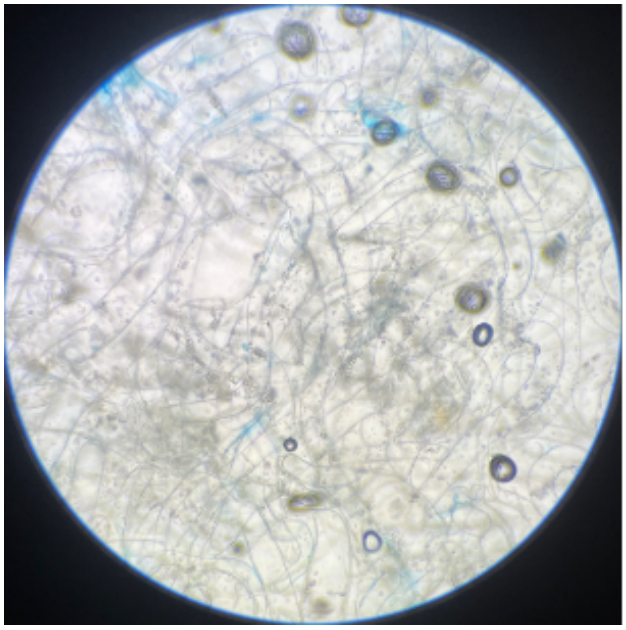
3. Astra Blue (indicator for lignin-free cellulose):
    - "Naive" cells (negative for cellulose):

image.png



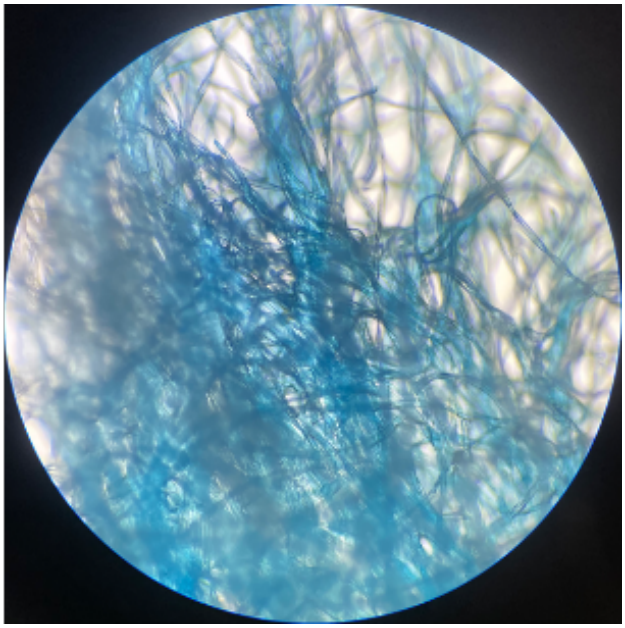
- Elongated cells (negative for cellulose):

image.png



- Mature fiber (positive for cellulose):

image.png



Vitality percentage (see Development and implementation of kinetic Resazurin vitality assay (Ideation Idea #2) **)**

image.png

SAMPLE	SLOPE FU/(mg*min)	StDev	Vitality, %	Vitality StDev, %
FJA-Davi, KD	0.580	0.048	100.0	8.2
FJA-Davi, G5 (21 d)	0.386	0.161	66.2	27.8
Blank	0.007	0.080	0.0	13.8

4. Major conclusions (Provide conclusions based on the results above).

1. FJA-Davidonis cells elongate in the presence of pro-elongation hormone: GA3 (5 uM)
2. Significant difference in elongation extent via manual (ca. 5-fold) vs automated (ca. 1.2-fold) measurements
3. Lignin in the naive cells, which dissipates in the elongated cells
4. 3-week elongation of FJA-Davidonis cells does not lead to a significant cellulose deposition (per Astra Blue staining)
5. The vitality of elongated cells (3 weeks) is only 66% of the "naive" cells

5. Describe all challenges encountered.

Some partially unclear staining results - need to optimize timing od staining and wash stringency

6. Does the original experimental plan need to be adjusted based on results to date? If so, how?

No

7. Outline the next steps (for final progress reports, include any fiber development process that resulted from this study and what the next steps are)

1. Check pH of the media and test both low (4.0) and moderate (5.5) pH for elongation
2. Evaluate BT (high potassium, high buffering capacity) media vs MS (low potassium, low buffering capacity) at low and moderate pH
3. Evaluate low (0.1 mM) moderate (1 mM) and high (3 mM) calcium
4. Evaluate low (2%), moderate (3%) and high (4%) carbon source (glucose and/or sucrose)

8. Scientist contributions(Please complete this table)

List all scientist involved in this project and extent of their contribution towards completion of the objective(s)

Table 3: Scientist role and effort towards achieving research objective

	A	B	C	D
1	Name	Title	Role on Project	% Effort /extent of contribution
2	Sergey Savinov	Senior Research Scientist	Experiment design and implementation	100%
3				
4				
5				

8. Personnel Changes (fill as needed)

Changes which occurred in the reporting period should be listed first. Planned changes should be listed next. Indicate the % effort of personnel departing with a "-" and those being added with a "+".

Table 4: Personnel changes(Fill as needed only)

	A	B	C	D
1	Name	Title	Role on Project	% Effort
2	None			
3				
4				
5				

9. Patents & Licenses

a) Has the program produced any patents, either pending or issued? **Y/N** _____

If so, please list:

Patent Applications:

Patents Issued:

b) Has the program generated any licensing, the outright sale of technology or rights, or any other monetary-based business agreements? **Y/N** _____

If so, please describe the nature of the agreement(s):