Effect of Shear Stress on Cell Health and Secondary Metabolite Production in *Taxus* Plant Cell Culture

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Abstract

Cancer is the second most common cause of death in many developed countries, making development of cancer treatments highly important. Paclitaxel is an FDA approved drug used for treatment of ovarian, breast, and lung cancer as well as Kaposi's sarcoma. Due to high demand for paclitaxel, it is important to have a robust and scalable production method. Plant cell culture (PCC) of *Taxus* species is the current state-of-the-art production of paclitaxel on an industrial scale.

One major drawback of producing paclitaxel using PCC is low and unstable yields. Thus, exploration of methods for increasing yield of paclitaxel in *Taxus* PCC is an ongoing field of research. When plant cells divide, they often form masses called aggregates that remain connected through their cell walls. Previous research in the Roberts laboratory has shown that large cellular aggregates produce less paclitaxel than smaller aggregates. We sought to investigate mechanical shearing of aggregates as a method for decreasing average aggregate size, which could be a method of increasing paclitaxel production. The three aims of this project are the following: determine how shearing affects *Taxus* secondary metabolism, including taxane, flavonoid, and phenolic biosynthesis, determine if stressors can make non-producing cell lines produce paclitaxel and design and perform tests with a novel device to implement continual shear stress on *Taxus* PCCs.

Both long-term and continuous shear increased the levels of paclitaxel production, with methyl jasmonate elicited sheared 48.82A.3s (paclitaxel producing) cell cultures accumulating the highest levels overall. Cell culture growth was not impacted by shearing in both paclitaxel producing (48.82A.3s) and non-paclitaxel producing (P093XC) cell lines for both long-term and continuous shearing, which was consistent with that observed in other *Taxus* cell lines in previous studies. Finally, global secondary metabolism (total flavonoids and phenolics content) was not impacted by long-term shear for 48.82A.3s and P093XC cell cultures.

Acknowledgement

I would like to thank my advisor Dr. Susan Roberts for giving me the opportunity to learn new skills and for her mentorship.

I would also like to thank Cassandra Brzycki for training me in the lab and aiding in experimental design.

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Chapter 1: Background

1.1 Paclitaxel

Tens of millions of people are diagnosed with cancer yearly, killing more than half of people diagnosed. Cancer is the second leading cause of death in most countries, following cardiovascular diseases. Due to significant improvements in treating and preventing cardiovascular diseases, many predict that cancer will soon take over as the number one cause of death (Ma, 2006).

Paclitaxel (generic), or Taxol®, is an FDA-approved chemotherapeutic for the treatment of ovarian, breast, and lung cancer, and Kaposi's sarcoma (Weaver, 2014). Due to the high demand for paclitaxel, it is important to have a robust and scalable production method. Initially, paclitaxel supply was met through natural extraction from the bark of *Taxus* (more commonly known as yew trees). However, there are several problems with this approach, including lack of sustainability, environmental toxicity, and inconsistent yields due to variations in growth conditions (Weaver, 2014).

Several approaches have been investigated for industrial-scale production of paclitaxel, including total chemical synthesis, semi-synthesis from harvested precursors, heterologous production in microbes and plant cell culture. Chemical synthesis is often successful for simple natural products but is difficult for more complicated natural products, such as paclitaxel, which have many chiral centers. While total chemical synthesis of paclitaxel is possible, yields are extremely low and the process uses many harsh chemicals, making it not environmentally friendly (Holton, 1994). Heterologous production is a better alternative to chemical synthesis because of its increased sustainability and established bioprocessing methods. However, heterologous production of paclitaxel is not currently possible due to incomplete knowledge of genes involved in the paclitaxel biosynthetic pathway in *Taxus* species. Even if production of paclitaxel in a heterologous host were possible, a high degree of engineering and strain optimization would likely be required to reach industrially feasible yields, due to the numerous enzymes involved in paclitaxel biosynthesis (Brzycki, 2021).

Plant cell culture (PCC) is a process that can be used to produce products that require a plant host due to chemical complexity or proper post-translational modifications. PCC lines can be created by treating a plant explant with hormones that cause it to dedifferentiate, allowing cells to be grown in both callus and suspension form. Suspension PCC can make use of bioprocessing advantages developed for microbial and other eukaryotic systems. Cells can be grown and maintained under sterile conditions in liquid media, which is referred to as "suspension" culture, or on solid media, which is referred to as "callus" culture (Furusaki, 2011). For the work presented in this thesis, cells were maintained in suspension cultures.

There are many benefits to producing certain natural products using PCC. Plant cells are the only native producers of alkaloids and anthocyanins and many of these secondary metabolites cannot be produced heterologously due to lack of genomic knowledge. For example, this method is often advantageous for production of unique and complex natural products from medicinal plants, including *Taxus* species and *Catharanthus roseus*, which produces the anti-cancer agents

vincristine and vinblastine (known as vinca alkaloids). There is also little risk of human infection when culturing plant cells, which can be a problem when using other production methods. Therefore, the current state-of-the-art industrial paclitaxel supply is production using *Taxus* plant cell culture (Furusaki, 2011).

1.2 Stress Response

One of the drawbacks to production of paclitaxel using PCC is low paclitaxel yields. When studying the production of paclitaxel, its precursors are also examined to better understand carbon flow through the biosynthetic pathway (shown in Figure 1). 10-deacetylbaccatin III (10-DAB) and baccatin III are late-stage precursors to paclitaxel and were quantified along with paclitaxel in the studies presented in this thesis.

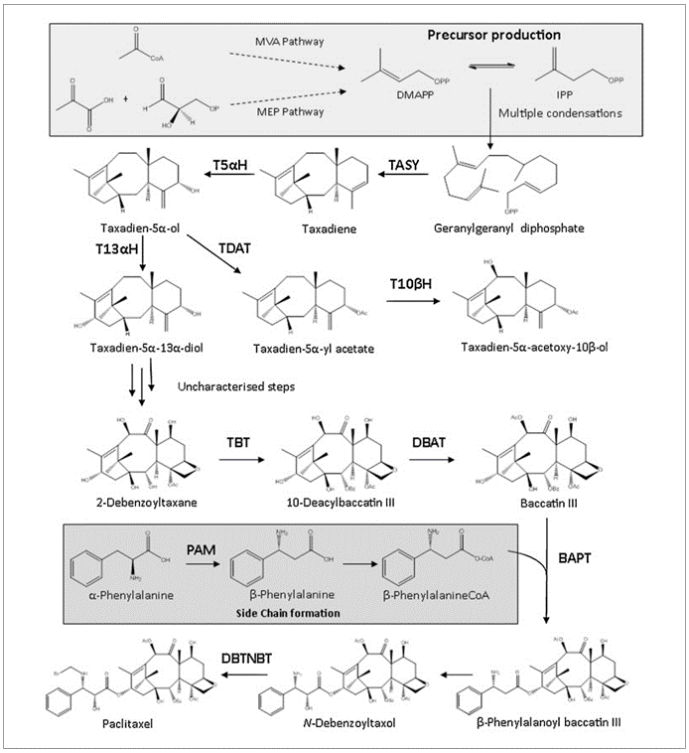


Figure 1: Paclitaxel Biosynthetic Pathway (Howat, 2019).

In nature, paclitaxel, and related precursors (known as secondary metabolites) are produced as part of an intricate defense system that protects *Taxus* from stressors in the environment (e.g., insects and animals). Secondary metabolites are not required for cell growth and are typically at their highest levels right after plant cell cultures are initiated, decreasing, and fluctuating as the plant cells mature in culture (Panche, 2020).

Environmental stressors can be mimicked in PCC through the addition of exogenous compounds (termed “elicitors”) or through mechanical stresses such as shearing (Wilson, 2020). Once

stressors are applied *in vitro* paclitaxel biosynthesis is "induced" and higher yields of taxanes are observed (Wilson, 2020).

There are many different mechanisms that can be exploited to induce a stress response (referred to as "elicitation") in plants, including application of light, heat, shearing, chemicals, and osmotic stress. Elicitation is one of the most effective and commonly used ways of increasing production of secondary metabolites. In particular, jasmonates (such as methyl jasmonate) are plant signaling hormones that are very commonly used in a variety of PCC systems to increase production of secondary metabolites (Gundlach, 1992). These hormones work by triggering a signal transduction cascade that results in activation of a defense response in the plant cells, causing upregulation of secondary metabolism, shown in Figure 2 (Halder, 2019). Methyl jasmonates often increase production of secondary metabolites by 2-8-fold, making them widely used in many different PCC systems, including *Taxus* species (Halder, 2019).

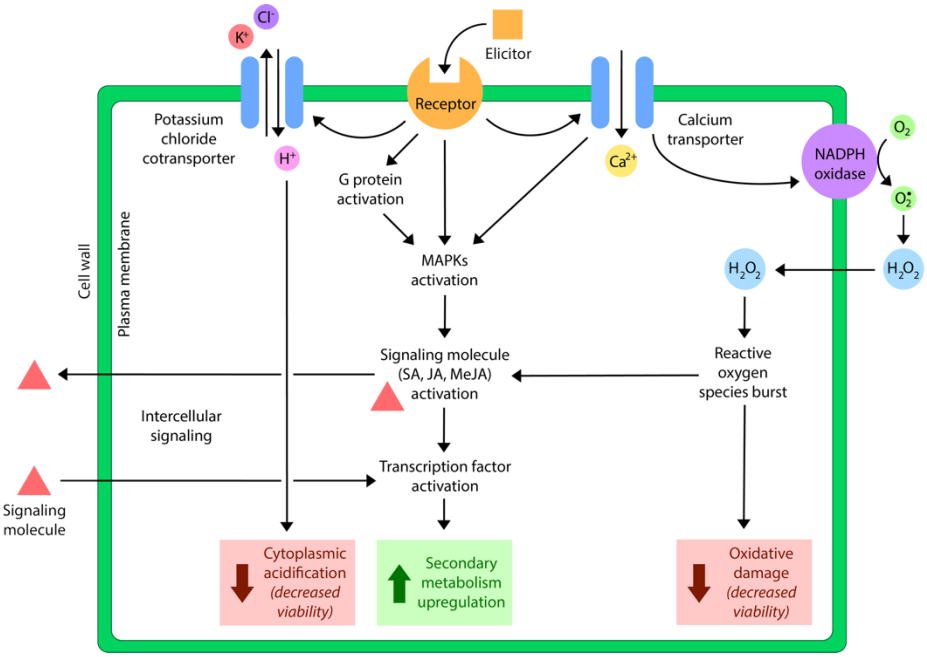


Figure 2: Signal transduction cascade that results in activation of a defense response in plant cells (Brzycki, 2021).

1.3 Shearing Cells

Cell aggregation occurs when plant cells divide improperly (Wilson, 2020). The cell wall does not fully split during division, forming aggregates that are connected by the middle lamella (Wilson, 2020). Studies have shown that smaller aggregates are associated with higher levels of paclitaxel, which can be achieved by breaking up the aggregates (Kolewe, 2010). Furthermore, smaller aggregate cultures over time are correlated with higher paclitaxel levels overall (Patil, 2012).

However, shear can have adverse effects on cell growth (Han, 2010). Alleviating the negative impact on cell growth is an important issue in bioengineering (Han, 2010). Previous studies show

that oxidative burst, nitric oxide generation, binding proteins, protein cascades, and phospholipid signaling all are involved in the response to shear stress (Han, 2010). Lipids are thought to be especially important to shear stress as they change membrane fluidity, and support many cellular processes (Han, 2010). Total phospholipid concentration was found to be decreased over a few days of shear, which could explain why shear can decrease viability (Han, 2010).

While the stress response induced by treatment with stress hormones, such as jasmonates, has been thoroughly studied in *Taxus* plant cell culture systems, other forms of stress have not been as closely investigated. There is evidence that mechanical stress induced by shearing cells could result in increased paclitaxel production because of research that shows that mechanical shearing causes smaller aggregate size (Wilson, 2020). Smaller aggregate size is proven to increase paclitaxel production, possibly due to increased shear stress inducing a stress response like the action of other elicitation mechanisms (Wilson, 2020).

Coulter-Counter analysis is robust method that can be used to differentiate plant cell aggregates based on size (Kolewe, 2010). The Coulter-Counter has some drawbacks when compared to other methods of determining aggregate size such as underestimating particle size (Kolewe, 2010). However, the relative sizes are consistent, so this makes the Coulter-Counter an accurate and reliable method to determine the relative aggregate size differences (Kolewe, 2010).

Previous data from the Roberts laboratory indicates that *Taxus* cells can be mechanically sheared by repeatedly drawing them through a serological pipette with no adverse effects on cell growth and health (Wilson, 2020). In the experiment conducted by Wilson et al, cells were sheared every 3-4 days using a serological pipette for a total of 8 generations of subculturing (112 days). By applying continuous shearing over multiple generations of subculturing, the average size of cell aggregates significantly decreased (Wilson, 2020). However, it is unknown whether this treatment increased paclitaxel production since the cell lines studied did not produce measurable levels of paclitaxel. Thus, we seek to understand the relationship between shear stress and paclitaxel production by replicating these experiments using a paclitaxel producing cell line. There is a known correlation between smaller aggregates and higher paclitaxel production (Kolewe, 2011). Based on the results of these two previous studies, it is hypothesized that continuous shearing will result in both a smaller average aggregate size and increased paclitaxel production.

Secondary metabolites in general are an important component of this research as well. Because there is not much known about the potential stress response induced by mechanical shearing, it is crucial to know if shearing induces a response in production of global secondary metabolites beyond paclitaxel and taxanes. Two important classes of secondary metabolites produced in plant species are phenolics and flavonoids. Phenolic compounds are the most pronounced secondary metabolites found in plant cells and are usually related to the defense response in plants (Lin, 2016). Flavonoids accumulate in diverse plant cell cultures, including *Taxus* (McKee, 2021). Furthermore, previous studies indicate that in some cell lines the accumulation of phenolics and flavonoids closely mirrors paclitaxel trends (McKee, 2021).

1.4 Research Aims

The primary aim for this thesis is to determine if treating *Taxus* cultures with continuous shear stress causes cells to produce increased levels of paclitaxel. Continuous shearing is hypothesized to increase paclitaxel production because it is known to result in a smaller average aggregate size, which is correlated with increased paclitaxel production. Mechanical shearing is also hypothesized to increase global secondary metabolite production because similar stressors (such as methyl jasmonate) are known to generally upregulate secondary metabolism, increasing production of compounds such as phenolics and flavonoids. Finding the long-term effects of mechanical shearing is important because it could show a consistent way to increase secondary metabolite production in PCC processes. The secondary aim for this thesis is to determine if stressors, such as shearing and methyl jasmonate elicitation, can produce paclitaxel in typically non-producing cell lines. The tertiary aim is to design and perform tests with a continual shearing device that can be used for further experimentation. To summarize, the three aims for this thesis are as follows:

Aim 1: Determine how shearing affects *Taxus* secondary metabolism, including taxane, flavonoid, and phenolic biosynthesis.

Aim 2: Determine if stressors can make non-producing cell lines produce paclitaxel.

Aim 3: Design and perform tests with a novel device to implement continual shear stress on *Taxus* PCCs.

Chapter 2: Methods

2.1 General Experimental Design

There were two main experiments performed in this thesis. The first experiment tested the long-term impacts of shearing and elicitation on paclitaxel and non-paclitaxel producing cell lines, 48.82A.3s and P093XC, respectively, as seen in Figure 3. Table 1 describes the overall protocol for this experiment and Table 2 shows the treatments applied to each cell line. This experiment was designed to determine the impact of long-term shear on viability, aggregate diameter distribution, paclitaxel concentration and global secondary metabolite concentrations. Samples for this experiment were taken on Days 0, 7, and 14 and repeated for each generation. One generation of cells is 14 days, with a new generation being propagated by subculturing cells into fresh medium. The experiments were repeated every 14 days. Therefore, Day 28 is Day 14 of the 2^{nd} culture cycle and Day 42 is Day 14 of the 3^{rd} culture cycle.

The second experiment tested the impacts of continual shearing on a paclitaxel producing cell line, 48.82A.3s, as seen in Figure 4. The overall protocol for this experiment is shown in Table 3. This experiment lasted two days to determine the impact of short-term continuous shearing. Viability, aggregate diameter distribution and paclitaxel concentration were measured. Samples were taken on Days 0, 1 and 2. On Day 0 samples were taken before continual shearing to obtain a baseline for each culture.

Note that all procedures mentioned in the overall protocols are further detailed below.

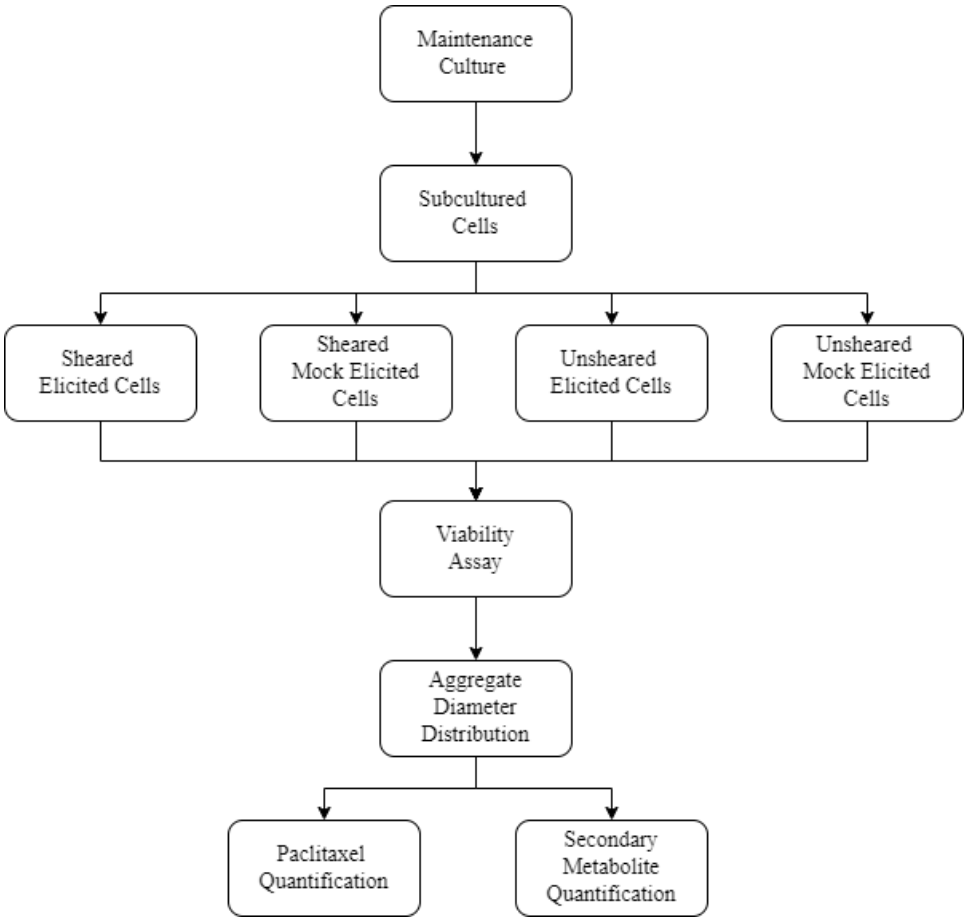


Figure 3: Overall methodology for the first experiment performed.

Table 1: Overall procedure detailing the general protocol for one generation of cells.

Days	Procedures
Day 0	Subculture cells. Shear cells 25 times with a pipette. Sample for paclitaxel, secondary metabolites, and viability, Coulter-Counter analysis to determine aggregate size distribution.
Day 4	Shear cells 10 times with a pipette.
Day 7	Shear cells 10 times with a pipette. Elicit (with 200 μM methyl jasmonate) or mock-elicit cells. Sample for paclitaxel, secondary metabolites, and viability.
Day 11	Shear cells 10 times with a pipette.
Day 14	Subculture cells. Shear cells 25 times with a pipette. Sample for paclitaxel, secondary metabolites, and viability, Coulter-Counter analysis to determine aggregate size distribution.

Note: all steps were performed sterilely to prevent contamination.

Table 2: Illustration of the four treatments applied to the 24 flasks used for this experiment. Each treatment was applied to three different flasks/cultures, representing biological replicates.

48.82A.11 Cell Line	P093X Cell Line
Sheared, MJ-Elicited	Sheared, MJ-Elicited
Sheared, Mock-Elicited	Sheared, Mock-Elicited
Not Sheared, MJ-Elicited	Not Sheared, MJ-Elicited
Not Sheared, Mock-Elicited	Not Sheared, Mock-Elicited

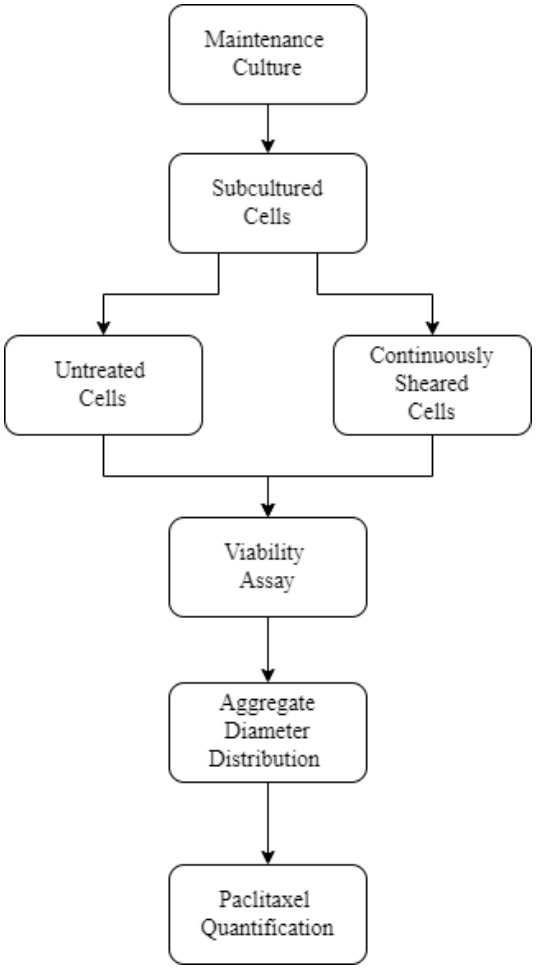


Figure 4: Overall methodology for second experiment performed.

Table 3: Overall procedure detailing general protocol.

Days	Procedures
Day 0	Subculture cells. Sample for viability, paclitaxel, and aggregate diameter distribution. Shear one flask/culture continuously for 24 hours.

Day 1	Sample for viability, paclitaxel, and aggregate diameter distribution. Shear the same flask/culture continuously for 24 hours.
Day 2	Sample for viability, paclitaxel, and aggregate diameter distribution. Discard continuously sheared flask at completion of experiment.

2.2 Shearing Cells

Cells were sheared by pipetting through a 10 mL polystyrene disposable serological pipette, ensuring that one shear had 10 mL of culture volume entering and exiting the pipette as described in Wilson et al. Cells were sheared 25 times on Days 0 and 14, before transferring or taking any samples. Cells were also sheared 10 times on Days 4, 7, and 11. They were sheared continuously for the continual shear experiment.

2.3 Subculturing Cells

48 mL of sterile antioxidant stock solution was added to 800 mL of sterile Gamborg B5 cell culture media in a laminar flow hood. The cell culture media consists of 3.21 g Gamborg B5 basal salts, 20 g/L sucrose, 2.7 µM naphthalene acetic acid, 0.1 µM benzylidene, and approximately 800 mL of nanopure water, enough to bring the total volume to 1 L (Kolewe, 2010). The media was then adjusted to pH 5.5 using KOH and HCl, then autoclaved (Kolewe, 2010). The sterile antioxidant stock solution consists of 150 mg/L citric acid, 150 mg/L ascorbic acid and 6.0 mM glutamine, which was filter sterilized (Kowele, 2010).

Using a sterile 50 mL Falcon tube, 40 mL of the media and antioxidant mixture was added to sterile 125 mL Erlenmeyer flasks with foam caps in the laminar flow hood. 10 mL of well-mixed cell suspension was then added to each flask using a serological pipette, with the tip broken off to ensure all size aggregates would be transferred. The cells were stored in an incubator at 23°C and 125 RPM and subcultured every two weeks (Kolewe, 2011).

2.4 Diameter Distribution Analysis

On Days 0 and 14, culture aggregate size distributions were measured using a Multisizer 3 Coulter Counter with a 2,000 µm aperture (Beckman Coulter), according to the previously established protocols (Kowele, 2010). Diluent made of 65:35 Isoton (1% NaCl, Beckman Coulter): Glycerol (Fisher Scientific) was vacuum filtered and reused throughout sampling (Kowele, 2010). Baseline noise was determined using only the diluent (Kowele, 2010). To analyze the cell cultures, 2 mL of well-mixed culture was collected using a cut 1 mL pipette tip and diluted into 380 mL of the diluent (Kowele, 2010). Samples were run for 60 seconds, using a flow rate of 5.1 mL/s (Kowele, 2010). The raw data from the Coulter Counter were then used to calculate mean aggregate diameter and biomass according to previously established correlations (Kowele, 2010).

2.5 Paclitaxel Extraction

On Days 0, 7 and 14, 1 mL of well mixed culture sample (cells plus media) was taken for taxane quantification, placed in a 1.5 mL microfuge tube and dried in the Eppendorf, Vacufuge plus evaporative centrifuge on the V-AQ setting until completely dry (at least six hours) (Naill, 2004). Each sample was resuspended in 0.01% acetic acid in 1 mL methanol and sonicated using a Fisher sonicating bath to further break up the dried sample for 20 minutes (Naill, 2004). To ensure that the samples were not overheated, ice was placed on top of the samples and in the sonicating bath. Samples were then centrifuged using an Eppendorf Centrifuge 5424 for 20 minutes at 15000 RPM (Naill, 2004). 800 µL of supernatant was removed and transferred to new 1.5 mL centrifuge tubes (Naill, 2004). The samples were dried again in the evaporative centrifuge on the V-AL setting until completely dry; at least 1 hour (Naill, 2004). The samples were resuspended in a 25:35:40 mixture of methanol/acetonitrile/water (Naill, 2004). The samples were filtered using a 0.22 µM PVDF filter and 1.0 mL syringe into a low-volume UPLC vial and then ran on a Waters Acquity UPLC H-Class system using a previously established protocol (Patil, 2012).

2.6 Elicitation with Methyl Jasmonate

On Day 7, the cells were elicited with methyl jasmonate or mock-elicited. The mock elicitation stock solution was prepared in a 1.5 mL centrifuge tube by combining 500 µL of ethyl alcohol with 500 µL of nanopure water. The methyl jasmonate stock solution was prepared in a 1.5 mL centrifuge tube by combining 42.1 µL of methyl jasmonate (95% Sigma Aldrich) with 457.9 µL of ethyl alcohol and 500 µL nanopure water. Both sterile solutions were filtered in the laminar flow hood using an autoclaved 0.22 µM PVDF filter and a 1.0 mL syringe into a sterile 1.5 µL centrifuge tube. A 200 µM elicitation was done by adding 56.6 µL of the appropriate solution to the flasks of cells (Patil, 2014).

2.7 Secondary Metabolites

Samples for secondary metabolites were taken on Days 0, 7 and 14 by taking a 1 mL well-mixed sample from the cells. To analyze phenolics, gallic acid standards were first made at differing concentrations; 0.20 mg/mL, 0.15 mg/mL, 0.10 mg/mL, 0.0075 mg/mL, 0.05 mg/mL, 0.025mg/mL, and 0 mg/mL (McKee, 2021). Then, 20 µL of the sample, 40 µL of 0.2N FC Reagent, and 160 µL of 700 mM sodium carbonate were added to a tube and allowed to incubate for 10 minutes (McKee, 2021). The tubes were spun in a vortex for 60 seconds at 16,000 RPM (McKee, 2021). 200 µL of the supernatant was transferred in triplicate to a 96 well plate and the absorbance was measured at 750 nm using a plate reader (McKee, 2021).

To analyze flavonoids, catechin standards were made at differing concentrations; 1.0 mg/mL, 0.8 mg/mL, 0.6 mg/mL, 0.4 mg/mL, 0.2 mg/mL, 0.1 mg/mL, and 0 mg/mL (Mckee, 2021). The following was added to well plates; 25 µL sample, 50 µL water, and 75 µL of $NaNO_2$ at a

concentration of 6 g/L (McKee, 2021). The samples were incubated for 30 seconds. Next, 75 µL of $AlCl_3$ (22 g/L $AlCl_3 \cdot 6H_20$) was added and incubated for 2 minutes (McKee, 2021). 75 µL of 0.8M NaOH was added (McKee, 2021). Finally, 200 µL of the supernatant was transferred in triplicate to a 96 well plate and the absorbance was measured at 490 nm using a plate reader (McKee, 2021).

2.8 Resazurin Viability Assay

When working with stressors, it is important to ensure that plant cell health is not negatively impacted. Plant cell viability can be analyzed using viability stains, such as propidium iodide (PI) and fluorescein diacetate (FDA), or simply through visual inspection. Healthy plant cells in suspension have several important characteristics: a light-yellow color, dense looking samples, no discoloration, no large clumps of cells, and no mold. When cells look the way that was previously described, they are often thought to be healthy. Plant cell health can be analyzed through cell viability assays. In this experiment, cell viability was tested using a resazurin assay. Resazurin assays detect cellular metabolic activity (Resazurin Assay Kit, 2021).

On Days 0, 7, and 14, 1 mL well-mixed samples were taken from the flasks to measure cell viability. First, the supernatant was removed and disposed of (Resazurin Assay Kit, 2021). 900 µL of PBS and 100 µL of resazurin were added to the samples, which was a modification from the used protocol (Resazurin Assay Kit, 2021). The samples were inverted so they were well mixed (Resazurin Assay Kit, 2021). Then, the samples were incubated at room temperature in a dark room for an hour, which was another modification from the original protocol (Resazurin Assay Kit, 2021). 100 µL of the samples were pipetted into a black 96 well plate in triplicates (Resazurin Assay Kit, 2021). Finally, the fluorescence was read at 545-590 nanometers (Resazurin Assay Kit, 2021).

2.9 Design of Continual Shear Device

The design of this device was based on that of a continuous shear device for mammalian cells (Zhang, 2014), with design and machining by Ian Anderson, machinist in the Department of Chemical Engineering. In both manual and continual shearing, one shear is defined as 10 mL of cell culture volume pipetted up and down using a serological pipette tip (Wilson, 2020). A flow rate of 7.3 mL/s was used for the shear device as this was found to have the best disaggregation efficiency in previous studies (Wilson, 2020).

This device will allow for cells to be sheared continuously, as opposed to several times throughout the week. The impact of continual shearing on cell heath, aggregate size, and paclitaxel production will be compared to the intermittent shearing model. Because the cell cultures are put under constant stress in a continual shearing device, it is hypothesized that aggregate size will dramatically and quickly decrease, and paclitaxel levels will increase. The

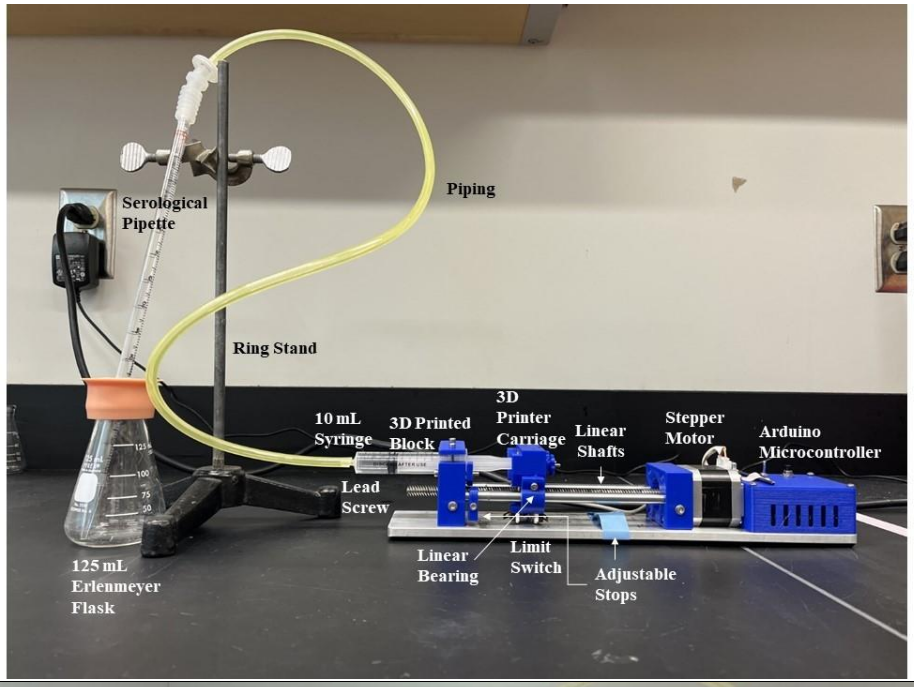
potential use of such a device at the end of a bioprocess run may be effective in increasing overall paclitaxel yields. In addition, the optimization of such a device can be used to strictly control aggregate size, which may be beneficial to overall product yields. To our knowledge, a continual shearing device has never been designed for plant cell culture.

Table 4 shows the list of equipment required to build the device and Figure 5 shows the labeled built device. This device uses an Arduino microcontroller as its control unit. It is programmed to accept inputs from its limit switches to correspond to its output for the stepper motor. The stepper motor is connected to a lead screw that drives the carriage containing the syringe plunger. As the motor turns, the carriage is driven in a linear direction, which in turn either depresses or retracts the syringe plunger. When one of the limit switches comes in contact with the adjustable stops, the Arduino is signaled to reverse direction. This allows for the device to run continuously without needing user input. The adjustable stops are adjustable to allow for a set amount of liquid to be moved in each stroke of the syringe plunger. In this experiment, 10 mL of cell media and aggregates were used to replicate one shear iteration. Piping is used to connect the syringe to the serological pipette to translate the flow of media and aggregates. Additionally, linear shafts and bearings are used to create a smooth, linear motion for the carriage.

Table 4: List of equipment required for continual shear device.

Equipment	Recommendations
Erlenmeyer Flask	Sterilize before use. 125 mL in size.
Foam Cap	Sterilize before use. Must fit on top of Erlenmeyer flask. Must have a hole in it to seal serological pipette tip.
Serological Pipette Tip	Use serological pipette tip that comes in sterile packaging. Inner diameter of 1.6 mm.
Ring stand	Must around the serological pipette tip to hold it up straight.
Piping	Use piping that has been sterilized. 0.9525 cm inner diameter.
Syringe	Must hold 10 mL.
3D Printed Block	Must fit around syringe.
3D Printed Carriage	
Linear Shafts	
Limit Switch	
Linear Bearing	
Adjustable Stops	Can be adjusted to ensure the appropriate amount of liquid is being injected.
Stepper Motor	
Arduino Microcontroller	Have programmed to know to accept inputs from limit switches.

(a)



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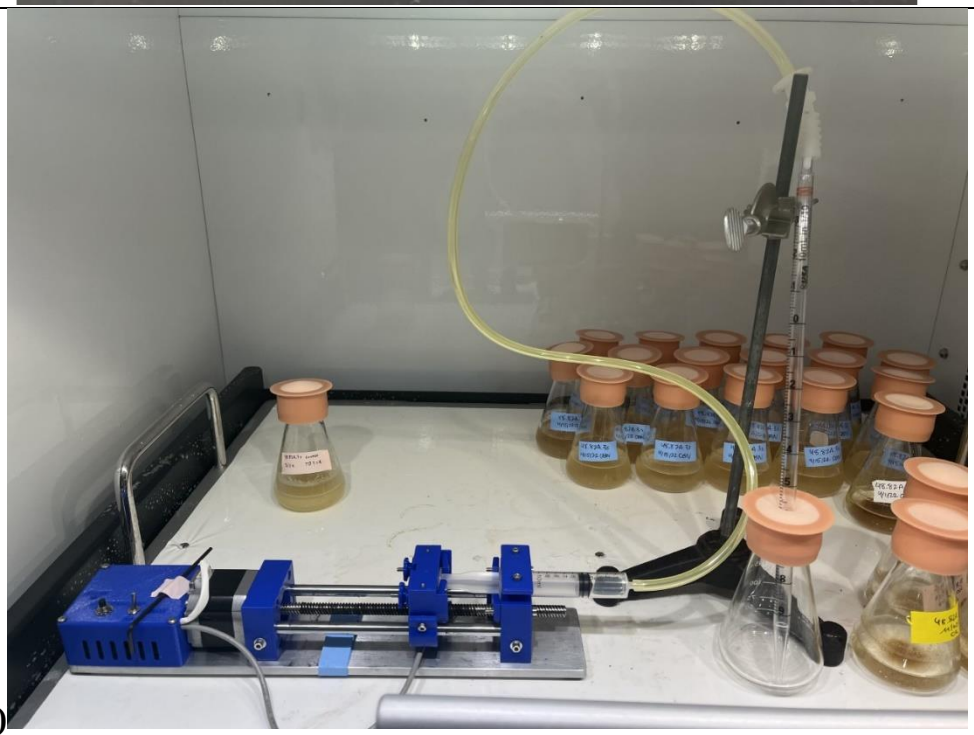


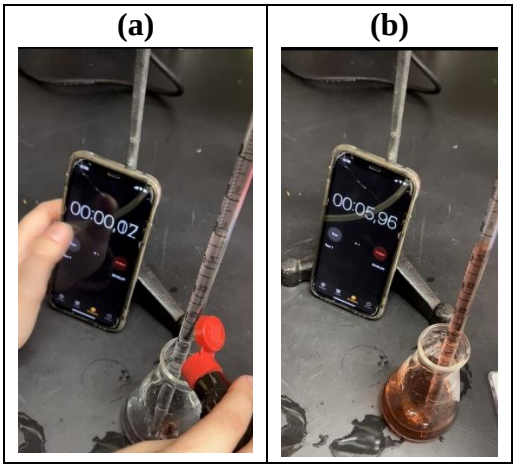
Figure 5: Continuous shearing device.

2.10 Use of Continual Shear Device

This device was used in short-term experiments to determine if paclitaxel production (and aggregate size) could be optimized more quickly and reliably than in the long-term experiments where intermittent shearing was performed.

The device was kept in the incubator (in the dark at 23°C shaking at 125 RPM) to ensure that the cells would be kept under proper growth conditions. All cell cultures were transferred into autoclaved flasks, with autoclaved caps using sterile media. Cells were sheared continuously in the incubator before all samples were taken. After samples were taken, the serological pipette tip was removed and replaced with a new one to ensure sterility. The syringe was also replaced daily to ensure that there was no buildup on the linear shaft.

Another consideration of the shearing device was how quickly the shearing device would be able to fully mix a sample. To estimate this, a drop of red food dye put into a 125 mL Erlenmeyer flask which was filled with 50 mL of water and a video was taken to capture the mixing of the food dye. Photograph 1 a shows the initial time point of the drop touching the water. Based on this photograph, it is difficult to tell whether the time was 0.07 seconds or 0.12 seconds. Therefore, it was estimated that the drop of red food dye touched the water at 0.095 seconds. Photograph 1 b shows the well mixed food dye and water solution. The solution was considered well mixed when the red food dye was no longer distinguishable from the water, which occurred at 5.96 seconds. Therefore, it took about 5.865 seconds for the sample to be fully mixed. Based on these results, it is likely that the cell cultures would also be quickly mixed. Therefore, after 24 hours of testing, the cell cultures would have been fully mixed.



Photograph 1: Estimation of Complete Mixing of Shearing Device

The final consideration of the device was how much shear stress would be applied. To calculate this the equation for shear stress of fluids was used: $\tau(y) = \mu\frac{du}{dy}$, where τ is the shear stress of a fluid, μ is the dynamic viscosity, u is the velocity of the flow and y is the height above the

boundary (Shear stress equation clearly explained - average, beam, impact, fluids shear stress). The viscosity of cell culture is unknown, so it was estimated using the following formula: $\mu = \frac{2\,(\rho\ aggregate - \rho\ media)gr^2}{9v}$ (Banas, 2020). A range of aggregate densities were used based on the biomass calculations done by the Coulter-Counter correlation to density (Kolewe, 2010). The radius used was calculated based on the range of average aggregate diameters shown in Chapters 3.2 and 3.7. The media was estimated to have the same density as water because water is the largest component of the media. The shear stress could range from $1.0 \times 10^{-4}\ {}^{N}/_{cm^2}$ to $1.38 \times 10^{-4}\ {}^{N}/_{cm^2}$.

2.11 Constraints of the Continual Shear Device

There are a few constraints with the continual shear device. First, the device can only shear one flask/cell culture at a time. Typically, three biological replicates are used experimentally, taking three technical replicates from each, which leads to nine total samples. In this case, there was only one flask being sheared, with three technical replicates for analysis. This design decreases the accuracy of experimentation and statistical validation. Furthermore, there because samples are not taken at the same time this could lead to batch-to-batch variability. While experimentation begins on Day 0 for all three replicate experiments, there are other factors that could contribute to variability. For example, contamination in the incubator could also contaminate the cell cultures being tested, which was hypothesized to occur in Figure 16 a. The contamination in the incubator caused the variability of all samples to be low when compared to the viability of the samples on other testing days. The aggregates could also get stuck in the pipette tip, which would block shearing for all aggregates. While this did not occur in experiment, this would be a large cause of variability as some aggregates would not be sheared for many hours. Additional devices should be constructed to enable biological replicates when evaluating shear.

Due to the simplicity of this machine's design, it can be modified to fit multiple form factors. This can be done by modifying the dimensions of the syringe used as well as the pitch of the thread on the lead screw. For example, both changes can be used to make the overall length of the machine shorter while still injecting the same volume of liquid into the serological pipette. Since an Arduino microcontroller is used to control the device, one device can control multiple pipette tips. To accomplish this, the device carriage can be modified to hold multiple syringes. Each of those syringes can then be piped to their respective pipettes. In this case, every pipette would be injected at the same time.

Another constraint is that cell aggregates can get stuck in the pipette tip, which blocks any other aggregates from being sheared. During the long-term experiments, this is something that happened but applying more pressure on the syringe quickly ejects the aggregates and eliminates

the problem. The continual shear device is not being constantly monitored; therefore, it is difficult to know at what point in the experiment shear had been stopped. In this experiment, the blockage happened while running preliminary testing, not while taking samples. However, the lack of shear for 24 hours would invalidate that round of testing.

One way to alleviate this issue is to introduce another sensor and actuator to the device. The sensor can be introduced as an infrared (IR) sensor positioned to look into the serological pipette. The machine can then be programmed to use the IR sensor to detect when no liquid has been injected into the serological pipette for a certain amount of time. The actuator can be input as a small air pump that is also piped into the serological pipette. When the IR sensor detects no liquid in the pipette, the machine can then turn on the air pump to flush aggregate from the serological pipette.

The final constraint with the shearing device is that not all parts were sterile. For future experimentation, the parts should be 3D printed using high heat resistant material so they can withstand the heat of the autoclave. All sterile parts were specified in Table 5. If some of these parts are not available sterilized, they can be 3D printed to ensure sterility.

Chapter 3: Determining How Shear Affects *Taxus* Secondary Metabolism

The results in this chapter are compared to two prior sets of experiments performed. The first set of experiments studied how aggregate size in *Taxus* can be controlled through manual shearing in a non-paclitaxel producing cell line (Wilson, 2020). This study aimed to understand if aggregation could be controlled with targeted shearing without negative effects on cell growth and viability. Results demonstrated that long-term shearing (weekly) does not negatively affect growth or viability (Wilson, 2020). A number of non-paclitaxel producing cell lines (P093X, P093XC, P991C, P93AF, P93AFC, and C093D) were evaluated; shearing effects on secondary metabolism and in the presence of elicitation with methyl jasmonate (MJ) were not assessed. The second study focused on the relationship between long-term shear, MJ-elicitation, and secondary metabolism (Dinicu, 2021). These experiments aimed to find the impact of long-term shear on secondary metabolism of a paclitaxel producing cell line, 48.82A.11 (Dinicu, 2021). The experiments presented in this thesis aimed to extend these studies in both the analysis of a paclitaxel-producing cell line and the establishment of an automated shearing device.

This chapter contains data collected throughout the duration of the two primary experiments described in the Methods chapter. First, the long-term impacts of shear on a paclitaxel-producing, 48.82A.3s, and non-paclitaxel producing cell line, P093XC, were studied. On Day 0 of the long-term shear experiment cell cultures were subcultured, sheared with a pipette 25 times, then samples were taken. On Day 4, cell cultures were sheared with a pipette 10 times. On Day 7, cell cultures were sheared 10 times with a pipette, MJ-elicited with 200 μM MJ or mock-elicited, and then sampled. On Day 11, cell cultures were sheared 10 times with a pipette. On Day 14, cell cultures were subcultured, sheared 25 times with a pipette, and then sampled. The experiments were repeated every 14 days. Therefore, Day 28 is Day 14 of the 2nd culture cycle and Day 42 is Day 14 of the 3rd culture cycle.

The second experiment tested the impact of continual shearing on a paclitaxel producing cell line, 48.82A.3s. On Day 0, the cell cultures were sampled, then one flask was continuously sheared for 24 hours, and the other cell cultures were left untreated as controls. On Day 1, samples were taken, and the continuously sheared cell cultures were sheared for another 24 hours. On the final day of experimentation, Day 2, samples were taken, and continuously sheared cell cultures were discarded.

The long-term shear experiment studied the impact of long-term shear on viability, aggregate diameter distribution, cell growth, paclitaxel production, and general secondary metabolite production (Chapters 4.1 - 4.5). The continual shearing experiments examined the impact of shear on viability, aggregate diameter distribution, cell growth and paclitaxel production (Chapters 4.6 - 4.9).

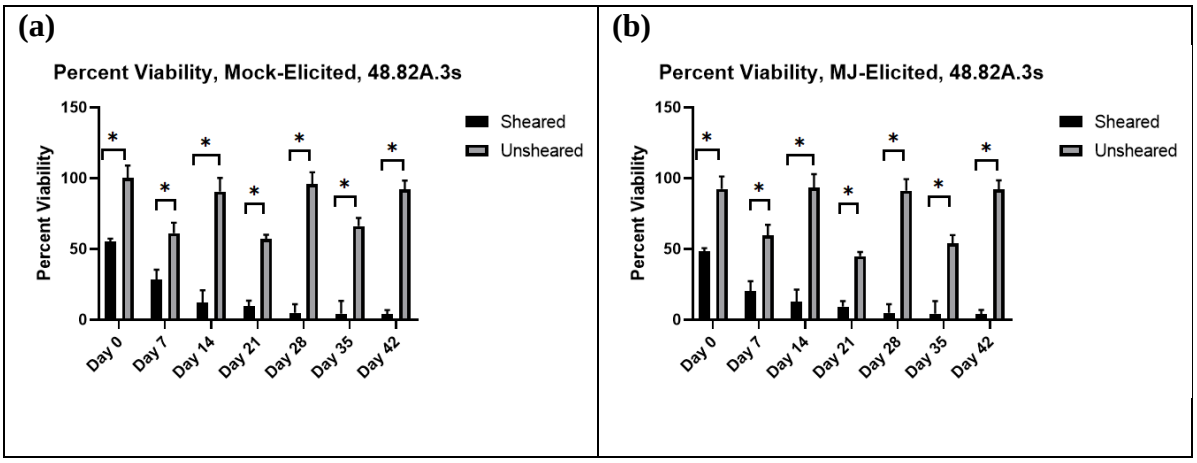
3.1 Effect of Long-Term Shear on Cell Viability

The effect of long-term shear on cell viability and health was studied using the resazurin viability assay. Based on previous studies, it was expected that long-term shear would not negatively impact cell health (Wilson, 2020).

Figure 6 shows the relationship between cell viability and long-term shear. Viability in unsheared cell cultures of 48.82A.3s is significantly higher than sheared cell cultures in all cases (Figure 6 a and b). However, the sheared cultures in this cell line had been subcultured using contaminated media, which was unknown while running the experiment. After Day 42, the sheared cells turned pink, which is very unusual and a clear sign of contamination and additional stress.

As seen in Figure 6 c and d, there were no detrimental effects of shearing on cell viability in P093XC cell cultures, which confirms data collected on other *Taxus* species and cell lines (Wilson, 2020).

The impact of MJ elicitation and shear on paclitaxel-producing lines had not previously been studied. Data show that mock-elicited, and MJ-elicited cell lines behaved similarly, which demonstrates that elicitation does not have a detrimental effect on cell viability and health in the presence of additional shear stress. Furthermore, these data suggest that 48.82A.3s cell cultures may be more sensitive to shear than P093XC cell cultures. However, because of the potential issue with contamination, additional experiments should be performed with the 48.82A.3s cell line before coming to this conclusion. As seen in previous work, the P093XC cell line is not greatly impacted by shear (Wilson, 2020).



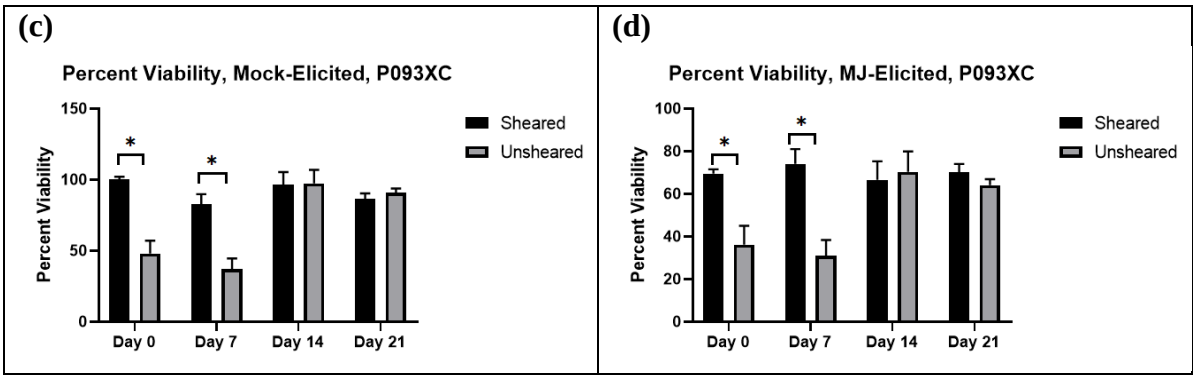
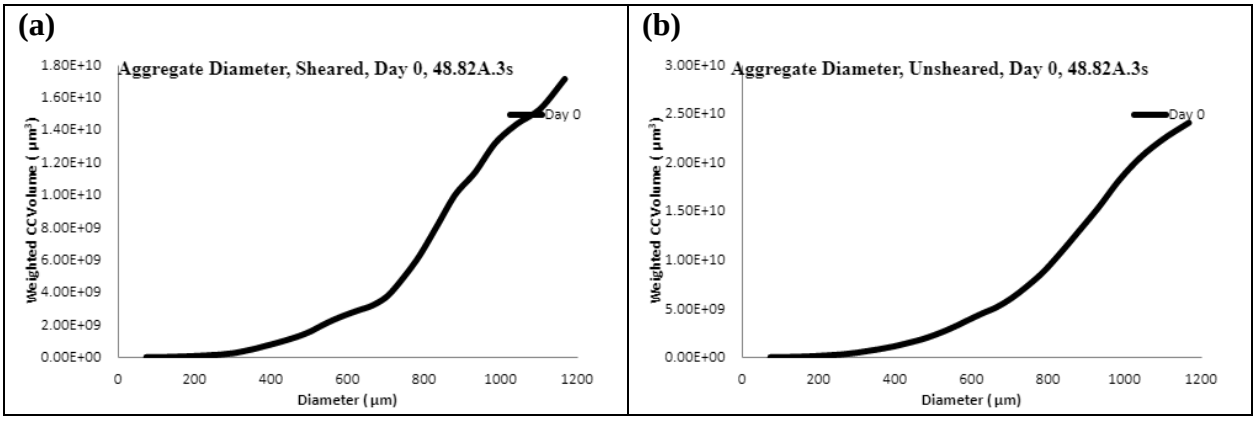


Figure 6

The impact of long-term shear on viability in two *Taxus* cell lines. Sheared 48.82A.3s cell cultures (a) and (b) had a significantly lower viability than the unsheared cell cultures throughout the 42 days of testing. Sheared P093XC cell cultures (c) and (d) had the same (or higher viability) than the unsheared cell cultures. Data represent the average of three biological replicates with standard deviation. A paired t-test was used to determine if the data were statistically significant using a confidence level of 95%. * represents data that are statistically significant.

3.2 Effect of Long-Term Shear on Aggregate Diameter Distribution

The impact of long-term shear on aggregate diameter distribution was studied. Overall, it was expected that long-term shear would decrease aggregate diameter distribution (Dinicu, 2021; Wilson, 2020). Figure 7 a and c show the impact of long-term shear on the 48.82A.3s and P093XC cell lines, respectively. All samples were subcultured and sheared prior to measuring the aggregate diameter distribution. At this point in the culture cycle cells had only been sheared 25 times via pipette, which is why there is not a drastic difference when compared to the aggregate diameter distribution of unsheared cells (Figure 7 b and d). The average aggregate diameters were very similar for all samples; for sheared 48.82A.3s cell cultures it was 967 ± 28 µm (Figure 7 a); for unsheared 48.82A.3s cell cultures it was 975 ± 36 µm (Figure 7 b); for sheared P093XC cell cultures it was 898 ± 42 µm (Figure 7 c); and for unsheared P093XC cell cultures it was 982 ± 39 µm (Figure 7 d).



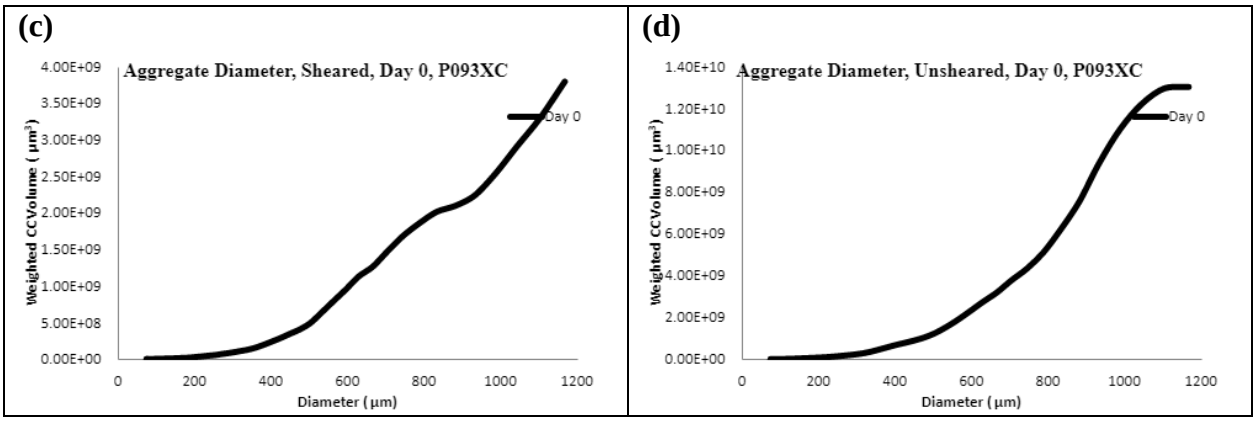
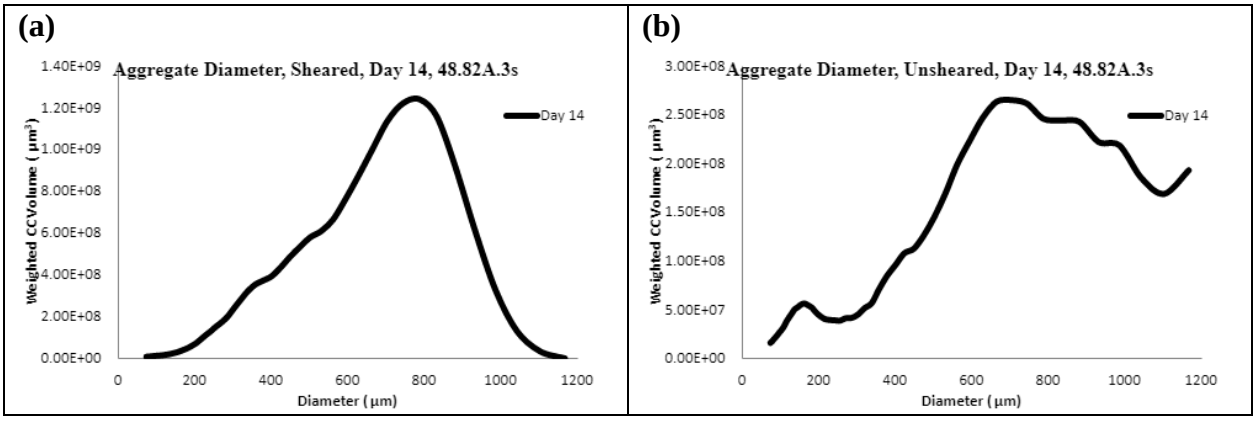


Figure 7

Aggregate diameter distribution on Day 0. On Day 0, cell cultures were subcultured, then sheared 25 times, then sampled for aggregate diameter distribution measurements. There were no major differences in the aggregate diameter distribution when comparing different cell lines and sheared versus unsheared cultures. Six biological replicates were taken to find the average diameter.

Figure 8 examines the relationship between long-term shear and aggregate diameter distribution on Day 14 of experimentation (last day of the 1st culture cycle). Before sampling for aggregate diameter distribution, cell cultures were subcultured and sheared as described in the Methods section. Figure 8 a and b shows the difference between aggregate diameter distribution for 48.82A.3s cells when long-term shear is involved. Sheared 48.82A.3s cell cultures have an average aggregate diameter of 764 ± 62 µm (Figure 8 a), while unsheared 48.82A.3s cell cultures have an aggregate diameter of 893 ± 57 µm (Figure 8 b). The sheared cell cultures have a smaller average diameter when compared to the unsheared cell cultures, which was expected from previous work (Dinicu, 2021; Wilson, 2020). Figure 8 c and d shows a similar relationship between sheared and unsheared cell cultures of P093XC. The average aggregate diameter in Figure 8 c was 794 ± 31 µm, while the average aggregate diameter for unsheared P093XC cell cultures was 997 ± 79 µm (Figure 8 d). Both the 48.82A.3s and P093XC cultures confirm the relationship that shearing creates smaller aggregate diameter distributions (Dinicu, 2021; Wilson, 2020).



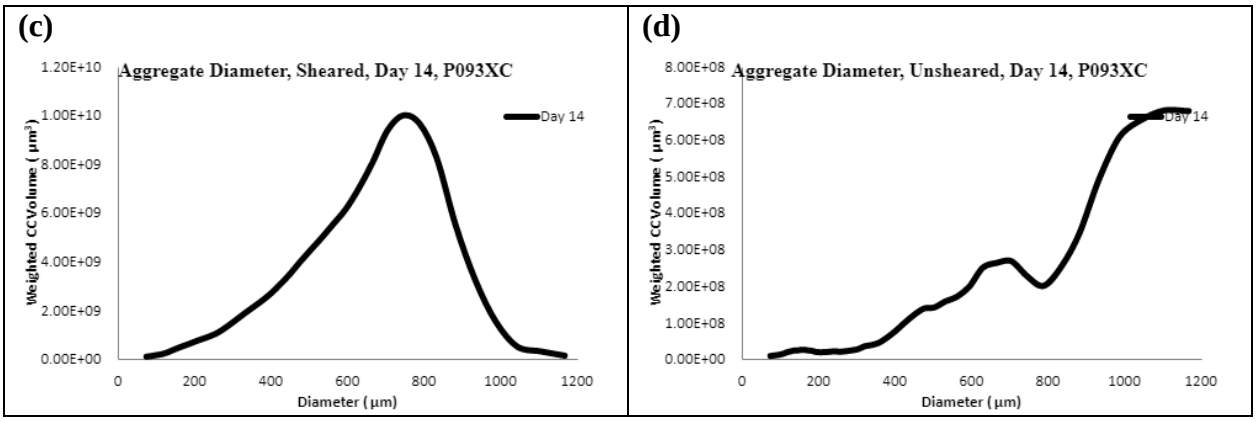


Figure 8

Aggregate diameter distribution on Day 14. The aggregate diameter distribution decreased in 48.82A.3s (a and b) and P093XC (c and d). Six biological replicates were taken to find the average diameter.

Figure 9 shows the aggregate diameter distribution for sheared and unsheared 48.82A.3s cell cultures on Day 28 (day 14 of the 2nd culture cycle) and displays similar trends as observed on Day 14 in culture (Figure 8), with shearing decreasing aggregate diameter distribution. The average aggregate diameter for sheared 48.82A.3s cell cultures was 584 ± 62 µm (Figure 9 a), while the average aggregate diameter distribution for unsheared 48.82A.3s cell cultures was 837 ± 81 µm (Figure 9 b). These data solidify the hypothesis that shearing decreases mean aggregate size (Dinicu 2021; Wilson, 2020).

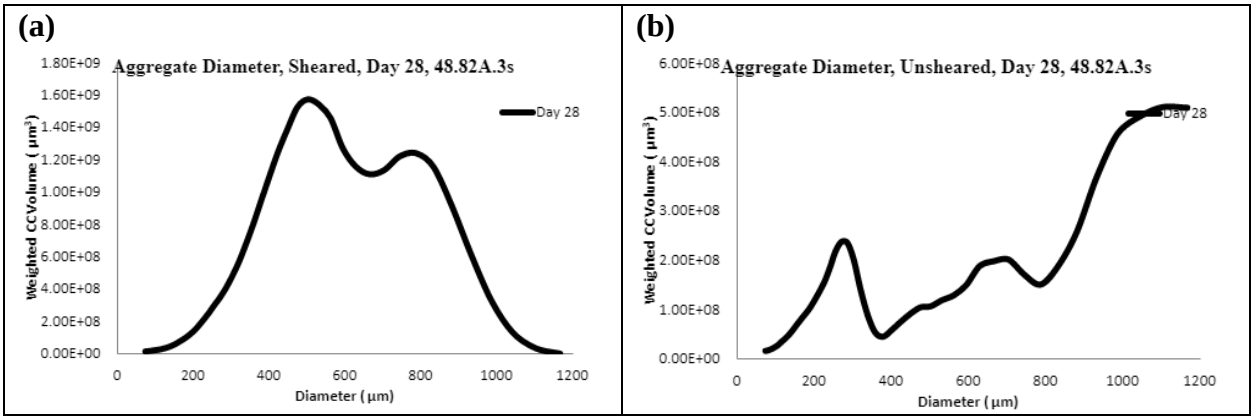


Figure 9

Aggregate diameter distribution on Day 28. There was a large difference in the mean aggregate size for sheared versus unsheared cells of 48.82A.3s. Six biological replicates were taken to find the average diameter.

Aggregation data were only collected through day 28 of culture due to complications with the Coulter-Counter.

3.3 Effect of Long-Term Shear on Cell Growth

Long-term shear has been found to have no impact on cell growth in previous studies (Wilson, 2020). To calculate cell growth, an established correlation between Coulter-Counter volume (mL/L) and biomass (g/L) was used, where Coulter-Counter volume = 2.5*(biomass) (Kolewe, 2010). Cultures should increase in biomass density during a generation of cell growth (Wilson, 2020). These data show cell growth is not impacted by long-term shear in both 48.82A.3s and P093XC cell cultures (Figure 10) as the biomass increases over one generation of cell culture. While there is a slight biomass drop on Day 28 (Figure 10 a and b), this has also been observed in other studies (Wilson, 2020) and is likely due to the subculturing phenomenon.

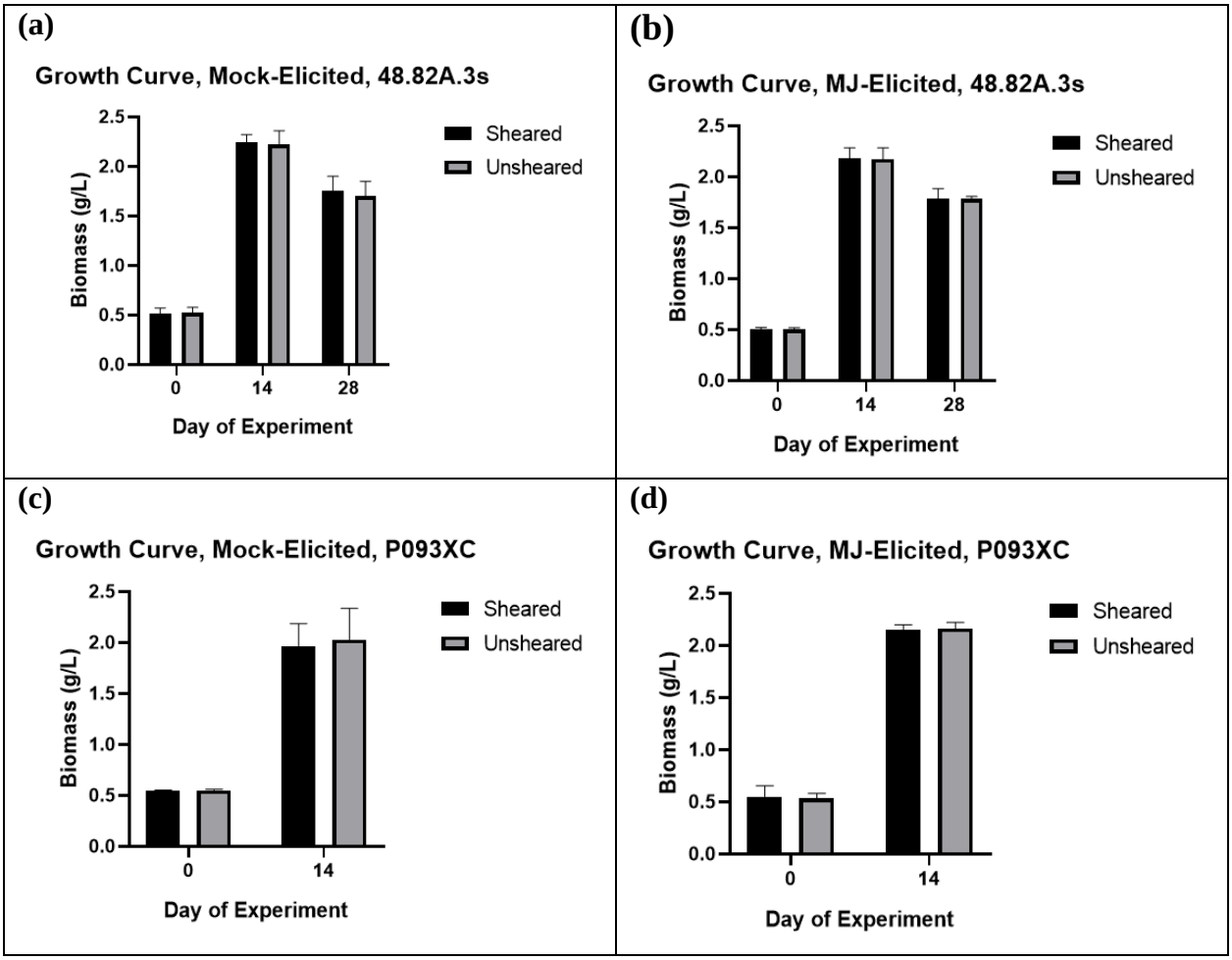


Figure 10

Long-term shear did not impact cell growth for 48.82A.3s and P093XC cell cultures. Data represent the average of three biological replicates with standard deviation shown. A paired t-test was used to determine if the data were statistically significant using a confidence level of 95%. * represents data that are statistically significant.

3.4 Effect of Long-Term Shear on Production of Paclitaxel and Related Precursors

The impact of long-term shear on 10-DAB, baccatin III, and paclitaxel accumulation was also examined. While the biosynthesis of paclitaxel is a multistep process, one important reaction is the acetylation of 10-DAB to baccatin III, a direct precursor to paclitaxel (Zocher, 1996). Because of this established relationship, it is hypothesized that 10-DAB and baccatin III will increase as an initial stress response, and then decrease as paclitaxel is produced and the two precursors are consumed.

As seen in Figure 11 a and b, long-term shear increased 10-DAB concentration in both mock-elicited and MJ-elicited 48.82A.3s cells.10-DAB production in P093XC cell cultures was not impacted by shear (Figure 11 c and d). It is possible that some of the 10-DAB was consumed to produce more paclitaxel, and then more 10-DAB was produced as the cell cultures were being stressed over time, resulting in unchanged observable levels.

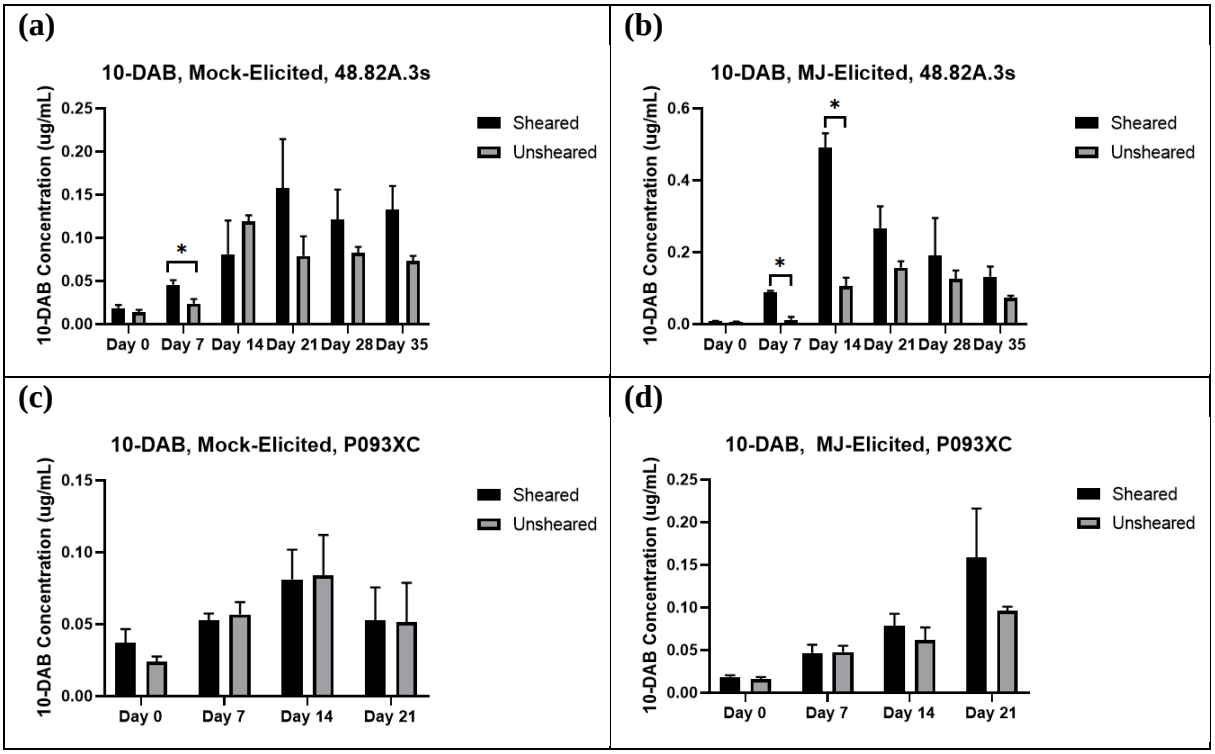


Figure 11

Impact of long-term shear on 10-DAB production in 48.82A.3s and P093XC. 10-DAB production is increased with shear in 48.82A.3s cell cultures (Figure 11 a and b), while production is unchanged in P093XC cell cultures (Figure 11 c and d). Data represent the average of three biological replicates with standard deviation represented. A paired t-test was used to determine if the data were statistically significant using a confidence level of 95%. * represents data that are statistically significant.

The impact of long-term shear was also examined for baccatin III concentration for both cell lines, as seen in Figure 12. Figure 12 a and b show the impact of long-term shear on the

48.83A.3s cell line. In the mock-elicited cells, the unsheared group produces higher levels of baccatin III. The MJ-elicited cells show mixed results; on Day 0 the unsheared cell cultures produced higher levels of baccatin III but on Day 7 the sheared cell cultures produced higher levels of baccatin III.

Figures 12 c and d show the impact of long-term shear on P093XC. In both mock-elicited and elicited, the sheared cells produced higher levels of baccatin III. The shear could have stressed the cell cultures and caused them to produce more of the paclitaxel precursor.

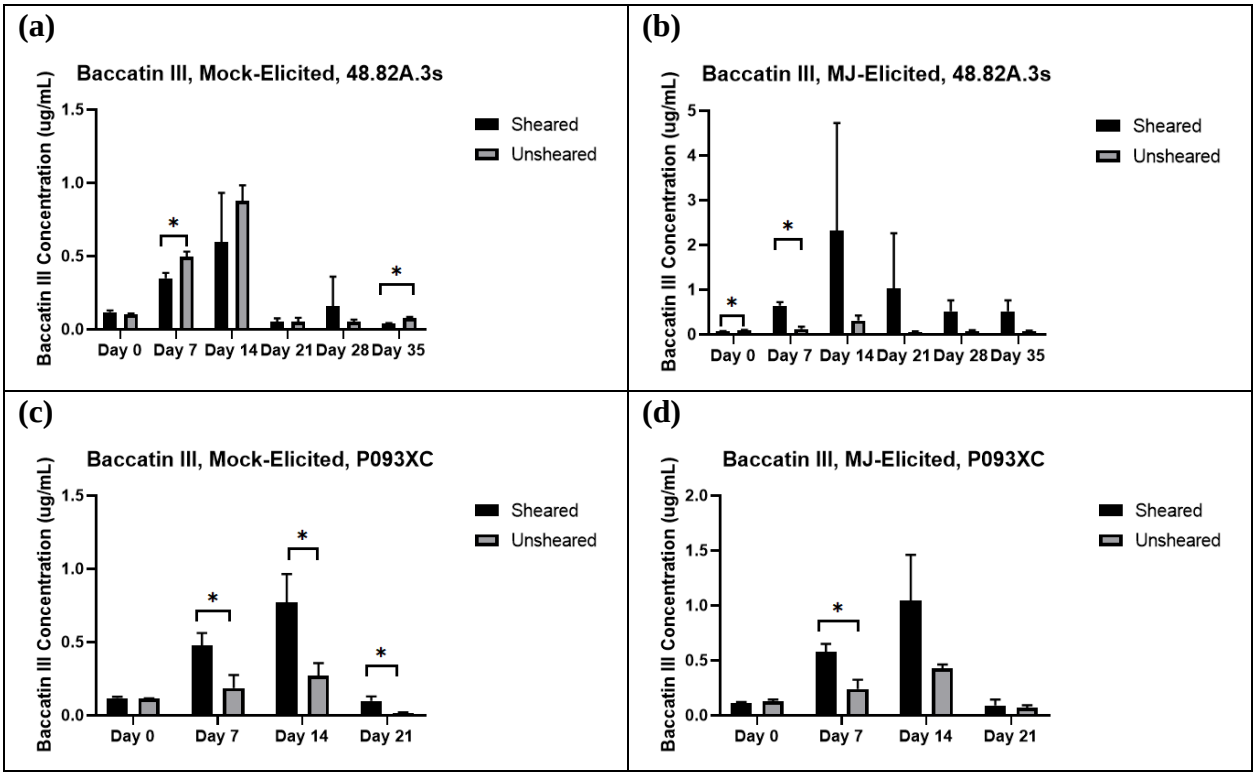


Figure 12

Impact of long-term shear on baccatin III production in 48.82A.3s and P093XC cell cultures. Unsheared mock-elicited 48.82A.3s cell cultures produced higher levels of baccatin III compared to sheared cultures (Figure 12 a). However, sheared MJ-elicited 48.82A.3s cell cultures produced higher levels of baccatin III (Figure 12 b). Both MJ-elicited and mock-elicited P093XC sheared cell cultures produced higher levels of baccatin III (Figure 12 c and d). Data represent the average of three biological replicates with standard deviation represented. A paired t-test was used to determine if the data were statistically significant using a confidence level of 95%. * represents data that are statistically significant.

Based on previous studies, it was expected that paclitaxel production would increase with shearing in paclitaxel producing cell lines (Dinicu, 2021). Figure 13 a and b show paclitaxel is increased with shearing in 48.82A.3s cultures. For the mock-elicited cells, paclitaxel concentration was higher on Days 0, 21, and 35. The MJ-elicited cells had higher concentrations of paclitaxel on Days 7, 14, 21, and 35. The higher paclitaxel concentration in sheared cells

could have been increased by the added stress caused by the contaminated media; experiments should be repeated to confirm these results.

As seen in Figure 13 c, unsheared cell cultures of P093XC had higher concentrations of paclitaxel on Days 7 and 14. Figure 13 d shows that the unsheared cell cultures produced higher levels of paclitaxel on Day 0. Because these cells are traditionally non-paclitaxel producing, this could explain why there was a discrepancy between the expected result and the experimental result. 48.82A.3s cell cultures exhibited higher levels of paclitaxel overall.

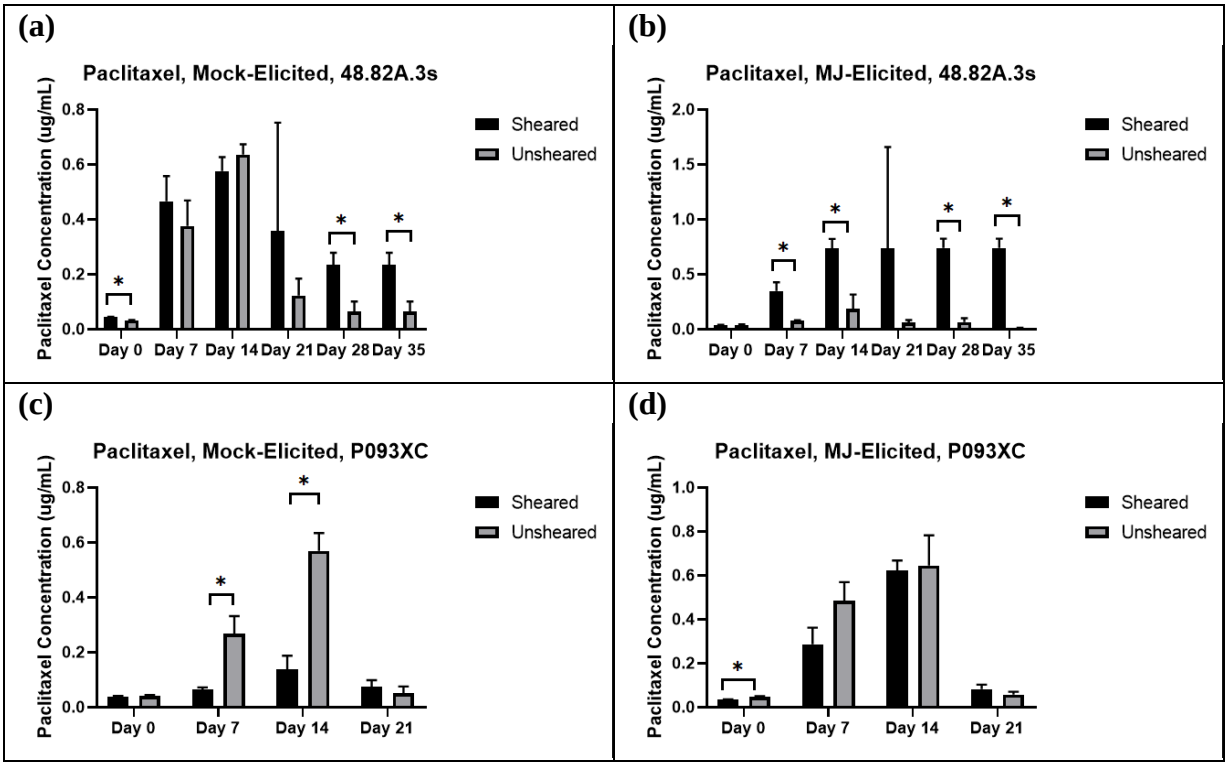


Figure 13

Impact of long-term shear on paclitaxel production. Paclitaxel production was increased in sheared 48.82A.3s cell cultures (Figure 13 a and b). MJ-elicited 48.82A.3s cell cultures provided the highest concentration of paclitaxel overall (Figure 13 b). Paclitaxel production was greatly increased in unsheared mock-elicited P093XC cell cultures (Figure 13 c). MJ-elicited unsheared P093XC cell cultures showed a greater paclitaxel production on Day 0 (Figure 13 d). Data represent the average of three biological replicates with standard deviation represented. A paired t-test was used to determine if the data were statistically significant using a confidence level of 95%. * represents data that are statistically significant.

3.5 Effect of Long-Term Shear on General Secondary Metabolism

The effect of long-term shear on general secondary metabolite accumulation was tested to determine the global cellular stress response to shear in culture. Figure 14 a, b, c and d show the impact of long-term shear on flavonoids in the 48.82A.3s and P093XC cell lines. There was no

statistically significant difference between the sheared and unsheared cell cultures for both cell lines, although trends suggest that shearing may increase flavonoid accumulation. The paclitaxel producing cell line should be impacted by shear and MJ-elicitation (Dinicu, 2021). Previous studies were performed to find the impact of long-term shear on paclitaxel-producing cell cultures using the 48.82A.11 cell line (Dinicu, 2021). As seen in Figure 14 e and f, secondary metabolite production increases with shearing in both mock-elicited and MJ-elicited 48.82A.11 cell cultures (Dinicu, 2021). It is possible that secondary metabolism in 48.82A.3s is not as sensitive to shear or MJ-elicitation as in cell line 48.82A.11.

Previous data show that flavonoid concentration is unchanged by MJ-elicitation in non-paclitaxel producing cell cultures (McKee, 2021), which is verified by these results. Therefore, shear stress might also not impact secondary metabolism in non-paclitaxel producing cell lines.

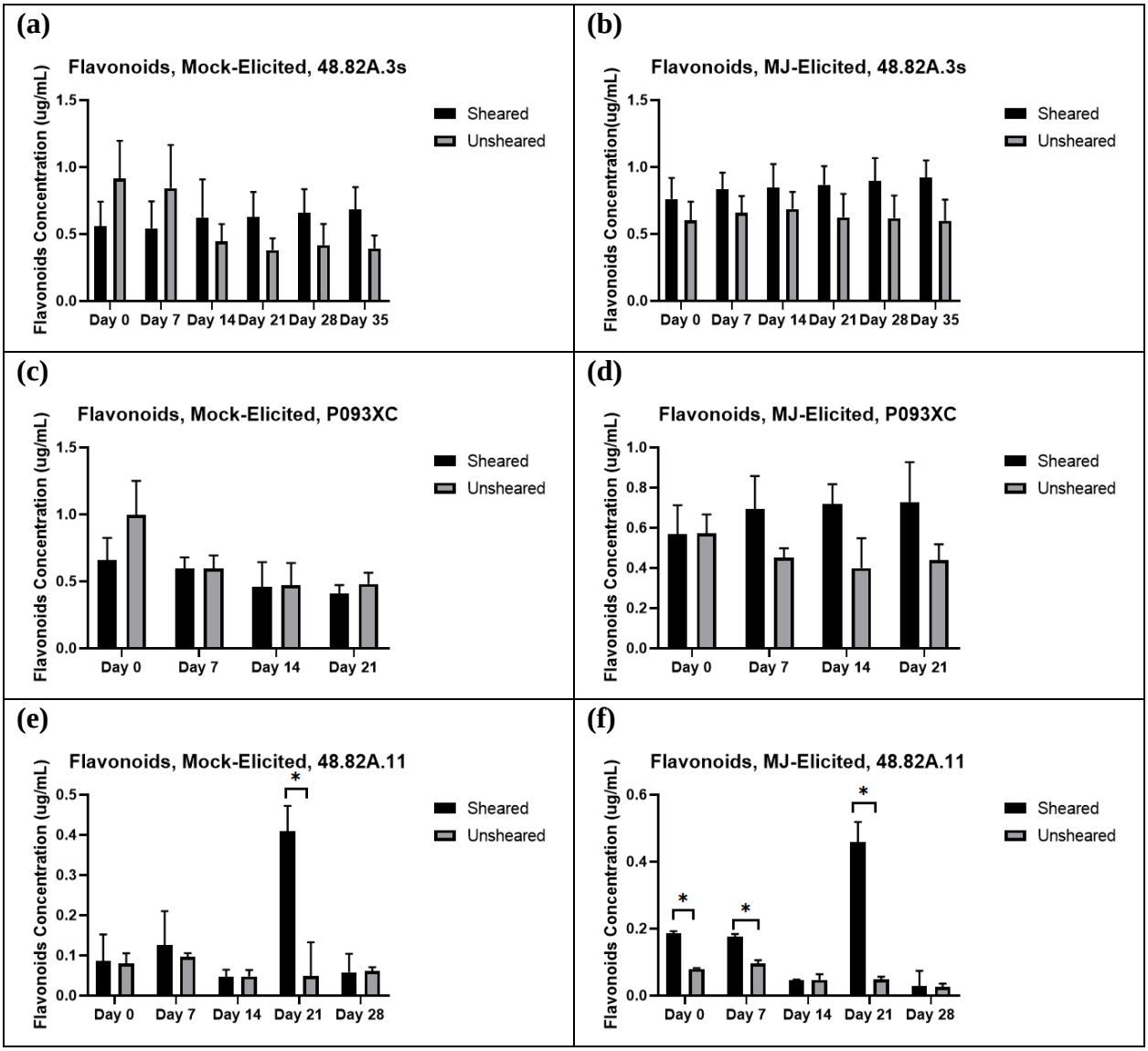
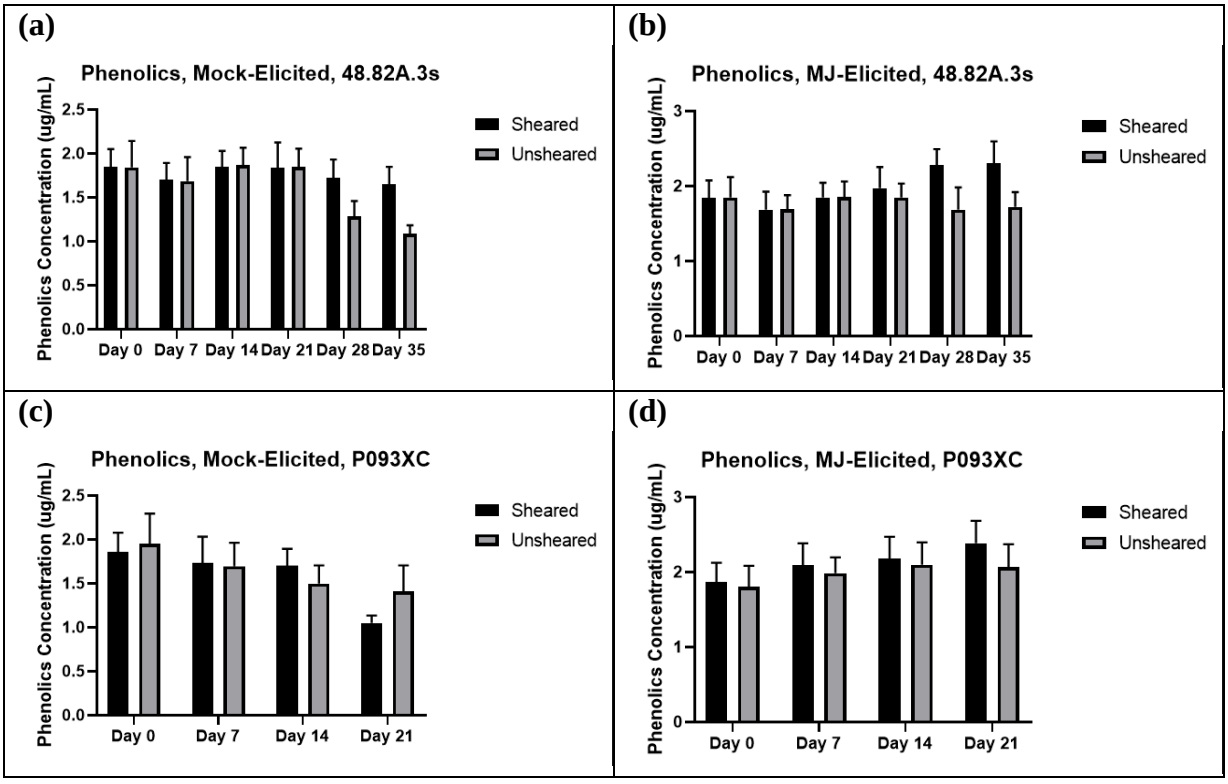


Figure 14

Impact of long-term shear on flavonoid production. Figure 14 a, b, c and d show that there was no statistically significant impact on flavonoid production caused by shearing or MJ-elicitation in 48.82A.3s and P093XC cell cultures, respectively. Figure 14 e and f show that shearing increases flavonoid production in 48.82A.11 cell culture. Data represent the average of three biological replicates with standard deviation represented. A paired t-test was used to determine if the data were statistically significant using a confidence level of 95%. * represents data that are statistically significant.

Phenolic concentrations showed similar results to flavonoid concentrations. Figure 15 a, b, c and d show that shearing did not impact phenolic accumulation. Previous experiments show that paclitaxel producing cell lines have increased phenolics when MJ-elicited or sheared, as seen in Figure 15 e and f (Dinicu, 2021). Because both flavonoid and phenolic concentrations were not impacted by shear or MJ-elicitation, this could further prove that the secondary metabolism of the 48.82A.3s cell line is not sensitive to shear or MJ elicitation.

Like flavonoids, phenolics in non-paclitaxel producing cell lines are not impacted by MJ-elicitation (McKee, 2021). Because shearing is another form of stress, this could explain why general secondary metabolism in the P093XC cell culture was not impacted by shear.



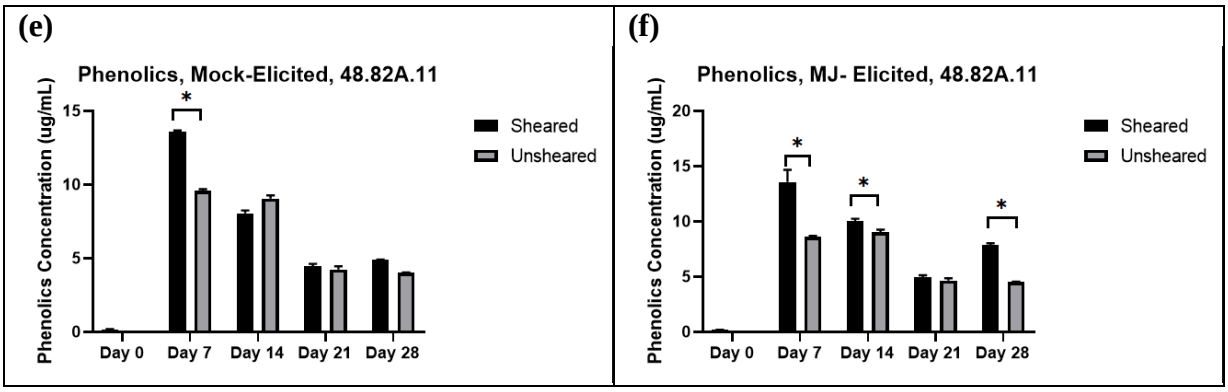


Figure 15

Impact of long-term shear on phenolics concentration. Figure 15 a, b, c, and d show that shear and MJ elicitation do not have an impact on phenolics concentration for 48.82A.3s and P093XC cell cultures, respectively. Figure 15 e and f show that sheared 48.82A.11 cell cultures produce higher phenolics concentrations when compared to unsheared cell cultures. Data represent the average of three biological replicates with standard deviation represented. A paired t-test was used to determine if the data were statistically significant using a confidence level of 95%. * represents data that are statistically significant.

3.6 Effect of Continuous Shear on Viability

All continuous shearing experiments were performed on the shearing device that was designed for this experiment, which is detailed in the Methods section. The shearing device defines one shear as 10 mL of cell culture volume pipetted up and down using a serological pipette tip and uses a flow rate of 7.3 mL/s (Wilson, 2020). The experiments were performed on cells from Day 0 of a culture cycle.

Three replicate experiments (labeled by dates in this section) were performed to determine the impact of continuous shear on viability of 48.82A.3s cell cultures, which were used for all continuous shear experiments. Figure 16 a shows the impact of continuous shear on viability over a two-day period on March 8-10. Overall, the viability is not impacted by continuous shear as there are no statistically significant results. However, the overall viability remained low in this experiment, which could have been caused by incubator contamination (Ryan, n.d.). During experimentation, other cell cultures that shared the same incubator showed signs of contamination, such as turning bright red and growing mold. It is possible that the contamination spread to these cell cultures and that physical signs of contamination could not be detected as they had such a short incubation period of 2 days.

The second experiment was performed on March 15-17 to gather more data on the relationship between short-term continuous shear and cell health, as shown in Figure 16 b. Figure 16 b shows that cell cultures had not been impacted by continuous shear. The third and final repeat experiment was performed on April 7-9 (Figure 16 c) and found that viability was not impacted by continuous shear.

Overall, there is not a significant reduction in the viability of the sheared cultures over time.

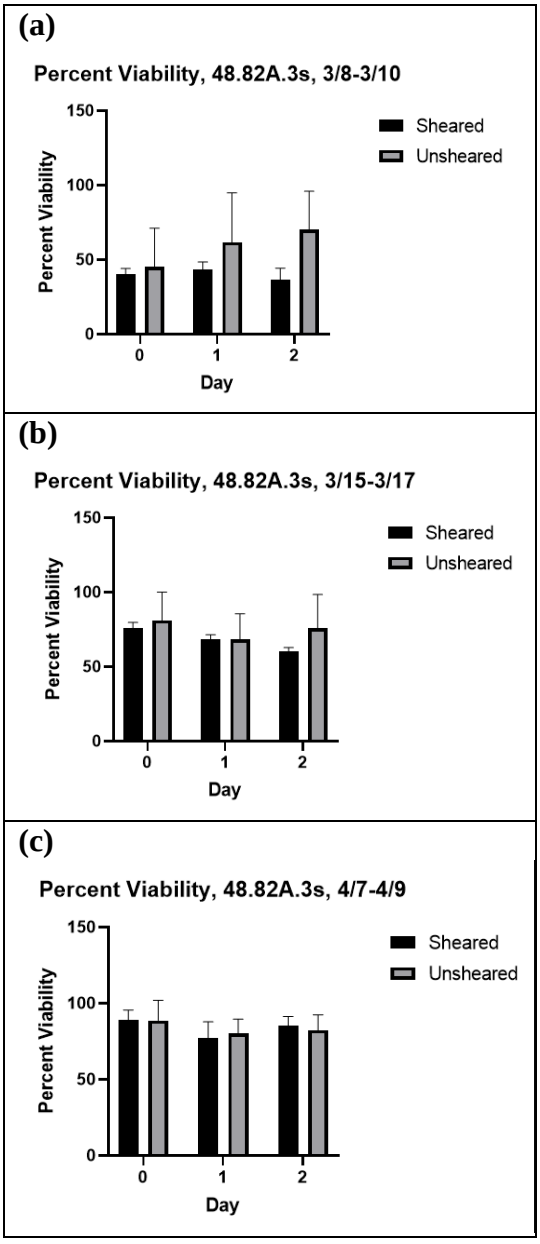


Figure 16

The impact of continuous shear on viability of 48.82A.3s cultures. Figure 16 a, b and c show that unsheared cells have higher viability. Data represent the average of three biological replicates with standard deviation for the unsheared cell culture. Only one biological replicate could be taken for the sheared cell culture. A paired t-test was used to determine if the data were statistically significant using a confidence level of 95%. * represents data that are statistically significant.

3.7 Effect of Continuous Shear on Aggregate Diameter Distribution

The effect of continuous shear on diameter distribution was evaluated. It was expected that continuous shear would have a more dramatic effect compared to long-term shear as the cultures are sheared more significantly in a shorter span of time (Dinicu, 2021). Figure 17 a and b shows the aggregate diameter distribution of cells. The aggregate diameter distributions were very similar between the sheared and unsheared cultures: sheared cell culture had an aggregate diameter distribution of 836 µm (Figure 17 a) and the unsheared group had an average aggregate diameter of 852 ± 74 µm (Figure 17 b). When samples were taken on Day 0, no shearing had been done. The purpose of these samples was to get a baseline to compare the sheared samples back to over time.

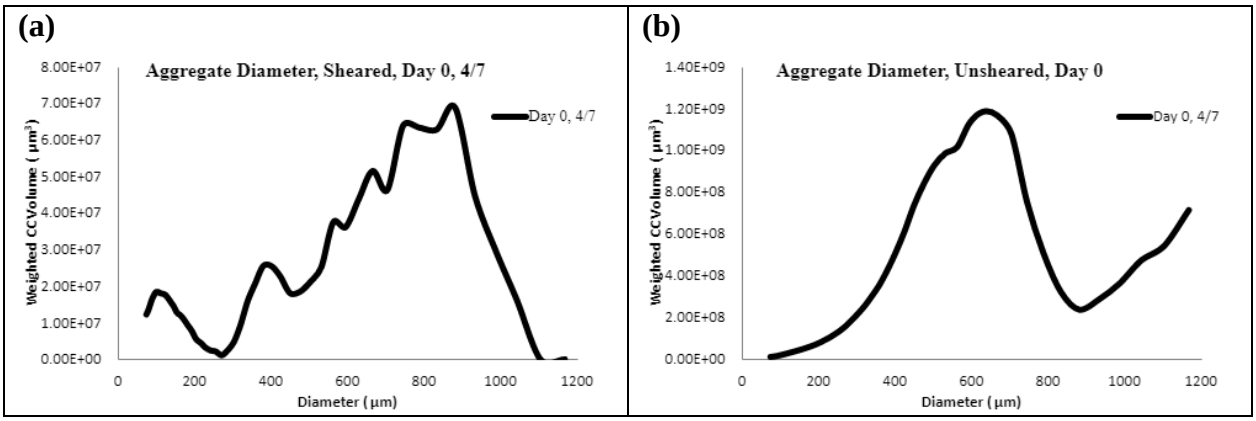


Figure 17

Impact of continuous shearing on aggregate diameter distribution on Day 0. Both Figures 17 a and b show a broad range of aggregate diameters. Data represent the average of three biological replicates with standard deviation for the unsheared cell culture. Only one biological replicate could be taken for the sheared cell culture.

Figure 18 a and b shows the diameter distribution of sheared versus unsheared cell cultures on Day 1. The diameter of the sheared cells dramatically decreased when compared to their own distribution on Day 0 and the unsheared cells on Day 1. The aggregate diameter of sheared cell cultures was 550 µm (Figure 18 a) and the unsheared average aggregate diameter was 789 ± 62 µm (Figure 18 b).

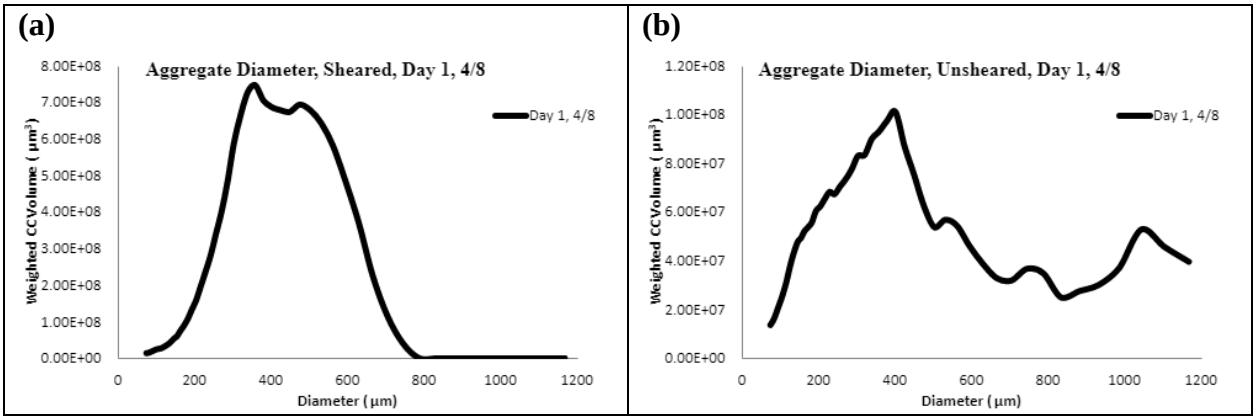


Figure 18

Impact of continuous shearing on aggregate diameter distribution on Day 1. Figure 18 a shows a smaller diameter distribution than 18 b. Data represent the average of three biological replicates with standard deviation for the unsheared cell culture. Only one biological replicate could be taken for the sheared cell culture.

Figure 19 expresses the relationship between continual shear for two days and diameter distribution. The diameter distribution decrease is even more dramatic than that of Figure 18. The average aggregate diameter of sheared cell cultures was 275 µm (Figure 19 a), while the average aggregate diameter of unsheared cell cultures was 732 ± 64 µm (Figure 19 b). The diameter distribution over two days of continuous shear shows how drastically the distribution can change. These data confirm the hypothesis that continual shear would decrease aggregate diameter very rapidly.

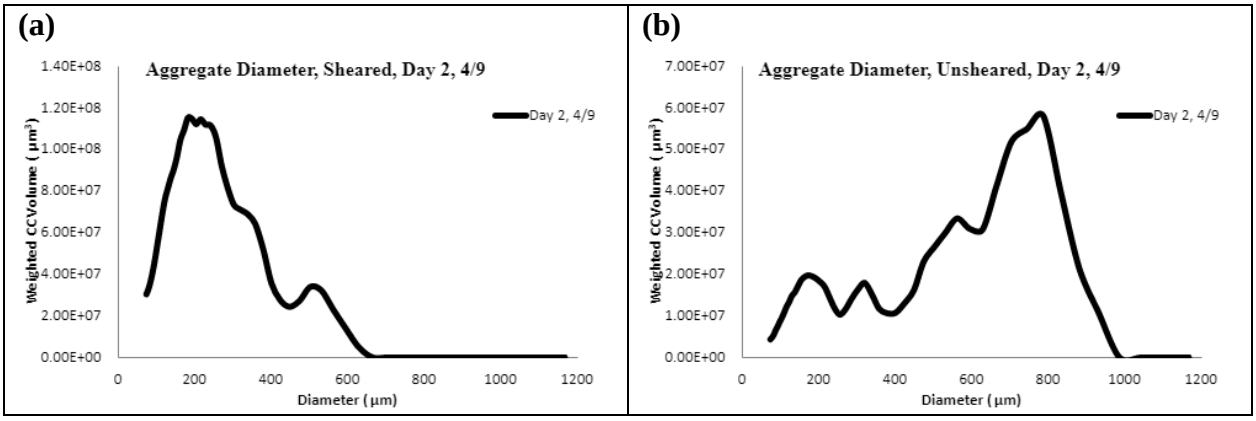


Figure 19

Impact of continuous shearing on aggregate diameter distribution on Day 2. Figure 19 a shows a dramatic change in size distribution when compared to Figure 19 b. Data represent the average of three biological replicates with standard deviation for the unsheared cell culture. Only one biological replicate could be taken for the sheared cell culture.

3.8 Effect of Continuous Shear on Cell Growth

The same Coulter-Counter volume to biomass conversion was used as seen in Chapter 4.3 (Kolewe, 2010). The growth curve of 48.82A.3s cell cultures showed that growth was not impacted by shear (Figure 20). To ensure that cell cultures are growing, there should be an increase in biomass over time (Wilson, 2020). Previous studies showed that growth for a few days of culture does not drastically increase biomass (Wilson, 2020). Based on the growth curve (Figure 20) and previous data (Wilson, 2020), data suggest that continuous shear does not impact growth.

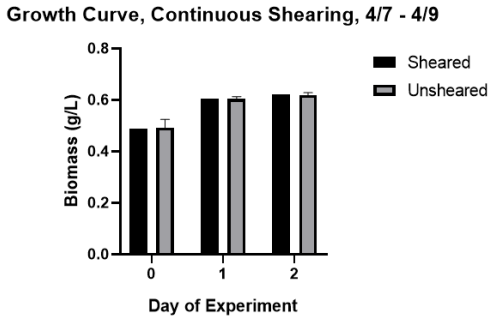
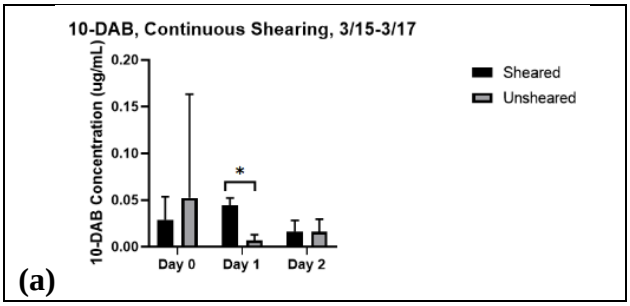


Figure 20

Continually sheared cell culture grows at the same rate as unsheared cell cultures. Data represent the average of three biological replicates with standard deviation for the unsheared cell culture. Only one biological replicate could be taken for the sheared cell culture. * represents data that are statistically significant.

3.9 Effect of Continuous Shear on Paclitaxel Production

Figure 21 shows the impact of continuous shear on taxane production for samples taken from March 15-17. Figure 21 a examines the relationship between 10-DAB and continuous shear, which shows that 10-DAB was higher for sheared cells on Day 1. On Day 2, the 10-DAB concentration in the sheared cells decreased from Day 1, which could indicate that it is being converted to paclitaxel as time progresses. Baccatin III levels follow similar patterns as 10-DAB levels (Figure 21 b). Figure 21 c shows the relationship between paclitaxel and continuous shear. Days 0 and 1 showed that the paclitaxel production was higher in sheared cells. No shearing was done when samples were taken on Day 0, which indicates that these cells could have been experiencing stress caused by something other than shear. It is possible that sharing the incubator with other contaminated cell cultures would have caused these cell cultures to experience some stress (Ryan, n.d.). However, after 1 day of shear, the paclitaxel production in is still higher than that of the unsheared cell cultures.



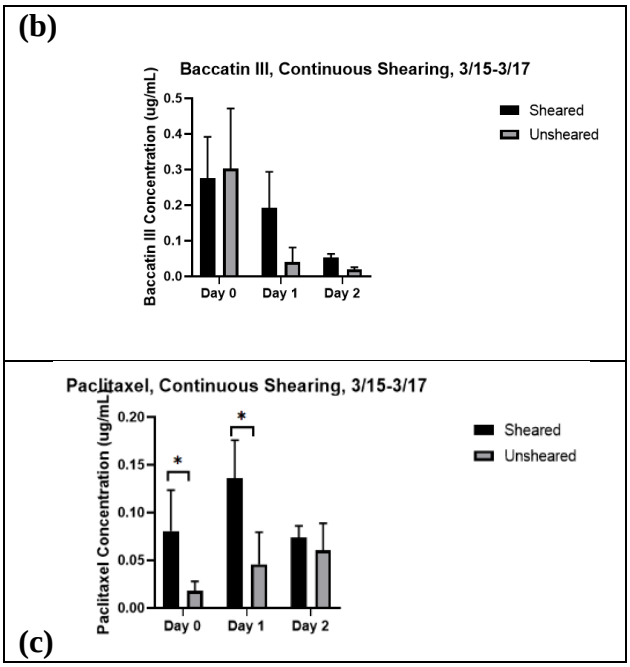
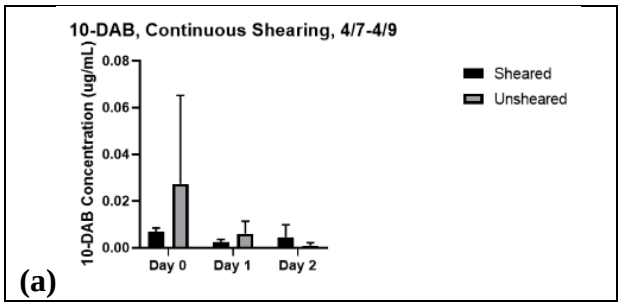


Figure 21

Impact of continuous shearing on the 10-DAB, baccatin III and paclitaxel production for cell culture samples taken from March 15-17. Figure 21 a shows that 10-DAB was increased in sheared cells on Day 1. There was no impact on baccatin III concentration (Figure 21 b). Paclitaxel concentration was higher on Days 0 and 1 in sheared cell culture (Figure 21 c). Data represent the average of three biological replicates with standard deviation for the unsheared cell culture. Only one biological replicate could be taken for the sheared cell culture. A paired t-test was used to determine if the data were statistically significant using a confidence level of 95%. * represents data that are statistically significant.

This experiment was repeated (April 7-9) and data are shown in Figure 22. Figure 22 a shows that 10-DAB production was not impacted by continual shear. Figure 22 b shows the impact of shear on baccatin III production. Baccatin III production over time decreases suggesting a potential conversion to paclitaxel or other taxanes. The relationship between paclitaxel and shear can be seen in Figure 22 c. Paclitaxel production was relatively low for both Days 0 and 1 but the sheared cells had a rapid increase on Day 2. Both Figures 21 c and 22 c provide evidence that continual shear increases paclitaxel production.



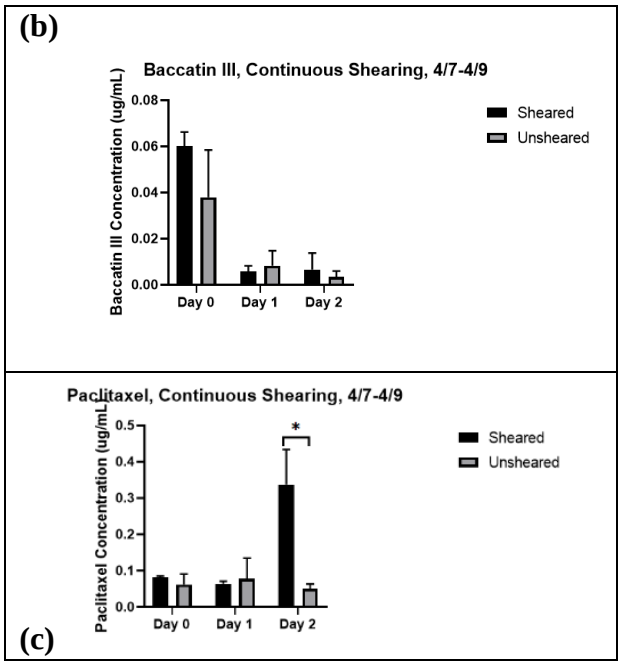


Figure 22

Impact of continuous shearing on 10-DAB, baccatin III and paclitaxel levels for cell culture samples taken from April 7-9. Both 10-DAB and baccatin III production were not impacted by continuous shearing (Figure 22 a and b). Paclitaxel concentration increased greatly on Day 2 due to shearing (Figure 22 c). Data represent the average of three biological replicates with standard deviation for the unsheared cell culture. Only one biological replicate could be taken for the sheared cell culture. A paired t-test was used to determine if the data were statistically significant using a confidence level of 95%. * represents data that are statistically significant.

In summary, there are several key findings found. First, it is possible that there was contamination in the incubator for the continually sheared samples, which would have skewed the results for viability and paclitaxel quantification. It was also found that the device was successful at reducing aggregate size significantly. Furthermore, cell growth was not impacted by sheared samples, and viability did not drastically drop. Preliminary data also show that paclitaxel levels increase due to continuous shearing, which is a promising result, but would need to be confirmed with further testing.

4. Conclusions and Recommendations

In summary, both long-term and continuous shear increased the levels of paclitaxel production, with MJ-elicited sheared 48.82A.3s (paclitaxel producing) cell cultures accumulating the highest levels overall. 10-DAB levels also increased in 48.82A.3s cultures with both long-term and continuous shear. Paclitaxel precursor baccatin III levels were less predictable, suggesting additional experimentation to discern the relationship with shear and MJ-elicitation. As expected, both long-term and continuous shear were shown to decrease aggregate diameter distribution. The impact of shear can be seen more dramatically in the continuously sheared cells, where mean aggregate size decreased by up to 63.43% (from 732 ± 64 μm to 275 μm) in just two days.

Cell culture growth was not impacted by shearing in both paclitaxel producing (48.82A.3s) and non-paclitaxel producing cell lines for both long-term and continuous shearing, which was consistent with that observed in other *Taxus* cell lines in previous studies (Wilson, 2019). P093XC cell culture viability was not affected by shear; however, 48.82A.3s cell culture viability decreased with shear in contaminated cultures and therefore the effects of shear cannot be accurately determined.

The majority of continual shear experiments showed that cell culture viability was not impacted by shear. In one experiment with the continuous shearing device, initial cell viability was lower than cell viability for control (unsheared) cultures. However, initial cell viability was measured prior to the start of continual shearing. Experiments should be repeated with careful attention to incubator contamination to ensure that cell viability is the same for all cultures at the start of the study.

Finally, global secondary metabolism (total flavonoids and phenolics content) was not impacted by long-term shear for 48.82A.3s and P093XC cell cultures. Previous testing on another paclitaxel producing cell line demonstrated that shearing does impact global secondary metabolism (Dinicu, 2021). However, non-paclitaxel producing cell lines are not impacted by MJ-elicitation (McKee, 2021), which could also explain why the P093XC cell line was not impacted by shear. Results here suggest that some cell lines may be more sensitive to shearing than others.

There are several recommendations that can be made to improve future experimentation. First, multiple biological replicates should be tested while running continuous shear experiments to ensure statistical validity. Multiple biological replicates could be achieved by having multiple shearing devices in the incubator or redesigning the shearing device to be able to shear multiple flasks simultaneously.

Second, continuous shear experiments should be expanded to additional cell lines and longer time periods to determine the impact of continuous shearing on both paclitaxel producing and non-producing cell lines. Third, different shearing protocols should be tested in long-term experiments where continuous shearing can be compared with both unsheared and intermittently sheared cultures. All these experiments should include both MJ-elicited and mock-elicited cultures. Data have shown that a combination of elicitation and shearing produces the highest amount of paclitaxel. MJ elicitation and long-term continuous shearing could be a way to

optimize paclitaxel production in bioprocesses. Finally, future work could examine the relationship between one cell to fully understand the relationship between aggregate size and shear.

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