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

by

**Caroline V. Rauber**

A Thesis

Submitted to the Faculty of the

WORCESTER POLYTECHNIC INSTITUTE

in partial fulfillment of the requirements for the

Degree of Master of Science

in Chemical Engineering

by

April 2023

Approved by :

_____

Pr. Susan Roberts, Ph.D.

Advisor

Department Head of Chemical Engineering, WPI

_____

Pr. Michael Timko, Ph.D.

Committee Member

_____

Pr. Christina Bailey-Hytholt, Ph.D.

Committee Member

## Abstract

Paclitaxel (or Taxol™) is a highly effective FDA-approved chemoterapeutic drug due to its effective cytotoxicity against different types of human and animal cancers. However, the limitation in the widespread use of paclitaxel is its very low production yield in nature, given the slow growth of the yew tree and the very low content of paclitaxel stored in the bark of the tree. Although there are currently different alternative methods to produce paclitaxel for clinical use, plant cell culture (PCC) is the one that is the most viable, stable and attractive commercially as it offers uniform and continuous paclitaxel quality and a renewable resource. PCC, however, faces some challenges such as aggregation of cells in culture, which induces cellular heterogeneity resulting in unpredictable changes in metabolism and growth. Previous research has demonstrated that smaller aggregated cultures accumulate higher levels of paclitaxel. Several techniques have been developed to reduce aggregate sizeusing either physical or chemical factors.

This thesis focused on the use of an automated shearing device to study both short-term and long-term shearing with varied parameters. Results were compared with previously established enzymatic digestion methods. The impact of long-term shearing combined with methyl jasmonate elicitation on cell health, production of paclitaxel and its precursors, global secondary metabolite synthesis and mean aggregate size was studied.

Both techniques of disaggregation were very effective in reducing aggregate size when applied for four hours, but continuous shearing via the automatic device was shown to have the most significant results in reducing aggregate size while still maintaining healthy and metabolically active cells. Preliminary data on the automated device showed that continuous shearing at the highest flowrate (8.6 mL/s) did not impact negatively cell metabolic activity and resulted in a significant decrease of the mean aggregate size. Long-term shearing showed increased levels of paclitaxel over time, the highest concentrations being achieved for sheared cultures elicited with methyl jasmonate. These promising results support the further study and adaptation of the automated shearing device for promotion of paclitaxel synthesis in PCC.

# Acknowledgement

I would first like to thank Pr. Susan Roberts, my advisor, for welcoming me into her laboratory only a few weeks after I joined WPI's graduate program and for her mentorship throughout this thesis.

I would also like to express my gratitude towards my lab mate Cassandra Newton, for training me to the different procedures in the lab and always taking time to answer my questions and providing help every time I needed it. I would also like to thank my other lab mates, Toni Sassano and Tahsin Rahi.

Finally, I would like to thank Ian Anderson for taking into consideration every thought I had to design and build the shearing devices in a way that allowed me to perform all the experiment described in this thesis.

# Table of contents

# Chapter 1 : Background

## 1.1 Paclitaxel and *Taxus* plant cell culture

Cancer is an important public health problem worldwide and it is the second leading cause of death in the United States (Siegel, 2023). In 2023, about 1,958,300 new cancer cases and 609,820 cancer deaths are projected to occur in the country. However, the death rate progressively declined in recent years (1.5% decrease from 201 to 2020), thanks to advances in treatments (Siegel, 2023).

Paclitaxel (or Taxol™) is a highly effective chemoterapeutic drug for treating various cancers due to its effective cytotoxicity against different types of human and animal cancers, such as breast, esophagus, ovarian, bladder, lung, endometrium, and cervix cancers, as well as Kaposi's sarcoma and lymphoma (Wilson, 2019). Paclitaxel is a diterpenoid pseudoalkaloid that was originally isolated from the bark of the Pacific yew tree, Taxus brevifolia (Wilson, 2020). Paclitaxel was approved by the FDA in 1994 to treat breast cancer, Kaposki's sarcoma, ovarian cancer and non-small cell lung cancer. Paclitaxel targets microtubules and prevents cell division by both promoting the assembly of stable mircrotubules from β-tubulin heterodimers and inhibiting their depolymerization (Zhong, 2002). Therefore, paclitaxel-treated cells are arrested in the cell cycle in the G2/M-phase and undergo apoptosis, as seen in *Figure 1*.

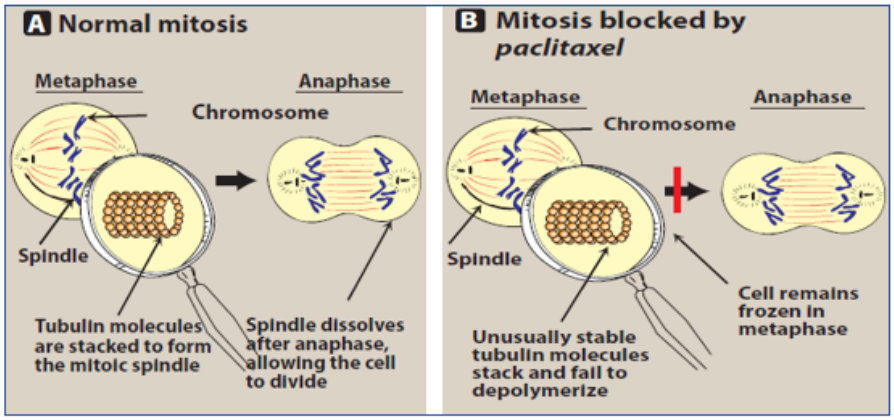



*Figure 1 : Mitosis with and without Paclitaxel treatment*

There are hundreds of different known taxoid structures, with some of them showing similar or superior activity to paclitaxel, and some useful as precursors for Paclitaxel production. However, the limitation in paclitaxel supply is its very low production yield in nature, given the slow growth of the yew tree and the very low content of paclitaxel stored in the bark of the tree (around 0.01% on a dry weight basis). There are currently four alternative methods to produce paclitaxel for clinical use: total synthesis, semi-synthesis using its natural precursor (10-deacetylbaccatin III), heterologic production by bacteria or fungi, and plant cell culture (Zhong, 2002). Given that paclitaxel is a complex tetracyclic diterpene, its biosynthetic pathway is believed to have nineteen steps, although certain transformations have not been fully characterized (Howat, 2014).

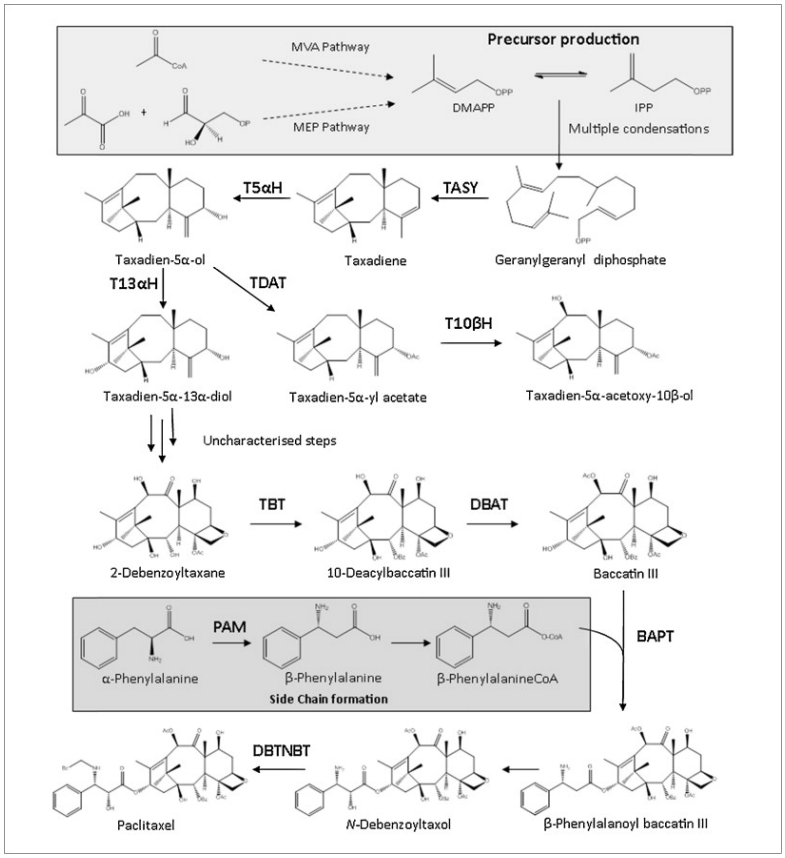



*Figure 2: Biosynthetic pathway of paclitaxel (Howat, 2014)*

An overview of the taxoid biosynthetic pathway is shown in *Figure 2*. The complexity prevents total synthesis from being economically feasible at large scale due to low yields and the use of environmentally unfriendly solvents. Semi-synthesis from the precursor 10-deacetyl baccatin III was previously used commercially by Bristol Myers Squibb, but has discontinued due to several disadvantages such as precursor production being dependent on environmental and epigenetic factors, and complex, environmentally unfriendly purification, which is very expensive. Several steps of the biosynthetic pathway of Paclitaxel have been transferred into bacteria and fungi such as *Saccharomyces cerevisiae*, or *Escherichia coli,* but full development of the pathway in those organisms is limited due to difficult expression of cP450 in microbial systems (Howat, 2014).

Plant cell culture (PCC) is an *in vitro* process with tremendous potential commercially because it can be used to synthesis products such as anthocyanins and alkaloids that animal cells or bacteria cells cannot produce (Furusaki, 2011). It can also allow biotransformation of biological compounds such as steroids or terpenoids. PCC is a technique in which cells or tissues are isolated from plants and cultivated in an artificial environment. This technique is used often in agriculture and was first developed by Haberlandt in 1902, who cultivated isolated cells. Although he found that his cells couldn't grow, the discovery that cytokinin and auxin are required for cell division and growth enabled isolated cells and tissues to be successfully proliferated in their artificial environment in many studies conducted in the 1950s (Furusaki, 2011). PCC is therefore considered to be the most viable, stable and effective method to produce paclitaxel offering several advantages over other processes such as uniform and continuous paclitaxel quality, production independent of seasonal and geographical variations, and an environmentally friendly and renewable resource (Howat, 2014). *Taxus* cell culture can eliminate the use of the limited natural resources of the *Taxus* species and provide a steady supply

of paclitaxel. Paclitaxel via PCC is currently produced on an industrial scale by two companies, Samyang Genex and Phyton Biotech USA. The culture process starts with the generation of callus, cell agglomerate grown In a specific media formulation from plant tissues. Callus cultures are a mixture of cells in different states of differentiation that have the metabolic capability to produce secondary metabolite such as paclitaxel. Plant cells can also be maintained in suspensions, with sugars, vitamins, hormones and inorganic salts, which plays specific functions in metabolic regulation (Furusaki, 2011). PCC however faces some difficulties, such as heterogeneous culture (i.e., all cells do not have the same metabolism), low growth rate and variable product yield (Howat, 2014).

Studies on elicitation have shown that it is an effective tool to enhance production of secondary metabolites in cultured *Taxus* cells (Brzycki, 2021). Elicitation involves application of a chemical or physical agent that stimulates a defense response. Since paclitaxel and related precursors, along with most secondary metabolites known as secondary metabolites, are produced most often in response to a specific environmental stimulus, elicitation causes plants to produce secondary metabolites with increased resistance to environmental stressors, as seen in *Figure 3*. Elicitors serve as signals, and the process of elicitation begins with elicitor-specific receptors on the plant cell membrane that detects the signal. This event is followed by the beginning of a signal transduction cascade, which changes the expression level of various regulatory transcription factors and rate-limiting genes of the secondary metabolic pathway genes, increasing the synthesis and accumulation of secondary metabolites (Halder, 2019). Depending on a number of variables, such as the species, culture age and elicitor concentration, treatment with elicitors frequently boosts synthesis of secondary metabolites by roughly 2- to 8+-fold at laboratory scales (Brzycki, 2021). Elicitation has the advantage of rapidly inducing complicated transcriptional reprogramming, which results in coordinated activation of all metabolic pathways just when desired enhanced production is wanted for secondary metabolites (McKee, 2021). Jasmonate acid and its methyl esther called methyl jasmonate are two of the most effective stressors. They are plant signaling hormones generated via the octadecanoid pathway that starts with linolenic acid that activates the synthesis of defense genes responsive to wounding. Many studies have shown that methyl jasmonate enhances production of paclitaxel (Brzycki, 2021). This regulatory compound activates secondary metabolism, therefore adjusting plants to environmental changes by the action of newly generated phytochemicals that increase the fitness of the species (Nielsen, 2019).

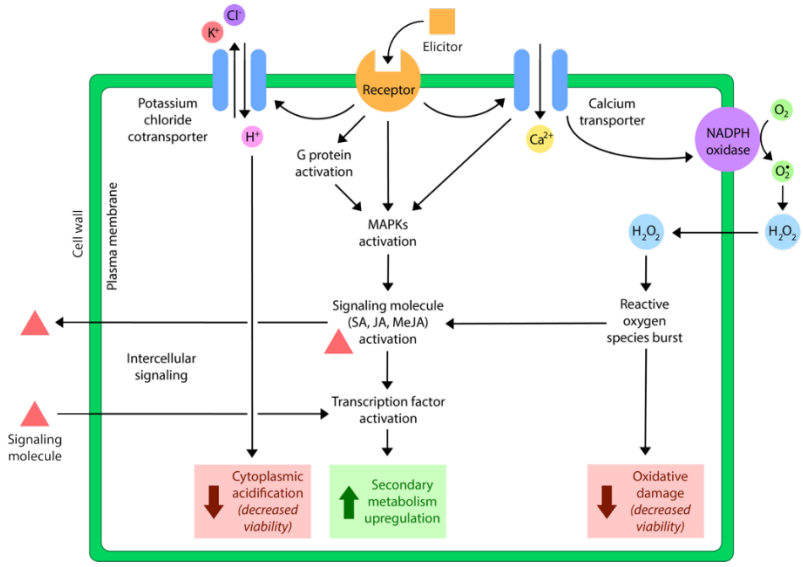



*Figure 3: Elicitation signal transduction cascade resulting in activation of a defense response in plant cells (Brzycki, 2021)*

Finally, another challenge in the application of plant cell culture is cell susceptibility to shear stresses in bioreactors and to aggregation of cells in culture, which results in transport issues.

### 1.2 Aggregation

One complication of plant cell systems is that as the cells divide, they tend to remain connected via cell walls following cytokinesis, resulting in aggregates of two or hundreds of cells that are connected by the middle lamella (Wilson, 2020). Taxus cell suspensions within larger aggregates, are periodically broken given that they are subject to shear forces from agitation, which results in aggregates that range from 100 μm to over 2,000 μm (Patil, 2012). The degree of cellular aggregation has been shown in previous studies to influence paclitaxel accumulation, culture heterogeneity and changes in metabolism, growth and differentiation among cells within a cluster (Wilson, 2020). Our laboratory has shown that smaller aggregated cultures produce higher levels of paclitaxel and related metabolites, making aggregation an important processing parameter (Kolewe, 2010). We hypothesize that increased shear stress due to the fact that more cells are in direct contact with the culture environment induces a stress response in the cells that mimics the elicitation mechanisms (Wilson, 2020).

### 1.3 Disaggregation techniques

Several techniques have been developed to break down aggregates, either using physical or chemical factors, but have resulted in limited success due to a decrease in cell growth and viability. Filtration using sheet mesh filters has been studied to break down aggregates, but resulted in modest success (Kolewe, 2011). The use of pressurized air thermostats, based on the principle of compressed air for aeration and agitation prevents the cells from clumping together, was shown to be successful on soybean suspension cultures (Kurz, 1971). Chemical techniques have also shown the ability to produce single cells or smaller clusters, including the use of proteins or enzymes. For the latter, cellulase and pectolyase were used to degrade cell wall components, similarly to protoplasting, and therefore dissociate *Taxus* aggregates (Naill, 2012). Given that cellulose is the main component of the primary cell wall and pectin of the middle lamella, they can be selectively degraded by the appropriate concentration of enzymes, specifically cellulase targeting the cellulose and pectolyase targeting pectin, while keeping the primary cell wall intact (Naill, 2004).

One of the most successful techniques for disrupting aggregates is mechanical shearing, using serological pipettes to break the aggregates. Previous studies in the Roberts laboratory developed a very efficient and low-cost method based on mechanical shear stress induced by pipetting the *Taxus* cultures suspensions with a 10 mL serological pipette; this method was proven to have no short-term impact on cell growth (Wilson, 2020). A long-term mechanical sharing experiment was also conducted in combination with methyl jasmonate elicitation where shearing was applied applied every three to four days to *Taxus* cultures over eight different generations of subculturing (each subculture cycle is two weeks), which resulted in a significant decrease in aggregates diameter (reduced from 730 $\mu m$ to 470 $\mu m$ ), and no impact on cell growth over the different generations (Wilson, 2020). The cultures used in these studies were not producing paclitaxel, so no information could be discerned on the influence of mechanical shearing on secondary metabolite productivity. To determine particle size distributions, an electrical resistance pule sizing technique, also known as the Coulter principle, can be used (Kolewe, 2010). According to this principle, particles suspended in a conducting salt solution will create a voltage pulse when passing through an aperture across which a continuous current is supplied, due to the displacement of electrolyte by the particle (Graham 2003). The voltage pulse's amplitude is then proportional to the particle's volume. Studies have shown that the use of the Coulter Counter is

a reliable technique, as it can quickly measure aggregate size distributions and total biomass as a total of all aggregates (Kolewe, 2010).

The effect of long-term continuous shearing on taxoid production and secondary metabolism is the focus of this research as it was not studied in previous work. The effect on general secondary metabolism is important to understand along with the effect on paclitaxel production directly.

**1.4 Aims of the research**

This thesis work has three main goals. Previous research conducted by Teodora Dinicu developed a first shearing device that could allow for continuous shearing at a fixed flowrate of 7.3 mL/s (Dinicu, 2022). While this preliminary device allowed testing the effect of continuous shearing on cell viability, taxoid production, and more global secondary metabolism, it had several constraints. First of all, typically three biological replicates are used for long-term experiments, and the previous device didn't allow experiments to be done in parallel. Second, the previous device had only one kind of settings, meaning that the flowrate was set and the number of pipetting times could not be changed automatically. In this research, the first goal was to create a device were the flowrate could be precisely controlled, the shearing could be set to continuous or intermittent and the number of pipetting cycles could be programmed. Different parameters were used to evaluate the disaggregation of different phases of the *Taxus* cell culture cycle (lag phase, exponential growth phase and stationary phase), to identifythe optimal conditions for shearing the cells. With those parameters determined, the second goal of this work was to determine the long-term impact of continuous mechanical shearing combined with methyl jasmonate elicitation on taxoid production, secondary metabolism and cell viability. This would allow to find a viable and reliable way to increase taxane production in plant cell culture processes.The third goal of this research was to further study the disaggregation protocol established by Naill et al., with 1.0 % (w/v) cellulase and 0.1 % (w/v) pectolyase Y-23 in an osmoticum of 0.5 M mannitol and 0.3 % dextran sulfate, and compare it to the shearing technique.[9] The two methods were to be tested over different time periods, and the aggregate size diameter and viability analyzed. To summarize, the aims for this thesis are as follows :

<u>*Aim 1 :*</u> Design a device allowing continual shear stress on *Taxus* cultures with selected parameters (flowrate, intermittent or continuous shearing, shearing time and pipetting cycles) and perform short-term tests to determine the optimized parameters for promoting optimal cell health and dissociation of aggregates.

<u>*Aim 2 :*</u> Determine the long term effect of continuous shearing combined with methyl jasmonate elicitation on taxoid production, global secondary metabolism and cell metabolic activity.

<u>*Aim 3 :*</u> Optimize the disaggregation protocol using enzymes and compare it to the shearing technique.

# Chapter 2 : Methods

## 2.1 General Experimental Design

Throughout this thesis, several experiments were carried out on the same paclitaxel producing cell line, *Taxus chinensis* 48.82A.3S. `

The first experiment as described in *Figure 4* consisted of a preliminary test of the shearing device at various shearing speeds. Previous research (Wilson, 2020) showed that the higher the shearing flowrate was, the better the aggregates were broken down. The highest flowrate investigated was 7.3 mL/s. The new shearing device allowed different flowrates. We evaluated 4.3 (speed 8), 5.2 (speed 9) and 8.6 (speed 10) mL/s. In this experiment four flasks, one unsheared, and the other three sheared 25 times, were evaluated at the three different speeds, and the metabolic activity and mean aggregate diameters were determined (details of all procedures are described below).

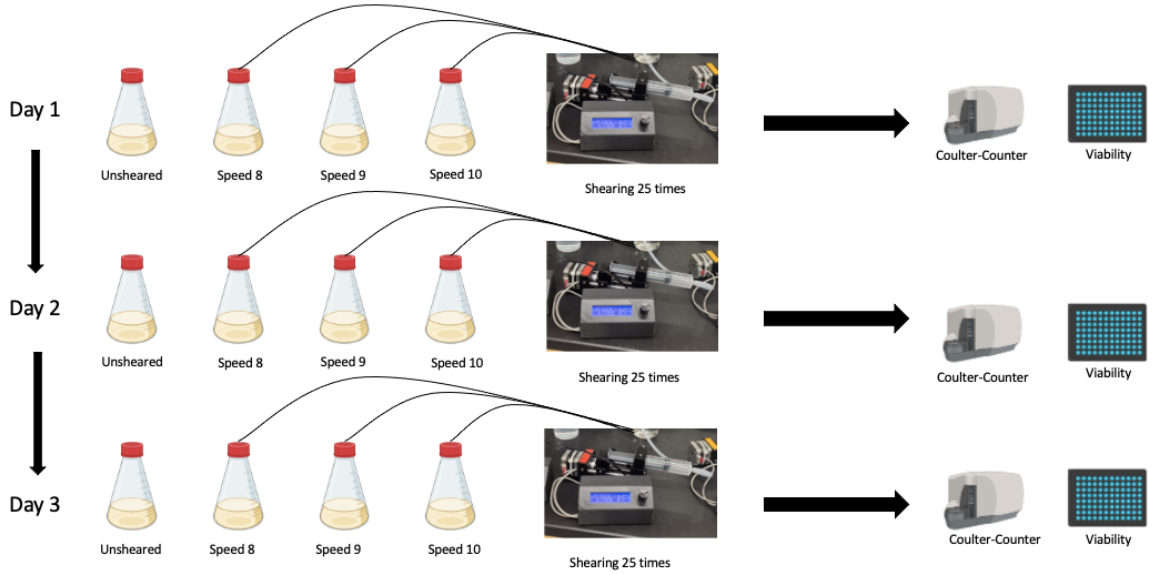
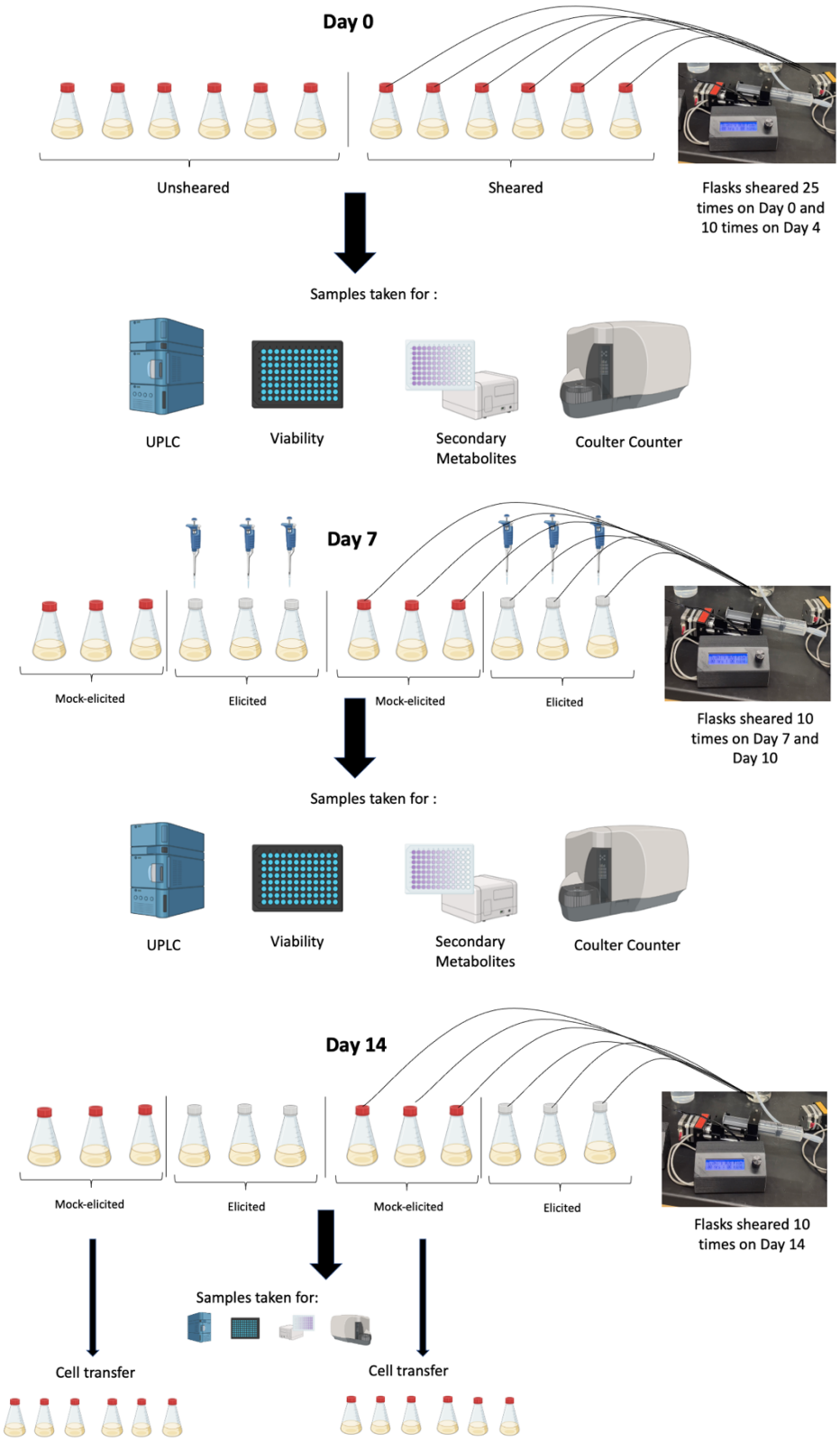



*Figure 4: Overall methodology for preliminary testing of the shearing device*

The second experiment described in *Figure 5* then tested the long-term impact of elicitation and continuous shearing on a paclitaxel-producing cell line, 48.82A.3S. This experiment was designed to determine the impact of shearing on cell viability, aggregate diameter distribution, taxoid production and global secondary metabolites synthesis. After transferring the cells on day 0, shearing was performed 25 times and repeated 10 times on days 4, 7, 10 and 14. Methyl jasmonate elicitation was done on day 7 and samples for the different analyses were taken on day 0 (before and after shearing), and days 7, 14 and 21. On day 14, half of the cell suspensions were transferred to continue the experiment on a new cycle, while the other half was kept until day 21 for analysis. Therefore, day 0 of the second cycle is day 14 of the first and day 28 is the first day of the third cycle.


Day 0
Unsheared
Sheared
Flasks sheared 25 times on Day 0 and 10 times on Day 4
Samples taken for :
UPLC
Viability
Secondary Metabolites
Coulter Counter
Day 7
Mock-elicited
Elicited
Mock-elicited
Elicited
Flasks sheared 10 times on Day 7 and Day 10
Samples taken for :
UPLC
Viability
Secondary Metabolites
Coulter Counter
Day 14
Mock-elicited
Elicited
Mock-elicited
Elicited
Flasks sheared 10 times on Day 14
Samples taken for:
Cell transfer
Cell transfer

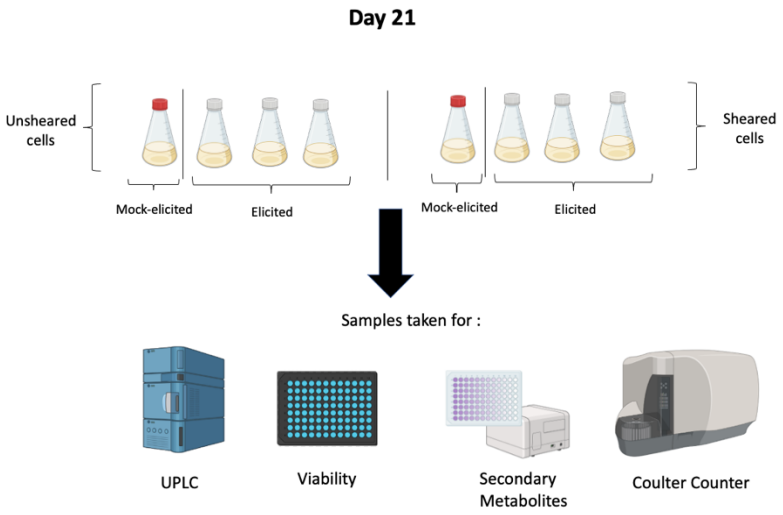



*Figure 5: Overall methodology for the long-term experiment*

The third experiment consisted of further testing of the shearing device. It was divided into two separate tests, the first one, as described in *Figure 6*, tested the effect of shearing on the different phases of the culture cycle as well as a different number of shearing times. As shown in *Figure 7,* previous data from the Roberts laboratory found that the *Taxus* culture cycle consists of three separate phases, the lag phase (days 0-2), the growth phase (days 3-12) and the stationary phase (days 13-14). To test the influence of shearing on all phases, the same experiment was repeated using cells at different ages. Five flasks, one unsheared, and the four others, sheared, respectively 25 times, 50 times, 75 times and 100 times, were tested over a three-day period.

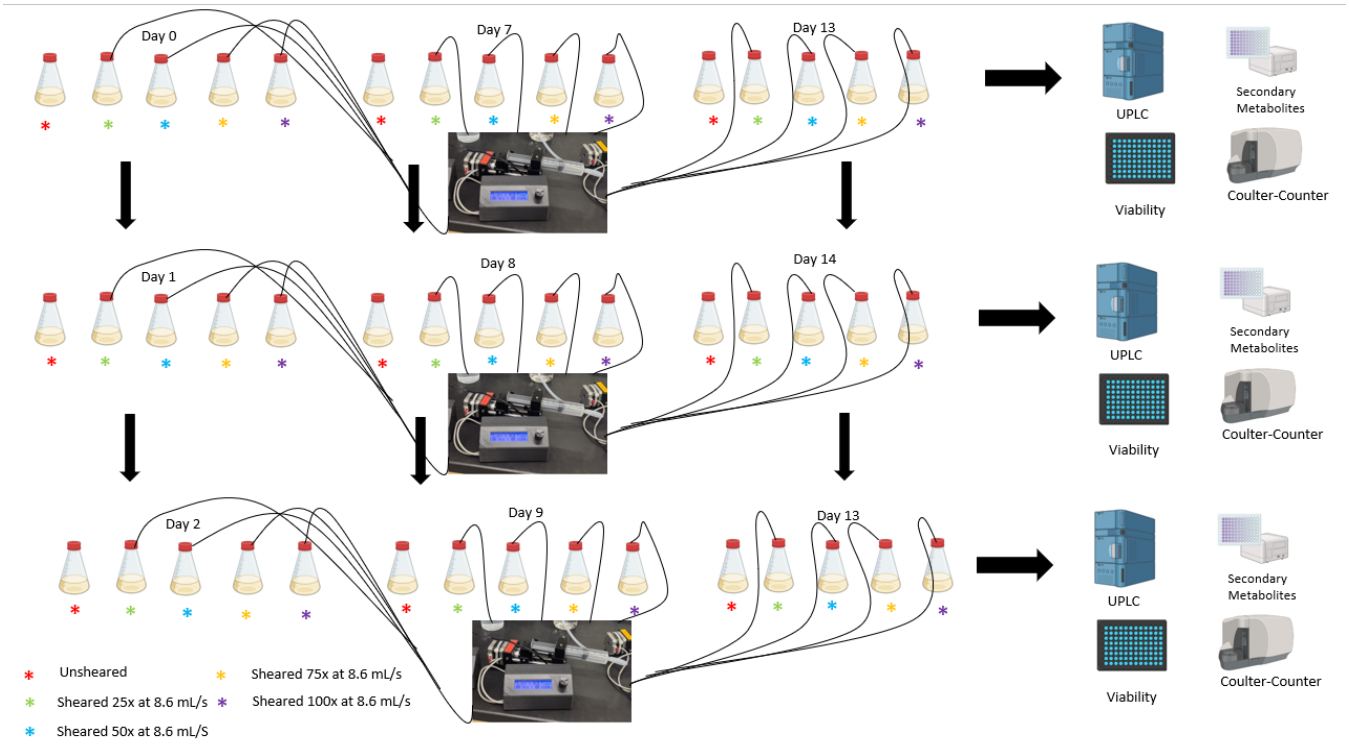



*Figure 6: Overall methodology for the culture cycle phases shearing test*

On the first day, the cell cultures were sheared continuously according to their respective shearing times number and sampled for viability, global secondary metabolite synthesis, paclitaxel synthesis, and aggregate size analysis. The same protocol was repeated 24 hours later on the second day and 48 hours on the third and final day. The first set of experiments was carried out during the lag phase on Day 0, after subculturing the cells, and continued on Days 1 and 2. The second set was carried out during the growth phase on Days 7,8 and 9. The third set was carried out during the stationary phase and Days 13,14 and 15.

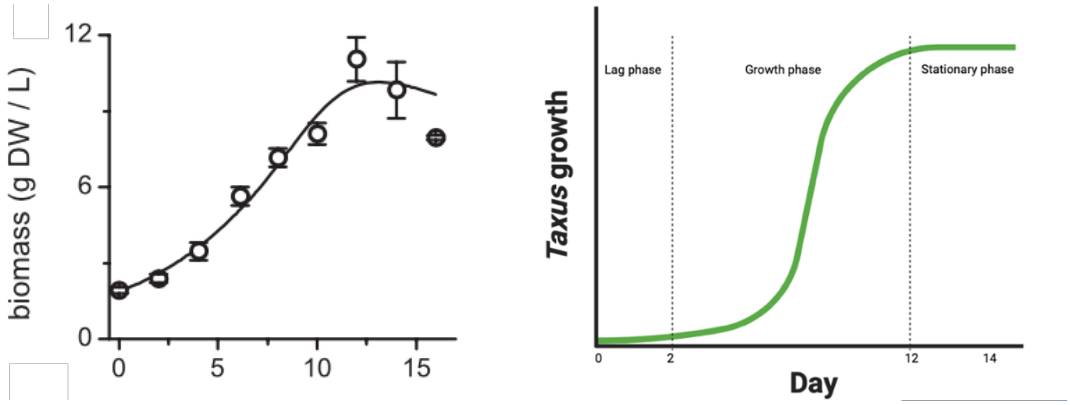



*Figure 7: Different phases of the* Taxus *culture cycle (Kolewe, 2011)*

The second phase of the culture cycle experimentation as described in *Figure* 8 was designed to test distinct days of the culture cycle as well as continuous shearing for different time periods (one or two hours). In the long-term shearing experiment carried out by Dr Wilson, days 4, 7 and 10 of the growth phase were chosen to shear the cells. Therefore, it was important to investigate the possibility of a different day of culture in this phase to be more appropriate for shearing. Three flasks, one unsheared, one sheared for one hour, and one sheared for two hours were tested over a period of one day, and the experiment was repeated on different days of the cycle: day 3,4,5,9,10 and 11. On each day, the cell cultures were sheared continuously according to their respective shearing time and sampled for viability, secondary metabolite synthesis , paclitaxel synthesis and and aggregate size distribution.

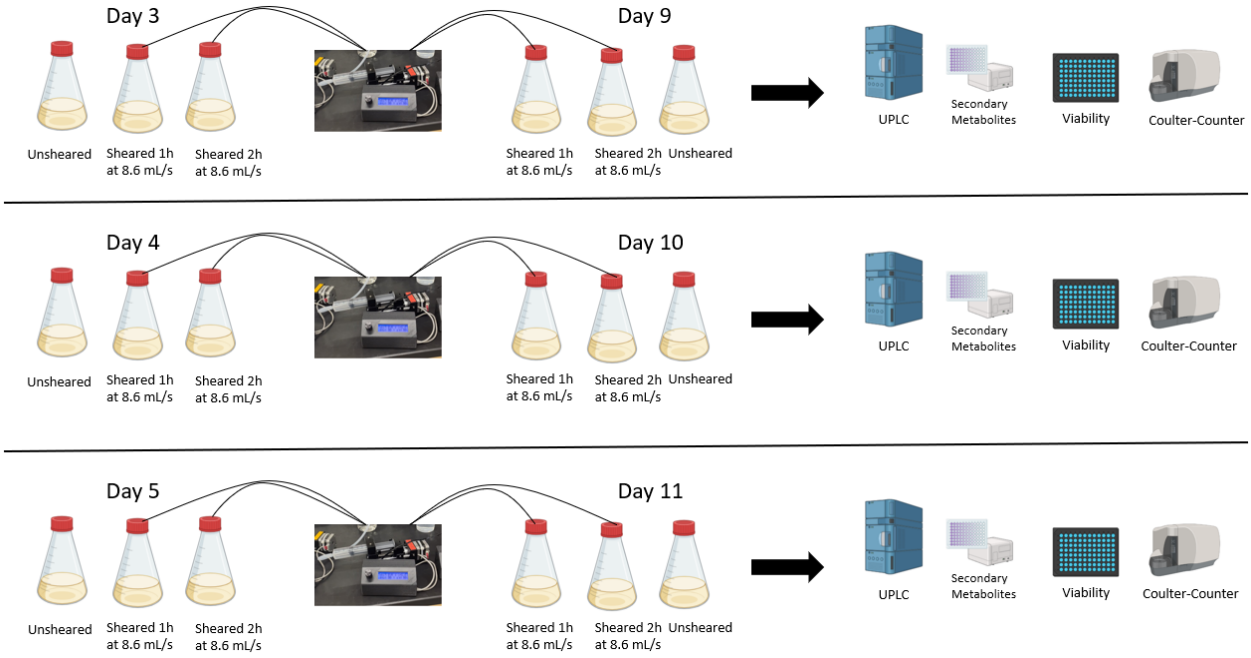



*Figure 8: Overall methodology for the testing of separate days of the growing cycle*

Finally, the fourth experiment as described in *Figure 9* consisted of confirming and optimizing the disaggregation protocol previously established by Naill et al. (2004) using 1.0 % (w/v) cellulase and 0.1 % (w/v) pectolyase Y-23 in an osmoticum of 0.5 M mannitol and 0.3 % dextran sulfate and comparing it to the mechanical shearing method, to determine which method resulted in better cell separation and viability. This experiment was carried out for a single day, and tested different treatment periods, to determine any impacts on cell viability. The experiment consisted of seven different flasks, one unsheared, one sheared for four hours and one sheared for five hours, one treated with enzymes for four hours and one for five hours, and finally one sheared and treated with enzymes for four hours and one for five hours. Samples were taken for viability and aggregate size distribution at the beginning and end of the experiment.

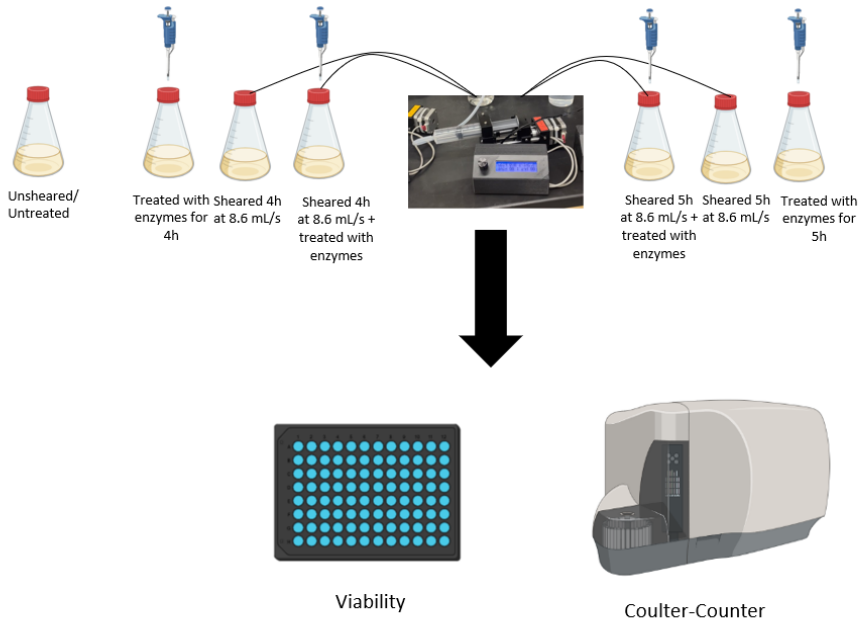



*Figure 9: Overall methodology for the comparison of disaggregation via enzymes and shearing*

**2.2 Culture initiation**

Fifteen needles were taken from a branch sampled directly from yew trees from the *Taxus chinensis* species at the Arnold Arboretum of Harvard University in June 2022 and put into a beaker filled halfway with water. After being shaken in the incubator for two to three minutes at 100 rpm, the water was discarded from the beaker and replaced with 100 mL of a diluted soap/detergent solution. The beaker was shaken for two to three minutes at 100 rpm, and the soap solution was discarded. The needles were then rinsed five times with 10 mL nanopure water (Gibson, 1993).

Under the laminar flow hood, 50 mL of a 70% alcohol solution were pipetted into a labeled sterile flask, where the needles were carefully placed using a tweezer. After letting the flask sit for 20 secs and shaking, the alcohol was removed from the flask using a 25 mL pipette. Using tweezers, the needles were carefully removed from the flask and put into another flask containing 50 mL of bleach. After letting the flask sit for 10 mins and shaking, the bleach was removed from the flask using a 25 mL pipette (Gibson, 1993). Using tweezers, the needles were carefully removed from the flask and put into another flask containing 50 mL of sterile water. This last operation was repeated five times. Using tweezers, each needle was carefully taken out of the flask, put onto a sterile petri dish (Fisher 50 mm x 0.8 mm) with solid media, and cut in two lengthwise using a scalpel. Each plate was then labelled, wrapped with Parafilm and placed into the 23˚C incubator at 125 rpm (Gibson, 1993). A 1 L solution of solid *Taxus* media at pH = 5.5 contains 3.21 mg of Gamborg B-5 basal medium, 16 g of sucrose, 96 µL of benzylidene, 2.16 mL of naphthalene acetic acid, 1.6 mL of Plant Preservative Mixture, 3g of PCC agar and approximately 1 L of nanopure water (Enaksha, 1994).

**2.3 Subculturing cells**

A month after harvesting the needles, the callus cultures, dedifferientiated cells that grew from the needles, were transferred to larger petri dish (Fisher, 100 mm x 15 mm). After maintaining the callus cultures and transferring them to fresh plates every three weeks, the cultures were ready to be transferred to liquid media, approximately three months after the initiation (Gibson, 1993).

To transfer from Callus to suspension cultures, cells were taken from the Callus plate using a sterilized spatula and transferred into a sterile 125 mL Erlenmeyer flask containing 40 mL of liquid media, under the laminar flow hood. A 1L solution of liquid *Taxus* media at pH = 5.5 contains 3.21 mg of Gamborg B-5 basal medium, 16 g of sucrose, 96 µL of benzylidene, 2.16 mL of naphthalene acetic acid, 1.6 mL of Plant Preservative Mixture, and approximately 1 L of nanopure water (Enaksha, 1994). After autoclaving the solution, 48 mL of filter sterilized antioxidant stock solution containing 150 mg/L ascorbic acid, 150 mg/L citric acid and 6.0 mM L-Glutamine was added under the laminar flow hood (Kolewe, 2010).

To transfer the suspension cultures, 40 mL of liquid media with antioxidant was added to a sterile 125 mL Erlenmeyer flask with a foam cap under the laminar flow hood using a 25 mL stereological pipette. 10 mL of well-mixed suspension culture was added to each flask using a 10 mL stereological pipette with a broken tip, to ensure that all size aggregates could be transferred. The flasks were then placed into an incubator at 23˚C and 125 rpm in the dark and subcultured (i.e. transferred to fresh media) every 14 days for maintenance (Kolewe, 2010).

**2.4 Design of the shearing device**

The design and machining of the shearing devices were performed by Ian Anderson, lab manager and advanced machinist for WPI's Chemical Engineering department. The cell shearing pump is a design adapted from an open-source syringe pump (Samokhin, 2020). To mirror manual shearing, continual shearing is defined as 10 mL of cell culture volume going up and back down a serological pipette. Given that typically, three biological replicates are evaluated experimentally to ensure statistical significance of results, three shearing devices were built to allow for simultaneous shearing of the culture replicates. The device shown in *Figure 10* was built almost exactly as the previous device built by Ian Anderson for Teodora Dinicu's research. It uses 3D printed mechanical components of Samokin's design with a custom code written in the Arduino integrated microcontroller that is programmed to make the stepper motor drive the carriage housing connected to a lead screw from one limit to another. Therefore, when one of the limit switches comes into contact with the adjustable stops (set for 10 mL of liquid), the syringe plunger is signaled to stop and go into the reverse direction which allows the device to run continuously. The Arduino microcontroller and electronic components are housed in a case with a single rotary knob to select all test parameters and an LCD to display the parameters and test results.

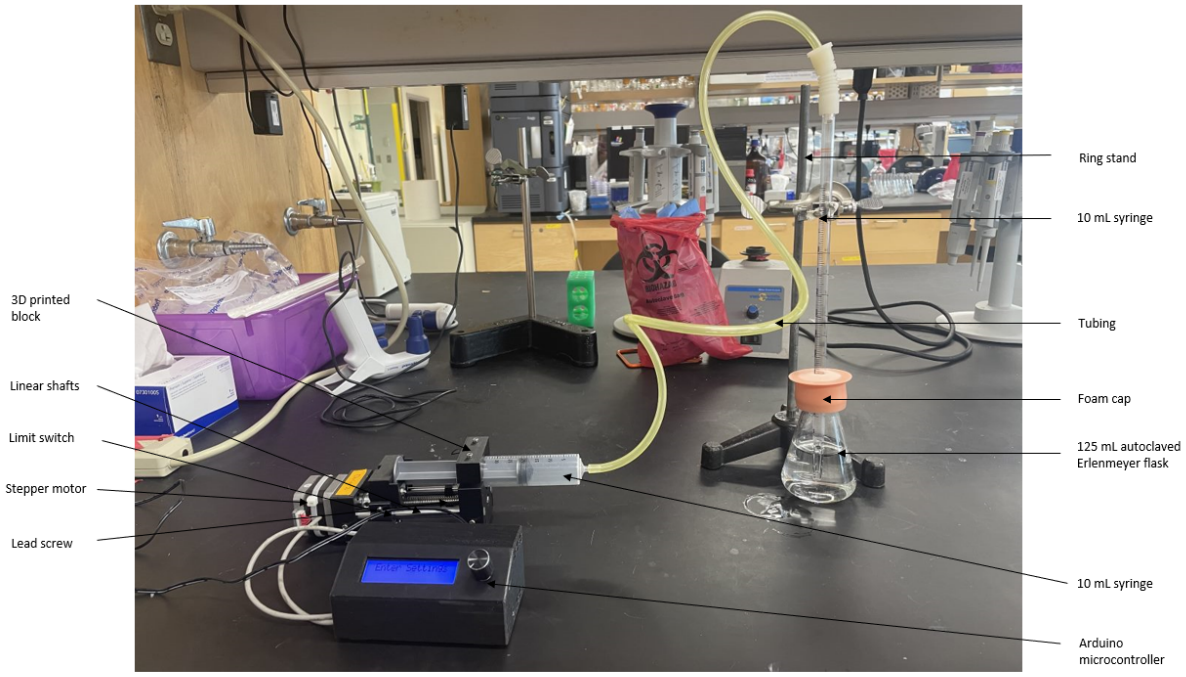



*Figure 10 : Detail schematic of the continuous shearing device*

The new devices were built so that different parameters could be chosen, such as the flowrate, the cycle count and test time. There are two types of modes of operation - continuous shearing, and intermittent shearing. The continuous shearing mode requires input of the pump speed 1 through 10. Speed 1 will move the plunger in and back out over approximately 20 seconds. Speed 10 will do the same in about 2 seconds. The test time is entered in 15-minute intervals up to 360 minutes (six hours). The second mode pumps a desired number of cycles at the set pump speed before resting for a set number of minutes until the test duration time has been met. This mode requires entering the number of cycles per set and rest time between sets in addition to the previous test parameters. During all tests, the LCD will display the elapsed test time and the calculated flow rate based on the time it takes for the syringe to move from one end to the other of its travel (assuming 10 mL). The instructions implemented in the Arduino microcontroller screen are shown in *Table 1*:

| | |
|---|---|
| Test Type<br>1 1=Cont, 2=Int. | Select “1” if you would like the pump to cycle continuously for the test duration.<br><br>Select “2” if you would like the pump to cycle a set number of times followed by a rest period. This will repeat until the test duration has expired. |
| Pump Speed<br>1 (1 through 10) | The pump speed can be adjusted between 1 and 10.<br><br>Speed 1 = 1.0 mL/s; Speed 2 = 1.1 mL/s; Speed 3 = 1.2 mL/s; Speed 4 = 1.4 mL/s; Speed 5 = 1.7 mL/s; Speed 6 = 2.2 mL/s; Speed 7 = 2.8 mL/s; Speed 8 = 4.2 mL/s ; Speed 9 = 5.2 mL/s ; Speed 10 = 8.6 mL/s |
| Test Duration<br>15 Minutes | Set the total desired run time of the test in 15 minutes intervals, 360 minutes being the maximum. |

| | |
|---|---|
|  | This number is the number of times the plunger will move in and back out before a rest period when test type 2 is selected. It will be ignored for test type 1. |
|  | This is the time from the start of one set to the start of the next set for test type 2. 100 minutes maximum in 5 minutes intervals. This setting will be ignored for test type 1. |
|  | Press the center button to start the test. |
|  | The approximate flowrate through the nozzle is calculated and displayed while the test is running. It is assuming that 10mL is being pumped each time and is based on the time it takes the plunger to move in one direction, not a full cycle. |

*Table 1 : Cell shearing pump operating instructions*

Although the devices are more advanced than the previous pilot device, they share some of the same constraints. The first constraint is that the devices aren't sterile, which can introduce contamination to the cultures. The 3D printed parts should be made with autoclavable parts which would significantly reduce the risk of contamination. Another constraint is the fact that cell aggregates can get stuck inside the pipette, which prevents the rest of the aggregates from being sheared and invalidates all results from an experiment, as it is impossible to determine at which point the cells got stuck. This issue is even more significant as the flowrate of shearing increases. To prevent this issue from happening, a sensor could be implanted into the devices and detect if liquid has been sucked into the pipette or not. The machine could then turn on the air pump to flush stuck aggregates from the pipette (Dinicu, 2022). Finally, another constraint of the devices is an error in the code implanted in the Arduino microcontroller. For the intermittent shearing program, if the number of shearing cycles exceeds one hundred, the shearing mode switches to continuous mode and doesn't know when to stop shearing. A simple rewriting of the code could fix that issue.

The shear force of the device can be calculated for the lowest and highest flowrates (1.0 mL/s and 8.6 mL/s). First, the Reynolds can be calculated to determine whether the flow is laminar or turbulent.

$$Re = \frac{\rho v_{avg} D}{\mu} = \frac{2\rho Q}{\pi R \mu}$$

Where $\mu$ and $\rho$ are respectively the viscosity and the density of the fluid which here will be considered to be water as it is the most important component of the media solution. Q is the flowrate and R is the radius of the 10 mL pipette.

For both flowrates, the flow is found to be laminar. For low flow in a cylindrical tube (here the 10 mL serological pipette), the velocity distribution is the following :

$$v_z = v_{max}\left(1 - \frac{r^2}{R^2}\right)$$

Where $r$ the distance from the center of the pipette and $v_{max}$ is the maximum velocity:

$$v_{max} = \frac{2}{\pi R^2} Q$$

Then, the shear stress is given as:

$$\tau_{rz} = -\mu \frac{dv_z}{dr} = 2\mu v_{max} \frac{r}{R^2} = \frac{4\mu r}{\pi R^4} Q$$

The maximum shear stress will be at the wall $\tau_{rz}|_{r=R}$:

$$\tau_{rz}|_{r=R} = \frac{4\mu}{\pi R^3} Q$$

This simple analysis ignores the presence of the aggregates, but it is a reasonable "first-order" approximation of the shear experienced in the experiment. The actual shear would likely be less than this, because some of the aggregates are close to the center where the shear is zero. So, it may be more accurate to compute the average shear stress over the 10 mL pipette:

$$\langle \tau_{rz} \rangle = \frac{1}{\pi R^2} \int_0^{2\pi} \int_0^R \tau_{rz}\, r dr d\theta = \left(\frac{Q}{\pi R^2}\right)\left(\frac{4\mu}{\pi R^4}\right) \int_0^{2\pi} \int_0^R r^2 dr d\theta = \left(\frac{Q}{\pi R^2}\right)\left(\frac{8\mu}{3R}\right)$$

The values obtained for both low and high flowrates are shown in *Table 2*:

| Q (mL/s) | Re | Flow | $\tau_{rz}\|_{r=R}$ (N/m$^2$) | $\langle \tau_{rz} \rangle$ (N/m$^2$) |
|---|---|---|---|---|
| 1.0 | 212 | Laminar | 0.0472 | 0.0313 |
| 8.6 | 1824 | Laminar | 0.406 | 0.270 |

*Table 2 : Reynolds number, maximum shear stress at the wall and average shear stress over the 10 mL pipette for a flowrate of 1.0 mL/s and a flowrate of 8.6 mL/s*

For both maximum shear stress and average shear stress, it varies linearly with the flowrate, so if the flowrate is doubled, then the shear stress is also going to double.

The binding force σ of the particles and the shear stress are linked through the following dimensionless number $N$ :

$$N = \frac{\sigma}{\tau} > 1$$

At a low flowrate of 1.0 mL/s, the device still disaggregates the *Taxus* cells, which means that the shear force $\tau$ is already more important than the binding force $\sigma$. In order to understand at which speed of the device the disaggregation begins, flowrates lower than 1.0 mL/s should be added to the device, to see when the binding force $\sigma$ is more important than the shear force $\tau$.

**2.5 Shearing cultures/cells**

For the long-term experiment, cells were first sheared 25 times on day 0 of the growth cycle. To shear the cells, a 10 mL disposable serological pipette was attached to the shearing device and a ring stand and inserted into the flask covered with a pierced cap. The intermittent cycle of the device

was chosen, at speed 10 (8.6 mL/s) and with a pipetting number dependent on the day of the cycle. On days 4, 7, 10 and 14, the cells were sheared 10 times (Wilson, 2020).

For the short-term experiments done to test the device, *Tables 3, 4* and *5* explain the number of times the cells were sheared, on which day of the *Taxus* growing cycle and at which speed.

| | **Day 1** | **Day 2** | **Day 3** |
|---|---|---|---|
| Flask 1 | Unsheared | Unsheared | Unsheared |
| Flask 2 | 25 times / Speed 8 | 25 times / Speed 8 | 25 times / Speed 8 |
| Flask 3 | 25 times / Speed 9 | 25 times / Speed 9 | 25 times / Speed 9 |
| Flask 4 | 25 times / Speed 10 | 25 times / Speed 10 | 25 times / Speed 10 |

*Table 3: Preliminary test (test n°1) of the shearing device*

| | **Days 0,1,2** | **Days 7,8,9** | **Days 13,14,15** |
|---|---|---|---|
| Flask 1 | Unsheared | Unsheared | Unsheared |
| Flask 2 | 25 times / Speed 10 | 25 times / Speed 10 | 25 times / Speed 10 |
| Flask 3 | 50 times / Speed 10 | 50 times / Speed 10 | 50 times / Speed 10 |
| Flask 4 | 75 times / Speed 10 | 75 times / Speed 10 | 75 times / Speed 10 |
| Flask 5 | 75 times / Speed 10 | 75 times / Speed 10 | 75 times / Speed 10 |

*Table 4: Test n°2 of the shearing device*

| | **Day 3** | **Day 4** | **Day 5** | **Days 9** | **Day 10** | **Day 11** |
|---|---|---|---|---|---|---|
| Flask 1 | Unsheared | Unsheared | Unsheared | Unsheared | Unsheared | Unsheared |
| Flask 2 | 1hour / Speed 10 | 1hour / Speed 10 | 1hour / Speed 10 | 1hour / Speed 10 | 1hour / Speed 10 | 1hour / Speed 10 |
| Flask 3 | 2 hours / Speed 10 | 2 hours / Speed 10 | 2 hours / Speed 10 | 2 hours / Speed 10 | 2 hours / Speed 10 | 2 hours / Speed 10 |

*Table 5: Test n°3 of the shearing device*

For each experiment, the shearing was either performed under the sterile hood (for short experiments), or in the incubator at 23˚C and 125 rpm to maintain sterility.

**2.6 Elicitation with Methyl Jasmonate**

For the long-term experiment, on day seven, cells were mock-elicited or elicited with methyl jasmonate. In a first autoclaved 1.5 mL microcentrifuge tube, the mock elicitation stock solution was prepared by mixing 500 µL of Mili-Q water and 500 µL of ethyl alcohol. In a second autoclaved 1.5 mL microcentrifuge tube, the elicitation solution was prepared by mixing 500 µL of Mili-Q water, 457.9 µL of ethanol and 42.1 µL of methyl jasmonate (95% Sigma Aldrich) (Patil, 2014). Both solutions were

filtered under the laminar flow hood using a$\mu M$ syringe and a 0.22 µM VDF filter $\mu L$ microcentrifuge tube. The elicitation was then done by adding 56.6 µL of the corresponding solution into the flasks that were then covered with aluminium foil to minimize evaporation of the volatile components and placed back into the 23˚C incubator at 125 rpm in the dark. The final concentration of methyl jamonate in all flasks were 200 µM (Patil, 2014).

**2.7 Disaggregation with enzymes solution**

For the shearing and enzymes disaggregation comparison experiment, 100 mL of stock enzyme solution was prepared by mixing 1.0 % (w/v) cellulase and 0.1 % (w/v) pectolyase Y-23 in an osmoticum of 0.5 M mannitol and 0.3 % dextran sulfate (Naill, 2012). Sterile flasks were filled with 5 mL of the stock solution. Using a Buchner funnel drapped with Miracloth, 1g of Taxus cells were filtered and poured into each flask using a sterile spatula (Naill, 2012). Half of the flasks were then incubated at 23˚C and 125 rpm for four hours and the other half for five hours. Once the incubation period was over, the content of each flask was poured into a 20 mL Falcon tube. The supernatant was then removed using a 10 mL steroloigcal pipette. Using a sterological pipette, 10 mL of fresh media was added to the tube, and then removed after letting the cells sit at the bottom of the tube. This last step was repeated one more time to wash the cells (Naill, 2012).

**2.8 Resazurin Metabolic Activity and Cell Viability Assay**

The viability of the cells is one of the most important parameters to analyze when adding mechanical and chemical stressors to the cells. Healthy cells appear light-yellow in color with a moderate density indicating growth. We check for the absence of excessively large aggregates and potential contamination (mold, bacteria, yeast, …). The resazurin assay is a quantitative assay that is fast, simple, accurate and can be used to determine cellular metabolic activity, which can roughly be correlated with viability. The protocol is based on the reduction of oxidized non-fluorescent blue resazurin to a red fluorescent dye (called resorufin) by the mitochondrial respiratory chain in live cells. Only viable cells with active metabolism can reduce resazurin into resorufin. Living cells are metabolically active and are also able to reduce via mitochondrial reductase, the nonfluorescent dye resazurin to the strongly fluorescent dye resorufin. The fluorescence output is then directly proportional to the number of living cells (Kuete, 2017).

For each experiment, 1 mL of well-mixed samples were collected from each culture flask to be evaluated. For the long-term experiment, samples were collected on Days 0,7,14 and 21 and it was collected on each of the three days for the short-term experiments. After removing the supernatant by pipetting the liquid media, 900 µL of PBS and 100 µL of Resazurin were added to the samples (Resazurin Assay Kit, 2019). The samples were then inverted to mix, covered in tin foil and incubated at room temperature for one hour. After the incubation period, the samples were centrifuged at highest speed for one minute to pellet and 100 µL were pipetted into a black 96-well plate in triplicates. The fluorescence was read at 570 nm on a Victor Nivo PerkinElmer microplate reader (Resazurin Assay Kit, 2019).

**2.9 Paclitaxel and Taxoid Extraction and Analysis**

One mL of well-mixed samples was collected for each culture for taxoid quantification. The samples were placed in 1.5 mL centrifuge tubes that were dried overnight in an Eppendorf, Vacufuge on V-AQ setting (Naill, 2004). The samples were then resuspended in 1 mL of 0.01 % acetic acid in methanol. The aggregates of dried materials were broken down with a spatula, and the samples were vortexed and sonicated for 20 minutes (Naill, 2004). This step was repeated one more time. The samples were then centrifuged for 20 minutes using a 5224 Eppendorf centrifuge at 15,000 rpm. 800 µL of supernatant were removed from the samples and pipetted into new 1.5 mL microcentrifuge

tubes (Naill, 2004). The samples were then dried again in the evaporative centrifuge on V-AL setting for 1 hour. The samples were then resuspended again with 25 µL of methanol$\mu L$ µL $\mu L$ acetonitrile and 40 µL of sterile water. In between the addition of each solvent, the samples were sonicated for 15 seconds (Naill, 2004)$\mu M$ 1.0 mL syringe and a 0.22 µM PVDF filter and poured into a low volume vial and ran on a Waters Acquity UPLC H-Class system (McKee, 2021).

**2.10 Secondary Metabolites Analysis**

One mL of well-mixed samples was collected. The samples were placed in 1.5 mL centrifuge tubes that were dried overnight in an Eppendorf, Vacufuge on V-AQ setting (McKee, 2021). The samples were then resuspended in 500 µL of 0.01% acetic acid in methanol. The aggregates of dried materials were broken down with a spatula, and the samples were vortexed, and centrifuged for 5 minutes in a 5224 Eppendorf centrifuge at 15000 rpm (McKee, 2021). To analyze phenolics concentration, Gallic acid standards were prepared at 1.0 mg/mL, 0.8, 0.6 mg/mL, 0.4 mg/mL, 0.2 mg/mL, 0.1 mg/mL and 0 mg/mL concentrations.$\mu L$ µL$\mu L$ of samples, 240 $\mu L$ of 0.2N Folin and Ciocaltue's phenol (FC reagent) and 960 µL of 700 mM sodium carbonate were then added to new 1.5 mL centrifuge tubes, that were incubated at room temperature for 10 minutes$\mu L$ ,000 rpm. 200 µL of supernatant was then transferred to plate wells in triplicates and read at 750 nm on a Victor Nivo PerkinElmer microplate reader (McKee, 2021).

To analyze flavonoids concentration, catechin standards were prepared at 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 concentrations. 200 µL of sterile water, 300 µL of $NaNO_2$ and 100 µL of samples were then added to new 1.5 mL centrifuge tubes. After an incubation period of 30 seconds, 300 µL of $AlCl_3$ were added to the samples (McKee, 2021). After an incubation period of 2 minutes, 300 µL of 0.8M NaOH were added to the samples that were then incubated at room temperature for 10 minutes and spun down for 60 seconds at 15,000 rpm. 200 µL of supernatant were then transferred to plate wells in triplicates and read at 490 nm on a Victor Nivo PerkinElmer microplate reader (McKee, 2021).

**2.11 Aggregate Diameter Distribution Analysis**

Aggregate size distributions were analyzed on a Multisizer 3 Beckman Coulter Counter with an aperture of 2,000 $\mu m$ . The complete procedure can be found in "Multi-scale characterization and engineering of *Taxus* suspension cultures" (Kolewe, 2011) and "A population balance model to modulate shear for the control of aggregationin Taxus suspension cultures" (Wilson, 2020). The diluent solution contains 65:35 Isoton:Glycerol and was filtered using a Buchner funnel and reused for all analyses to minimize waste (Kolewe, 2010). Using this diluent solution, the background noise was determined and subtracted from the samples analysis. 2 mL of well-mixed samples were collected using a 1mL cut pipette tip and poured into 380 mL of the diluent solution and placed into the Coulter Counter acting as a batch reactor (Kolewe, 2010). Samples were then run for 60 seconds with a flowrate of 1.5 mL/s. The data from the Coulter Counter were then used to calculate biomass and mean aggregate diameter with previously established correlations (Kolewe, 2010).

**2.12 Microscopy**

For the shearing and enzymes comparison experiment, 500 $\mu L$ of cultures were collected from each flask and transferred into a 1.5 mL tube, where 500$\mu L$ of fresh media were added. The tube was then inverted to mix. In parallel the 10x stain solution was prepared by diluting 1 $\mu L$ of Fluorescein Diacetate and 1 $\mu L$ of Propidium Iodide in a 1.5 mL centrifuge containing 98 $\mu L$ of nanopure water (Jun Kim, 2005). After inverting to mix, 10 $\mu L$ of the 10x stain solution was added to 90 $\mu L$ of the diluted cells into a new 1.5 mL centrifuge tube. After a period of incubation of 2 mins, 30 $\mu L$ stained cells were placed on a microscope slide covered with a glass coverslip, which was then sealed with wax (Jun Kim,

2005). The slide was then viewed under the fluorescent microscope to determine the effect of shearing and enzyme treatment on aggregate size and determine the presence or absence of smaller aggregates or single cells.

# Chapter 3 : Results and Discussion

My thesis began with a continuation of studies using continuous shearing mode with a prototype shearing device on a paclitaxel-producing cell line, 48.82A.3S (Dinicu, 2022). In this previous study, continuous shearing for a period of 72 hours at a flowrate of 7.3 mL/s (with samples being taken every 24 hours) showed a significant decrease in aggregate size and a promising increase in paclitaxel production. The first experiment presented in this work aimed to further test the impact of continuous shear on a paclitaxel-producing cell-line by considering several factors such as flowrate, phases of the *Taxus* culture cycle, shearing time, and the impact of shearing on mean aggregate size, aggregate size distribution, paclitaxel synthesis, global secondary metabolite production and cell viability and growth. The data for these sets of experiment can be found in chapters 3.1, 3.3 and 3.4.

The results of long-term shearing was compared to two previous research reports (Wilson, 2020; Dinicu,2022). The first report monitored long-term growth and aggregation of sheared cultures (Wilson,2020). Eight cultures of a non-paclitaxel producing cell line (P93AF) were maintained for eight generations (each generation representing a 14-day growth cycle). Flasks were manually sheared using a pipette 25 times on Day 0 and 10 times on Days 4,7,10 and 14 and elicited with 200 µM methyl jasmonate (MeJa) on Day 7. Results showed that the culture growth was stable and unaffected by shear over time and the mean aggregate diameter decreased in a constant manner for sheared cultures. The effect of shear on global secondary metabolite and paclitaxel accumulation was not studied due to the fact that the cell lines evaluated did not accumulate measurable levels of these compounds. The second report replicated the first one, but focused on the study of the comparison of manual long-term effect of shear on a non-paclitaxel producing cell line (P093XC) and a paclitaxel producing cell line (48.82A.3S) combined with MeJa elicitation and the impact of shear on cell viability and growth, mean aggregate size and paclitaxel and global secondary metabolite production (Dinicu, 2022). This research showed increased levels of paclitaxel and its precursors (baccatin III and 10-DAB) in MeJa-elicited paclitaxel producing cell cultures. A decrease in aggregate diameter distribution was also observed, up to 63.43 %. However, in this research contamination affected the cultures and therefore decreased their viability.

Work in this thesis aimed to replicate the shearing parameters (shearing iterations and days of shear) presented in the previous works, using an automated shearing device at the highest flowrate allowed (8.6 mL/s) instead of manual shearing. The work presented here tested the impact on mean aggregate distribution, paclitaxel synthesis, global secondary metabolite production, and cell viability and growth. The data for this experiment can be found in chapter 3.2.

The data presented in chapters 3.5, 3.6 and 3.7 compared the viability and aggregate size distribution of cells continuously sheared for a total of four and five hours to cells treated with 1.0 % (w/v) cellulase and 0.1 % (w/v) pectolyase Y-23 in an osmoticum of 0.5 M mannitol and 0.3 % dextran sulfate for the same amount of time.

### 3.1 Preliminary testing of the shearing device

Preliminary testing was conducted on the automated shearing device. This work was a follow-up to research done by Dr. Sarah Wilson testing different flowrates of continuous manual shearing showing that the highest speed tested (7.3 mL/s) resulted in the lowest mean aggregate diameter and

the fastest rate of disaggregation, reducing the mean aggregate size of the culture from a starting mean diameter of 1,050 μm to a final mean diameter of 550 μm (Wilson, 2020). Here, the three highest speeds of the shearing device (4.3 mL/s, 5.2 mL/s and 8.6 ml/s) were tested over a period of three days with a number of 25 iterations per day. Samples for mean aggregate size diameter and viability (roughly correlated as metabolic activity by the resazurin assay) were taken before and after shearing.

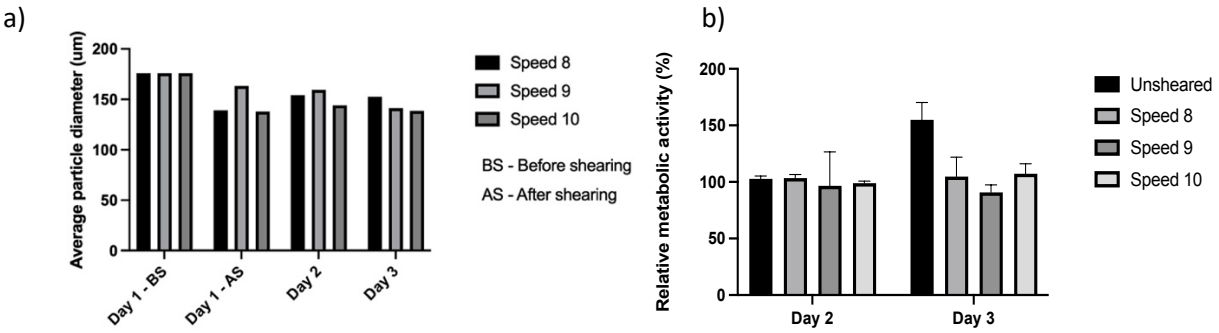



*Figure 11. (a) Mean particle diameter of cultures on Day 1 before and after shearing and Days 2 and 3. There is only one sample replicate for each flask (b) Relative metabolic activity of Day 2 compared to Day 1 and Day 3 compared to Day 2 of cultures unsheared (control) and sheared at different speeds of the device (Speed 8: 4.3 mL/s; Speed 9: 5.2 mL/s; Speed 10: 8.6 mL/s) Error bars represent three technical replicates taken from the same flask. Relative metabolic activity is proportional to the number of viable cells.*

The mean aggregate size of *Taxus* suspension cultures was successfully reduced by pipetting the culture with the shearing device through a 10 mL pipet. One single iteration of shear refers to the uptake and release of 10 mL of culture. Through shearing, the mean aggregate diameter was reduced from 176 μm on Day 1 to 152 μm for Speed 8, 141 μm for Speed 9 and 138 μm for Speed 10 on Day 3, as seen on *Figure 11.a*. Different flowrates did not impact viability, as it remained constant throughout the experimentation period (*Figure 11b*).

Considering these results, a correlation can be made between the speed of the device and the level of shear by graphing trend lines on the average particle diameter over time, as shown in *Figure 12*.

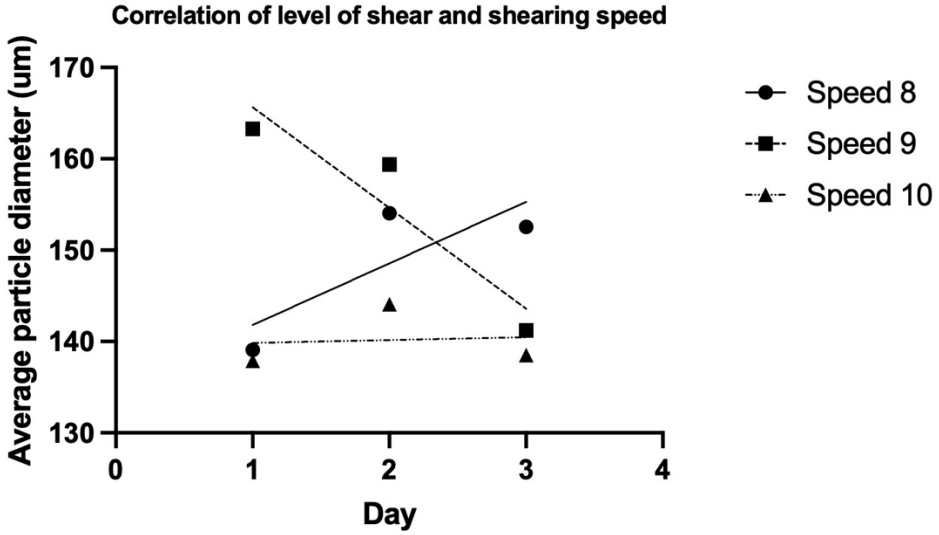



*Figure 12 : Correlation of level of shear with the average particle diameter in μm and shearing speed over time*

As is seen in *Figure 12*, no correlation between level of shear and shearing speed can be made as there are no consistent trends amongst the different shearing speeds employed. This lack of correlation maybe be due to the fact that the amount of shear applied in this experiment was relatively low, with 25 shearing iterations per day (which is about 60 seconds for the highest speed). To define a reliable correlation between level of shear and shearing speed, continuous shearing should be applied for at least an hour (which equates to around 1100 shearing iterations at the highest speed).

### 3.2 Effect of long-term shearing on cell metabolic activity, paclitaxel synthesis, global secondary metabolite production, and aggregate size

Using the data on the shearing device as described below, the highest speed with a flowrate of 8.6 mL/s was used to carry out the long-term experiment; specifics are explained in the methods section.

#### *3.2.1. Effect of long-term shearing on cell metabolic activity*

The effect of long-term shearing on cell health and metabolic activity was studied using the resazurin metabolic activity assay. Results suggested that shearing at a flowrate of 8.6 mL/s would have no negative impact on cell viability. However, in this experiment, a decrease in metabolic activity was observed over time, as shown in *Figure 13*. Unsheared cells both mock-elicited and elicited with MeJa, have a slightly higher metabolic activity than sheared cells over time (50% viability against 20% for both elicited and mock-elicited cultures). Given that the viability dropped so significantly on Day 0 right after culture and decreased during the first subculture cycle, the cells were not subcultured on Day 14 and the experiment was stopped on Day 21. Previous data showed that MeJa elicitation had no impact on cell health (Dinicu, 2022). Results here were consistent, given that after elicitation on Day 7, the metabolic activity of the cultures remained constant and didn't drop any further, as seen in *Figure 13 (b).* It's also important to note that for every type of culture, the error bars are significant, which means that there were some cultures with high viability throughout the experiment.

a)

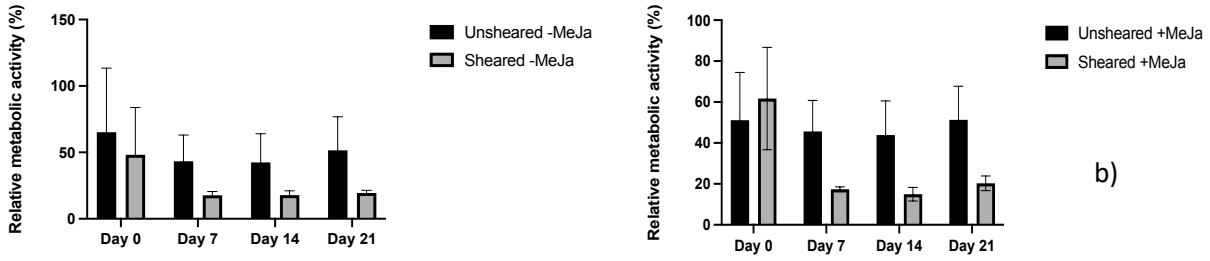


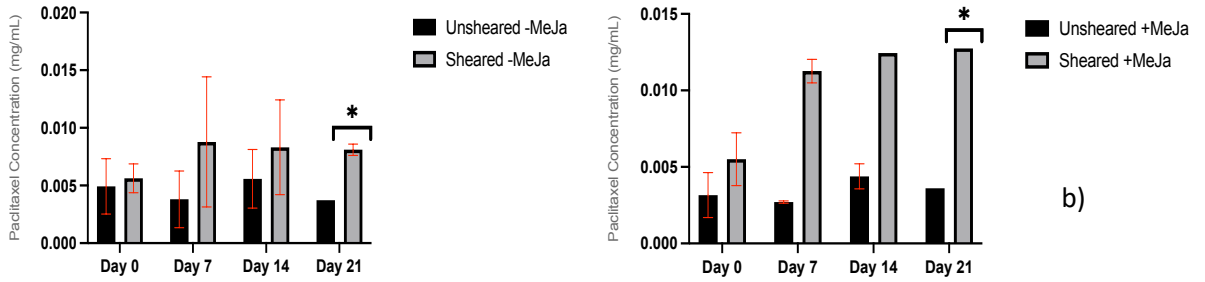

*Figure 13. (a) Long-term relative metabolic activity of three biological replicates of unsheared cells mock elicited and three biological replicates of sheared cells mock elicited. Cells were sheared 25 times on day 0, 10 times on day 4, day 7, day 10 and day 14. (b) Long-term relative metabolic activity of three biological replicates of unsheared cells Meja elicited and three biological replicates of sheared cells MeJa-elicited on day 7. The relative metabolic activity on day 0, days 7, 14 and 21 compares the fluorescence of the cells after shearing to cells on day 0 before transfer. Error bars represent the average of three biological replicates with standard deviation. +MeJa indicates that cells were elicited on Day 7 with methyl jasmonate; -MeJa indicates the cells were mockelicited. Relative metabolic activity is proportional to the number of viable cells.*

#### *3.2.2 Effect of long-term shearing on production of paclitaxel and related precursors*

The impact of long-term shearing on paclitaxel accumulation and its precursors, 10-DAB and baccatin III was also examined, using UPLC. One of the most important steps in the complex synthesis of paclitaxel, is the acetylation of 10-DAB into baccatin III, given that it is a direct precursor to the production of paclitaxel (Zocher, 1996).

As seen in *Figure 14 (a)* and *(b)*, there is a major difference in paclitaxel concentration between sheared and unsheared cultures. On day 0, both types of cultures have a similar concentration at around 0.005 mg/mL, but as the concentration in unsheared cultures remains almost always constant (slight increase in the growth phase between days 7 and 14), the concentration for sheared cultures keeps increasing during the culture cycle, up until day 14 where it stabilizes and remains constant up to day 21. There is also a difference between MeJa-elicited and mock-elicited cultures. On day 21, the concentration for sheared MeJa-elicited cultures is higher than sheared mock-elicited cultures. This correlates with previous data showing that MeJa elicitation increases paclitaxel levels. For all cultures, almost no concentration of precursors, 10-DAB and baccatin III were found, which means that it's possible that the precursors were consumed to produce more paclitaxel over time.

a)



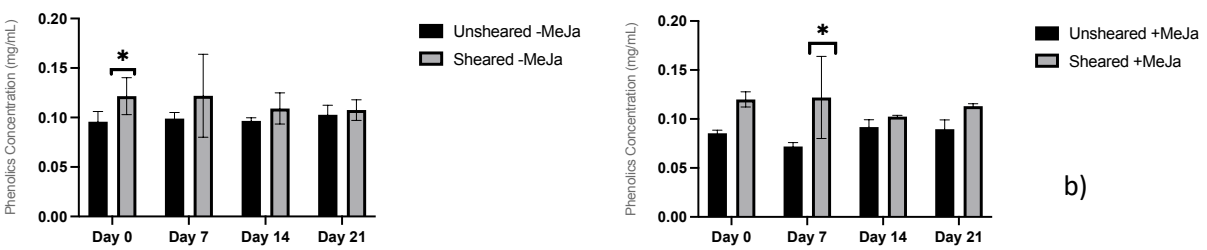

b)

*Figure 14. Paclitaxel concentration (mg/mL) over time in (a) unsheared and sheared mock-elicited cultures (b) unsheared and sheared MeJa-elicited cultures. A Student's t test determined the paclitaxel production for unsheared cells to be statistically different from sheared cells after 21 days for both MeJa-elicited and mock-elicited cultures (*) Error bars represent the average of three biological replicates with standard deviation. +MeJa indicates that cells were elicited on Day 7 with methyl jasmonate; -MeJa indicates the cells were mock-elicited.*

### *3.2.3 Effect of long-term shearing on global secondary metabolism*

The effect of long-term shear on global secondary metabolite accumulation was tested to determine the general cellular stress response to shearing. *Figures 15 (a)* and *(b)* show a slight difference in phenolics levels between unsheared and sheared cultures, with sheared cultures having a higher concentration. *Figures 15 (c)* and *(d)* show that flavonoids accumulation was not impacted by shearing, as both unsheared and sheared levels remain the same all throughout the experiment. MeJa elicitation was also shown to have no further impact on phenolics and flavonoids accumulation, as there is no difference in concentration between mock-elicited and MeJa-elicited cultures. This correlates with previous studies, showing that the elicitation of the 48.82A.3S cell line has no impact on phenolics and flavonoids accumulation (Dinicu, 2021). However, the lack of increase of general secondary metabolites synthesis with MeJa elicitation is unusual, as increases are typically observed in elicited *Taxus* cultures. (McKee, 2021).

a)




b)

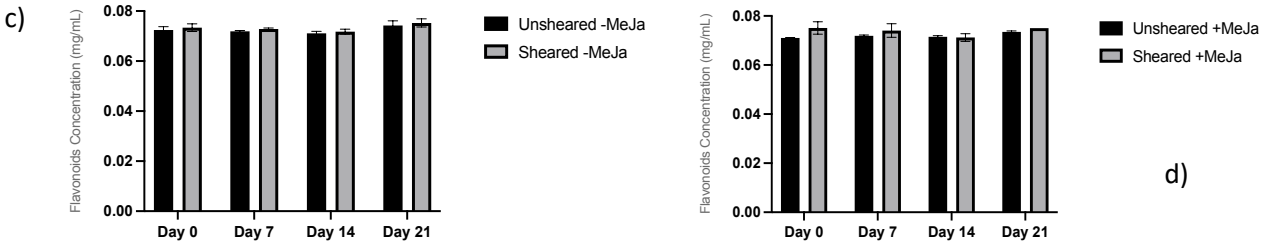



*Figure 15. Phenolics concentration (mg/mL) of sheared and unsheared (a) mock-elicited cultures (b) MeJa elicited cultures. Long-term shear had only a minor impact on phenolics levels, showed by a higher concentration in phenolics for sheared cultures than for unsheared cultures. Elicitation had no impact on phenolics concentration. Flavonoids concentration (mg/mL) of sheared and unsheared (c) mock-elicited cultures (d) MeJa elicited cultures. Shear and elicitation had no impact on flavonoids levels. Error bars represent the average of three biological replicates with standard deviation. A Student's t test determined the paclitaxel production for unsheared cells to be statistically different from sheared cells after 21 days for both elicited and mock-elicited cultures (*) +MeJa indicates that cells were elicited on Day 7 with methyl jasmonate; -MeJa indicates the cells were mock-elicited.*

### *3.2.4 Effect of long-term shearing on aggregate diameter distribution*

The impact of long-term shearing on aggregate diameter distribution was studied using the Coulter-Counter. Samples were taken on day 0, before and after shearing, and on days 7, 14 and 21 after shearing. A significant decrease in mock-elicited sheared cells was observed in *Figure 16 (a)* from day 0 before and after shearing. The mean aggregate size dropped from 400 µm to 220 µm and continued to decrease until day 21 where it reached a value of 145 µm. On day 0, the cultures were sheared with the highest number of iterations, 25, while culture were only sheared 10 times over the rest of the culture cycle. This difference explains why the decrease in mean aggregate size is more significant on day 0 before and after shearing, then from day 7 compared to day 0, day 14 compared to day 7 and day 21 compared to day 14.

The decrease in aggregate size for sheared MeJa-elicited cultures was less significant as the mean aggregate size before shearing was 240 µm, but it reached a value of 135 µm on day 21. To further understand the differences in aggregate sizes achieved with shearing, the experiment should be reproduced on a longer period, with multiple subcultures cycles.

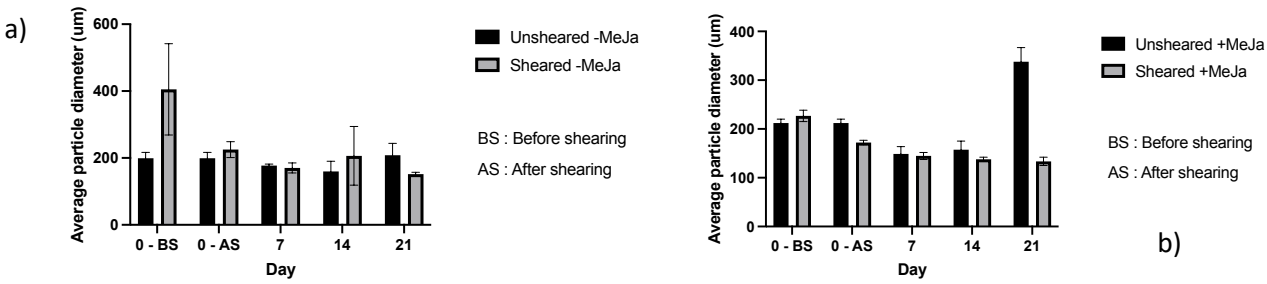



*Figure 16. Mean aggregate size of (a) mock-elicited sheared and unsheared cultures (b) MeJa elicited sheared and unsheared cultures. A decrease in mean aggregate size over time was observed for sheared cultures. Error bars represent the average of three biological replicates with standard deviation. +MeJa indicates that cells were elicited on Day 7 with methyl jasmonate; -MeJa indicates the cells were mock-elicited.*

### *3.2.5 Effect of long-term shearing on cell growth*

In order to understand the origin of the decrease of viability of cells over time, we can graph the biomass in g/L of the MeJa-elicited and mock-elicited sheared cultures. To calculate cell growth, an established correlation was used, Coulter-Counter volume (mL/L) =2.5*biomass (g/L),(Kolewe,2010). During a culture cycle, the biomass of the cultures should increase over time until it reaches stationary phase. The data shown in *Figure 17* demonstrates a decrease over time in culture growth. Before transfer, sheared cultures already possess a very small amount of biomass, meaning that they have a very low density. After transfer, the biomass decreased considerably. To look at the impact of shearing alone, we can consider the growth between Day 0, after transfer and shearing, and Day 21. For both mock-elicited and MeJa-elicited cells, the biomass decreased slightly between Day 0 and Day 7, but then remained constant up until the end of the experiment. This result demonstrates that the shearing and elicitation did not have a major impact on cell growth and that the decrease in viability is likely due to the low biomass of the cells and/or the stress from subculture at the beginning of the experiment. The same pattern of decrease in biomass was observed in unsheared cultures, both MeJa-elicited and mock-elicited. To further understand the variation in viability from long-term shearing and MeJa elicitation, this experiment should be replicated with cells with starting higher density and/or different subculture methods.

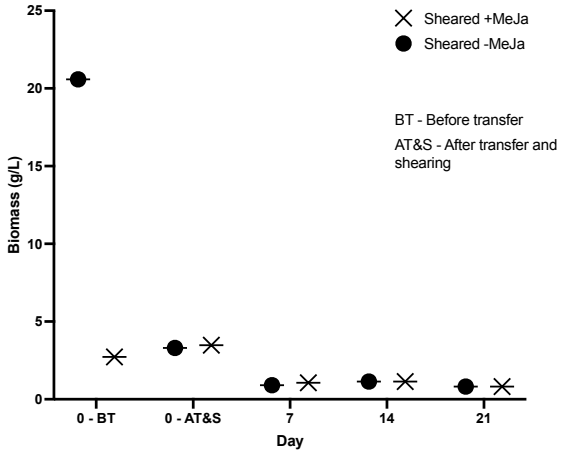



*Figure 17. Biomass (g/L) of both MeJa-elicited and mock-elicited cells decreased over time. Shearing and elicitation were shown to have only a minor impact on cell growth. Error bars represent the standard deviation between three biological replicates. +MeJa indicates that cells were elicited on Day 7 with methyl jasmonate; -MeJa indicates the cells were mock-elicited.*

## 3.3 Effect of intermittent shearing on the different phases of the growth cycle

To further determine the different factors that affect disaggregation using the shearing device, several parameters were tested, including the day of culture and iterations of shear. Data shown in this chapter tested four separate shear iterations, 25, 50, 75 and 100 over a period of three days. This experiment was replicated in the three phases of the culture cycle. Days 0, 1 and 2 focused on the lag phase; days 7, 8, and 9 on the growth phase; days 13, 14 and 15 on the stationary phase.

### *3.3.1 Effect of intermittent shearing on cell metabolic activity*

The metabolic activity and viability of the cells was measured using the resazurin assay. Samples were taken on day 0 before and after shearing and on days 1 and 2 after shearing. The fluorescence on days 0, 1 and 2 after shearing was compared to the fluorescence on day 0 before

shearing to obtain the relative metabolic activity presented in *Figure 18*. This experiment was reproduced on days 7, 8 and 9 with the metabolic activity on day 7 being taken before and after shearing; it was also replicated on days 13, 14, 15 with the metabolic activity on day 13 being taken before and after shearing. During the lag phase, from day 0 to day 2, the metabolic activity increased for all levels of shearings. The lowest metabolic activity was observed on the first day of the experiment; this makes sense as the cells were under stress after having been just transferred/sub-cultured. The highest metabolic activity was achieved on day 2 by cells sheared 75 times. During days 7 to 9, the metabolic activity of all types of sheared cells remained constant around 100%. During the stationary phase the metabolic activity of all types of sheared cells was unstable, but increased for cells sheared 25 times and 50 times, and reached 100% on day 15. During the stationary phase, the density of the cells is very high, nutrients deplete and toxic products accumulate. In addition, it is more challenging to shear cultures of higher cell density as some can clog the 10 mL pipette. For future experiments, shearing of the cells should be performed during the growth phase, as viability is less likely to decrease.

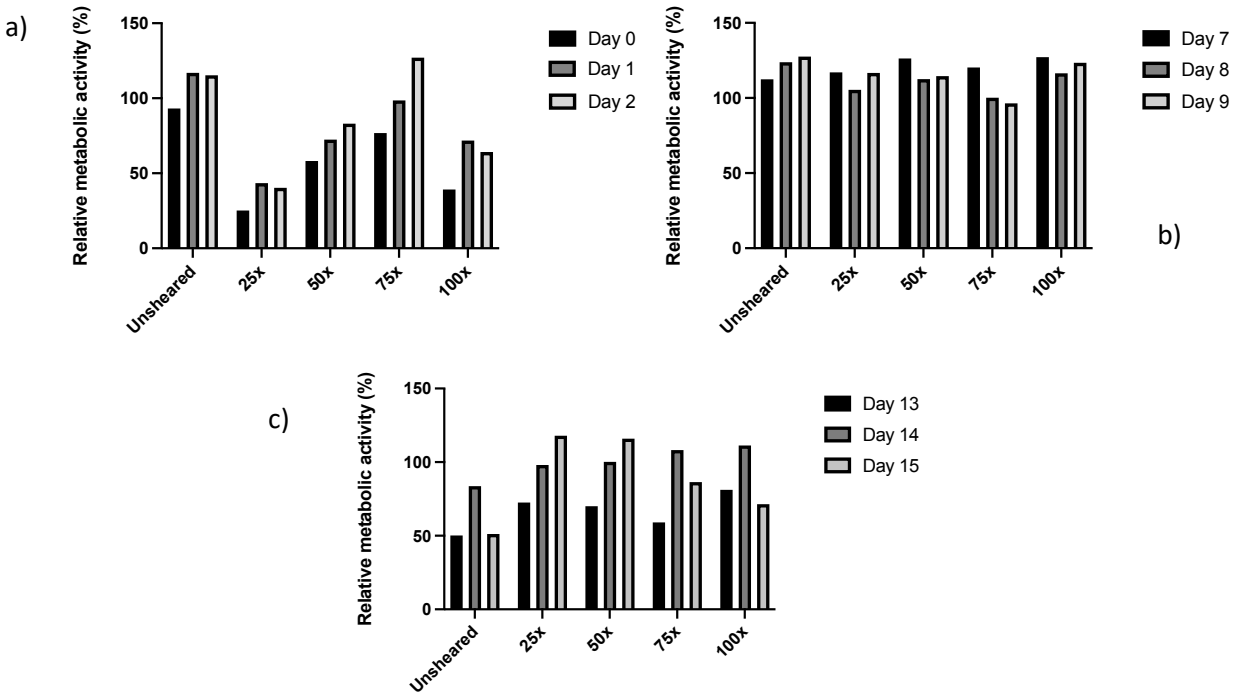



*Figure 18. Relative metabolic activity during (a) the lag phase (b) the exponential growth phase (c) the stationary phase. The highest metabolic activity was achieved during the exponential growth phase where it remained constant for all levels of shearing at around 100%. 25x, 50x, 75x and 100x represent the number of shearing cycles applied each day on the cultures. Only one replicate was sampled for each flask. Relative metabolic activity is proportional to the number of viable cells.*

### *3.3.2 Effect of intermittent shearing on paclitaxel, 10-DAB and baccatin III production*

Samples for measuring the concentration of paclitaxel and its precursors were taken at the end of each three-day experiment, on day 2, day 9 and day 15. Results, as seen in *Figure 19*, were inconclusive, as only a couple of samples were found to have paclitaxel and 10-DAB, and the UPLC peaks were very low. However, baccatin III was found in almost all of the samples, at a very low concentration, except on day 9 for a number of shearing cycles of 25 and 100. Given that only one replicate of each flask was taken, it is not possible to tell from these data an accurate baccatin III

concentration on day 9 for 50 and 75 shearing cycles. However, as the concentration for the sheared culture is more than four times higher than the concentration for the unsheared culture, it is reasonable to conclude that continuous shearing does increase baccatin III concentration. The lack of paclitaxel in the cells can be explained by the absence of MeJa elicitation in this experiment, which is used to enhance paclitaxel production above undectable levels in mock-elicited cultures.

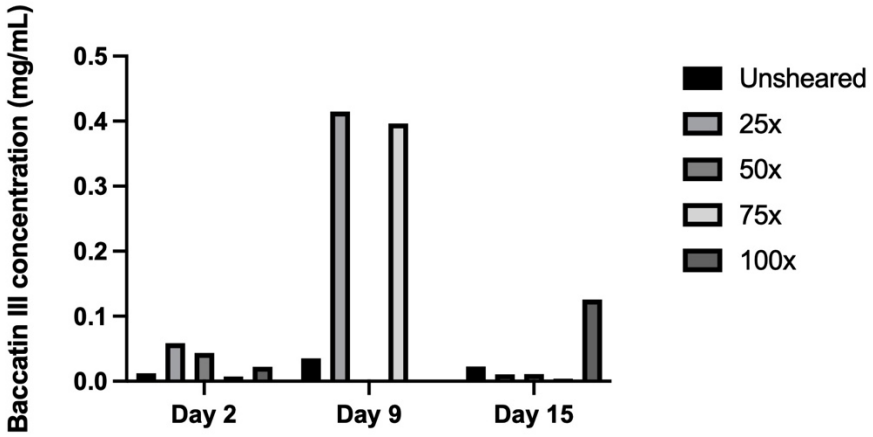
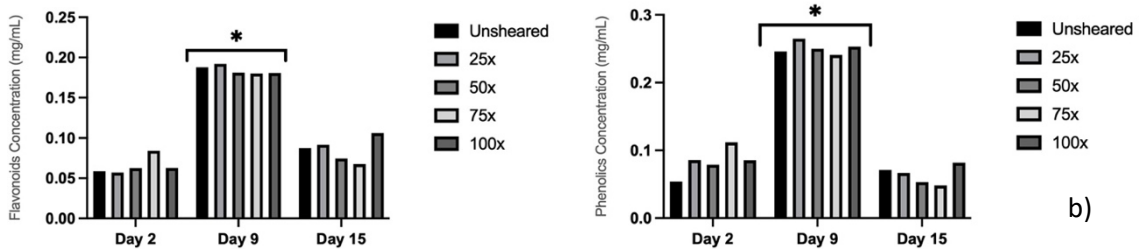



*Figure 19. The highest levels of baccatin III (mg/mL) were found during the growth phase on Day 9. Only one replicate was sampled for each culture, but the sheared culture wqx found to have higher levels of baccatin III than the unsheared culture. 25x, 50x, 75x and 100x represent the number of shearing cycles applied each day on the culture cycles.*

### *3.3.3 Effect of intermittent shearing on global secondary metabolism*

Samples for measuring the concentration of phenolics and flavonoids were taken at the end of each three-day experiment, on day 2, day 9 and day 15. As seen in *Figure 20 (a)* and *(b)*, there is a significant increase in phenolics and flavonoids concentration during the growth phase, where the concentration is almost three-fold higher than that measured in the lag and stationary phases. It is also important to note that shearing does not impact negatively the concentration of global secondary metabolites, as the levels of phenolics and flavonoids remain at the same level as unsheared cultures for each day.

a)




b)

*Figure 20. (a) Flavonoids and (b) phenolics concentration (mg/mL) of unsheared and unsheared cultures. Higher levels of phenolics (mg/mL) and flavonoids (mg/mL) were found for all types of cultures during the growth phase on Day 9. There was only one replicate culture for each condition. 25x, 50x, 75x and 100x represent the number of shearing cycles applied each day on the cultures.*

### *3.3.4 Effect of intermittent shearing on aggregate diameter distribution*

The mean aggregate diameter of the cells was measured using the Coulter Counter. Samples were taken on day 0 before and after shearing and on days 1 and 2 after shearing. This experiment was reproduced on days 7, 8 and 9 with mean aggregate diameter on day 7 being taken before and after shearing; it was also replicated on days 13, 14 and 15 with the mean aggregate diameter on day 13 being taken before and after shearing. As seen in *Figure 21 (b)*, the lowest aggregate size can be observed from day 7 to 9, during the growth phase, where unsheared cells have a mean aggregate size of 220 $\pm$ 20 μm. These data confirm previous reports that smallest aggregated cultures have the highest levels of global secondary metabolites (Kolewe, 2011; Patil, 2014). *Figure 21* shows that the largest decrease in mean aggregate size was achieved for culture sheared with the highest number of cycles (100x). As seen in *Figure 21 (c)*, both sheared and unsheared cultures had the highest aggregate size at the end of the culture cycle, during the stationary phase. Unsheared cells had a diameter of 350 $\pm$ 50 μm during this phase, against a diameter of 180 $\pm$ 30 μm for cells in the lag or exponential growth phase.

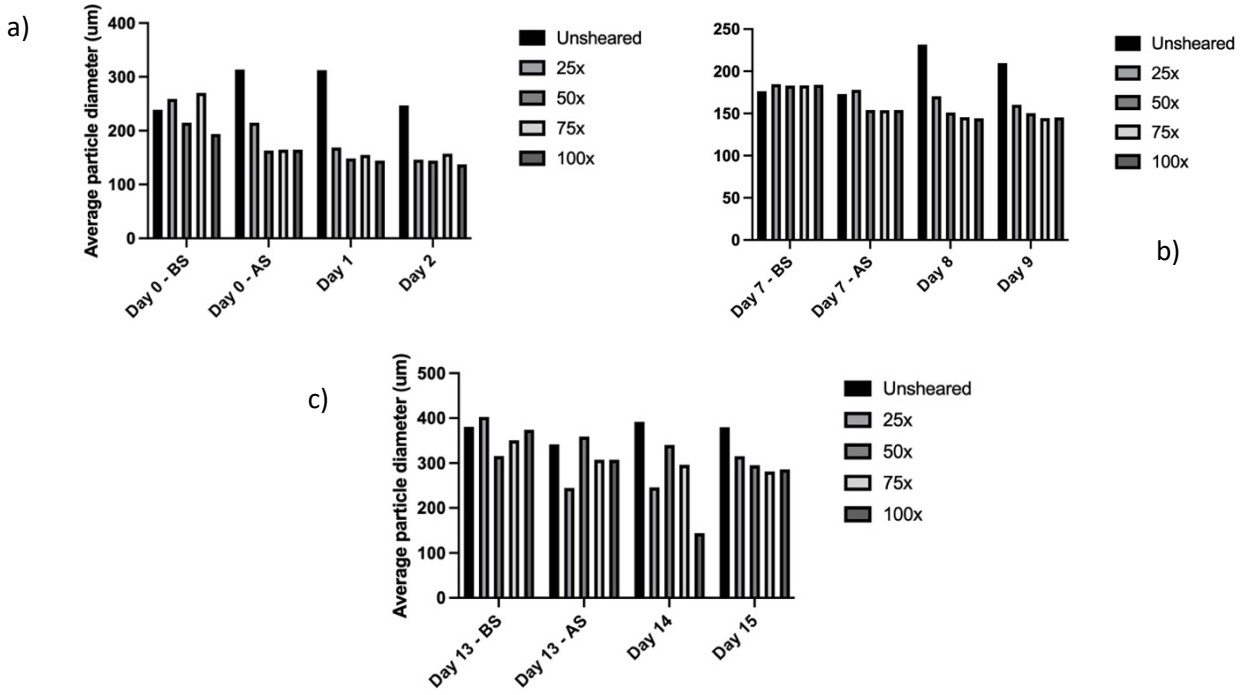



*Figure 21. Mean aggregate size decreased for sheared cultures during the (a) lag phase, (b) exponential growth phase, and (c) stationary phase. Mean aggregate size is higher during the stationary phase, being at a diameter up to 400 μm. There was only one replicate culture for each condition. 25x, 50x, 75x and 100x represent the number of shearing cycles applied each day on the cultures.*

### *3.3.5 Effect of intermittent shearing on cell growth*

To determine cell growth, an established correlation was used, Coulter-Counter volume (mL/L) =2.5*biomass (g/L), (Kolewe,2010). *Figure 22* shows that shearing had no impact on cell growth, as the cells kept growing up until day 15 and there were no significant differences observed between cultures with different shearing levels sampled on the same day. The highest growth was achieved by cells sheared 25 times on each of the three days of the experiment. The biomass concentration of the cultures with the highest shearing cycles applied (100x) was typically the lowest when compared to

the unsheared cultures or cultures with a lower number of shearing cycles. These data suggest to use lower numbers of shearing cycles (25 or 50) to avoid a negative impact on cell growth and health over time.

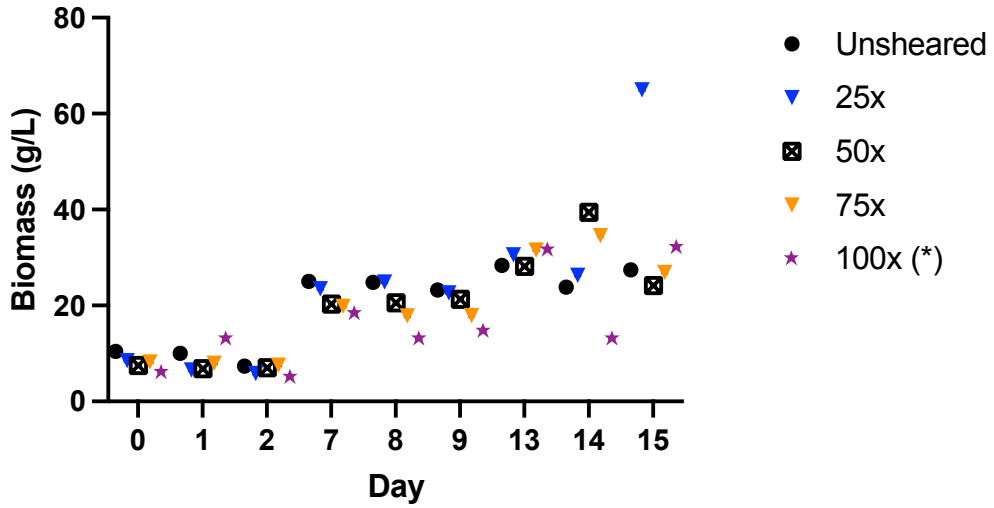



*Figure 22. Biomass concentration (g/L) increased for all cultures throughout the culture cycle. 25x, 50x, 75x and 100 represent the number of shearing cycles applied to the cultures on each day of the experiment. A Student's t test determined the paclitaxel production for unsheared cells to be statistically different from sheared cells (*)*

### 3.4 Effect of continuous shearing on select days of the growth phase

This experiment was designed to test select days of the growth phase of the culture cycle. Previous studies chose days 4, 7 and 11 of the subculture cycle to shear the cells, as it was shown that intermittent shearing with a small number of shearing iterations (e.g. 25x or 10x) had no impact on culture growth or health. Data in this chapter show results for metabolic activity, mean aggregate diameter, secondary metabolites synthesis and production of paclitaxel and its precursors for continuous shearing at a flowrate of 8.6 mL/s for a period of one hour and two hours on days 3, 4, 5, 9, 10 and 11 of the culture cycle. Samples were taken at the end of each day.

#### *3.4.1 Effect of continuous shearing on cell metabolic activity*

Samples for relative metabolic activity were taken each day before and after shearing and the fluorescence after shearing was compared to the level before shearing. Confirming results of the intermittent shearing experiment (section 3.3.1), the highest viability was achieved on day 9. However, all throughout the days tested of the growth phase, the metabolic activity remained closed to 100% for most cultures. The metabolic activities compared between cultures sheared one hour and two hours were variable during the different days, therefore a clear trend is not apparent. We can conclude from these data that there is not a large difference on the impact on cell health and metabolic activity between the two shearing times. More tests should be conducted with a higher number of biological replicates and varying shearing times.

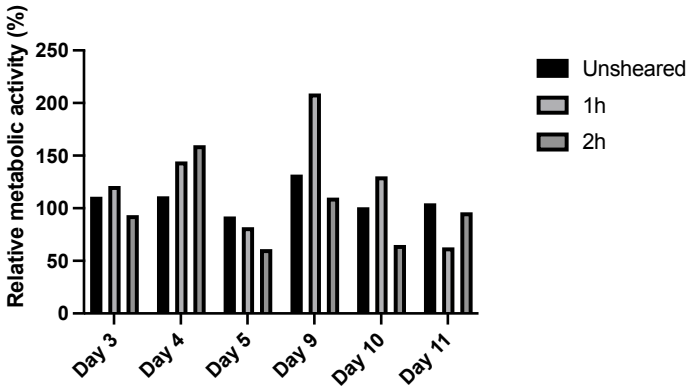



*Figure 23. Impact of continuous shearing for one hour and two hours on relative metabolic activity on days 3, 4, 5, 9, 10 and 11 of the culture cycle. There was only one replicate culture for each condition. On each day, metabolic activity after shearing was compared to metabolic activity before shearing. The highest metabolic activity was achieved on day 9. Relative metabolic activity is proportional to the number of viable cells.*

### *3.4.2 Effect of continuous shearing on paclitaxel, baccatin III and 10-DAB production*

Samples for concentration of paclitaxel and its precursors were taken at the end of each days of the experiment. As in chapter 3.3.2 only a couple of samples were found to have paclitaxel and 10-DAB levels, as was expected without MeJa elicitation. However, baccatin III was found in almost all of the samples, at a very low concentration (*Figure 24*). Given that only one replicate of flask was cultured for each condition, it is not possible to conclusively determine from these data the differences in baccatin III concentration amongst the sheared cultures.

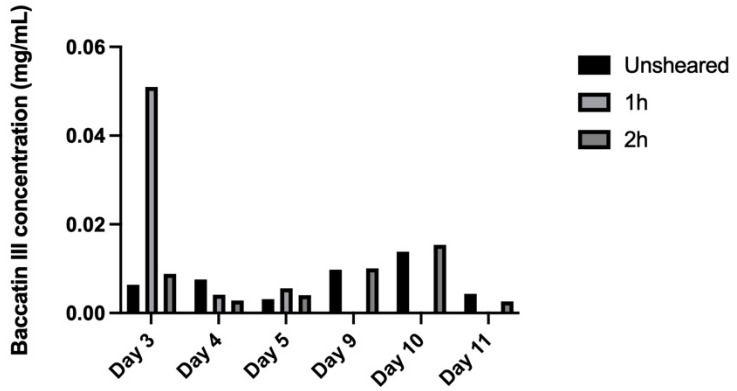


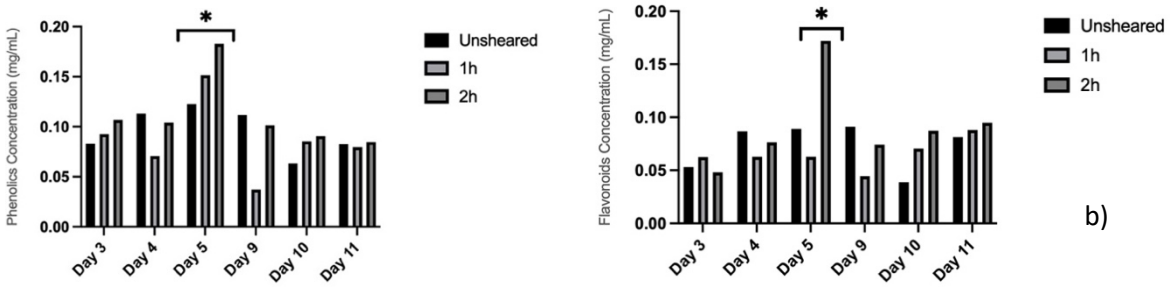

*Figure 24. The levels of baccatin III (mg/mL) in cultures that were unsheared and sheared for one hour and two hours, samples on days 3, 4, 5, 9, 10 and 11 of the culture cycle.*

### *3.4.3 Effect of continuous shearing on global secondary metabolism*

Samples for concentration of phenolics and flavonoids were taken at the end of each days of the experiment. As seen in *Figure 25 (a)* and *(b)*, there is a significant increase in phenolics and flavonoids concentration during the growth phase, confirming results presented in Chapter 3.3.3. The concentration of both global secondary metabolites is higher during day 5 than during day 9. As days 5, 6, 7 and 8 were not tested in the previous intermittent experiment which showed higher levels of secondary metabolites during days 9, samples should be taken in future experiments between days 5 and 9 to determine the day of maximal secondary metabolic activity. It is also important to note that there is a slight increase in almost all days of cells sheared continuously for two hours compared to cells sheared for one hour and cells unsheared. This result suggests that continuous shearing for long periods of time during the growth phase might increase global secondary metabolite accumulation.

Future experiments should be conducted to test this hypothesis and with multiple biological replicates to enable statistically significant conclusions.

a)



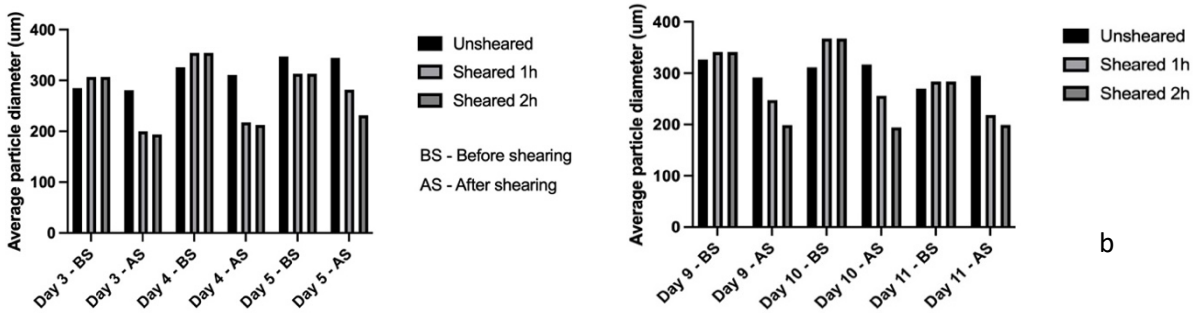

b)

*Figure 25. Higher levels of (a) phenolics (mg/mL) and (b) flavonoids (mg/mL) were found for all types of cultures during the growth phase on Day 5. There was only one replicate culture for each condition.*

### *3.4.4 Effect of continuous shearing on aggregate diameter*

Samples for the mean aggregate size were taken each day before and after shearing. As seen in *Figures 26 (a) and (b)*, mean aggregate size dropped significantly after shearing, all the cells having a decrease in mean aggregate size of 100 μm or more. The difference between mean aggregate size sheared one hour and two hours is more significant during days 9 to 11 where, cells sheared for two hours have a lower mean aggregate size, reaching a diameter of 198 $\pm$ 4 μm.

a)




b

*Figure 26. Mean aggregate diameter of unsheared and sheared cells during days (a) 3, 4 and 6 of the culture cycle and (b) days 9, 10 and 11 of the culture cycle. During days 9 to 11, the mean aggregate size decreased more significantly from one hour to two hours of continuous shearing. There was only one replicate culture for each condition*

## 3.5 Comparison of continuous shearing and enzymes disaggregation on cell metabolic activity

Using the enzymatic disaggregation protocol designed by Naill et al. (2004), a first test was carried out with only two *Taxus* culture flasks, one untreated and one treated for four hours with 1.0 % (w/v) cellulase and 0.1 % (w/v) pectolyase Y-23 in an osmoticum of 0.5 M mannitol and 0.3 % dextran sulfate. At the end of this experiment, the treated cells were found to have a relative metabolic activity of 3%, which means the cells did not survive. To try to increase the viability of the cells, the experiment was reproduced in increased sterile conditions. In parallel, different types of treatment were tested for this experiment. Cultures were simultaneously treated and sheared for either four or five hours. As shown in *Figure 27*, at the end of this experiment the viability decreased for all types of treatments. However, cells sheared for four hours and cells treated with enzymes for four hours both had the highest metabolic activity observed, 43%. Cultures treated and sheared for four or five hours did not survive the stress of both treatments, with only 6% metabolic activity. After five hours, sheared cultures remained at a viability of 40%, but enzyme-treated cells had a significant decrease in metabolic activity down to 8%.

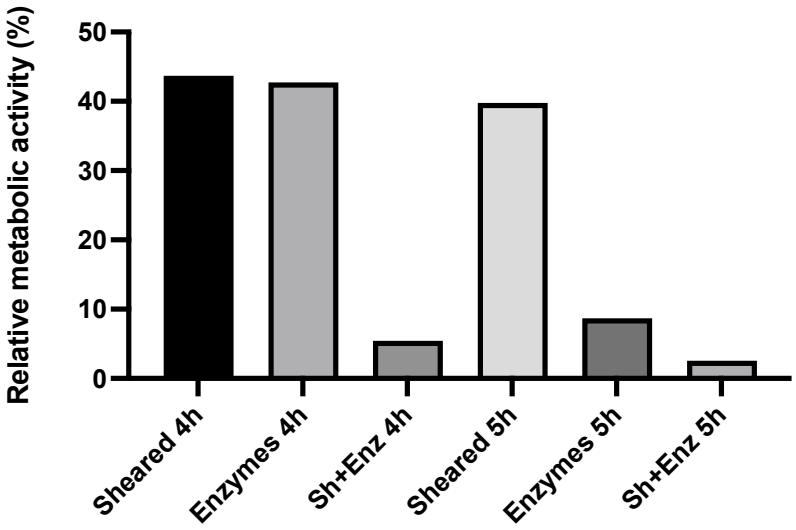
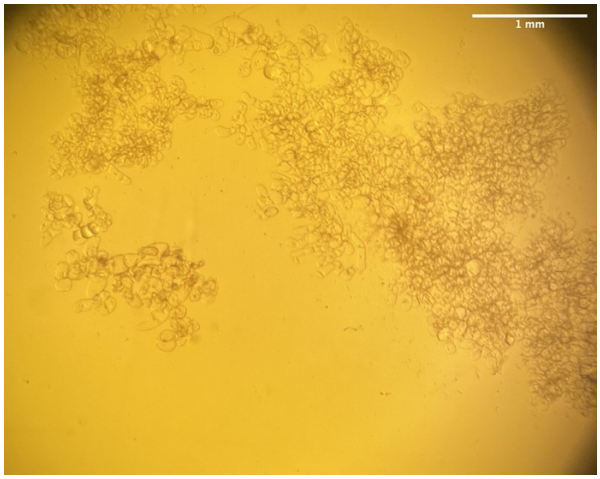



*Figure 27. Relative metabolic activity after different types of treatment were applied for four and five hours : shearing at 8.6 mL/s ; treatment with 1.0 % (w/v) cellulase and 0.1 % (w/v) pectolyase Y-23 in an osmoticum of 0.5 M mannitol and 0.3 % dextran sulfate; shearing and treatment with both enzymes. For each type of treatment, there is only one biological replicate. Relative metabolic activity is directly proportional to the number of viable cells.*

### 3.6 Effect of enzyme disaggregation and shearing on cell separation

After four hours of treatment, sheared cells and cells treated with 1.0 % (w/v) cellulase and 0.1 % (w/v) pectolyase Y-23 were viewed under the microscope and compared to unsheared and untreated cells. *Figure 28 (a)* shows untreated and unsheared cells, with aggregates of hundreds of cells clumped together. After continuously shearing *Taxus* cultures for four hours, aggregates were reduced significantly in size, as seen in *Figure 28 (b).* Some single cells were also observed. After a four-hour treatment with enzymes, the cell aggregate size was significantly reduced, also with also some single cells present, as shown in *Figure 28 (c).* Both methods of disaggregation were successful in reducing aggregates size and producing single cells. However, the decrease in aggregate size was more significant with sheared cells. Continuously sheared cells should be observed under the microscope after a longer period of time (24 hours continuous shearing for example), to determine if aggregate size can be reduced even further.

a)



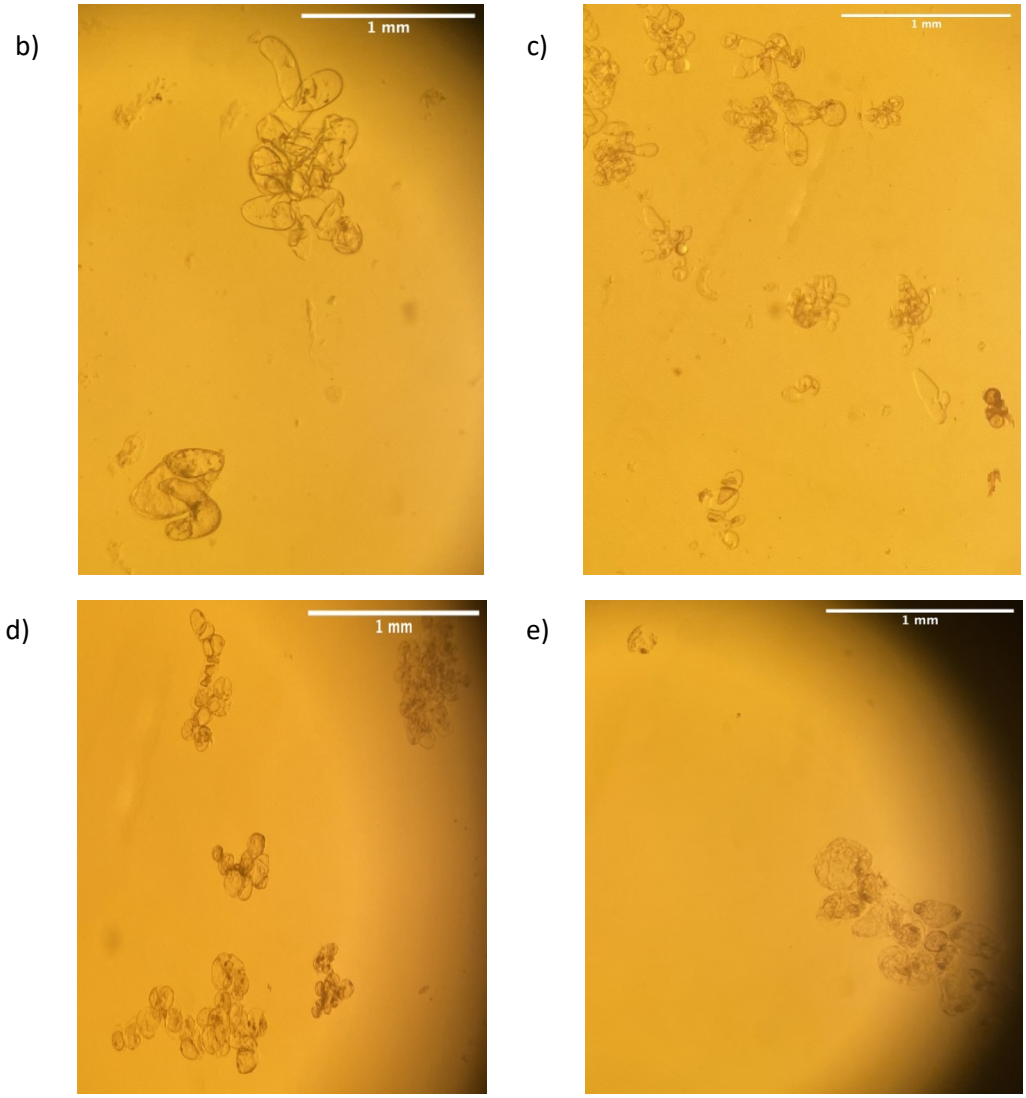



*Figure 28 : Bright-field view under the fluorescent microscope of (a) unsheared cells (b) and (c) cells continuously sheared for four hours (d) and (e) cells incubated for four hours in an enzymatic solution of 1.0 % (w/v) cellulase and 0.1 % (w/v) pectolyase Y-23 in an osmoticum of 0.5 M mannitol and 0.3 % dextran sulfate*

### 3.7 Comparison of continuous shearing and enzymes disaggregation on aggregate diameter distribution

For all different treatments, the mean aggregate size was measured using the Coulter-Counter, before and after the treatment was applied. The most significant decrease in diameter was observed for sheared cells for four and five hours, that both had a diameter of 242 µm before shearing, and a diameter of 96 µm after treatment (*Figure 29*). The decrease in mean aggregate diameter for cells treated for four and five hours with the enzyme solution had a less significant decrease, with cells measuring an average diameter of 123 µm after the five hours treatment. Shearing cells while treating them with the enzymes was shown to have no further impact on decreasing aggregate size as with one of the treatments alone, with the mean aggregate diameter of 98 µm after four hours of shearing.

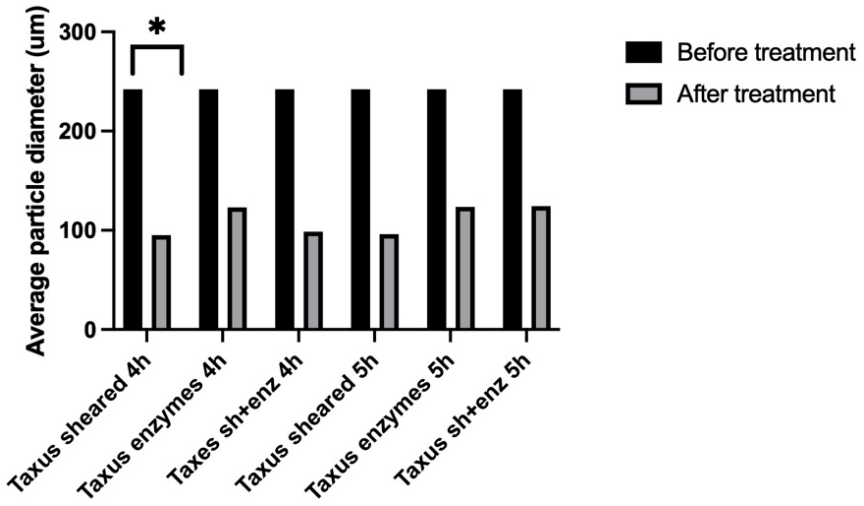



*Figure 29. Decrease of mean aggregate size after different treatments where applied for four hours and five hours: shearing ; treatment with 1.0 % (w/v) cellulase and 0.1 % (w/v) pectolyase Y-23 in an osmoticum of 0.5 M mannitol and 0.3 % dextran sulfate; shearing and treatment with both enzymes. For each type of treatment, there is only one biological replicate.*

## Chapter 4 : Conclusion and recommendations

This thesis aimed to design and test an automated device for shearing *Taxus* plant cells and compare this method to both past studies using manual shearing methods as well as enzymatic disaggregation. A successful automated shearing device was created and showed promising results. The different short-term experiments provided preliminary data on the most appropriate parameters to use. Results showed that continuous shearing at the highest flowrate (8.6 mL/s) did not negatively impact cell metabolic activity and resulted in a significant decrease of the mean aggregate size. This is important because smaller aggregated cultures have previously been shown to produce more secondary metabolites, such as paclitaxel and are therefore desired for bioprocessing applications. Intermittent shearing on the different phases of the culture cycle (lag, exponential and stationary) showed that the most effective phase to shear the cells is the exponential growth phase, especially between days 5 and 9. During this phase, the aggregate size is lower, and levels of global secondary metabolites are also higher. To test levels of paclitaxel during this phase, methyl jasmonate, or another elicitor, should be added to the cultures to induce synthesis. This work demonstrated that a relatively lower number of shearing cycles (e.g., 25x or 50x) works efficiently and does not negatively affect cell health. 100x cycles were shown to be detrimental to cell viability, likely due to extreme stress. Results suggest that the aperture of the pipette used in the automated device should also be increased, to avoid clogging of the cells inside the pipette and the disaggregation of larger size aggregates. Results of the different tests on the shearing device can be summarized in *Table 6*:

| Shearing parameters | Mean aggregate size decrease | Paclitaxel and precursors' production | Flavonoid accumulation | Phenolics accumulation | Metabolic activity |
|---|---|---|---|---|---|
| Speed 8 (4.2 mL/s) | + | / | / | / | + |
| Speed 9 (5.2 mL/s) | + | / | / | / | + |
| Speed 10 (8.6 mL/s) | ++ | / | / | / | + |
| Lag phase (at speed 10) | + | - | - | - | - |
| Exponential growth phase (at speed 10) | ++ | ++ | ++ | ++ | ++ |
| Stationary phase (at speed 10) | - | - | - | - | + |
| 25 cycles (at speed 10) | - | ++ | **No impact** | **No impact** | + |
| 50 cycles (at speed 10) | + | / | **No impact** | **No impact** | + |
| 75 cycles (at speed 10) | + | ++ | **No impact** | **No impact** | + |
| 100 cycles (at speed 10) | ++ | / | **No impact** | **No impact** | - |
| 1h (at speed 10) | + | + | + | + | + |
| 2h (at speed 10) | ++ | + | ++ | ++ | + |
| 4h (at speed 10) | +++ | / | / | / | - |

*Table 6: Summary of the results for the different tests on the shearing device. +, ++, +++ indicate a positive impact of varying degrees. / indicates no tests were performed. – indicates a negative impact. No impact indicates that no changes in the parameter were observed.*

Both techniques of disaggregation were shown to be very effective in reducing aggregate size when applied for four hours, but continuous shearing via the automatic device was shown to have the most significant result in reducing aggregate size while still promoting healthy and metabolically active cells. To further understand the impact of enzymatic disaggregation on cell health and metabolic activity, different concentrations of pectolyase and cellulase should be tested along with different lengths of treatment. Different combinations of both shearing and enzymatic disaggregation techniques should be applied together (e.g., shearing for one or two hours and then using enzymatic disaggregation) to determine if there are positive synergistic effects.

In addition, it would be useful to evaluate both processed for the formation of single cells, which can then be further studied using techniques such as flow cytometry to better understand cellular heterogeneity in plant cell culture environments.

The long-term experiment shed light on the impact of cell transfer on cell metabolic activity. Before transfer, cultures are shaken in the incubator continually which allow proper transfer of oxygen from the culture surface to the media and eventually to the cells. Therefore, the media levels of oxygen are relatively high and sufficient for promoting cell health. When cultures are transferred, they are removed from the shaker for a period of time and pipetted into fresh liquid media that has been kept out of the incubator in a stagnant environment, which likely contains significantly less dissolved oxygen due to the lack of agitation. Therefore, the stress caused by subculture/transfer along with the lack of oxygen in the new environment, results in lower relative metabolic activity. Recall that the resazurin assay measures mitochondrial metabolism. Lower levels of metabolic activity were measured immediately after transfer in both the long-term experiment and the intermittent shearing experiment. In the latter experiment, after cells recovered from the stress, their metabolic activity increased on the following days. The same results were not seen for the long-term experiment, but the decrease in metabolic activity and viability can be explained by the low density of the cells; cells require a certain density to be optimally metabolically active.

The final take away of the long-term experiment is the increased levels of paclitaxel measured upon shearing. However, it remains unclear if it is continuous shearing or the reduction of the size of aggregates that allowed this increase in paclitaxel levels. Two long-term experiments should be carried out in the future to confirm the connection between shearing and increased levels of paclitaxel. The first experiment should be carried out over a long period of time, around six months, with many cell generations. In this experiment, cultures would be sheared continuously, every 3 to 4 days, mirroring the long-term experiment carried out in this thesis, and paclitaxel levels should be regularly measured over time upon induction with MeJa. The second experiment should also be carried out over a long period of time, around six months, with the cultures being sheared continuously for the initial generations. This experiment design would allow an important decrease in aggregate size. After that, cells would be subcultured and paclitaxel levels would also be measured upon induction with MeJa. By comparing those two experiments, we would be able to conclude if increased levels of paclitaxel are the result of continuous shearing or simply the result of increased paclitaxel synthesis via smaller cellular aggregates.

Overall, continuous shearing via an automated device is a very promising technique to reduce aggregate size and increase secondary metabolite synthesis. Manual shearing through a pipette is not a viable solution on an industrial level; however, a modified automated device such as that designed and tested in this thesis could be an effective method to optimize bioprocessing and production at scale.

# References


Brzycki C., “Secondary Metabolite Production in Plant Cell Culture: A New Epigenetic Frontier” https://doi.org/10.1007/978-3-030-58271-5_1

Dinicu A. (2022) Master’s thesis : “Effect of Shear Stress on Cell Health and Secondary Metabolite Production in Taxus Plant Cell Culture”

Enaksha R.M., et al., (1994) « Taxus Cell Suspension Cultures: Optimizing Growth and Production of Taxol” https://doi.org/10.1016/S0176-1617(11)80541-9

Furusaki S., et al., (2011) “ Plant Cell Culture”. Retrieved March 13th 2023, from https://www.sciencedirect.com/topics/immunology-and-microbiology/plant-cell-culture

Gibson D.M., et al., (1993) “Initiation and growth of cell lines of Taxus brevifolia (Pacific yew)” https://doi.org/10.1007/BF00236091

Graham M.D., et al., (2003) « The Coulter Principle: Foundation of an Industry” https://doi.org/10.1016/S1535-5535-03-00023-6

Halder M., et al., (2019) “Elicitation: A biotechnological tool for enhanced production of secondary metabolites in hairy root cultures” DOI: 10.1002/elsc.201900058

Howat S., et al., (2014) “Paclitaxel: biosynthesis, production and future prospects” https://doi.org/10.1016/j.nbt.2014.02.010

Jun Kim B., et al., (2005) “Relationship of Viability and Apoptosis to Taxol Production in Taxus sp. Suspension Cultures Elicited with Methyl Jasmonate” DOI : 10.1021/bp050016z

Kampam M., et al., (2015) “Paclitaxel and Its Evolving Role in the Management of Ovarian Cancer” https://doi.org/10.1155/2015/413076

Ketchum R., et al., (1999) “The kinetics of taxoid accumulation in cell suspension cultures of Taxus following elicitation with methyl jasmonate” https://doi.org/10.1002/(SICI)1097-0290(19990105)62:1<97::AID-BIT11>3.0.CO;2-C

Kolewe M., et., (2011) “A population balance equation model of aggregation dynamics in Taxus suspension cell cultures” https://doi.org/10.1002/bit.23321

Kolewe M., et al., (2011) “Analysis of aggregate size as a process variable affecting paclitaxel accumulation in Taxus suspension cultures” https://doi.org/10.1002/btpr.655

Kolewe M., et al., (2010) “Characterization of aggregate size in *Taxus* suspension cell culture” https://doi.org/10.1007/s00299-010-0837-5

Kuete V. (2017) “Anticancer Activities of African Medicinal Spices and Vegetables” https://doi.org/10.1016/B978-0-12-809286-6.00010-8

Kurz W.G.W, (1971) “A chemostat for growing higher plant cells in single cell suspension cultures” https://doi.org/10.1016/0014-4827(71)90104-2

McKee M., et al., (2021) “The Interface amongst Conserved and Specialized Pathways in Non-Paclitaxel and Paclitaxel Accumulating Taxus Cultures” https://doi.org/10.3390/metabo11100688

Naill M. (2012) “Paclitaxel uptake and transport in Taxus cell suspension cultures” https://doi.org/10.1016/j.bej.2012.01.006

Naill M., (2004) “Preparation of singke cells from aggregated Taxus suspension cultures for population analysis” https://doi.org/10.1002/bit.20083

Nielsen E., et al., (2019) “Improvement of phytochemical production by plant cells and organ culture and by genetic engineering” https://doi.org/10.1007/s00299-019-02415-z

Patil R., et al., (2013) “Cellular aggregation is a key parameter associated with long term variability in paclitaxel accumulation in Taxus suspension cultures” https://doi.org/10.1007/s11240-012-0237-3

Resazurin Assay Kit (Cell Viability) (ab129732) (Updated In 2019) https://www.abcam.com/products/assay-kits/resazurin-assay-kit-cell-viability-ab129732.html

Samokhin A. S. (2020) “Syringe Pump Created using 3D Printing Technology and Arduino Platform” https://doi.org/10.1134/S1061934820030156

Siegel R., et al. (2023) “Cancer statistics, 2023” https://doi.org/10.3322/caac.21763

Wilson S., et al. (2020) “A population balance model to modulate shear for the control of aggregation in Taxus suspension cultures” https://doi.org/10.1002/btpr.2932

Zhong J., (2002) “Plant Cell Culture for Production of Paclitaxel and Other Taxanes” https://doi.org/10.1016/S1389-1723(02)80200-6