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Synchronization and Media Exchange in Large-Scale Caenorhabditis elegans Cultures

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SYNCHRONIZATION AND MEDIA EXCHANGE IN LARGE-SCALE

CAENORHABDITIS ELEGANS CULTURES

by

Jason Daniels Brown

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Biological Engineering

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2009
ABSTRACT

Synchronization and Media Exchange in Large-Scale

*Caenorhabditis elegans* Cultures

by

Jason Daniels Brown, Master of Science
Utah State University, 2009

Major Professor: Dr. Kamal Rashid
Department: Biological and Irrigation Engineering

The nematode *Caenorhabditis elegans* is a model organism for understanding sensory molecules of multicellular organisms. Ovulating hermaphrodites produce putative pheromone(s) that cause male attraction. Because pheromones are produced in such small quantities, adult conditioned-media from large-scale synchronous culture is necessary to analyze these pheromones. Current protocols for culture synchronization have volume constraints that limit large-scale synchronous cultures and current methodology for adult conditioned-media production is impractical.

Modification of Tangential Flow Filtration (TFF) systems was investigated for use as a method to increase the volume limits of bleach egg harvest for *C. elegans* culture synchronization. Also, an adult retention device built within the culture vessel was investigated to optimize the environment for aseptic conditioned-media production from dense large-scale *C. elegans* cultures.
During this investigation, we have shown that synchronous *C. elegans* cultures for adult conditioned-media production can be grown at scales larger than reported before, with potential for further scale up. Our growth methodologies have also yielded denser cultures than previously achieved at large scales. Since rapid bleach harvesting appears to be the bottleneck for large-scale production of synchronous *C. elegans* cultures, our approach of using modified TFF systems with mesh to retain *C. elegans* eggs increased the amount of eggs that could be bleach harvested at one time. Using this method we have been able to achieve up to $5 \times 10^3$ synchronous *C. elegans* per mL at a 50L scale. Since scale-up of TFF is straightforward, our results suggest that the technique reported here can easily be applied to larger scale systems for production of adult conditioned-media from *C. elegans*. Further, the adult retention device within the culture vessel can ensure that the whole process remains aseptic.

(78 pages)
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I thank all those involved with the project for their expertise, hours of work, and resources. I thank my advisory committee, Dr. Kamal Rashid, Dr. Sridhar Viamajala, and Dr. David Britt, for their time, support, and constructive advice during the course of investigation. I want to recognize Mark Signs for his skill with bioreactor and harvest techniques and Dr. Jamie White for his expertise in small-scale C. elegans culture techniques.

I thank my beautiful supportive wife, Kelly, for allowing me to work extensive hours on this project while we dated and during the first months of our marriage. I love her!

Jason Daniels Brown
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### Abbreviation Key

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<tr>
<td>ARF</td>
<td>Adult retention filter</td>
</tr>
<tr>
<td>GPC</td>
<td>Gel permeation chromatography</td>
</tr>
<tr>
<td>ID</td>
<td>Inside diameter</td>
</tr>
<tr>
<td>$k_{l,a}$</td>
<td>Rate of oxygen mass transfer</td>
</tr>
<tr>
<td>M9</td>
<td>M9 Salt buffer</td>
</tr>
<tr>
<td>N</td>
<td>Normal</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NGM</td>
<td>Nematode growth media</td>
</tr>
<tr>
<td>ppm</td>
<td>Parts per million</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>slpm</td>
<td>Standard liters per minute</td>
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<td>TFF</td>
<td>Tangential flow filtration</td>
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CHAPTER 1
INTRODUCTION

*Caenorhabditis elegans*, a species of nematode (round worm), has become a model organism for understanding the complexities of multicellular life forms (Brenner 2002). A current interest of *C. elegans* research is to understand its nervous system and sensory molecules and utilize these molecules to advance medical, physiological, and biological research investigations. In response to their environment *C. elegans* produce small sensory molecules called pheromones to communicate conditions of the environment (Schroeder 2006). The pheromones signal a response in sensory pathways of the receiving organism, affecting development, behavior, or movement (Bargmann 2006). Recent research with *C. elegans* has shown that ovulating hermaphrodites produce putative pheromone(s) that cause male attraction (White et al. 2007). Since such sensory molecules are ubiquitous among higher life forms, it is anticipated that an improved understanding of *C. elegans*’ male attractant pheromone will establish insights into sensory pathways of other complex organisms.

Pheromones are produced in such small quantities that large-scale culture is necessary for analysis of *C. elegans* pheromones. To identify dauer pheromones 22.5mg of active dauer pheromone fraction was extracted from 300L of dauer culture. Pheromone was purified from culture by centrifugation, filtration, ethanol and ethyl acetate extraction, silica gel column chromatography, Gel Permeation Chromatography (GPC) column, followed by a worm activity assay, and then analyzed with Nuclear magnetic resonance (NMR) for structure. About 1mg of the dauer pheromone fraction was used for each NMR structural assays (Jeong et al. 2005), which mean about 13.5L
was required for each NMR analysis. It is assumed that similar amounts of male attractant pheromone are produced per litter by adult hermaphrodites.

Two technical hurdles must be overcome to collect sufficient male attractant pheromone for analysis and identification. First, a large synchronous culture of *C. elegans* must be cultivated. A synchronous culture optimizes the media components and time necessary to produce milligrams of pheromone product. Large cultures have been done, but current protocols for synchronization have volume constraints that eliminate large-scale synchronization as a possibility. Second, analysis interference must be minimized by the use of adult conditioned-media. Adult conditioned-media refers to media from synchronous cultures of *C. elegans* during just the adult stage. Male attractant pheromones are only produced during the adult stage (Chasnov et al. 2007). To eliminate metabolites produced during previous growth, the media is replaced when the worms reach the adult stage to facilitate simpler purification of target pheromones produced exclusively by adults (White 2007, personal communications). The adults are cultured for another period of time to complete induction and production of pheromones and the media is subsequently harvested.

Current methodology for synchronization and conditioned media production uses mesh screens or rotor centrifuges both of which do not maintain favorable growth conditions or a closed system. A method needs to be developed for aseptic conditioned-media retrieval from 2-100L of dense *C. elegans* culture without stressing the worms excessively. Overcoming these hurdles is critical for preparing sufficient amounts of male attractant pheromones for research purposes.
In the longer term, proteomics research will require cultivation of developed transgenic *C. elegans* in mass synchronous cultures (White 2007, personal communications). As nematodes have become a research tool it is possible that they will be more optimal than yeasts and *E. coli* for production of functional complex proteins. Because the worms are more similar to higher organisms than microorganism they have the cellular machinery that is necessary to correctly fold and produce active proteins. This may reduce the downstream processing steps involved in protein production. Also, due to the optimal growth of nematodes being room temperature, compared to the body temperature for most microorganisms, the cost of production can be optimized by reducing heating costs. The protein products from nematodes can be different depending on the stage of growth. Alfred L Fisher from the Division of Geriatric Medicine, University of Pittsburgh, found that the second larval stage of strain Alf4 *C. elegans* is optimal for expression of some proteins of interest. To produce sufficient protein for sequencing it is necessary to culture 5L of synchronous Alf4 worms until they reach the L2 stage. Thus, a synchronous cultivation would optimize nematode protein production.

*C. elegans* have been used to understand the biological process which has brought an understanding of human disease and therapeutic intervention concepts. *C. elegans* have been used as a discovery tool for drug targets and compound screening. *C. elegans* was used to develop RNA interference for pharmaceutical use as well as crop protection (deVGen 2009).

Other closely related nematodes such as *Steinernema* and *Heterorhabditis*, have agricultural uses for integrated pest management practices where a synchronous culture must be achieved to optimize the process of insect infectious worm production (Ehlers
A method of maintaining the culture synchronous would optimize substrate utilization and thus production.

The results of this investigation will support future scientific advances in human therapeutics for disease treatment and prevention.

**OBJECTIVES**

The main objective of this investigation was to demonstrate production of 100L of conditioned-media from *C. elegans* cultures at densities of $5 \times 10^3$ to $1 \times 10^4$ gravid adults per mL with negligible contaminants and growth byproducts. We believe that demonstration at this scale is a critical first step to validate the potential for large-scale production of synchronous *C. elegans* cultures. To accomplish this goal, the following specific objectives needed to be achieved:

- **Produce large-scale synchronous *C. elegans* cultures**
  - Develop a method for large-scale egg retention and synchronization.
  - Design a filter unit to allow synchronization of 1L, 5L, and 10L to produce 5L, 50L synchronous cultures.
- **Produce sufficient conditioned-media for analysis**
  - Develop a method for large-scale adult *C. elegans* retention and media exchange without causing undue stress to the organisms.
  - Design a filter unit within the bioreactor to allow media exchange up to 50L of synchronous cultures.
CHAPTER 2
LITERATURE REVIEW

*Caenorhabditis elegans* are considered a model organism for various reasons. They were one of the first complex organisms to have their genome mapped that enables tracing of the fate of every somatic cell. They have several stages of development. In the adult stage they can reproduce sexually. Although nearly microscopic, *C. elegans* have a nervous system, and communicate through small sensory molecules. Because of these facts, studies with *C. elegans* can give fundamental insights into the genetics, development, aging, nervous system, and other aspects of higher multicellular life. (Brenner 2002)

Large-scale *C. elegans* cultures are necessary for sufficient pheromone production for analysis. Recently, Butcher et al. (2007) isolated and analyzed several small-molecule pheromones produced by *C. elegans* that cause a state of dormancy called dauer. In order to isolate sufficient dauer pheromones for identification using NMR and mass-spectroscopy it was necessary to culture about 50L of dauer conditioned culture medium (Butcher et al. 2007).

The life stages of *C. elegans* begin as an embryo or egg (Figure 3-1). When the egg hatches, the first larval stage, L1 emerges. If conditions are optimal the worm develops from an L1 into an L2, L3, and L4 followed by the final adult stage in about 5 days at 20°C (Lewis and Fleming 1995). As a young adult the hermaphrodites produces the male attractant pheromone as it ovulates becoming a gravid adult.
If food is not available, the L1 will not mature and arrest in this stage for about 24 h (Altun and Hall 2006). If the worm receives no food within this time, it enters the dauer state during or at the end of the L2 stage (Golden and Riddle 1984). The larvae will also enter this state of dauer when exposed to temperatures above 25°C (Golden and Riddle 1984) or if the population is above $10^4$ worms per mL (Butcher et al. 2006). The worms produce the dauer pheromone under unfavorable conditions, which will cause other worms to also enter the dauer state if they detect it. In the dauer state, the worm’s skin thickens, movements decrease, and pumping through the digestive tract stops (Golden and Riddle 1984). The worm can survive lower moisture conditions and live about 4 months in the dauer state. If food becomes available, temperature returns to
optimal, or the dauer pheromone concentration depletes, the worm leaves the dauer state and enters the young adult state (Golden and Riddle 1984).

White et al. (2007) reported that adult hermaphrodite C. elegans produce a pheromone that attracts males and that there are sex-specific neurons. Because sex attractant pheromones are adult stage-specific, the need to synchronize the culture becomes important to generate sufficient mass of pheromone product. Once the nematodes reached the young adult stage, the salt based media was replaced with fresh media without E. coli. When they became gravid, the conditioned-media was collected for male attraction analysis. Adult conditioned-media was placed on culture plates with an adult male or hermaphrodite to determine attraction. Results showed that C. elegans males are attracted to conditioned-media from adult hermaphrodites.

Isolation of male attractant in sufficient quantities for analysis necessitates a synchronous culture of about $10^4$ C. elegans per mL in about 10L of media to produce about a mg for a single NMR analysis and about 100L for complete analysis (Jeong et al. 2005). Large volume culturing of C. elegans is a process that takes up to several weeks. At 25°C the life cycle from embryo to gravid adult requires about 45 h (Lewis and Fleming 1995). The nematodes are first cultured on nematode growth media (NGM) agar plates. From the plates, eggs are harvested with alkaline bleach from adults and transferred into small liquid cultures. The size of the culture can be increased about tenfold with each generation. The final culture is desired to have a concentration of at least 5,000 gravid adults per mL to optimize pheromone production (White 2007, personal communications). From asynchronous plate cultures it would take about 5 to 10 bleach harvests to produce a 1L synchronous culture.
In culturing *C. elegans*, synchronization is the process of getting the whole culture in the same development stage. This process is constrained by a series of time-sensitive steps using a 15mL conical tube. Once the hermaphrodites become gravid (developing eggs are visible in their abdomen), the culture is harvested in 10mL increments. With lower density cultures several 10mL volumes can be centrifuged together to bring the pellet to about 1mL. The worms are washed with buffer to remove excess debris until the wash buffer comes out clear. Then they are resuspended in 9mL of alkaline bleach solution (1.26% hypochlorite, 0.25N NaOH) for three to four minutes while being vortexed. This step is repeated to insure digestion of the adult tissue. If the worms have been in the dauer state it takes more exposure to alkaline bleach to digest its body (White 2007, personal communications). This process releases the eggs from the adult bodies.

The timing is critical; if the culture is bleached too long, fewer viable eggs are recovered, while if it is not bleached long enough, the eggs may be trapped within adult debris. The bleach step is followed by three consecutive rapid rinses to remove the bleach and small debris before the eggs lose viability. The isolated eggs are seeded into fresh media with a density of about $10^4$ eggs per mL. The eggs are then cultured for 16-24 h, allowing them to hatch and arrest in the first larval stage. After this time they are fed *E. coli*. This produces a culture of synchronous worms (White 2007, personal communications).

Existing synchronization protocols produce limited volumes of synchronous nematodes. Although mass cultures of *C. elegans* have been reported in volumes up to 250 liters with kilograms of worms produced (Lewis and Fleming 1995), these cultures were not synchronous. Fabian and Johnson (1994) described a method for synchronization of nematodes with a similar alkaline hypochlorite method that created
populations up to $10^7$ synchronous worms on agar plates. They also described a method that produced 800mL cultures of 800 synchronous worms per mL. Current methods from the worm community database (wormbook.org) describe protocols for synchronous culturing *C. elegans*. Up to one liter of volume of culture can be produced using 10 mL in a 15mL conical tubes for manual bleach harvest of eggs (Shaham 2006). To produce a culture of one liter would take several 15mL conical tube harvests till $5 \times 10^3$ or more eggs mL is achieved.

Another protocol describes harvesting eggs from a liter of culture of asynchronous worms and washing out the bleach with a 250mL conical centrifuge tube. The liter culture is filtered through 35um mesh, washed with water, and then treated with 100mL of alkaline-bleach for 5-10 minutes on a stir plate (Shaham 2006). Once the adults start to break open the solution is centrifuged at $2 \times 10^3$ rpm and then washed by centrifugation with M9 twice. Large centrifuges take more time to settle eggs and several minutes for the rotor to stop. This excess time reduces the number of viable eggs and thus would not be optimal to scale up the volume.

There are a couple non-bleach methods to create a synchronous culture of *C. elegans*. An aging study reported use of a mutant strain with a temperature-sensitive sterile mutation that inactivates sperm blocking reproduction (Lund et al. 2002). This method only allows egg development for certain time period and thus the eggs hatch developing relatively synchronous. The number of eggs developed in this time period would be inadequate to scale up.

An alternate method starts with a large asynchronous dauer culture and allows it to go without food for several days. After an extended time only dormant worms in the
dauer state survive. The culture is then fed and the worms develop into young adults becoming gravid synchronously (Politz et al. 1990). This method causes the worms to produce dauer pheromones and other stress sensory molecules that would complicate isolation of male attractant pheromones.

Another method uses size screening to get cultures of the desired size for a specific development state (Mutwakil et al. 1997). The screen method does not guarantee that all worms that fit through the mesh are at the same larval stage and it was noted worms at different stages of growth co-existed in this culture.

A major issue with these non-bleach methods is contamination; growth debris and sensory molecules from previous worm generations that may interfere with the pheromone analysis may still exist in the growth media. With a long culture period of 3 to 6 days contaminating organisms could outgrow the normal *E. coli* food source for the nematodes and could cause the worms to starve or suffer from lack of oxygen. The bleach egg harvest method would remove the conflicting compounds, debris, and contamination.

Synchronization is a tedious and precise procedure that is limited in feasibility of large-scale culture. Synchronizing nematodes into cultures greater than 1L with current methods is impractical and could produce other sensory molecules that would be detrimental to male attractant analysis. Development of a large-scale synchronization method is necessary for successful male attractant pheromone analysis.

Factors that may hinder analysis must be eliminated from media prior to the final harvest. In the lab *E. coli* is used as the source of food for *C. elegans*, repeated feedings are required during the organism’s growth (Fabian and Johnson 1994). Because *C.
C. elegans culturing require long incubation periods, there is an increased possibility for bacterial or fungal contamination. Growth byproducts also build up as the substrates are used. Any contamination would be amplified without elimination between generations and could foul analysis of the pheromones. Culture synchronization after each generation eliminates contamination, growth debris, and conflicting chemical signals. For a good culture for pheromone harvesting it is important to synchronize the culture after each generation before increasing the culture volume. To eliminate these complicating factors before the final harvest of the last culture it is necessary to exchange the media and culture the worms for an additional 16 h (White 2007, personal communications).

Media exchange before the last 16 h of culture is currently limited to small C. elegans culture volumes for conditioned-media cultivation. Once the worms reach the young adult stage (begin to ovulate), the media must be removed and replaced with fresh media free of the bacterial food source. The current method for nematode separation for media exchange is done by centrifugation of the culture for 30-60 sec in a 15 mL conical tube. With larger volumes separation of worms from media is done by allowing the worms to settle out of solution. The longer time necessary for settling exposes the worms to unfavorable culture conditions. The environmental stress due to the lack of oxygen and nutrients can cause the worms to produce dauer pheromones or become dormant. Once new media is introduced, the worms may stay in this dormant state and not produce the male attractant desired for analysis. This research developed a method to aseptically exchange the media while reducing worm stress.

Producing large cultures of C. elegans is an extensive process and requires bioprocess engineering research to optimize adequate pheromone production. Scale-up
factors must be considered for the synchronization steps and for vessel conditions. Dissolved oxygen, temperature fluctuations, and food availability are problematic volume scale-up issues that may cause the worms to enter the dauer state. In a shaker flask the surface to volume ratio is sufficient for oxygen dispersal but in a reactor vessel, this ratio is greatly reduced due to the larger volume and corresponding slower mass transfer rates (Shuler and Kargi 2002). Some areas of the vessel may be well oxygenated while other regions are anaerobic (Shuler and Kargi 2002).

Worm viability is one of the most important factors to consider in the scale-up process optimization. The bubble size, impeller dimensions, and tip speed affect the shear as well as the oxygen available (Doran 1995). High shear would decrease the viability of the worms. The possible shear from impellers is great due to the large size of an adult of about 1mm long and 50µm wide. The shear may damage and kill the worms or cause the worms to produce stress sensory molecules. Because the agitation speed required may be low compared to microbial fermentation the rate of oxygen mass transfer will also decrease. To increase the available oxygen and rate of oxygen mass transfer the bubble size should be decreased, thus increasing the number of bubbles while maintaining the air flow rates (Doran 1995).

To produce optimal conditions and maintain productivity sparge stones could be used in place of the fermentation spargers, rushton impellers could be replaced with marine blade impellers. Marine blade impellers have lower shear rates than Rushton impellers and would thus, decrease the shear. Sparge stones decrease the bubble size while maintaining the same air flow rates reducing bubble implosion shear while
increasing bubble surface area and thus oxygen mass transfer. Aeration and agitation factors can be optimized for *C. elegans* culture scale-up.

The most important scale-up factor for worm viability during bleach egg harvests is the exposure time to bleach. The more time exposed to bleach the less viable the *C elegans* eggs (Lewis and Fleming 1995). As the volume of gravid adults increases the difficulty of egg collection increases and thus, decreases the eggs viability. Current protocols use centrifugation or mesh screens with gravity filtration to collect eggs, thus, as the volume of eggs increases the time required to harvest increases. With more eggs the gravity filter becomes clogged (Millipore 2003) or with greater volume centrifugation takes more time to separate.
CHAPTER 3
SYNCHRONOUS CULTURE PRODUCTION

Abstract

The alkaline bleach method was scaled up using Tangential Flow Filtration (TFF). A nylon mesh used as the exclusion barrier of the TFF units adequately retained nematode eggs. TFF was used to quickly concentrate eggs and allow bleach dilution during bleach egg harvest steps from 100mL to 5L gravid C. elegans cultures. TFF simplified large culture synchronization allowing scale-up synchronous C. elegans cultivation.

It was observed from an inverted microscope at 200X magnification (CK Olympus Tokyo, Japan) with an ocular micrometer insert (Nikon Stage Micrometer Type A, Nikon Instech Co., Ltd., Kanagawa, Japan) that eggs are about 22µm by 30µm. Mesh sizes were purchased based on estimated egg size. Mesh with a pore size of 20µm was predicted to retain the eggs. Available nylon mesh with pore sizes of 30, 20, 10, and 5µm (Small Parts, Miramar, FL) were investigated for egg retention during TFF. TFF was analyzed as a new method for bleach egg harvest synchronization.

Materials And Methods

The synchronization and culture optimization research was undertaken in the fermentation laboratory of the Center for Integrated BioSystems. One, 5, 10, and 100 L bioreactor’s impellers and spargers were modified to allow for C. elegans culture. Fermentation spargers and rushton impellers were replaced with sparge stones and marine blade or pitched blade impellers. kLa studies were undertaken with the
BioFlow3000 5L vessel to understand the oxygen mass transfer rates with Rushton (3in diameter 6 paddles 0.6in tall x 0.76), Marine blade (3in diameter, 0.7in tall, ~22.5° pitch), and Pitched blade (4in diameter, 3in tall, 45° pitch) impellers (New Brunswick Scientific, Edison, NJ) under C. elegans culture conditions. The B. Braun 10L Biostate-E (Melsungen, Germany) vessel cooling system was insufficient for constant temperatures below 25°C. The 10L vessel was connected to a temperature control refrigeration unit, which controlled the temperature in the required ranges for optimal growth.

**Media formulation and preparation**

Basal Media (Table 3-1) components i)-iii) were dissolved in 800 mL volumes and then diluted to 1L with double deionized H2O. Basal Media solution [with components i)-iii)] and individual liquid components iv)-vii) were sterilized separately with an autoclave at 121°C for 30 min (or more depending on the volume). Liquid components were added aseptically in a laminar flow hood. The cholesterol ethanol solution was sterile filtered through a 0.2µm filter. Sterile components iv)-vii) were added aseptically post sterilization. One mL of component viii) was added just after eggs had been harvested and 1mL was added during each day of culture up to 5mL/L.

M9 Buffer (Table 3-3) components i) - iii) were dissolved in 800mL and then diluted to 1L with double demonized H2O. Sterile component iv) was added aseptically post sterilization. M9 Buffer [with components i) - iii)] and liquid component iv) were stylized separate by autoclaving at 121°C for 30 min (or more depending on the volume).
Table 3-1: Basal Media (Stiernagle 2006)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount / L</th>
</tr>
</thead>
<tbody>
<tr>
<td>i) KH$_2$PO$_4$</td>
<td>6.00g</td>
</tr>
<tr>
<td>ii) K$_2$HPO$_4$</td>
<td>1.00g</td>
</tr>
<tr>
<td>iii) NaCl</td>
<td>5.85g</td>
</tr>
<tr>
<td>iv) 1 M Potassium Citrate</td>
<td>10.0mL</td>
</tr>
<tr>
<td>v) Trace Metals (Table 3-2)</td>
<td>10.0mL</td>
</tr>
<tr>
<td>vi) 1 M CaCl$_2$</td>
<td>3.0mL</td>
</tr>
<tr>
<td>vii) 1 M MgSO$_4$</td>
<td>3.0mL</td>
</tr>
<tr>
<td>viii) Cholesterol (5mg/mL) in Eth</td>
<td>5.0mL</td>
</tr>
</tbody>
</table>

Table 3-2: Trace Metals

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount / L</th>
</tr>
</thead>
<tbody>
<tr>
<td>i) Disodium EDTA</td>
<td>1.860g</td>
</tr>
<tr>
<td>ii) FeSO$_4$ • 7 H$_2$O</td>
<td>0.690g</td>
</tr>
<tr>
<td>iii) MnCl$_2$ • 4 H$_2$O</td>
<td>0.200g</td>
</tr>
<tr>
<td>iv) ZnSO$_4$ • 7 H$_2$O</td>
<td>0.029g</td>
</tr>
<tr>
<td>v) CuSO$_4$ • 5 H$_2$O</td>
<td>0.025g</td>
</tr>
</tbody>
</table>

Table 3-3: M9 Buffer (Stiernagle 2006)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount / L</th>
</tr>
</thead>
<tbody>
<tr>
<td>i) KH$_2$PO$_4$</td>
<td>3.00g</td>
</tr>
<tr>
<td>ii) Na$_2$HPO$_4$</td>
<td>6.00g</td>
</tr>
<tr>
<td>iii) NaCl</td>
<td>5.00g</td>
</tr>
<tr>
<td>iv) 1 M MgSO$_4$</td>
<td>1.0mL</td>
</tr>
</tbody>
</table>

C. elegans Cultures and Conditions

*C. elegans* were cultured on Nematode Growth Media (NGM) agar plates and transferred into liquid Basal Media (Table 3-1) via manual bleach egg harvest.
synchronization. Manual bleach egg harvest was performed with a 15mL conical tube as described previously with the culture being synchronized with food after all the viable eggs had hatched. Optimal growth temperatures for *C. elegans* range from 16 to 25°C (Lewis and Fleming 1995) with 20 to 22°C as most optimal. The temperature was varied between 16 up to 25°C to optimize harvest times. A culture at 25°C could be harvested after three days and a culture at 16°C could be harvested after about 6 days of starting the culture. Most often the cultures were grown at 22°C and harvested every 3.5 days. Eggs from 16, 20, and 25°C cultures hatch after about 24, 15, and 12 h from being laid (Lewis and Fleming 1995). To finalize the synchronize the culture, *E. coli* was introduced as the only food source at 28, 20, and 16 h post egg seeding for 16, 20, and 25°C cultures, respectively.

Liquid cultures from $5 \times 10^3$ to $10^4$ mL were grown in shaker flasks at 200 rpm. The cultures were grown with a 25mg/mL concentration of cholesterol to reduce the number of worms entering the dauer state (White 2007, personal communications). The culture volume was increased from generation to generation with TFF bleach harvest synchronization steps until sufficient synchronous $5 \times 10^3$ to $10^4$ gravid worms per mL was achieved for culture in the 5L bioreactors. Five-liter cultures were initiated with 5mg of cholesterol per liter. The agitation was set at 200 rpm with a program to ramp the agitation to 400 as needed to maintain 30% dissolved oxygen. An additional 5mg of cholesterol per liter was added daily. At point of feeding for synchronization and daily there after, 10µL samples of the liquid culture were observed at 100X and 200X magnification (CK Olympus Tokyo, Japan) for synchronism.
An automatic feed was developed to control *E. coli* addition as a continuous feed for the 5L vessel. A program for scheduled *E. coli* addition was developed using New Brunswick’s Bio-Command bioreactor control software. A cell culture spinner flask was modified with a withdrawal tube and used as an *E. coli* feed vessel. In comparison to a stir bar, the spinner flask reduces mechanical sheer to the *E. coli* cells while keeping the solution in suspension. The bottom of a 4cm thick Styrofoam cooler was heat formed with a glass flask heated to 200°C. The shape in the center of the cooler was just larger than the spinner flask leaving the foam only about 1cm thick. Thinning the cooler allowed for continual stirring of the stir flask while keeping it cooled to about 2°C.

HB101 *E. coli* pellet was resuspended in the stir flask with M9 buffer (Table 3-3) at mixing concentration of 1g of pellet brought to 4mL volume with M9. The flask was filled with 160mL of *E. coli* suspension and set in the Styrofoam cooler on a stir plate. The stir plate was set to turn the stir flask impeller at 60 rpm. The cooler bottom was filled with ice packs to just below the lid of the stir flask to be able to visually assure the flask was stirring. As the days passed the ice packs were replaced to maintain the temperature below 4°C and above freezing.

For a *C. elegans* culture of 5x10^3 worms per mL at 22°C the program increased feed volume based on the schedule for expected food uptake. The expected food uptake was based on maintaining about 10^9 *E. coli* per mL or an optical density between 0.7 and 0.9 at A_600 (Fabian and Johnson 1994). The program was as follow: After 24 h of culture, the expected time for all viable eggs of a culture to have hatched and still be in the first larval stage, 10mL of feed per liter of culture was introduced into the vessel. The feed program then added 1mL of feed per hour per 5L beginning at 26 h, ramping to
2mLs per hour by 96 h, and then ramping down to 1mL per hour by 121 h where the feed program turned off.

From this scale-up investigation it has been seen that it takes about 22 days and five harvests to reach 5L of \(5 \times 10^3\) \(C.\) \(elegans\) per mL. It was estimated that a 100L of culture, starting from NGM plates, would require about 32 days and eight synchronizations.

**TFF Theory**

TFF is a method of filtration where there is a constant flow across the surface of the particle exclusion barrier (Millipore 2003). In normal flow filtration debris builds up clogging the exclusion barrier (Figure 3-1). In TFF the tangential flow of filtrate across the membrane surface reduces build up on the exclusion barrier (Figure 3-1). A TFF filter can easily be cleaned and reused for multiple filtrations. The ability to reuse the filter is an advantage of TFF that improves processing and lowers costs (Kacmar 2006).

**Figure 3-1:** Normal Flow Filtration and Tangential Flow Filtration (Adapted from Millipore 2003).
The Minitan and Pellicon TFF systems for micro filtration cartridges were purchased from Millipore (Milllerica, MA). For egg retention a cartridge would not be optimal. Therefore, the Minitan and Pellicon TFF systems were modified and tested for synchronization optimization with 1, 5, and 10L cultures.

**TFF unit setup and harvest procedure**

The basic set up of the TFF systems for egg retention is as depicted below in Figures 3-2. More detailed pictures (Figures 3-4, 3-9, and 3-11) and diagrams of the assembly of each TFF unit used can be found in the Appendix.

To harvest eggs, gravid adults are introduced into the holding vessel of the TFF system. Two and one-half volumes (one volume equals that of the concentrated gravid adult solution) of alkaline bleach solution are introduced into the system in Flushing Mode. Running the alkaline bleach through the whole system assures that the system is sterile once the eggs are released from the digested adult bodies. The hold vessel is shaken for 3 min and then 8.5 volumes more of alkaline bleach solution is added to the

![Diagram](image)

**Figure 3-2:** Basic flow diagrams of the TFF system. TFF Mode is for concentrating the *C. elegans* eggs post bleach exposure. Flushing Mode is for flushing the eggs out of the TFF systems. The Flushing Mode diagram shows the basic set up of the TFF unit to remove the eggs from the mesh filter after egg harvest. In Flushing Mode the TFF unit’s Permeate line is run backwards into the TFF unit alternating flow out the Feed and Retentate lines till there are no visible debris on the mesh. The flushing solution is changed depending on the flushing step during the egg harvest process.
holding vessel. This is shaken until the majority of the adults are seen broken in half and many eggs released as seen from 10µL samples spread on glass slides (max bleach time of 7 min). At this point the eggs have been released sufficiently from the digested adult bodies so the bleach is rapidly removed while concentrating the eggs in TFF Mode (Figure 3-2).

Once most of the liquid is removed in TFF Mode, the system is switched to Flushing mode. The volume in the holding vessel is brought back to 12 volumes or greater with M9. TFF Mode is then repeated followed by Flushing Mode until the bleach is reduced to 10 parts per million (ppm) of bleach content or lower. Then the eggs are flushed into a bioreactor vessel in the Flushing Mode with Basal media without cholesterol. TFF Mode and Flushing Mode with Basal media are repeated until the desired egg concentration and volume are achieved in the bioreactor vessel.

There are some alterations not shown in Figure 3-2, to optimize flow that would make the diagram confusing. The system only needs one pump, which is placed between the holding vessel and TFF unit (more pumps could be used but may complicate the process unless automated). Tubing is connected with valves (or clamps for the Minitan) so flow can rapidly be switched from TFF Mode to Flushing mode. During Flushing Mode a valve is switched to change flow passing from the permeate side backwards through either the feed or retentate lines back into the holding vessel. Changing the flow from one line to the other removes buildup that has slowly accumulated during TFF Mode. The Permeate line converts into the feed line during Flushing Mode. A valve (or clamps for the Minitan) changes the flow from the waste line to the feed line. Other
variations of the tubing and valves optimize flow as the *C. elegans* TFF filter is scaled up for harvesting large cultures.

The Millipore Minitan TFF system is about 3 times smaller than the Pellicon TFF system. The Minitan TFF system was used to scale up egg harvest to larger numbers than that produced by the 15mL Manual centrifugation harvest method. The Pellicon TFF system was used to scale up egg harvest to numbers larger than capable with the Minitan TFF system. The Minitan and Pellicon TFF systems scale up the number of eggs harvested to produce large-scale dense synchronous cultures.

**Minitan TFF**

The Minitan TFF system was modified for egg retention. A channel was machined in the retentate plate to modify the plate from use with filter cartridges for use with a single membrane (Figure 3-3). This channel allows filtrate to flow across the

![Figure 3-3](image)

**Figure 3-3:** The bottom acrylic plate of the Minitan system. The L shaped channel is the retentate exit line. The L channel was machined to allow use of the TFF system with a single membrane (or exclusion barrier). The channel parallel to the long side of the L channel is the filtrate feed channel. The nylon mesh separated this plate from the permeate plate which appeared identical except for without the machined channel. The Permeate exit line was on the same side as the long side of the machined L channel.
surface of the mesh (Figure A-1). The modified Minitan filter surface area is 7.6cm x 10.2cm or 77.5cm².

After culturing a liter of synchronous worms, the 30, 20, 10, and 5µm meshes were tested for egg retention. The meshes were cut and fitted to the Minitan TFF unit. The culture was divided into four equal volumes of 200mL. Each mesh size was used individually in the Minitan TFF system (Figures 3-4 and A-1) to concentrate eggs after the adult bodies had been digested in bleach for about 7 minutes or until bodies were mostly digested. An inverted microscope at 100X magnification (CK Olympus Tokyo, Japan) was used to observe 10µL samples periodically during digestion. Sterile M9 salt buffer was flushed through the permeate side to flush eggs off the mesh. The eggs were then concentrated again by TFF. The bleach was washed from the concentrated eggs by repeating the M9 buffer flush step till the waste exit solution tested for less than 10ppm of bleach content. Ten-microliters samples were taken every 10 sec and observed for eggs permeating mesh. The harvest vessel and waste reservoir were also examined for eggs.

Figure 3-4: Minitan TFF setup for bleach harvesting C. elegans eggs.
The mesh sizes were further investigated for optimal egg retention. A gravid culture was harvested using the 10µm mesh in the Minitan TFF unit and the final concentration of eggs was $6 \times 10^3$ eggs per mL. This solution of eggs was divided into three equal volumes and filtered individually with the Minitan TFF with the 10, 20, and 30µm mesh sizes. The permeate beaker was agitated and the suspension counted for eggs from 10µL samples.

Clogging during dense harvests runs with the 10µm mesh necessitated a more open mesh size. An 11µm mesh with a six percent open area, three times the open area of the 10µm mesh, was purchased to optimize flow. No pore sizes between 11µm and 20µm were available. The 11µm mesh was tested with gravid cultures under TFF harvest conditions with the Minitan system with samples collected every 10s. Also the permeate reservoir was observed for eggs.

**Minitan TFF vs. Manual**

To test for the efficiency of the modified Minitan TFF, a comparison was undertaken. Ten milliliters from the cultures were removed prior to the Minitan synch and a manual synch (15mL conical tube) was performed. The eggs expected yield and the percent viability were compared. This comparison is seen in Figure 3-12 of the results. The populations of the harvested and viable eggs are shown in Table 3-5.

**Pellicon TFF modifications**

The Pellicon TFF system had to be modified for egg retention to allow cultures to be bleach harvested with a single mesh membrane. In the Minitan system the feed and retentate lines are in the bottom plate and the permeate line is in the top plate. With the
Figure 3-5: The base acrylic plate of the Pellicon TFF system. The center threaded hole on the front of the plate is the permeate exit line. The other two threaded holes are the feed and retentate lines. The odd holes on each end of the top are feed or retentate feed bores. The even bores have square channels that lead to the center permeate exit line as depicted. The even holes are not connected to the odd holes.

Pellicon system the feed, retentate, and permeate line connections are all in the bottom plate (Figure 3-5). The flow from the feed line is split in the acrylic bottom plate into nine bores that then enter into a polypropylene plate. The polypropylene plate further divides up the flow into nineteen parallel channels that flow across the surface of a filter cartridge and then out the retentate line in the same design as the feed (Figure 3-6). Permeate that enters through the cartridge membrane is redirected to one of eight bores that channel to the permeate exit line.

To modify the system for use with a single membrane (or mesh barrier) a polypropylene plate was modified to direct flow from the permeate side of the mesh to these eight permeate exit bores. The nine feed and nine retentate bores were filled with polypropylene. New channels were machined to direct flow to the permeate bores (Figure 3-6). With this plate in place on the permeate side of the mesh the Pellicon system now allowed for a single membrane for TFF. The modified Pellicon filter surface
Figure 3-6: The unmodified vs. modified Pellicon polypropylene permeate plates. The plate on the left is an unmodified polypropylene plate used for the feed and retentate side of the mesh (mesh surface channels facing up). The plate on the right is the modified polypropylene plate (mesh surface channels facing down). The two plates are identical except the modifications.

area is 14.9cm x 14.6cm or 218cm². With the modified permeate polypropylene plate the system was tested for egg harvest.

A leak was found during egg concentration and bleach dilution tests. It was hypothesized that the leak was in the modified polypropylene plate (Figure 3-6). To test that this plate did have a leak a rubber sheet was used in place of the plate. The lines were connected as normal and the same pressures applied as in Minitan operation.

The leak was further investigated. There was a silicon sheet as a barrier between the feed/retentate plate and the acrylic plate. There are eight square channels across the surface of the acrylic plate that direct the flow from the four permeate bores on each end of the TFF unit (Figure 3-5). It was hypothesized that the silicon sheet flexed into the channel creating a new channel just long enough from the polypropylene feed/retentate plate to the permeate exit channels. An acrylic plate was cut and machined with nine bores on each end to fit the acrylic plate (Figure 3-7). This new plate was inserted into
Figure 3-7: 18 hole acrylic plate. This 3mm thick acrylic plate was used to seal the leak that formed as the rubber seal formed secondary channels from the polypropylene feed plate to the channels of the permeate in the base acrylic plate.

the system with an extra seal (Figure A-2). The system was tested for a leak and then for egg retention.

To improve pressure resistance, shorten bleach dilution times, and help eliminate seal blowouts, the thin edge of the polypropylene plates were engraved with a razor blade to create strait ridgelines in the polypropylene plastic parallel to the seal and channels (long edge of both plates in Figure 3-6). Later a new seal with an adhesive side was fitted to the two polypropylene plates (Figure A-2). The Pellicon system seal was tested for failure during dense harvests.

Pellicon TFF vs. Manual

To test for the efficiency of the modified Pellicon TFF system, a comparison was undertaken against Manual bleach egg harvest synchronization. Ten mL from each culture were removed prior to the Pellicon harvest synch and a Manual harvest synch in a 15mL conical tube was performed. The expected egg yield and the percent viability were
compared and are shown in Figure 3-13 of the results. The populations of the harvested and viable eggs are shown in Table 3-6.

The efficiency of the Minitan and Pellicon systems was also compared. One fourth of each culture was harvested with the Minitan system and the remaining three fourths was harvested with the Pellicon system. The viability of the eggs harvested is shown in Figure 3-14 of the results. The populations of the harvested and viable eggs are shown in Table 3-7.

50L synchronous culture

Three synchronous 5L cultures produced previously from one harvest with the Pellicon system was sufficient to seed 28L with 5x10^3 eggs per mL. Once gravid these three 5L cultures were harvested one after the other with the Pellicon system and the eggs were flushed into a 100L (B. Braun, Melsungen, Germany) working volume vessel. About 10L of Basal media was used to flush each culture into the 100L vessel. The initial harvest was flushed with an additional 20 liters to bring the volume high enough for the sparge stones to cause mixing and aeration. The time elapse from one culture to the next was about an hour. The final volume was about 50L, which allowed agitation by the bottom of two marine blade impellers along with contact with the temperature control probe and the dissolved oxygen probe. The dissolved oxygen levels were sufficient at this point. Cholesterol was added at 5 mg per liter and the culture was allowed to hatch.

TFF visualization modifications

Being able to see the mesh during egg harvest was hypothesized to enable optimization of the TFF. The system had to be optimized to allow for scale-up dense
synchronous cultures. Pressure forces during egg retention are sufficiently less than micro filtration parameters the Minitan system was built for. The less pressure the less torque necessary for a seal. With this lower pressure conditions it was hypothesized that the acrylic cover feed plate would not flex if the steel plate were left out of the setup of the Minitan TFF system.

**Figure 3-8:** Visually modified Minitan TFF unit. As seen in the picture, the steel top of the TFF unit has been left out. The clear acrylic allows the mesh to be seen through the plate. Originally there was a steel plate that impeded visualization of the mesh or flow of the TFF system.

The cover plate of the Minitan system was a stainless steel plate over the clear acrylic permeate plate. The steel plate was left out of the system (Figure 3-8). Spacer washers were installed in place of the 316 steel cover plate and the hand nuts were tightened down. The Minitan system was tested with pressure and flow conditions similar as well as just beyond those of the bleach dilution or egg dilution steps.

The visualization modifications were duplicated similarly with the Pellicon system. Since the feed, retentate, and permeate hose connections in the bottom plate for
the Pellicon system, there was no thick acrylic plate before the steel top plate. For this unit a new cover plate was constructed using 1.27cm acrylic plastic. The plastic was machined to match the surface of the steel top plate. Then three acrylic ribs of the same thickness and 3.81cm tall were glued to the machined acrylic plate with methylene chloride. The constructed clear plate was tested with water using clamps to produce similar flow pressures of that seen during bleach dilution steps (Figure 3-9).

Figure 3-9: Modified Pellicon TFF setup with clear acrylic top plate.

Scale-up TFF design

To scale up to 10L harvests, a larger TFF was developed to wash and retain eggs. The original Millipore TFF systems followed a scale-up ratio of 3.333 from the Minitan to the Pellicon system. The in house modified Millipore TFF systems have a scale-up ratio of 2.8 times the surface area from the Minitan system to the Pellicon system. Because available acrylic plates come in square foot increments, a convenience ratio of 3 was used for scale up of the new TFF.
Figure 3-10: New *C. elegans* TFF designed acrylic feed/retention and permeate plates. Measurements are displayed in inches.

Two one-foot-square acrylic plates, two rubber sheets with adhesive on one side, Pharmed tubing, pipe thread to tube adaptors, and 11µm mesh were purchased for the construction. Channels 14.3mm wide and 2.38mm deep were machined into both of the acrylic plates two identical plates (Figures 3-10 and A-3). The rubber sheets were adhered to the plates and the channels cut out. The filter surface area of the new *C. elegans* TFF system is 631.6cm², which is 2.89 times the size of the Pellicon TFF. One plate was the retentate plate and one as the permeate plate (Figures 3-11 and A-4).
Tubing was connected appropriately for flushing, concentrating, and recirculating steps. Either side of the mesh could be observed during TFF (Figures 3-11 and A-5).

![Image](image.jpg)

**Figure 3-11:** Picture of new *C. elegans* TFF system.

**New *C. elegans* TFF vs. Manual**

The new *C. elegans* TFF was simultaneously tested with a gravid culture against the manual method for the viability and number of eggs harvested. The results are summarized in Figures 3-15 and Table 3-8.

**Results and Discussion**

The initial work was done to understand feeding schedules and substrate concentrations to optimize large-scale cultivation. In preliminary research, cholesterol separated out of the medium. Excessively small bubbles from sparging may have caused
the component separation from the media. As the culture came to an end point, excessive foaming occurred. Surplus substrate feed may have caused the foaming. The feeding schedule was evaluated to optimize substrate introduction in cultures larger than 1L with a continuous *E. coli* and cholesterol feed. It was determined that cholesterol added manually and aseptically daily eliminated the foaming and cloudiness due to high cholesterol concentrations.

Three available impellers were compared for $k_L\alpha$. The standard conditions in the 5L vessel were with an airflow rate of 2.5slpm and temperature of 22°C. Only the impeller and shaft speed were altered. The results are summarized in Table 3-4. From the results it was determined that a marine blade or pitched blade impeller could provide sufficient aeration in comparison to the rushton impeller. These $k_L\alpha$ results helped develop agitation rates and cascade limits to maintain desired dissolved oxygen levels for the marine and pitched blade impellers. To decrease possible shear a marine blade was used for agitation of *C. elegans* cultures.

<table>
<thead>
<tr>
<th>Impeller</th>
<th>Shaft Speed</th>
<th>$k_L\alpha$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pitched Blade</td>
<td>100 rpm</td>
<td>18.7 min$^{-1}$</td>
</tr>
<tr>
<td></td>
<td>200 rpm</td>
<td>35.6 min$^{-1}$</td>
</tr>
<tr>
<td>Marine Blade</td>
<td>250 rpm</td>
<td>17.8 min$^{-1}$</td>
</tr>
<tr>
<td></td>
<td>500 rpm</td>
<td>29 min$^{-1}$</td>
</tr>
<tr>
<td>Rushton</td>
<td>100 rpm</td>
<td>16.9 min$^{-1}$</td>
</tr>
<tr>
<td></td>
<td>250 rpm</td>
<td>28.9 min$^{-1}$</td>
</tr>
</tbody>
</table>

Observations for synchronism at the point of feeding and daily thereafter from 10µL samples showed that the cultures were synchronous. Visibly the worms were in the same stage of growth. The worms became gravid simultaneously and contained
relatively the same number of eggs within their body at the point of the next bleach harvest.

**Minitan TFF results**

The Minitan harvest vessel and waste reservoir examined for eggs showed the following: From samples during concentration steps it was observed that the 30µm mesh allowed eggs to permeate during the first two bleach concentration steps. After bleach had been diluted below 10ppm by flushing steps, few or no eggs were retained as observed in the 30µm mesh harvest flask. The 20µm mesh allowed several eggs to permeate during TFF. From the harvest reservoir it was noticed that the 20µm mesh retained a lot fewer eggs than expected and many eggs were seen in its permeate waste reservoir. The 10µm mesh size retained all eggs. No eggs were seen in permeate samples taken every ten seconds during concentration steps with the 10µm mesh. Debris settled at bottom of waste beakers were also observed. Eggs were observed in the 20 and 30µm mesh waste reservoirs. No eggs were noticed from the 10µm mesh bleach harvest. Results showed that the 10µm mesh was most optimal for egg retention during TFF.

From the further investigation of the 10, 20, and 30µm mesh sizes with the Minitan TFF and 6x10³ eggs per mL concentration the following results were observed: The 10µm pore size with a two percent open area was found to retain all eggs and none were seen in the permeate beaker. The 20µm mesh size allowed 1.4x10³ eggs per mL to penetrate the mesh with just one pass of the egg concentration. The 30µm mesh size retained few or no eggs. The 10µm mesh size was determined to be the mesh for egg retention in TFF harvest conditions.
Under actual TFF egg harvest conditions it was found that the 10µm mesh retained all eggs, but when initial gravid concentrations were increased it was found that at times the mesh would clog and impede timely dilution of the bleach solution after adult carcass digestion. As bleach dilution time increases egg viability decreases excessively. To overcome this an 11µm mesh with a 6 percent open area, three times the open area of the 10µm mesh, was purchased to optimize flow. No pore sizes between 11µm and 20µm were available. The 11µm mesh was found to retain all eggs while allowing optimal flow. Clogging was less prevalent than with the 10µm mesh. The 11µm was used for TFF systems developed and used in this research for egg retention.

**Minitan TFF vs. Manual results**

The Minitan method on average harvested 78±20% expected eggs from the gravid cultures and 66±19% of the expected eggs hatched (Figure 3-12). The manual method on average harvested 96±4% of the expected eggs and 67±33% of the expected eggs hatched (Figure 3-12). The manual method on average harvested 1% more viable eggs per mL than the Minitan.

Even with a lower viability than the manual method, the Minitan method reduces the number of harvests performed to synchronize as low as a 100mL culture to produce a 1L synchronous culture. Although the manual method produced 1% more viable eggs than the Minitan method, the Minitan method averaged 55 fold more eggs, and 57 fold more viable eggs were observed (Table 3-5). The time saved is even more substantial to produce larger numbers of synchronous larvae. The Minitan system allows for bleach synchronization of eggs from 1L cultures to produce 5L to 10L synchronous cultures.
The system performed most optimally when harvesting eggs from gravid cultures of 100 to 500mLs.

**Table 3-5:** Results for Minitan TFF vs. Manual harvest method comparison. The table shows the average (±SEM) results for total eggs produced from three cultures harvested with both the Minitan TFF and Manual methods simultaneously.

<table>
<thead>
<tr>
<th>Method</th>
<th>Harvested in Million</th>
<th>Viable in Million</th>
<th>% Harvested of Expected</th>
<th>% Viable of Expected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minitan TFF</td>
<td>27.06 ± 7.57</td>
<td>22.86 ± 7.24</td>
<td>78 ± 20%</td>
<td>66 ± 19%</td>
</tr>
<tr>
<td>Manual</td>
<td>0.49 ± 0.11</td>
<td>0.40 ± 0.21</td>
<td>96 ± 4%</td>
<td>67 ± 33%</td>
</tr>
<tr>
<td>Difference</td>
<td>55 fold</td>
<td>57 fold</td>
<td>18 %</td>
<td>1 %</td>
</tr>
</tbody>
</table>

**Figure 3-12:** Manual vs. Minitan TFF viability. The graph shows the results for percent harvested and percent viable from three cultures. The Manual method was used to harvest 10mL and the Minitan method was used to harvest the rest of each culture. The first bar for the method and date shows the percent of the average number of eggs harvested from that of the expected. The second bar shows the percent of the average number L1 or hatched eggs from that of the expected number of eggs.
Pellicon TFF modification results

During egg concentration and bleach dilution steps with the modified polypropylene plate in the Pellicon TFF setup, the number of eggs slowly decreased until there were little or no eggs on the retentate and feed side of the TFF system. With just a rubber sheet used in place of the modified polypropylene plate, water was found to come out the permeate line even when no opening was available to the permeate holes from the feed or retentate lines. The eggs were not passing through the modified polypropylene plate.

The leak was further investigated to determine how water penetrated the system from the feed and retentate side to the permeate exit line without an obvious flow path during water pressure tests. There was a silicon sheet as a barrier between the feed/retentate plate and the acrylic plate. There are eight square channels across the surface of the acrylic plate that direct the flow from the four permeate bores on each end of the TFF unit (Figure 3-5). The silicon sheet may have flexed into the channel creating a new channel just long enough from the polypropylene feed/retentate plate to the permeate exit channels.

With the inserted acrylic plate machined and an extra seal inserted into the system corrected the leak. The new plate and seal addition showed that the eggs bypassed the mesh filter system. A secondary channel was formed as the silicon seal flexed under required harvest pressure.

During dense 5L cultures the seal next to the mesh of the Pellicon system would fail occasionally. The ribs etched into the sides of the acrylic plate reduced seal failure. The rubber seal with an adhesive side eliminated seal failure.
It was later noted if the system bolts were not loosened once the harvest was complete, then the adhesive rubber seal would creep and adhere permanently out of place allowing leaks under high-pressure conditions. The seal did not fail completely but only leaked.

**Pellicon TFF vs. Manual results**

The Pellicon method on average harvested 76±14% of the expected eggs from gravid cultures and 28±14% of the expected eggs hatched (Figure 3-13). The Manual method on average harvested 78±16% of the expected eggs and 60±22% of the expected eggs hatched (Figure 3-13). The manual method harvested 32% more viable eggs per mL.

The Pellicon system allows for bleach synchronization of eggs from 1-5L cultures to produce 5-20L synchronous cultures and performed most optimally when harvesting eggs from gravid cultures of 1L-2.5L. The Pellicon method produced an average of 208 fold more eggs than the manual method, however, due to bleach dilution time only 55 fold more viable eggs were observed (Table 3-6).

**Table 3-6:** Results for Pellicon TFF vs. Manual harvest method comparison. The table shows the average (±SEM) results for total eggs produced from three cultures harvested with both the Pellicon TFF and Manual methods simultaneously.

<table>
<thead>
<tr>
<th>Method</th>
<th>Harvested in Million</th>
<th>Viable in Million</th>
<th>% Harvested of Expected</th>
<th>% Viable of Expected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pellicon TFF</td>
<td>43.62 ± 9.39</td>
<td>11.23 ± 1.40</td>
<td>76 ± 14%</td>
<td>28 ± 14%</td>
</tr>
<tr>
<td>Manual</td>
<td>0.21 ± 0.07</td>
<td>0.20 ± 0.14</td>
<td>78 ± 16%</td>
<td>60 ± 22%</td>
</tr>
<tr>
<td>Difference</td>
<td>208 fold</td>
<td>55 fold</td>
<td>2 %</td>
<td>32 %</td>
</tr>
</tbody>
</table>
Figure 3-13: Pellicon TFF vs. Manual viability. The comparison results of three cultures harvested 10mL with the Manual and the rest with the Pellicon bleach harvest methods simultaneously.

A Pellicon TFF harvest produced sufficient eggs from one dense culture to seed 28L with $5 \times 10^3$ eggs per mL. Because an automatic feed was not available for the 100L vessel at the time, the eggs were seeded into three 5L vessels at a density of about $9 \times 10^3$ eggs per mL in each vessel with 5L of media.

Pellicon TFF vs. Minitan TFF results

From the repeated experiments the following results were observed: The Pellicon method harvested $43 \pm 3\%$ expected eggs from the gravid cultures and $20 \pm 4\%$ of the expected eggs hatched (Figure 3-14). The Minitan method harvested $52 \pm 13\%$ of the expected eggs and $17 \pm 8\%$ of the expected eggs hatched (Figure 3-14). The Pellicon harvested 9\% less eggs per mL but 3\% more viable eggs per mL than the Minitan. Due
to the volume increase the Pellicon method averaged 2.2 fold more eggs and 2.5 fold more viable eggs than the Minitan method (Table 3-7).

Table 3-7: Results for Pellicon TFF vs. Minitan TFF harvest method comparison. The table shows the average (±SEM) results for total eggs produced from three cultures harvested with both the Pellicon and Minitan methods simultaneously.

<table>
<thead>
<tr>
<th>Method</th>
<th>Harvested in Million</th>
<th>Viable in Million</th>
<th>% Harvested of Expected</th>
<th>% Viable of Expected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pellicon TFF</td>
<td>84.35 ± 25.90</td>
<td>37.90 ± 14.01</td>
<td>43 ± 3%</td>
<td>20 ± 4%</td>
</tr>
<tr>
<td>Minitan TFF</td>
<td>38.93 ± 7.02</td>
<td>15.33 ± 8.15</td>
<td>52 ± 13%</td>
<td>17 ± 8%</td>
</tr>
<tr>
<td>Difference</td>
<td>2.2 fold</td>
<td>2.5 fold</td>
<td>9 %</td>
<td>3 %</td>
</tr>
</tbody>
</table>

Figure 3-14: Pellicon TFF vs. Minitan TFF viability. The graph shows the results for three cultures compared with the Pellicon and manual bleach harvest methods simultaneously.

The Pellicon system encountered flow issues with dense 5L cultures. At times the system clogged with the number of eggs harvested from a 5L culture of $5 \times 10^3$ gravid worms per mL. Clogging was most prevalent when debris buildup was noticed in the
culture. The debris could have been either from the growth stage molts of the worms as they grew, growth byproducts, bacterial clumping, and also possibly fungal contamination. Clogging caused slow bleach dilution and low viability. The Pellicon system was found to be inadequate for bleach synchronization of 10L synchronous gravid cultures.

During some harvest experiments it was observed that the Pellicon silicon seal would burst under abnormally high pressure. This pressure most often occurred as the result of clamping the wrong tube with a high pump speed. In order to improve the concentration or bleach dilution time, the operator would restrict the retentate line to increase the flow rate out the permeate line. This restriction helped speed up the egg concentration with the Minitan system but also increased clogging. In the Pellicon system it was an almost instantaneous cause of the seal to blowout. When this occurred about half or more of the culture was lost and half of the remainder was lost due to the extra time exposed to bleach and unfavorable oxygen conditions.

Etched ribs and adhesive seal reduced seal failure. The engraved edges of the polypropylene plates parallel to the seal were found to help reduce blow out incidents. The seal modifications of the Pellicon system favorably handled pressures beyond that of the concentration steps necessary for timely bleach dilution.

50L synchronous culture results

From the three Pellicon harvests producing the 50L synchronous culture in the 100L vessel it was observed that a larger TFF would be required for efficient scale-up. Of the $4.9 \times 10^3$ eggs per mL only $1.2 \times 10^3$ per mL were seen to have hatched. Because the
culture was produced from 3 harvests there was about an hour of time between each. The first and second harvested eggs were under less favorable conditions than the third and for an hour or two longer. A larger TFF unit would allow the eggs to receive uniform and optimal conditions to produce a larger synchronous culture.

**TFF visualization modifications results**

To optimize the Minitan TFF process it was evident that it is necessary to observe TFF flow conditions of the filter area during bleach dilution. Pressure tests showed that the Minitan TFF system without its steel cover plate did not leak under pressures and flow conditions were beyond those of the bleach dilution or egg dilution steps. Hence, it was concluded that the steel plate was not necessary.

Using the Minitan system without the steel cover plate allowed for visualization of clogging during egg concentrating steps and bleach dilution. This led to modification of the flow paths into the cover plate and modification of flushing steps for optimization of the bleach dilution steps. During flush steps the debris could be observed and flow patterns changed to dislodge or break up clumps. Visualization allowed for flow management, which improved bleach dilution times and egg viability.

The visualization modifications duplicated similarly with the Pellicon system were found to perform as well as the steel plate. Leaks only occurred under pressure when the retentate line was completely closed, at which point the rubber seal would burst in the same manner as with the steel plate. The pressure tests showed that this new setup did not leak under max pressures and conditions seen during bleach dilution steps. The
modified Pellicon system with acrylic plate proved to function as well as with the steel plate.

The presence of a visual window to the mesh membrane allowed the setup of the TFF system to be monitored during egg concentration steps. The Minitan system appeared to create a dead space where debris was retained. The Pellicon system had channel systems that did not allow the mesh to lie against the surface of the plastic thus there was less debris buildup. The clear top plates allowed visualization of any buildup during concentration. If accumulation was noticed, the flow could be redirected to avoid buildup. Being able to visualize the mesh in the TFF units helped recover all eggs into the culture vessel during flushing steps after the harvest.

Need for TFF scale-up

Three separate harvests of three 5L gravid cultures into a 100L fermentor did not allow for favorable conditions for culture synchronization. Many of the larvae died or became dormant. The whole process of setting up for more than one harvest with the TFF caused about an hour delay between bleach harvests. After the first and second harvests the media levels were too low to allow adequate aeration. The hour delay between bleach steps with unfavorable conditions lowered the number of viable eggs harvested. The final culture had enough eggs for a 50L culture but only 1.2x10 eggs per mL hatched. A larger TFF unit would have to be used to allow for the culture to be synchronized with one use of the TFF and allow the batch to receive optimal aeration conditions. The batch of eggs would be in favorable conditions upon completion of the egg collection. From the observations it was decided that a larger TFF system would
save time and allow bleach harvest of 10-20L cultures to optimally synchronize eggs for 100L cultures.

**TFF scale-up design**

In the scale-up TFF design two options were available for consideration. A larger unit could be purchased and modified for egg retention or a larger TFF system could be built from observations of the modified smaller TFF systems. After considering both options it was decided to build the TFF. Most TFF systems are built for high pressures and thus, have steel plates eliminating visualization of the flow paths. Building the scale-up TFF system would allow for the system to be visually optimized. Also building the TFF system would allow for it to be designed for the mesh to retain the *C. elegans* eggs. Building the TFF system is most optimal for scale-up egg harvest.

**New *C. elegans* TFF vs. Manual results**

From repeated experiments the following results were observed: The new *C. elegans* TFF method harvested 78±12% expected eggs from the gravid cultures and 31±7% of the expected eggs hatched (Figure 3-15). The Manual method harvested 86±13% of the expected eggs and 79±20% of the expected eggs hatched (Figure 3-15). Due to the volume increase the new *C. elegans* TFF method averaged 258-fold more eggs and 101-fold more viable eggs than the Minitan method (Table 3-8).
Table 3-8: Results for new *C. elegans* TFF vs. Manual harvest method comparison. The table shows the average (±SEM) results for total eggs produced from three cultures harvested with both the 1/2 New TFF and Manual methods simultaneously.

<table>
<thead>
<tr>
<th>Method</th>
<th>Harvested in Million</th>
<th>Viable in Million</th>
<th>% Harvested of Expected</th>
<th>% Viable of Expected</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/2 New TFF</td>
<td>64.43 ± 34.91</td>
<td>19.80 ± 4.38</td>
<td>78% ± 12%</td>
<td>31% ± 7%</td>
</tr>
<tr>
<td>Manual</td>
<td>0.25 ± 0.11</td>
<td>0.20 ± 0.09</td>
<td>86% ± 13%</td>
<td>79% ± 20%</td>
</tr>
<tr>
<td>Difference</td>
<td>258 fold</td>
<td>101 fold</td>
<td>8 %</td>
<td>48 %</td>
</tr>
</tbody>
</table>

Figure 3-15: Half New TFF vs. Manual viability. Results from three cultures compared simultaneously for the average percent harvested of the expected. 10mL was harvested with the Manual method and the rest of each culture was harvested with half of the new *C. elegans* TFF method.

Conclusion

To overcome current constraints of *C. elegans* synchronization, TFF systems were modified or built with a nylon mesh to retain eggs. The 11µm nylon mesh with a 6% open area retained *C. elegans* eggs optimally during large-scale bleach harvest for culture
synchronization. The TFF system recirculates the retentate over the membrane, thus reducing fouling of the mesh while allowing a much smaller surface area to be used to harvest. The unit also provides turbulence that assisted in breaking up debris during the concentration steps. The TFF allowed rapid dilution of bleach while retaining the eggs.

A scale-up bleach harvest method and equipment to produce large-scale dense synchronous cultures of *C. elegans* were developed during this investigation. Two TFF units were modified to harvest *C. elegans* eggs, and have been shown to produce synchronous cultures of $5 \times 10^3$ *C. elegans* per mL in 5L cultures. One of the harvests with the Pellicon TFF unit was sufficient in number to seed 28L with $5 \times 10^3$ eggs per ml. Three harvests with the Pellicon TFF seeded a 50L synchronous culture with $5 \times 10^3$ eggs per ml. A third scale-up TFF unit was designed and built to harvest *C. elegans*. Tests showed that the scale-up TFF improved viability and increased the total population when using just half the TFF unit.

To optimize production of a protein of interest at concentrations highest in the second larval stage, a 5L synchronous culture of *C. elegans* strain Alf4 was produced. The scale-up synchronization methods developed during this investigation were used to culture 5L of $6 \times 10^3$ Alf4 *C. elegans* per ml. Thirty million (16.1g) Alf4 in the second larval stage were recovered for protein extraction. This project showed the value of this method for production of stage specific proteins of interest from *C. elegans*.

Results of this investigation led to the development of methods and instrumentation for producing large-scale synchronous *C. elegans* cultures. Sufficient eggs were harvested from 12L of gravid culture to produce 50L of about $5 \times 10^3$ eggs per mL. Synchronous *C. elegans* cultures up to 50L in volume in a 100L fermentation vessel
were achieved. Larger synchronous cultures could be produced using the developed scale-up bleach harvest method and *C. elegans* TFF equipment. The methods developed from this research allow for scaled-up bleach egg harvest enabling large-scale dense cultivation of synchronous *C. elegans*, which optimizes production of stage specific products.
CHAPTER 4
MEDIA EXCHANGE AND CONDITIONED-MEDIA PRODUCTION

Abstract

A filter prototype built within the culture vessel was built to allow aseptic media exchange and conditioned-media production. A nylon mesh would adequately retain nematodes during media exchange for conditioned-media production. It was estimated from microscope observation that a gravid nematode had the diameter of about 50µm. Mesh sizes were purchased based on estimated worm size. Mesh with a pore size of 50µm was estimated to retain the adults. Available nylon mesh with pore sizes of 50, 36, 30, 20, and 10µm (Small Parts, Miramar, FL) were purchased and analyzed for use in adult conditioned-media production.

Materials and Methods

To produce sufficient conditioned-media for analysis, a mesh size was found to adequately retain adult C. elegans for processing. Mesh sizes were purchased based on expected retention from estimated adult worm cross sectional diameter. From microscope calibrations it was estimated that a young adult and gravid adult C. elegans have the diameter of about 30-50um. Mesh with a pore size of 50µm was hypothesized to retain the adults.

Adult retention was tested with live cultures of C. elegans. A liter of synchronous worms was cultured and divided into 200mL aliquots. The 50, 36, 30, and 20µm meshes were cut and fitted to a screen exchangeable funnel filter. Each mesh was individually tested in the funnel filter for gravid adult retention with 200mL of gravid culture. The
filter permeate was observed for any gravid adults. The funnel filter was cleaned between mesh size tests.

To test the 30 and 50µm mesh under pressure conditions of adult retention, the mesh were cut and fit to the Minitan TFF. A $7.6 \times 10^3$ per mL gravid culture was suspended and divided into two 200mL aliquots. Under Minitan TFF concentrating conditions the culture was concentrated down till the volume was gone. The permeate reservoir was counted for gravid adults.

1-5L vessel ARF

To address conditioned-media production, a 2.84cm by 2.84cm (16cm²) dual sided filter prototype was built for 1L cultures (Figure 4-1). This prototype is a scaled down version of the media exchange mesh filtration designed for the 100L vessel for use in 1L cultures with a 2.5L vessel. The prototype was constructed for each of the 20, 30, 36, and 50µm mesh. The Adult Retention Filter (ARF) prototypes were tested using a peristaltic pump to draw through conditioned-media. The test would mimic conditions similar to those within the vessel during media exchange.

**Figure 4-1:** 1L in vessel ARF. Dual sided 2.84cm by 2.84cm (16cm²) filter prototype was built for retaining adults from of up to 1L cultures within the bioreactor.
The 20, 30, and 36µm ARF prototypes were tested with adult synchronous cultures in the 5L vessel. A 6.35cm by 6.35cm (80.65cm²) 5L vessel version (Figure 4-2) of the 20, 30, and 36µm ARF were constructed from a scaled-down design for the 100L bioreactor. The 1L and 5L two sided ARF prototypes were installed below the sparge stones of the vessels of their according working volume. Eggs from bleach harvest synchronizations were seeded into 1L to 5L cultures depending on the number of eggs harvested. Once the worms reach the young adult stage medium was exchanged with fresh media without food using the ARF. The filter permeate was observed for adults. The worms were cultured for another 16 h. The medium was again harvested using the ARF.

**Figure 4-2:** 5L in vessel ARF. Dual sided 6.35cm by 6.35cm (80.65cm²) filter prototype was built for retaining adults from of up to 5L cultures within the bioreactor during culture.

The 30µm mesh ARF was assembled into the 5L vessel and tested under culture conditions and then media exchange. A 4L culture of 5,500 adults with 2-4 eggs each was concentrated down to a thick slurry of worms. Samples of 2mL were taken from the
permeate line every half liter and the number of adults that permeated the mesh calculated.

**Funnel ARF**

A funnel ARF was built to allow for cleaning and the method was modified to reduce debris accumulation. M9 and Basal media have little nutrients other than salt buffer for osmotic neutrality. Once the growth media has been removed and the adults rinsed with sterile media, no contaminants should grow. It is possible that an axenic culture could be maintained even if the culture vessel had to be opened for media exchange. A funnel filter was built with 30µm mesh attached to the large end of a 6.35cm ID funnel (Figure 4-3). The 30µm mesh surface area was 0.4 times the size of the ARF built with in the vessel but it could easily be rinsed to remove clogs.

![Figure 4-3: Funnel ARF. 6.35cm diameter (31.6cm²) filter prototype was built for retaining adults from cultures of up to 5L. Image on right shows the Funnel ARF after concentrating a 5L culture.](image)

The small funnel end was attached to a tube and a peristaltic pump. When the cultures reached the young adult stage, the agitation was stopped and the head plate
removed. The airflow from the sparge stones was left constant. The ARF funnel was lowered into the media and the pump activated. When exit flow became constricted the pump was turned off and the funnel was lifted till perpendicular with the media surface. Adult bodies were washed from the mesh with squeeze bottle with M9 buffer. Filtration and mesh rinsing was continued till the culture was concentrated down to a thick slurry of adults (Figure 4-3). The 6cm funnel ARF method was tested with gravid adults cultures prior to egg harvest. The adults were transferred to harvest vessel with M9.

To improve time constraints of the 6.35cm funnel a 12.7cm funnel (126.7cm²), was modified with a 30µm mesh on the large end. This was tested with a 5L culture.

Screen ARF

To address the issues of the funnel ARF an open pressure free large screen may optimize adult retention. A large screen of 50, 36, and 30µm mesh was purchased. A 4L beaker (17.5cm ID) and 15L bucket (Figure 4-4) were cut 10cm below the rim. Another 5cm ring was cut off the bottom of these tops. This ring fit tightly into the top rims. With the mesh inserted the inch ring made a tight seal.

Figure 4-4: Screen ARF. Built from a 30.5cm diameter 15L bucket to form 28.5cm ID filter with a mesh surface area of 638.7cm² for adult retention.
This adult sieve was tested with 30, 36, and 50µm mesh for adult retention as a pre filter before egg harvest. Cultures of 3 to 5L were slowly pored onto the mesh. Periodically the sieve was swirled to help speed fluid penetration. Permeate was suspended and a 10µL sample was taken and counted for gravid adults.

The 36µm adult sieve was further tested with chilled adult for retention. Three cultures were chilled in at 4ºC. A chilled culture was filtered as before and the filter washed before the next culture was collected. A 10µL sample was taken from the suspended permeate and counted for gravid adults.

**Results and Discussion**

With the screen exchangeable filter funnel adult retention testing of 50, 36, 30, and 20µm meshes with live synchronous *C. elegans* cultures, the 50µm mesh retained the majority of gravid worms and the smaller mesh sizes retained all.

The 30 and 50µm mesh under TFF pressure conditions during adult retention with a 7.6x10³ per mL gravid culture showed that 1.8x10³ gravid adults per mL were counted in the suspended 50µm permeate and no adults were observed in the 30µm permeate.

The media exchange mesh filtration prototype under negative pressure conditions for the 20, 30, 36, and 50µm mesh gave the following results. The test revealed that the 50µm mesh inadequately retained the adult larvae under media exchange conditions. The 36µm and 30µm mesh retained the majority of adult worms. Even some worms were noticed in the permeate of the 20µm mesh. Under sucking forces some adults can be forced through small mesh pore sizes. The 20µm mesh took the longest time to
concentrate the adult worm solution. The results indicate that the 30µm mesh allowed for sufficient flow for scale-up parameters without excessive clogging.

1-5L vessel ARF

The 30µm mesh ARF assembled into the 5L vessel and tested under culture conditions followed by media exchange gave the following results: The ARF concentrated the first two large-scale cultures and allowed for optimal aeration during media exchange. A 4L culture of 5.5x10³ adults per mL with 2-4 eggs each was concentrated down to just a thick slurry of worms. Samples of 2mL were taken from the permeate line at half liter intervals and the number of adults to have permeated the mesh calculated. There were no adults counted in the first, second, and fifth samples. Only one adult was counted in the third and fourth samples per 10µL counted. Two, seven, and five adults were counted in the sixth, seventh, and eighth 500mL samples, respectively. From the sum of the calculated worms per mL there was about a total of 5.6x10⁵ gravid worms that permeated the 30µm mesh under concentration forces during media exchange. The volume of the culture was brought up to two liters with fresh Basal media and the density calculated at 9.3x10³ gravid adults per mL.

The culture started with 22x10⁶ gravid adults and ended with 18.6x10⁶. Actual counts showed that 5.6 x10⁵ were lost in the permeate waste and 2.75x10⁶ were unaccounted for. It was noticed that there were many worms crawling along the glass surface above the new level of fluid in the vessel. These worms on the bioreactor wall may account for some of the unaccounted worms. Partial counts made during concentration may have been inaccurate and could have missed some of the worms but
the counts seem reasonable when the gravid adults could be seen on the glass surface. By swirling the vessel some worms washed of the walls and a recount was taken. $10.1 \times 10^3$ gravid per mL were counted so only $1.15 \times 10^6$ were unaccounted for and may have still been those seen on glass surface.

After 14 h the culture was counted and it was found that the culture had about $8.4 \times 10^3$ gravid adults per mL or $16.8 \times 10^6$ adults. The other $3.2 \times 10^6$ may have been on the glass surface again. The $30 \mu m$ mesh ARF with in the vessel adequately retained the adults for media exchange and produced two liters of conditioned-media from dense large-scale *C. elegans* culture.

The $20 \mu m$ mesh ARF in the 5L vessel from a 5 day synchronous culture gave the following results: $6 \times 10^3$ adults per mL were counted in 2.5L. The culture was concentrated down to $7.3 \times 10^3$ adults per mL in 1.5L until it clogged. The culture noticeably had debris, some previous culture adult debris, and $1.1 \times 10^4$ eggs that had not hatched. The ARF needs to be able to function under these conditions and so it was determined that the $20 \mu m$ mesh was inadequate for timely adult retention.

Some adults were noticed in the settled conditioned-media removed with the $20 \mu m$ mesh. Either they were from larvae that were trapped in the ARF from younger growth stages or under sucking forces adult worms were able to squeeze through $20 \mu m$ mesh.

The third use of the $30 \mu m$ ARF for filtration took longer than the previous two. Contamination was observed in the culture. The culture was concentrated from 5L to 1L and then diluted to bring it back to the desired volume. With the forth use it was noted that concentration was very difficult. After the harvest the unit was disassembled and the
ARF cleaned. The 1L and 5L ARF designs do not allow cleaning of both sides of the mesh. Also during cultivation of the nematodes growth debris or contamination accumulated on the filter and inhibit flow. Debris accumulating inside of ARF could not be removed and may harboring spores that could survive autoclaving if the partial debris size was sufficient. The ARF needed to be redesigned to allow for cleaning. The method would also have to be modified so debris could not build up prior to the media exchange step.

The in vessel ARF units were useful for increasing the density of a culture to that desired for adult conditioned-media production. Cultures of lower density than $5 \times 10^3$ per mL were concentrated down as young adults and resuspended in Basal Media calculated to have a density of $5 \times 10^3$ per mL or higher.

**Funnel ARF**

The 6.35cm funnel ARF with 30µm mesh was tested with gravid adults cultures prior to harvest. The cultures were concentrated down to a thick slurry of adult worms. The adults were transferred to harvest vessel with Basal media. When flow slowed due to clogging, the mesh was easily cleared with a laboratory squeeze bottle of M9. The funnel ARF was seen to be able to repeatedly concentrates the adults of a culture and removes growth media. In between adult concentrations the filter could be cleaned with a brush on both sides of the mesh. With cultures larger than 5L the funnel ARF may take to much time for optimal conditions.

The 12.7cm diameter funnel modified with a 30µm mesh was shown to be inadequate for nematode retention. The funnel folded in on itself as adults covered the
surface of the mesh restricting the flow. As the funnel folded in on itself the surface area of the mesh decreased because the mesh came in contact with the funnel surface. Filtering took as long or longer than the 6.35cm diameter funnel ARF.

Screen ARF

The 30.5cm diameter screen ARF was tested with 30, 36, and 50µm mesh for adult retention as a pre filter before egg harvest. The 30µm mesh took a long time to filter, it was estimated that this might have stressed the adults for oxygen. The 50µm mesh filtered quickly but many adults penetrated. The 36µm mesh retained most of the adults and liquid drained quickly. The adults noticed in the debris that settled at bottom of the buckets for each sieve on the permeate side of the mesh. The amount appeared to increase respectively with the mesh size. The 36µm mesh was found to be most optimal for adult sieve filtration.

The 36µm adult sieve was further tested with adults chilled to 10°C prior to filtration. The mesh retained all adults and the permeate liquid drained quickly. No adults were noticed in debris of mesh permeate. Chilling the culture prior to filtration caused the worms to be less motile and thus, the 36µm mesh retained all adults. The bucket screen adult filter can be used in the event that the ARF clogs.

Conclusion

An ARF system within a culture vessel was developed during this investigation to maintain optimal conditions for conditioned media production. A prototype for a 5L vessel was developed that retained adults from large-scale synchronous cultures for adult conditioned media production. The accumulative total of adult conditioned media
produced from this research is sufficient to enable *C. elegans* attractant pheromone analysis.

To remove conflicting compounds prior to conditioned media production, the media was exchanged with fresh media using a mesh separation prototype within 5L working volume bioreactors. A nylon mesh with openings smaller than the width of adult worms retained the adult worms in the vessel during media exchange and conditioned media harvest. The ARF prototype designed for this purpose removes the media while allowing normal agitation and aeration. The mesh allows adequate aeration during media transfer even when worm concentrations become high due to reduced volume. In the event the ARF clogs, an alternate funnel ARF or external screen adult filter can be used to retain the adults for adult conditioned media production. Worms are kept in near-optimal conditions during the media exchange.

Results of this investigation led to the development of methods and instrumentation for producing adult conditioned-media from large-scale synchronous adult cultures. Methods for young adult retention for media exchange maintaining a closed system and production of 5L of conditioned-media were developed. Overall, more than 100L of gravid adult *C. elegans* conditioned-media was produced providing male attractant pheromone for further analysis and proteomic research. The methods developed from this research enabled aseptic harvest of soluble *C. elegans* products from large-scale dense synchronous *C. elegans* cultures.
CHAPTER 5

SUMMARY

*Caenorhabditis elegans* is a model organism for understanding multicellular organisms. As certain proteins and sensory compound products are higher in concentration during a specific stage of a *C. elegans* life cycle, production of these molecules is optimized with a synchronous culture. Ovulating hermaphrodites produce putative pheromone(s) that cause male attraction. Pheromones are produced in such small quantities, that adult conditioned-media from large-scale synchronous culture is necessary to analyze these pheromones. This research has overcome the volume constraints of previous protocols for culture synchronization and improved methodology for adult conditioned-media production.

Tangential Flow Filtration (TFF) systems were built and modified for this investigated for use as a method to increase the volume limits of egg harvest for *C. elegans* culture synchronization. An Adult Retention Filter (ARF) was designed and built for use within a culture vessel for aseptic conditioned-media production.

The main objective of this design research was to demonstrate production of 100L of conditioned-media from *C. elegans* cultures at densities of $5 \times 10^3$ to $1 \times 10^4$ gravid adults per mL with negligible contaminants and growth byproducts. To achieve this synchronous cultures of *C. elegans* were produced in 5, 10, and 50L cultures with densities of $5 \times 10^3$ worms per mL or more, and conditioned-media from adult *C. elegans* was produced.

*C. elegans* products were optimized due to this research. These methods of harvesting eggs for synchronization and adult media exchange optimally produces male
attractant pheromone. The method for egg harvest was also shown to produce $3 \times 10^7$ synchronous L2 *C. elegans* for optimal stage-specific protein production.

Future work would further test the new TFF unit to investigate egg harvest capabilities for larger synchronous cultures. Other mesh sizes could be investigated for retaining other stages of nematodes for media exchange in order to produce other stage-specific products.

The TFF and ARF methods developed from this research allow for large-scale egg harvest and aseptic adult media exchange. Techniques reported here can easily be applied to larger scale systems for production of synchronous cultures of *C. elegans* and adult conditioned-media. Which in turn will lead to important products for pheromone and proteomic research.
REFERENCES


APPENDIX
**Figure A-1:** Modified Minitan TFF unit assembly diagram. The Minitan TFF for *C. elegans* egg harvest is assembled as seen in Figure A-1 from bottom to top: Base stainless steel plate, modified acrylic base plate, silicone rubber seal with middle ribs cut out, 11µm nylon mesh, silicone seal/nylon screen, top acrylic plate, 4 washers on each head plate bolt, and finally a hand nut for each of the four head plate bolts. The hand nuts are tightened alternating, as to assure a good seal by maintaining the top acrylic plate parallel with the modified base acrylic plate. As seen in figures 3-3, 3-4, and 3-8, the feed and retentate lines are in the base acrylic plate and the permeate line is in the top acrylic plate.
Figure A-2: Modified Pellicon TFF unit assembly diagram. The Pellicon TFF for *C. elegans* egg harvest is assembled as seen in Figure A-2 from base to top: Base steel plate, acrylic base plate, 19 hole silicone rubber seal, 19 hole acrylic plate, 19 hole silicone rubber seal, 19 hole polypropylene channel plate, 19 hole silicone adhesive rubber seal with center cut out around channels (adhered to previous part), 11µm pore size 6% open nylon mesh, 8 hole silicone adhesive rubber seal with center cut out around channels (adhered to next part), 8 hole modified polypropylene channel plate, silicone rubber seal, top 3 rib acrylic plate, spring loaded spacer, a washers on each head plate bolt, and finally a brass nut for each of the four head plate bolts. The nuts are tightened alternating with a torque wrench (10.56Nm or 90inlb) an eighth of a turn each, as to assure a good seal by maintaining the top acrylic plate parallel with the modified base acrylic plate.
Figure A-3: 3D image of new *C. elegans* TFF acrylic feed/retentate or permeate plate.

Figure A-4: New *C. elegans* TFF assembly diagram.
Figure A-5: Picture of new *C. elegans* TFF assembled for use. The New TFF for *C. elegans* egg harvest is assembled as seen in Figures A-4 and A-5: A silicon rubber sheet with an adhesive side was adhered to the acrylic channel plate. The channels were cut out of the rubber sheet with a dissecting knife following the acrylic channel bellow. This was repeated for the opposing acrylic channel plate. A 30.5cm by 30.5cm (12in by 12in) 11µm pore size 6% open nylon mesh was set between the two acrylic channel plates. A heated nail was used to punch holes for the bolts to pass through the mesh. The stainless steel bolts and hand nuts were inserted with a nylon washer between the hand nut and acrylic channel plate. The hand nuts were tightened in an alternating cross pattern till they were hand tight, as to assure a good seal by maintaining the top acrylic plate parallel with the modified base acrylic plate.