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**DEVELOPMENT OF PLANT CELL CULTURE PROCESSES TO PRODUCE  
NATURAL PRODUCT PHARMACEUTICALS: CHARACTERIZATION,  
ANALYSIS, AND MODELING OF PLANT CELL AGGREGATION**

A Dissertation Presented

by

MARTIN E. KOLEWE

Submitted to the Graduate School of the  
University of Massachusetts Amherst in partial fulfillment  
of the requirements for the degree of  
DOCTOR OF PHILOSOPHY

September 2011

Department of Chemical Engineering

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Department of Chemical Engineering

## **DEDICATION**

To my wife, Christina

## **ACKNOWLEDGEMENTS**

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## **ABSTRACT**

# **DEVELOPMENT OF PLANT CELL CULTURE PROCESSES TO PRODUCE NATURAL PRODUCT PHARMACEUTICALS: CHARACTERIZATION, ANALYSIS, AND MODELING OF PLANT CELL AGGREGATION**

SEPTEMBER 2011

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Directed by: Professor Susan C. Roberts

Plant derived natural products represent some of the most effective anti-cancer and anti-infectious disease pharmaceuticals available today. However, uncertainty regarding the feasibility of commercial supply due to the limited availability of many plants in nature has resulted in a dramatic reduction in the use of natural products as leads in modern drug discovery. Plant cell suspension culture, consisting of dedifferentiated plant cells grown *in vitro* and amenable to large scale industrial biotechnology processes, is a production alternative which promises renewable and economical supply of these important drugs.

The widespread application of this technology is limited by low product yields, slow growth rates, challenges in scale-up, and above all, variability in these properties, which is poorly understood.

Plant cells grow as aggregates in suspension cultures ranging from two to thousands of cells (less than 100  $\mu\text{m}$  to well over 2 mm). Aggregates have long been identified as an important feature of plant cell culture systems, as they create microenvironments for individual cells with respect to nutrient limitations, cell-cell signaling, and applied shear in the *in vitro* environment. Despite its purported significance, a rigorous engineering analysis of aggregation has remained elusive. In this thesis, aggregation was characterized, analyzed, and modeled in *Taxus* suspension cultures, which produce the anti-cancer drug paclitaxel. A technique was developed to reliably and routinely measure aggregate size using a Coulter counter. The analysis of aggregate size as a process variable was then used to evaluate the effect of aggregation on process performance, and the analysis of single cells isolated from different sized aggregates was used to understand the effect of aggregation on cellular metabolism and heterogeneity. Process characterization studies indicated that aggregate size changed over a batch cycle as well as from batch to batch, so a population balance equation model was developed to describe and predict these changes in the aggregate size distribution. This multi-scale engineering approach towards understanding plant cell aggregation serves as an important step in the development of rational strategies aimed at controlling the process variability which has heretofore limited the application of plant cell culture technology.



## TABLE OF CONTENTS

	Page
<b>ACKNOWLEDGEMENTS.....</b>	<b>v</b>
<b>ABSTRACT.....</b>	<b>vi</b>
<b>LIST OF TABLES.....</b>	<b>xi</b>
<b>LIST OF FIGURES.....</b>	<b>xii</b>
<b>CHAPTER</b>	
<b>1 INTRODUCTION AND BACKGROUND.....</b>	<b>1</b>
1.1 Introduction .....	1
1.2 Production Options for Natural Products.....	2
1.3 Plant Suspension Cell Culture Technology.....	6
1.3.1 Traditional Strategies to Improve Cell Culture Yields.....	8
1.4 Towards Understanding and Controlling Variability in Product Accumulation...	13
1.4.1 Cellular Heterogeneity as a Basis of Variability.....	16
1.5 Metabolic Engineering and Directed Biosynthesis .....	21
1.5.1 Metabolic Engineering Tools.....	21
1.5.2 Metabolic Engineering Strategies .....	25
1.6 Future Directions in Engineering Supply.....	27
<b>2 CHARACTERIZATION OF AGGREGATE SIZE IN <i>TAXUS</i> SUSPENSION</b>	
<b>CELL CULTURE .....</b>	<b>34</b>
2.1 Introduction .....	35
2.2 Materials and Methods.....	38
2.2.1 Cell Cultures .....	38
2.2.2 Filtration .....	39
2.2.3 Electrical Resistance Pulse Amplitude Sizing .....	39
2.2.4 Image Acquisition and Analysis .....	40
2.3 Results and Discussion .....	41

2.3.1 Standard Methods to Measure Aggregation .....	41
2.3.2 Electrical Resistance Pulse Amplitude Sizing .....	43
2.3.3 Total Biomass Correlation.....	45
2.3.4 Validation of Aggregate Size Measurement.....	47
2.3.5 Batch culture process monitoring .....	49
2.4 Conclusions .....	50
<b>3 ANALYSIS OF AGGREGATE SIZE AS A PROCESS VARIABLE AFFECTING PACLITAXEL ACCUMULATION IN <i>TAXUS</i> SUSPENSION CULTURES .....</b>	<b>57</b>
3.1 Introduction .....	58
3.2 Materials and Methods.....	61
3.2.1 Cell cultures .....	61
3.2.2 Aggregate Fractionation and Paclitaxel Analysis .....	61
3.2.3 Aggregate Size Distribution Manipulation and Total Culture Studies .....	62
3.3 Results.....	63
3.3.1 Biomass-Associated Paclitaxel in Aggregate Fractions .....	63
3.3.2 Aggregate Size Manipulation and Total Paclitaxel Accumulation.....	64
3.4 Discussion .....	67
3.4.1 Limitations of Aggregate Fractionation .....	67
3.4.2 Aggregate Size as a Process Variable .....	69
3.5 Conclusion.....	72
<b>4 A POPULATION BALANCE EQUATION MODEL OF AGGREGATION DYNAMICS IN <i>TAXUS</i> SUSPENSION CELL CULTURES.....</b>	<b>81</b>
4.1 Introduction .....	82
4.2 Materials and Methods.....	85
4.2.1 Experimental.....	85
4.2.2 Model Formulation.....	85
4.3 Results.....	91
4.3.1 Stationary phase: breakage frequency and partitioning kernels .....	92
4.3.2 Initial growth phase: growth rate kernel.....	92
4.3.3 Entire batch: substrate-dependent growth .....	93
4.3.4 Predicted effect of breakage rate on paclitaxel accumulation .....	95

4.4 Discussion .....	96
4.4.1 Kernel functions provide qualitative insight into aggregation phenomena	96
4.4.2 Utilization of the model as a predictive tool to guide operating strategies	98
<b>5 ON THE RELATIONSHIP OF SINGLE CELL HETEROGENEITY TO AGGREGATION .....</b>	<b>110</b>
5.1 Introduction .....	110
5.2 Materials and Methods.....	110
5.2.1 Cell Culture Maintenance and Biomass Measurements .....	114
5.2.2 Analysis of single cell paclitaxel accumulation.....	114
5.2.3 Analysis of cell cycle participation .....	115
5.3 Results and Discussion .....	118
5.3.1 Heterogeneity of paclitaxel accumulation .....	118
5.3.2 Analysis of cell cycle heterogeneity .....	120
5.4 Conclusion.....	125
<b>6 IMPACT AND FUTURE WORK.....</b>	<b>131</b>
6.1. Impact.....	131
6.2. Future Work .....	136
<b>BIBLIOGRAPHY .....</b>	<b>141</b>

## LIST OF TABLES

Table	Page
<b>Table 1.1</b> Commercial plant cell culture processes for supply of natural products with pharmaceutical applications.....	31
<b>Table 1.2</b> Natural product biosynthesis in native species: A comparison of secondary pathways .....	32
<b>Table 3.1</b> Aggregate size and biomass prior to elicitation for cultures initiated with different aggregate size distributions. ....	74
<b>Table 3.2</b> Aggregate size and paclitaxel accumulation for cultures initiated from large aggregate distribution .....	74
<b>Table 4.1</b> Nominal functions and parameter values used for Figures 4.6-4.9. ....	101
<b>Table 5.1</b> Comparison of cell cycle activity measured by both DNA content and EdU incorporation in terms of percent increase in largest aggregates ( > 1320 $\mu\text{m}$ ) compared to smallest aggregates ( < 310 $\mu\text{m}$ ).. ....	101

## LIST OF FIGURES

Figures	Page
<b>Figure 1.1</b> Initiation of a plant suspension cell culture from explant material. ....	32
<b>Figure 1.2</b> Aggregate of <i>Taxus cuspidata</i> P991 cells in suspension culture.....	33
<b>Figure 1.3</b> Unsorted (a) and sorted (b) <i>T. cuspidata</i> P991 cells. ....	33
<b>Figure 2.1</b> Typical size and morphology of aggregates in <i>Taxus cuspidata</i> suspension culture.....	51
<b>Figure 2.2</b> Aggregate size distributions obtained using a Coulter counter. ....	52
<b>Figure 2.3</b> Suitability of the Coulter counter method to measure total biomass. ....	53
<b>Figure 2.4</b> Validation and correlations of Coulter counter size measurements. ....	55
<b>Figure 2.5</b> Process monitoring of cell cultures over batch subculture period.....	56
<b>Figure 3.1</b> Typical aggregates in <i>T. cuspidata</i> suspension culture.....	75
<b>Figure 3.2</b> Fractionation and analysis of aggregates of <i>T. cuspidata</i> P991 post elicitation with methyl jasmonate in two similar experiments.....	75
<b>Figure 3.3</b> Overview of filtration process to initiate cultures with differing aggregate distributions.....	76
<b>Figure 3.4</b> Detailed time course profiles of aggregate size distributions and paclitaxel accumulation in <i>T. cuspidata</i> P93AF. ....	77
<b>Figure 3.5</b> Aggregate size distributions of <i>T. cuspidata</i> immediately prior to elicitation with methyl jasmonate for (a) cell line P991C and (b) cell line P93AF. ....	78
<b>Figure 3.6</b> Aggregate size distributions measured for <i>Taxus cuspidata</i> P991C after subculture initiation in which aggregates used for inoculum were filtered using a 1000 $\mu\text{m}$ filter. ....	79
<b>Figure 4.1</b> Extended Hill-Ng binary power-law product partitioning function, where the parameter $q$ determines the shape of the distribution.....	102
<b>Figure 4.2</b> Experimental data used to structure and parameterize the model. ....	103
<b>Figure 4.3</b> Effect of the breakage kernel function on predicted distributions for a breakage only model, where model input was the Day 12 experimental distribution. ...	104
<b>Figure 4.4</b> Effect of growth kernel size dependence on predicted distributions from the initial growth phase, Day 0 to Day 6, using the breakage combinations from the breakage-only model and fitting the specific growth rate, $\mu_{\text{max}}$ , to minimize the residual error, $\Psi$ . ....	104
<b>Figure 4.5</b> Simulation of growth from Day 0 through Day 8. ....	105

<b>Figure 4.6</b> Comparison of experimental data and model predictions for the entire batch process. ....	106
<b>Figure 4.7</b> Comparison of experimental data and model predictions for the entire batch process. The model is compared to a non-fitted dataset with a significantly smaller initial aggregate distribution than that used for parameter estimation (Fig. 6).....	107
<b>Figure 4.8</b> Comparison of experimental data and model predictions for the entire batch process. The model is compared to a non-fitted dataset obtained one year earlier than that used for parameter estimation (Fig. 6). ....	108
<b>Figure 4.9</b> Simulated paclitaxel accumulation under different shear conditions. ....	109
<b>Figure 5.1</b> Analysis of paclitaxel content in single cells isolated from different sized aggregates within a culture. ....	126
<b>Figure 5.2</b> Average initial distributions for cultures initially filtered at 710 $\mu\text{m}$ . ....	127
<b>Figure 5.3</b> Cell cycle activity of aggregate fractions isolated from <i>Taxus cuspidata</i> cultures at several time points.....	128
<b>Figure 5.4</b> Cell cycle activity of aggregate fractions isolated from <i>Taxus canadensis</i> cultures at several time points.....	129
<b>Figure 5.5</b> Comparison of cell cycle activity between small aggregate cultures and large aggregate cultures of (a),(c) <i>Taxus canadensis</i> and (b),(d) <i>Taxus cuspidata</i> at several time points. ....	130

## CHAPTER 1

### INTRODUCTION AND BACKGROUND

#### 1.1 Introduction

Plants are a tremendous source of natural diversity in the multitude of compounds that they synthesize, which humans have utilized in varied ways including flavor enhancers, agricultural chemicals, and perhaps most importantly, medicines. Most of these active ingredients can be classified as secondary metabolites, broadly defined as any product which a plant synthesizes that is not necessary for basic life functions such as growth or replication. Secondary metabolites are a large, varied, and sometimes mysterious group of molecules; while some are most likely extraneous byproducts of metabolic pathways due to promiscuous enzyme activity, many do serve important functions in defense or protection *in planta*, and thus can be considered biologically active. It is a result of this biological activity that these molecules can be useful as applied treatments for human ailments; this has been recognized for centuries as plants have been used in traditional medicine across the world. Natural products, in the form of plants and plant extracts, have been used for millennia as the primary medicine for treating illnesses and ailments for most of the world's population. It is no surprise then that plants have also been used throughout history to treat cancer, with at least 3000 species reported to have been used (Hartwell 1982), though the actual number is probably higher (Graham 2000). Since the advent of chemotherapy to treat cancer in the 1940's, the search for chemicals to kill the abnormally and rapidly proliferating cells which invade normal tissue and form tumors has often turned to the natural world as both a starting point to develop effective chemotherapeutics, as well as a source of the actual chemical to be used as a final drug product. Whether from traditional medicine, serendipity, or large scale screening efforts,

nature has been the source of some of our most effective and successful cancer therapies. In fact, of the small molecule anti-cancer drugs developed between 1981 and 2008, almost 63% contained either a natural product or natural product derivative (Cragg et al. 2009). Over the past 100 years, as specific metabolites have been chemically isolated and identified, commercial production of these biologically active phytochemicals has presented an enormous challenge. Most secondary metabolites are present in extremely low amounts in the plant, often less than 1% of the total carbon (Bourgaud et al. 2001). This paucity can make natural harvestation impractical for bulk production, especially in the case of slow growing species. The significant engineering challenge is then to find a means by which to produce the desired natural products in a way that is both sustainable and financially feasible.

## **1.2 Production Options for Plant Natural Products**

Chemical synthesis of natural products is possible and commercially feasible, particularly for those with relatively simple chemical structures such as aspirin (derived from the natural product salicylic acid) and ephedrine (Wink et al. 2005). In many cases, however, the metabolite has a complex structure, which can include multiple rings and chiral centers, so that a synthetic production process becomes prohibitively costly. Many natural products used in cancer treatment, including compounds such as paclitaxel, vinblastine, and camptothecin, fall into this latter class, so an alternative method of supply is necessary.

Depending on the nature of the plant, extraction directly from harvested plant tissue may be an option. Especially if a plant can be cultivated en masse, this can be attractive on a commercial basis. The anticancer drugs vincristine and vinblastine, among other medicinally valuable metabolites such as ajmalicine and serpentine, are found in the



Madagascar periwinkle *Catharanthus roseus* (Liu et al. 2007). Even though these important alkaloids, particularly vincristine and vinblastine, naturally occur at very low levels in *C. roseus* – less than 3 g per metric ton – the fast growing nature of the periwinkle makes field cultivation most practical at the present time (Wink et al. 2005). However, the relative inefficiency and high cost of whole plant extraction implies that an improved method of supply would be useful for these valuable anti-cancer agents.

When natural supply is limited due to a combination of low yields and slow growth rates, an alternative method of supply is necessary, and is a significant engineering challenge. With the dramatic increase in tools for genetic manipulation and metabolic engineering, a synthetic biology approach is one alternative that has attracted considerable attention in the past 10 years (Withers and Keasling 2007; Ajikumar et al. 2008; Zhang et al. 2008). This strategy involves identifying biosynthetic pathway genes as well as precursor and support pathway genes, and transferring these genes into microbial hosts to produce the final product of interest. The advantages of this approach are the availability and ease of implementation of tools for the manipulation of microbes, from both the metabolic and process engineering perspectives. One notable success is the production of artemisinin, an important anti-malarial agent, in *E. coli* (Keasling 2008). In this particular example, rather than identify and transfer all of the genes involved in the biosynthetic pathway for artemisinin production in *Artemisia annua*, a critical step in the pathway was carried out by the *de novo* engineering of an enzyme, which ultimately yielded a precursor that could be readily converted via synthetic chemistry into the final product. The details of the artemisinin success underscore the primary limitation of the synthetic biology approach, which is that the identification of all the genes involved in secondary metabolite biosynthesis is very difficult, and has not been accomplished for most medicinal plant species (see below). Recent advances in the engineering of *E. coli* to

produce the anti-cancer drug paclitaxel which is naturally found in *Taxus* spp. have been widely publicized (Ajikumar et al. 2010), but they have only reported the successful transfer of the precursor isoprenoid pathway and the first 2 of 19 putative steps of the dedicated paclitaxel biosynthetic pathway. Particularly in systems such as *Taxus* where secondary biosynthetic pathways are complex and unknown, the utility of the synthetic biology approach is unlikely to ultimately be successful in ensuring an adequate and cost-effective supply of natural product pharmaceuticals (Roberts and Kolewe 2010).

In cases where no other scalable commercial production technology is available, *in vitro* cultures provide an attractive alternative. Most plant species can be cultured *in vitro* in either an undifferentiated or differentiated state. As many secondary metabolites are produced by specialized cells, organ cultures such as shoots or roots can exhibit similar metabolite profile patterns compared to the native plant (Verpoorte et al. 2002), whereas undifferentiated cultures often accumulate secondary metabolites to a lesser extent, and sometimes not at all. The anticancer compound camptothecin, produced by the ornamental tree *Camptotheca acuminata* as well as *Nothapodytes foetida* and *Ophiorrhiza pumila* among other species, has been shown to accumulate in undifferentiated cultures in very low or even undetectable amounts (Paqua et al. 2006), compared to root cultures in which production levels were comparable to the intact plant (Lorence and Nessler 2004). Similarly, no artemisinin, a potent anti-malarial drug, was found in cell suspension cultures of *Artemisia annua*, while trace amounts were detected in shoot cultures (Liu et al. 2006). Root cultures can be transformed into hairy roots using the soil dwelling bacteria *Agrobacterium rhizogenes*, resulting in cultures which are genetically stable, capable of unlimited growth without additional hormones, and have an increased capacity for secondary metabolite accumulation (reviewed most recently in Srivastava and Srivastava 2007; Guillon et al., 2006). The commercial potential of hairy

root cultures has been limited primarily due to challenges in cultivating hairy roots in a large scale system. Hairy roots form a complex, non-homogeneous, solid matrix, and present enormous difficulties in terms of bioreactor configuration (Srivastava and Srivastava 2007). While significant effort is currently focused on novel approaches to bioprocess and reactor design, such as a system in which roots are grown on racks and continuously sprayed with medium (Wildi et al. 2004) and an acoustic mist bioreactor in which a nutrient mist is generated by an acoustic field (Suresh et al. 2005), difficulties associated with scale up have generally prevented widespread commercial application of this technology.

Undifferentiated suspension cultures, which can be more easily scaled to levels suitable for commercial production, have been studied for producing useful metabolites since the 1950's with mixed success. There are currently 14 plant cell culture processes which have been commercialized for production of secondary metabolites (Frense 2007), including products used in applications other than pharmaceuticals such as food and cosmetics.

Table 1 presents an overview of secondary metabolites specifically used in pharmaceutical applications produced on a commercial scale. There are relatively few commercial processes due in part to the fact that many metabolites simply do not accumulate in undifferentiated cultures. Presumably, the biosynthetic pathways for many secondary metabolites are under strict control during developmental regulation leading to spatial separation of pathway segments in different types of tissues. In *A. annua*, artemisinin has been shown to accumulate at different levels in shoots, seeds, leaves, and flowers, with the highest levels in the flowers (Weathers et al. 2006). Undifferentiated cultures lack these specific organs or tissues, which may be associated with critical

portions of the biosynthetic pathways. Additionally, compartmentalization of secondary metabolite biosynthetic pathways also occurs at the subcellular level, and cells cultured *in vitro*, which have higher growth rates than soil-grown plants, may lack fully developed compartments such as plastids and vacuoles, which most likely contribute to the lower productivity of these cultures (Pasquali et al. 2006). Even in those cultures which do produce the compound of interest, yields are often low and highly variable (Ketchum and Gibson 1996; Naill and Roberts 2005), and most engineering work is focused on understanding and improving limitations associated with these two factors. Despite the challenges of using plant cell suspension cultures, advantages such as ease of scale-up and simpler purification schemes due to product secretion have prompted extensive research into facilitating commercialization of this technology.

### **1.3 Plant Suspension Cell Culture Technology**

Production of metabolites via plant cell suspension culture is renewable, environmentally friendly, and from a processing standpoint, amenable to strict control, an advantage in regards to meeting Food and Drug Administration manufacturing standards. Technology developed for other cell culture and fermentation systems (e.g., mammalian and yeast) can be readily adapted for large scale applications with plant cells, easing difficulties associated with scale-up. A notable example of the success of plant cell culture systems, due in large part to innovative research and the application of novel technologies, is paclitaxel synthesis and supply. Paclitaxel, produced by *Taxus* spp., is an important anticancer agent used as a first line treatment for several types of cancer, including breast, ovarian, and non-small cell lung cancer, and has also shown efficacy against AIDS-related Kaposi sarcoma (Cragg and Newman 2005). Production of paclitaxel via cell culture technology has been studied since the 1980's as an alternative

supply source to harvest of the slow growing yew tree, since a single dose of 300 mg requires the sacrifice of a 100 year old tree (Tabata 2004). A combination of process engineering and directed biosynthesis approaches ultimately led to significant improvement of yields in cell culture systems, to the extent that commercial success has been achieved by Phyton Biotech, in supplying Bristol-Myers Squibb paclitaxel for its Taxol<sup>®</sup> formulation (Tabata 2006) (see section 1.3.2).

Suspension cell cultures are initiated from an explant that has been isolated from plant material (e.g., embryo, needle, bark, stem). This explant can be plated on a solid growth medium (Figure 1), which must be tailored specifically for different species. Growth medium typically consists of a carbon source, minerals, phytohormones, and antioxidants. Under suitable conditions, the explant will grow into a proliferating mass of dedifferentiated cells known as a callus culture. The callus can subsequently be transferred from solid to liquid medium, resulting in a suspension cell culture that is incubated under agitation and controlled temperature. The dedifferentiated cells are considered to be totipotent – that is they have the ability to differentiate into any somatic cell type, and under suitable conditions, these cultures can be used to regenerate fertile plants in most species. However, this dedifferentiated state is not stable, and evidence suggests that all cultures lose the ability to regenerate into plants over time (Zhang and John 2005). To this point, little is known about how the developmental state of cells change over time in culture, which is of particular interest concerning the long term maintenance of suitable cell lines used for metabolite production (see below).

The primary challenges impeding regular commercial application of plant cell culture technology are low and variable yields of metabolite accumulation. As mentioned above, some metabolites do not accumulate in appreciable quantities in dedifferentiated

cells. In these cases, manipulation of genes within the biosynthetic pathway is needed to utilize plant cell cultures for bulk production, which is often unrealistic due to a lack of complete knowledge regarding secondary metabolic pathways and their regulation in most plant systems (see below). In the case that metabolite accumulation occurs in low yields, traditional strategies based on similar approaches in other types of cell culture and fermentation systems have been successful in improving metabolite yields to suitable levels for commercial production, as described below. Controlling variability in product accumulation has often been neglected in favor of improving yield, but more recently, the recognition of variability as a key limiting factor (Roberts 2007), especially in the common case in which metabolite accumulation decreases as a cell line ages (Deusneumann and Zenk 1984; De Jesus-Gonzalez and Weathers 2004; Qu et al. 2005) has led to a renewed focus on understanding its basis as a fundamental goal in current plant cell culture research.

### **1.3.1 Traditional Strategies to Improve Cell Culture Yields**

To date, much of the work on plant cell cultures that has translated into commercial success involves optimization strategies similar to those developed for other cell culture and fermentation processes. This type of process engineering approach includes manipulation of culture operating parameters such as media composition, cell line selection, and gas phase composition (reviewed in Kieran et al. 1997; Roberts and Shuler 1997). These strategies are a necessary starting point in many cases, especially when initiating a new cell line. The following section presents a brief overview and recent applications of some approaches that have traditionally been of interest to plant cell culture process development. While many of these ideas may be considered outdated in favor of newer metabolic engineering perspectives, their successful application is

essential for the eventual success of plant cell culture technology, and therefore remain active areas of research.

Perhaps the most notable strategy for improving metabolite yields is elicitation. An elicitor can be defined as any compound that induces an upregulation of genes. Some elicitors target secondary metabolic genes, which are often associated with defense responses to perceived environmental changes. Elicitors include natural hormones, nutrients, and many fungi-derived compounds. In particular, jasmonic acid and its methyl ester methyl jasmonate (MJ), are naturally occurring hormones involved in the regulation of defense genes as part of a signal transduction system (Gundlach et al. 1992). Applied exogenously, they have been shown to induce secondary metabolic activity and promote accumulation of desired metabolites in numerous plant systems, including *Taxus* spp. (Mirjalili and Linden 1996; Yukimune et al. 1996) and *C. roseus* (Aerts et al. 1994; Lee-Parsons and Royce 2006). Different elicitors may act on different segments of the biosynthetic pathway. For instance, MJ elicitation compared to salicylic acid elicitation in *Taxus* spp. cultures resulted in different relative increases of metabolic intermediates (Ketchum et al. 1999; Wang et al. 2004), suggesting that each elicitor preferentially directs flux towards, and possibly away from, different intermediate taxanes. While many of the specific targets of elicitors have yet to be conclusively identified, elicitation can be an extremely useful tool in conjunction with gene expression profiling for identifying rate influencing steps in secondary biosynthetic pathways (see below).

Product removal *in situ* has received considerable interest over the years, especially when using transgenic plant systems for the expression of foreign proteins which may be degraded post synthesis (Doran 2006). Metabolite accumulation in cell cultures may be limited by feedback inhibition and product degradation, so “two-phase”

systems present obvious advantages, including simpler downstream recovery. Many secondary metabolites may also be toxic to cultures at artificially high levels induced by elicitation, making product removal necessary for continued growth and biomass accumulation. More recently, the use of extraction resins and adsorbents has been shown to increase productivity in several systems, including anthraquinones from *Morinda elliptica* (Chiang and Abdullah 2007), and ajmalicine from *C. roseus* (Wong and Lee-Parsons 2004). A combination of an external extraction column with a high-rate perfusion bioreactor has been developed (De Dobbeleer et al. 2006) in a scalable design, indicating the practical applicability of this approach.

Immobilization of plant cell cultures has long been considered for increasing metabolite accumulation, as the potential of higher cell densities, continuous removal of products/inhibitors, and protection for shear-sensitive plant cells provide a number of advantages (Dornenburg and Knorr 1995). Immobilization can be simply achieved using a gel matrix such as alginate; however this becomes costly at a larger scale, especially when the product of interest is not secreted and must be released using sonication or treatments with an organic solvent (Verpoorte et al. 2002). Recently, immobilization of *T. baccata* cells in calcium-alginate beads was shown to produce one of the highest reported levels of paclitaxel accumulation among academic laboratories (43 mg/L) (Bentebibel et al. 2005). Immobilization also has the potential to simplify product extraction and purification, as immobilized cultures of *Linum usitatissimum* excrete the pharmaceutically active metabolite dehydrodiconiferyl alcohol-4- $\beta$ -d-glucoside (DCG) to a greater extent than suspension cultures (Attoumbre et al. 2006).

Plant cell suspension cultures in large scale bioreactors are subject to the hydrodynamic forces resulting from mechanical agitation, and many reactor designs have



been suggested over the years for minimization of these detrimental effects, often using airlift or bubble column designs to replace mechanical impellers (Bourgaud et al. 2001).

Plant cells are much larger than mammalian cells or microbes which make them extremely susceptible to shear forces in the surrounding fluid. Different types of plant cells exhibit different responses related to shear forces, and detailed studies have been performed on individual species evaluating a variety of effects relative to shear forces, including reduction in viability, release of intracellular components, changes in metabolism, and changes in morphology (Zhong 2002). Generally, excessive shear forces can lead to cell lysis and reduced viability. More recently, efforts have been focused on understanding the processes and underlying mechanisms involved in the cellular responses to shear. Traditionally, research focused on understanding cellular response in order to optimize bioreactor designs. Current research is being directed towards metabolic modification by either genetic transformation or optimizing specific environmental conditions, to allow cells to be less vulnerable to the negative effects of shear forces.

Plant cells may actually adapt to high shear environments over time. For example, suspension cultures of *T. cuspidata* show significant differences in response to shear stress at different culture ages, as measured by reactive oxidative species (ROS) concentration, extracellular pH, and membrane fluidity, all of which have been previously shown to be early defense responses to mechanical stress (Gong et al. 2006). Nitric oxide (NO), in combination with ROS, triggers biological responses including host cell death. NO generation and the suppression of glutathione S-transferase, a critical enzyme responsible for eliminating ROS, have been strongly correlated to shear stress in *T. cuspidata* cell suspensions in a Couette-type reactor (Gong and Yuan 2006).

Furthermore, the mechanism of plant cell mechanoreception appears to be analogous to integrins, which serve a similar function in animal cells through recognition of a family of

extracellular glycoproteins via an Arg-Gly-Asp (RGD) motif. Based on studies which utilized a synthetic RGD peptide to effectively disrupt communication between the extracellular matrix and cell interior, as evidenced by monitoring physiological responses to shear stress, such as ROS, the existence of similar RGD binding proteins in plants has been proposed (Gao et al. 2007). No homologue to integrin has been identified in the model plant *Arabidopsis thaliana* as of yet, but the eventual identification of the molecular structure and function of these unknown proteins in plant cells may provide an opportunity to regulate cellular physiological response to mechanical stress.

Efficient bioprocessing technologies will aid in the commercialization of plant cell culture processes. The high costs of industrial biomanufacturing facilities are due in large part to the expensive equipment used in culture processes, such as stainless steel bioreactors and support equipment, as well as the demands associated with aseptic processing, including clean utility generation and equipment for cleaning and sterilization. Disposable technology is increasingly being used in the scale up stages of industrial cell culture processes (Heath and Kiss 2007), but relatively few studies have examined the suitability of this equipment for plant cell cultures. Disposable reactors are typically pre-sterilized plastic reactors that eliminate the need for separate cleaning and sterilization cycles, and can reach working volumes of up to several hundred liters. Two types of these reactors: a wave undertow reactor consisting of a flexible plastic container on a horizontal platform that is intermittently raised to induce wave formation, and a slug bubble column consisting of a flexible, vertical cylinder in which large bubbles are periodically generated that rise to the top of the column, were recently evaluated using soya and tobacco cell cultures (Terrier et al. 2007). Apparent growth rates for both culture systems in reactors up to 100 L were comparable to those obtained in Erlenmeyer flasks and a stirred-tank bioreactor, and oxygen transfer rates were also relatively high,

pointing towards the suitability of these designs for high density cell cultures. Shear forces are drastically reduced in these disposable designs, especially in the wave bioreactor in which sufficient oxygen transfer is achieved without a mechanical impeller or sparge gas aeration.

#### **1.4 Towards Understanding and Controlling Variability in Product Accumulation**

Much improvement has been made in increasing yields of secondary metabolites which accumulate at low levels. However relatively little progress has been made in understanding and controlling the unstable secondary metabolite production patterns. The maintenance of consistently high production levels has proven to be difficult, and gradual loss of secondary metabolite productivity over time has long been known as an obstacle in the development of commercial plant cell culture production systems (Deusneumann and Zenk 1984; Qu et al. 2005). Unlike mammalian cells, which are routinely stored in liquid nitrogen, relatively few storage methods have been developed for plant cells, and those methods that do exist are typically characterized by low viability and long lag periods before recovering a rapidly growing suspension culture (Menges and Murray 2004). While cryopreservation methods can be optimized by using cryoprotectants, efficient freezing programs including cold acclimation and vitrification, and adding components such as calcium (see for example Lardet et al. 2007; Kadkade et al. 2004), most cell lines are maintained via periodic subculture. As a cell line effectively ages, the loss of desirable characteristics and instability of secondary metabolism is likely caused by a combination of factors, including: mutations that are likely to predominate in cell populations that have been repeatedly propagated in an undifferentiated state, varying ploidy levels which have been shown to affect secondary metabolism in several systems, occurrence of distinct subpopulations within a culture related to prolonged exposure to

microenvironments within the culture system, and inherent variability associated with the repeated subculture process (see below for more detail). In particular, the implications of subpopulations related to culture behavior have received considerable interest in recent years, and is the focus of much work in our laboratory.

Plant cell suspension cultures consist of dedifferentiated cells derived from explant tissue. While these cultures can be indefinitely maintained in a generally dedifferentiated state with the addition of phytohormones to the media, such as auxins and cytokinins, they are not maintained in a state of genetic or epigenetic stability. Older cultures of *Nicotiana plumbaginifolia* exhibited faster growth rates than newer cultures, which is attributed to the high proportion of cells with mutations that elevate cell dependant kinase (CDK) activity (Zhang and John 2005). This type of mutation accelerates proliferation and obstructs processes related to organ development and differentiation, to the point that older cultures were incapable of regenerating into plants. As many metabolic pathways are compartmentalized, particularly to the plastids (Shanks 2005), the lack of fully developed organelles could have profound effects on the ability of cells to operate and regulate these pathways.

Ploidy levels are commonly known to be variable in plants, and suspension cultures exhibiting varying degrees of polyploidy have been linked with the ability of a cell to differentiate (Schween et al. 2005) and regenerate into shoots (Shiba and Mii 2005). Increasing ploidy levels have been correlated with gene silencing in several plant systems (Pikaard 2001). Similar epigenetic silencing of regulatory genes may contribute to variable accumulation of secondary metabolites in *Taxus* spp. (Vongpaseuth and Roberts 2007), and may help to explain the relationship between ploidy variation and metabolite accumulation. Hypericin content in *Hypericum perforatum* decreased with

increasing ploidy, particularly between diploid and polyploid populations (Kosuth et al. 2003), whereas tetraploid cultures of *A. annua* hairy roots had a higher specific artemisinin concentration, which was partially offset by a slower growth rate (De Jesus-Gonzalez and Weathers 2003). Studies aimed at investigating the long term genomic stability have revealed that a majority of *Taxus* cell lines were putatively aneuploid over a two year period (Baebler et al. 2005). As much of the ploidy variation is cyclic, it may correlate with the short term irregular cycling patterns of metabolite accumulation often observed (Hirasuna et al. 1996) before accumulation gradually disappears (Qu et al. 2005). Differences in ploidy level were manifest in segments of the total cell population, as it was found that *Taxus* subpopulations with different ploidy levels coexisted in culture for extended periods of time (Baebler et al. 2005). Studies relating ploidy levels to metabolite accumulation only indirectly correlate the effect of aneuploidy with secondary metabolic activity, since metabolite accumulation is taken as a culture average, rather than relative to the individual subpopulations. Ploidy variations can be induced using mitotic inhibitors (Wallaart et al. 1999) and by altering plant growth regulators (Mishiba et al. 2001), providing another method to alter cell culture dynamics. A more fundamental understanding of the relationship between ploidy and secondary metabolism may prove to be beneficial to design a strategy aimed at controlling metabolism through manipulation of ploidy levels.

The periodic subculture process by which all *in vitro* lines are maintained also contributes to some degree of variability. After controlling for different types of media, inoculum size, and flask variation in *Taxus* cultures, variability in both growth and secondary metabolism was still present, leading to the conclusion that variability is inherently induced by the subculturing process (Kim et al. 2004).

A more mechanistic understanding of culture instability is necessary before variability in secondary metabolism can be adequately addressed. Metabolic instability is clearly related in some way to the genetic and epigenetic factors mentioned above, and analysis of specific secondary pathways, such as the paclitaxel pathway (see below), at the level of gene expression is needed to better understand the evolution of metabolic regulation as a culture ages. Gene expression profiles at different time points, especially at time points which correspond to high or low metabolite production levels in cells of the same lineage, will provide better clues as to what specific genes may be affected by the aforementioned factors. These genes could then be considered as objectives for targeted metabolic engineering, to stabilize productivity over time. Our laboratory has recently profiled gene expression in paclitaxel accumulating *Taxus* cell lines (Nims et al. 2006); a similar study is underway using a cell line that does not accumulate paclitaxel.

#### **1.4.1 Cellular Heterogeneity as a Basis of Variability**

Most research focused on understanding culture variability relies on measures of culture averaged parameters, such as cell density and metabolite concentrations. These types of measurements are essentially averages of properties over millions of cells, and neglect variation at the single cell level; while all cell cultures display some extent of heterogeneity as a result of normal statistical distributions, distinct subpopulations within cultures have been identified that have profound implications on culture behavior. For instance, differences in bulk anthocyanin production in *C. roseus* cultures were primarily dependant on the percentage of producer cells rather than the production level of each cell, determined by visual identification of the colored pigment (Hall and Yeoman 1987), and similar findings were reported regarding paclitaxel accumulation in *Taxus* cultures using flow cytometry (Naill and Roberts 2005a). To predict the effect of different elicitors

on taxane accumulation, a subpopulation induction model was developed based on the assumption that only certain subpopulations would be stimulated by two different elicitors (methyl jasmonate and ethylene); combined with enzyme kinetics-derived relationships to describe the production rate of paclitaxel and other intermediate taxanes, the model accurately predicted trends of metabolite accumulation under specified elicitation conditions (Senger et al. 2006). As subpopulation dynamics have been shown to exert considerable influence on culture behavior as a whole, understanding the basis of these subpopulations is key.

Due to the nature of plant cell division in which daughter cells often remain connected through a shared cell wall, aggregates of anywhere from two to several hundred cells can be found in suspension culture (Figure 2). These aggregates can reach sizes up to several mm, and result in different microenvironments with respect to nutrient and oxygen availability between inner and outer regions. Cellular metabolism is altered in the presence of these differential local environments, with implications for the formation of subpopulations regarding secondary metabolite accumulation and cell proliferation. There have been conflicting reports as to the effect of aggregate size on metabolite accumulation. Studies in *Tagetes petula* (Hulst et al. 1989) and *Fragaria ananassa* (Edahiro and Seki 2006) indicate that production of secondary metabolites increases with increasing aggregate size. Jaceosidin accumulation increases as aggregate size increases in suspension cultures of *Saussurea medusa* up to 4 mm, after which jaceosidin production decreases (Zhao et al. 2003). Oxygen transport models indicate that aggregates larger than several mm face oxygen depletion at their center, which has been proposed to stimulate secondary metabolic activity directly (Hulst et al. 1989; Kessler et al. 1999). Conversely, aggregation has been shown to inhibit ursolic acid accumulation in *Salvia officinalis* compared to single cells in suspension culture (Bolta et al. 2003), and

aggregates were shown to synthesize about the same amount of ajmalicine in *C. roseus* suspension cultures (Kessler et al. 1999). The lack of clarity about the effects of aggregates on the culture as a whole underscores the fact that very little is known about the actual nature of the microenvironments or their effects on individual cells within aggregates.

Techniques for analyzing properties of single cells can provide insight into the nature of these aggregates by identifying subpopulations of cells from different regions with a common characteristic, and can also provide information regarding subpopulations that may develop as a result of other factors, such as cell signaling. Flow cytometric population analysis methods have been employed by many groups studying plant cell suspension cultures, and have been used to identify subpopulations such as a group of non-cycling cells in *Taxus* cultures (Naill and Roberts 2005b) and *Solanum avicular* (Yanpaisan et al. 1998). Non-cycling cells may be specialized for secondary metabolism (Naill and Roberts 2005b; Yanpaisan and Doran 1999), and a clearer understanding of this relationship will provide targets for strategies aimed at increasing secondary metabolite yields in culture.

Our laboratory has developed techniques for analyzing properties of individual *Taxus* spp. cells, and is continuing work to establish relationships between key cellular parameters in order to describe culture behavior in context of a mathematical framework. As plant cells grow in aggregates, methods for isolating single particles are essential for subsequent flow cytometric analysis. Protoplasts and nuclei have been prepared from many plant species for this end, including *Taxus* spp. (Roberts et al. 2003; Aoyagi et al. 2002). However, a significant amount (30-60%) of paclitaxel accumulates in the cell wall (Roberts et al. 2003); as the cell wall is removed during protoplast preparation,



information regarding paclitaxel accumulation is lost. To overcome this limitation, we developed a method using the cell wall digesting enzymes cellulase and pectolyase to isolate single cells with an intact cell wall (Naill and Roberts 2004). The procedure provides a high single cell yield with minimal changes in cell physiology as determined through measuring both peroxidase, a cell wall localized protein, and paclitaxel distributions before and after single cell preparation (Naill and Roberts 2004). These single cells were then analyzed for paclitaxel content (Naill and Roberts 2005a) and protein accumulation (Naill and Roberts 2005c) using flow cytometry. A non-paclitaxel accumulating subpopulation was identified in methyl jasmonate-elicited cultures consisting of almost 20% of the total population. Ongoing work is aimed at correlating both paclitaxel and non-paclitaxel accumulating subpopulations with other factors, such as cell cycle participation, size, and cellular protein content.

These subpopulations can be isolated and further cultivated to propagate superior cell lines. Flow cytometers can be equipped with a sorting functionality, allowing for the study and re-culturing of distinct plant cell populations. In particular, much effort has focused on the selection and purification of protoplasts for further experimentation in culture (Galbraith et al. 2005). Under appropriate sorting and re-culture conditions, protoplasts remain metabolically active, and can subsequently regenerate cell walls and begin cell division. This has been achieved with heterokaryons produced by induced protoplast fusion, as well as following transformation (Galbraith 1990). Similarly, our group has investigated optimal re-culture conditions for isolated *Taxus* cells, and has achieved a growth rate similar to aggregated suspension cultures after using a combination of high seeding density and conditioned medium (Naill and Roberts 2005d).

Plant cells present several obstacles to successful sorting due to their large size. Most flow cytometers are designed to analyze and sort microbial and mammalian cells (1 – 10  $\mu\text{m}$ ). Because plant cells typically range from 20-60+  $\mu\text{m}$  in size, specialized equipment and methods must be utilized. Our group has recently investigated the feasibility of sorting *Taxus* cells, initially based on size. Single cells of *T. cuspidata* were prepared following standard protocols (Naill and Roberts 2004) and analyzed on a Becton Dickinson (San Jose, CA) FACSVantage<sup>TM</sup> flow cytometer, custom equipped for sorting plant cells with a MacroSort option and 200  $\mu\text{m}$  nozzle. A region defined by high values of forward scatter (FSC) and side scatter (SSC) was used as sorting criteria, and cells falling within this region were collected and further analyzed using a Becton Dickinson LSRII flow cytometer. A comparison of the sorted and unsorted samples shows that a distinct population of large cells was isolated (Figure 3). The primary obstacle for sorting plant cells is the instability of the flow stream when using a larger nozzle size. We have demonstrated the feasibility of sorting *Taxus* cells, which to our knowledge is the first report of sorting intact plant cells as opposed to plant derived particles such as protoplasts or nuclei. Ongoing experiments are aimed at sorting based on more relevant criteria, such as paclitaxel accumulation. Specific populations with an increased ability to produce paclitaxel can be isolated and further propagated, and eventually be used to establish stable, high-accumulating *Taxus* cell lines for use in bioprocesses. Differential gene expression analysis of high and low producing subpopulations also has the potential to lend considerable insight into the metabolic basis of different subpopulations. A fluorescence activated sorting approach has recently been used to isolate different cell types from *Arabidopsis* roots, and the resulting map of gene expression, which correlates groups of genes to specific cell fates, facilitates research on uncovering the regulatory mechanisms in organ development (Birnbaum et al. 2003). Similarly, expression of ROP

GTPases was compared between embryogenic and pollen-like cells that were isolated using flow cytometric sorting techniques (Chan and Pauls 2007).

## **1.5 Metabolic Engineering and Directed Biosynthesis**

The engineering of biosynthetic pathways within a plant cell to enhance accumulation of a constitutively produced metabolite is an appealing strategy in which exciting progress has been made in the past decade. Secondary metabolic biosynthetic pathways are extremely complex, and still remain at least partially undefined in most cases. Few plant genomes have been fully sequenced, and those that have been are model systems in which secondary pathways are not of as acute interest regarding natural product accumulation, and as a result, a system wide analysis is sorely lacking in most medicinal plant species. Furthermore, many aspects of global metabolism remain unknown in addition to the biosynthetic pathways, including product transport and degradation, and regulatory elements such as transcription factors for pathway genes or other signaling mechanisms. Difficulties applying traditional genetic transformation methods to plant cells have impeded progress in many systems. Nonetheless, significant advancements in overcoming some of these key challenges have been made in recent years, and are highlighted in the following sections.

### **1.5.1 Metabolic Engineering Tools**

A metabolic engineering approach involves the manipulation of targets within a cell. Techniques are therefore needed both for the identification of these targets (i.e. genes, proteins, metabolites) as well as for their exploitation. As many secondary pathways are still partially undefined, elucidating pathway genes and their control elements is an active research area. The subsequent identification of rate-influencing steps within a biosynthetic pathway can then be useful in providing targets for a rational

engineering strategy. A variety of tools have been employed to both identify unknown genes and characterize secondary metabolite pathway regulation, including precursor feeding, gene overexpression, application of metabolic inhibitors, and mutant selection. Additionally, elicitation, discussed above in relation to improving bulk yields in cell culture, can also be used as a powerful tool to investigate pathway regulation based on gene expression. Plant cell cultures, including both suspension cultures and hairy root cultures, have proven to be an extremely useful platform for metabolic studies, as a fast growing and renewable source of material. Whole plants can also be valuable, particularly as models to study complex spatial and temporal control mechanisms associated with environmental stimuli and morphogenesis from a global metabolic perspective (Pasquali et al. 2006).

Several approaches have been used to identify the enzymes and their corresponding genes which catalyze biosynthetic pathway steps. For the paclitaxel pathway, a successful approach utilized by the Croteau laboratory incorporated feeding cell free *Taxus* extracts with precursors to isolate and identify intermediate metabolites and enzymes. This approach led to the identification of taxadiene synthase, which catalyzes the first committed step of the taxane pathway (Koepp et al. 1995). Genes were subsequently identified from a cDNA library using PCR amplification based on degenerate primers designed to recognize conserved regions from homologous enzymes in other plants whose DNA sequences were known (Wildung and Croteau 1996). Differential display methods (via reverse transcription and PCR) comparing mRNA transcripts between elicited and unelicited cells supplemented with a homology-based search of a cDNA library from elicited cells (Kaspera and Croteau 2006), as well as random sequencing of the same induced library (Jennewein et al. 2004), have also proven

to be extremely effective in gene discovery (for a comprehensive review of molecular genetics in *Taxus* see Croteau et al. 2006).

An examination of the mRNA expression profile of known genes, either by northern blotting or RT-PCR, can provide insight into pathway regulation. By comparing the time course of expression of 11 known genes in elicited and unelicited *Taxus* spp. cultures to total taxane accumulation, potential bottlenecks were identified based on low levels of mRNA transcript accumulation in MJ-elicited cells (Nims et al. 2006). In this particular study, two late pathway steps were identified as likely rate influencing, and provide an objective for a targeted metabolic engineering approach by overexpressing these particular genes. In branched pathways such as the paclitaxel biosynthetic pathway, this type of analysis can also provide clues as to the predominate direction of flux (Nims et al. 2006). Transcript profiling can also be used to evaluate the effect of differentiation conditions on gene expression. A rotation culture system was devised for *C. roseus* cultures in which cell lines were maintained as either suspension cultures or differentiated calli (Dutta et al. 2007). Analysis of mRNA levels of genes in the precursor pathways and secondary (terpene indole alkaloid or TIA) pathways, as well as activators and repressors, showed a reduction or loss of expression of several key TIA pathway genes and activators in proliferating suspension cultures (Dutta et al. 2007). These levels subsequently rose when differentiated cultures were re-initiated, confirming the close relationship between secondary biosynthetic pathway gene expression and cellular differentiation. In model systems such as *Arabidopsis* for which microarrays containing probes for thousands of genes are available, a more detailed transcript analysis has been performed to examine effects of nutrient stress conditions (Contento et al. 2004) and cell cycle progression (Menges et al. 2003).

In order to implement a target metabolic engineering approach by overexpressing a pathway gene, a reliable transformation technology must be available to integrate foreign DNA into plant cells. A range of methods proven to work in plant species are available, and can be divided into two general classifications: *Agrobacterium*-mediated and direct gene transfer. *Agrobacterium* contains a Ti plasmid, a portion of which (T-DNA) is integrated into the plant genome after bacterial infection of the host cell. The T-DNA in wildtype bacteria contains genes to promote proliferation. Strains of bacteria where these tumor inducing genes have been replaced with genetic sequences of interest have allowed investigators to transfer genes by leveraging the native bacterial machinery (Taylor and Fauquet 2002). *Agrobacterium*-mediated transformation has become the method of choice due to its low cost and ease of use, though many plant species, including commercially valuable species such as gymnosperms and cereal grains, are recalcitrant to this transformation method (Gelvin 2003). ‘Super-virulent’ strains of *Agrobacterium* containing additional virulent proteins have been used to overcome this limitation (e.g. Torisky et al. 1997), and more recently, the manipulation of genes in the host plant associated with the transformation process has shown promise in improving the transformation efficiency of hard-to-transform plant species (Tzfira and Citovsky 2006). Host proteins involved in particular steps of the transformation process such as the initial host-bacteria contact, nuclear import of the T-complex, and integration, have been identified, and it may be possible to transport these proteins to the host cell using the same *Agrobacterium*, which can transport proteins independently of T-DNA (Tzfira and Citovsky 2006). Direct gene transfer techniques include polyethylene glycol (PEG)-mediated DNA uptake, silicon carbide fibers, electroporation, and microparticle bombardment. In particular, particle bombardment, in which micron sized metal particles are coated with DNA and accelerated into the target cells at a sub-lethal velocity capable

of penetrating the cell wall, is the transformation method of choice for those systems resistant to *Agrobacterium* (Taylor and Fauquet 2002). *Agrobacterium*-mediated transformation has been successfully applied to a number of medicinal species, including *C. roseus* (Goddijn et al. 1995) and *A. annua* (Vergauwe et al. 1996). *Taxus* spp. systems lacked a reliable transformation method until recent reports of a stable *Agrobacterium*-mediated transformation (Ketchum et al. 2007) and a transient particle bombardment transformation developed by our laboratory (Vongpaseuth et al. 2007).

### 1.5.2 Metabolic Engineering Strategies

Biosynthetic pathways can be divided into two major stages: pathways common to most plants, which produce universal precursors for a class of products (e.g. flavonoids, terpenoids), and divergent pathways for specific products within a particular class (e.g. terpenoid subclasses taxanes and carotenoids). While both of these pathways are targets for metabolic engineering, much more is known about precursor pathways, since they are universal and have been studied in multiple different systems. For instance, all terpenoids are derived from the precursor isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). These precursors are supplied via two pathways: the plastidial 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway, named after the first committed precursor, MEP (also commonly referred to as the deoxyxylulose-5-phosphate or DXP pathway), and the cytosolic mevalonate (MVA) pathway. All of the genes in both of these pathways have been identified (Withers and Keasling 2007). In contrast, the biosynthetic pathway from IPP to paclitaxel consists of 20 putative steps, for which 14 genes have been cloned (Croteau et al. 2006), and no transcription factors identified. Various secondary pathways have been defined to different extents, and an overview of

the relative knowledge regarding the biosynthetic pathways for four important pharmaceutically active natural products is shown in Table 2.

Provided a suitable genetic transformation method is available, overexpression of genes associated with precursor pathways, to increase available precursor pools, and secondary pathways are common strategies to increase end product accumulation. While precursor pathways are better defined in terms of individual pathway steps, there are often multiple precursor pathways (such as the MEP and MVA pathways), about which many aspects of global metabolism, such as flux between the pathways, are unknown. Inhibitors of these precursor pathways can be used to investigate the source of precursor supply. Recent studies in *A. annua* for production of artemisinin (Towler and Weathers 2007) and *Taxus* for production of paclitaxel (Cusido et al. 2007) indicate that precursors are supplied via both pathways. This approach is limited in that it cannot track precursor exchange between compartments to which each pathway is localized, and so cannot determine the proportion supplied by each pathway. Labeling studies can provide more definitive evidence as to the origin of precursor supply, and have been applied to show that salvinorin A is derived from the MVA pathway in *Salvia divinorum* (Kutrzeba et al. 2007), and that gaudichaudianic acid is derived from both the MVA and MEP pathways in *Piper gaudichaudianum*, with transport of specific intermediates across the plastid boundary (Lopes et al. 2007). Overexpression of genes in precursor pathways has proven to be successful in increasing end products of secondary metabolism such as monoterpene essential oils in both peppermint (Mahmoud and Croteau 2001) and lavender (Munoz-Bertomeu et al. 2006), but has also been shown to have a limited impact on end products due to tight regulation of metabolite accumulation, as evidenced by a substantial improvement of intermediates but limited accumulation of alkaloid end products in *C. roseus* (Hughes et al. 2004). The direct overexpression of genes associated with



dedicated secondary pathways has shown to be more effective in increasing alkaloid accumulation in *C. roseus* (Canel et al. 1998), though the effect appears to be temporary, most likely as a result of the same factors which induce variability in non-transgenic plants (see above).

Transcription factors are difficult to identify in non-model species, but can be a more powerful tool to control metabolic flux since they can regulate multiple steps, and also offer the potential to regulate steps for which the component enzymes are unknown (Broun 2004). A classical genetics approach, which does not require information about genes or metabolic intermediates, has proven to be useful in non-model species for which this information is still only partially known. T-DNA can be used as an activator causing overexpression of genes flanking the insert, which results in dominant gain-of-function mutations (Memelink 2005). Mutant phenotypes can then be screened based on properties such as pigmentation or resistance to a toxic metabolic derivative. This approach was successful in identifying the octadecanoid-derivative-responsive *Catharanthus* APETALA2 (AP2)-domain3 (ORCA3) transcription factor in *C. roseus* cells (van der Fits and Memelink 2000), and subsequent overexpression of this gene upregulated several genes involved in TIA biosynthesis and increased overall accumulation of TIAs. The lack of prerequisite tools for this approach, such as a reliable genetic transformation method, has limited its application to other systems, such as *Taxus*, to this point.

## **1.6 Future Directions in Engineering Supply**

Significant advancements have been made in understanding metabolite production dynamics in plant cell cultures on a number of levels, from bulk culture analysis and process design, to the recognition of subpopulations and heterogeneity at the single cell

level, to metabolic pathways and their regulation within the single cell. An integrated approach within each level of analysis and relating all three levels will be needed to fully exploit the potential of plant cell culture systems to reliably and consistently supply metabolites at a sufficient yield to make these systems economically viable.

At the metabolic level, efforts would be enhanced by a system-wide analysis of plants, both in differentiated and undifferentiated tissues. The lack of complete information about the genomes of most medicinal plants make traditional “omics” approaches difficult to apply to these systems. However, the rapid advances in high-throughput sequencing technology have been applied to many agriculturally important crops in recent years and will likely spread to medicinal plants as well. In 2008, only three plants had fully sequenced genomes (rice, maize, and *A. thaliana*), but by 2011, that has increased to 14 plants with fully sequenced genomes (<http://www.plantgdb.org>). In spite of the many recent advances, the information needed to annotate plant genes solely based on sequence homology to genes of known or predicted functions in other systems is simply not available in many cases. The complexity and redundancy of many pathways coupled to incomplete knowledge of their regulation can lead to unpredictable results from a targeted metabolic engineering approach. Overexpression of phytoene synthase, the first committed enzyme of carotenoid synthesis in tomato, led to growth defects due to flux diversion from gibberellin synthesis (Fray et al. 1995). This demonstrates the importance of understanding the implications of increased flux through one step of a pathway on the entire system. Transgenic manipulation of pathway enzymes has proven to be unstable, as gene expression and alkaloid accumulation eventually returned to the level of non-transgenic cultures in *C. roseus* (Whitmer et al. 2003), which may result from regulatory factors further downstream of the targeted genes. Transcription factors are more likely to be effective in controlling flux through a specific pathway or branch;

however it is often necessary to increase precursor availability using this approach, and a complete understanding of the coordination of multiple branches and sections of metabolic pathways is likely to require an integrated metabolic model including information from genomics, transcriptomics, proteomics, and metabolomics. A system wide analysis of metabolic flux can be quantified through labeling experiments (Sriram et al. 2007), which facilitates the construction of predictive models. Integrated systems biology approaches including genome-wide functional genomics models have been largely limited to model plant systems, though recent progress has been made in developing gene-to-gene and gene-to-metabolite networks in *C. roseus* (Rischer et al. 2006).

An integrated approach will most likely be necessary in successful engineering efforts to reliably increase metabolite production. For instance, as many secondary metabolites are toxic due to their native biological functions, artificially high induced levels of production via metabolic engineering may be detrimental to primary cell function, but may be mitigated in a product removal or cell encapsulation scheme. Population dynamics within a culture due to environmental effects may also prove to be the decisive factor in metabolite accumulation, as manipulation of secondary pathways may only affect a small subpopulation of cells. Despite the complexities of plant metabolism and open questions regarding population dynamics, substantial progress has been made in characterizing and utilizing native medicinal plant species for the production of valuable natural products. Although paclitaxel is a notable commercial success, plant cell culture is still an emerging technology. Continued advancements in the understanding of secondary metabolism within cells and within cell cultures are critical to eventual viability of plant cell culture as a means to supply vital natural products, which may have no alternate means of supply.

## 1.7 Research Goals

The motivation of this research is the facilitation of plant cell culture technology for the commercial supply of valuable secondary metabolites. Specifically, we have identified plant cell aggregation as a critical property of plant cell culture processes, and directed work towards characterizing, analyzing, and modeling this property from a multi-scale perspective. We utilized *Taxus* (yew) spp. cell culture systems for the production of the important anti-cancer drug paclitaxel (Taxol<sup>®</sup>). Paclitaxel is a multi-billion dollar chemotherapeutic with an increasing demand and persistent uncertainty regarding its sustainable, long-term supply capability. Furthermore, as plant cell culture has been demonstrated as commercially successful for the production of paclitaxel, *Taxus* plant cell cultures are one of the most widely studied medicinal plant cell culture systems, with a substantial knowledge base in both the published and patented literature. Nevertheless, a comprehensive understanding of aggregation in *Taxus* spp. as well as for plant cell culture systems in general is lacking, and the methods and results from this research can be extended to any plant cell culture system.

Research on aggregation in *Taxus* has focused on several specific areas, and has explored the effect of aggregation at both the cellular and process scale:

- (1) The development of a rapid and reliable method to characterize aggregate size distributions using a Coulter counter, which can be employed as a routine process measurement (Chapter 2) (Kolewe et al., 2010)
- (2) An analysis of the effect of aggregate size on paclitaxel accumulation at the process scale, in which both aggregate size fractionation and the initiation of separate cultures with distinct aggregate size profiles were used to assess process performance (Chapter 3) (Kolewe et al., 2011)

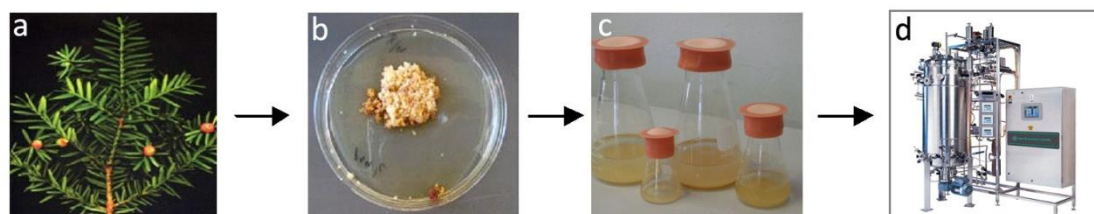
- (3) The development of a population balance equation (PBE) model to describe changes in the aggregate size distribution in batch culture, which provided insights into the nature of the phenomenological processes underlying aggregation and which was also used to guide the development of control strategies aimed at managing aggregate size (Chapter 4)
- (4) An investigation into the relationship between aggregation and single cell heterogeneity, in which single cells were isolated from aggregates of different sizes and assessed for characteristics of both primary and secondary metabolism (Chapter 5)

Metabolite	Species	Application	Manufacturer
Scopolamine	<i>Duboisia</i> spp.	Anti-cholinergicum	Sumitomo Chemical Industries (Japan)
Podophyllotoxin	<i>Podophyllum</i> spp.	Anti-tumor	Nippon Oil (Japan)
Protoberberines	<i>Coptis japonica</i> <i>Thalictrum minus</i>	Anti-biotic, anti-inflammatory	Mitsui Petrochemical Industries (Japan)
Paclitaxel	<i>Taxus</i> spp.	Anti-tumor	Phyton Biotech (USA) Samyang Genex (Korea)
Rosmarinic acid	<i>Coleus blumei</i>	Anti-inflammatory	Nattermann (Germany)
Ginseng	<i>Panax ginseng</i>	Dietary supplement	Nitto Denko (Japan)
<i>Echinaceae</i> polysaccharides	<i>Echinacea purpurea</i> <i>Echinacea augustifolia</i>	Anti-inflammatory, immunostimulant	Diversa (Germany)
Shikonin	<i>Lithospermum erythrorhizon</i>	Anti-HIV, anti-tumor, anti-inflammatory	Mitsui Petrochemical Industries (Japan)
Geraniol	<i>Geramineae</i> spp.	Anti-tumor	Mitsui Petrochemical Industries (Japan)

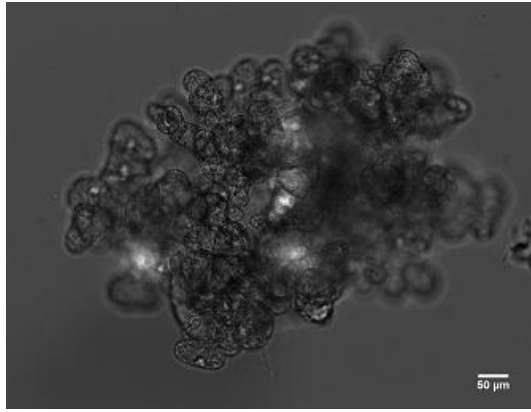
**Table 1.1** Commercial plant cell culture processes for supply of natural products with pharmaceutical applications (adapted from Eibl and Eibl 2002)

Secondary metabolite	Species	Application	Commercial production method	Metabolite classification	Secondary pathway genes identified	Expressed sequence tags	Regulatory elements identified
Artemisinin	<i>Artemisia annua</i>	Anti-malarial	Plant	Sesquiterpene lactone	3 of >7 <sup>15</sup>	52 <sup>a</sup>	No
Camptothecin	<i>Camptotheca acuminata</i>	Anti-tumor	Plant	Monoterpenoid indole alkaloid (TIA)	4 of >11 <sup>7</sup>	N/A	No
Paclitaxel	<i>Taxus</i> spp.	Anti-tumor	Suspension cell culture and semi-synthesis	Diterpenoid	14 of 20 <sup>87</sup>	22 <sup>a</sup>	No
Vincristine	<i>Catharanthus roseus</i>	Anti-tumor	Plant	Monoterpenoid indole alkaloid (TIA)	10 of 18 <sup>4</sup>	9042 <sup>b</sup>	Yes

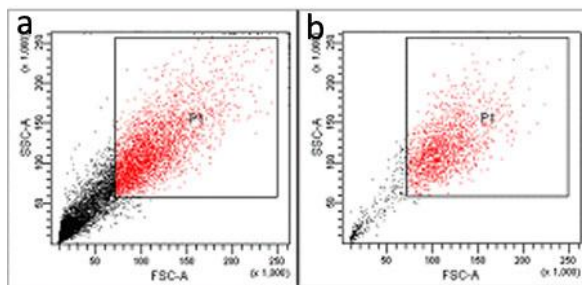
**Table 1.2** Natural product biosynthesis in native species: A comparison of secondary pathways



**Figure 1.1** Initiation of a plant suspension cell culture from explant material. Plant tissue (a) plated on solid growth induction medium will yield a callus culture (b), which can then be transferred to a liquid medium to obtain a suspension culture (c) and ultimately scaled-up for commercial production (d).



**Figure 1.2** Aggregate of *Taxus cuspidata* P991 cells in suspension culture.



**Figure 1.3** Unsorted (a) and sorted (b) *T. cuspidata* P991 cells. Gated region P1 was used as sorting criteria. 84% of particles fell within the P1 region of the sorted sample, compared to 36% in the unsorted sample, demonstrating that a distinct population was isolated.

## CHAPTER 2

### CHARACTERIZATION OF AGGREGATE SIZE IN *TAXUS* SUSPENSION CELL CULTURE

Plant cells grow as aggregates in suspension culture, but little is known about the dynamics of aggregation, and no routine methodology exists to measure aggregate size.

In this study, we evaluate several different methods to characterize aggregate size in *Taxus* suspension cultures, in which aggregate diameters range from 50  $\mu\text{m}$  to 2000  $\mu\text{m}$ , including filtration and image analysis, and develop a novel method using a specially equipped Coulter counter system. We demonstrate the suitability of this technology to measure plant cell culture aggregates, and show that it can be reliably used to measure total biomass accumulation compared to standard methods such as dry weight.

Furthermore, we demonstrate that all three methods can be used to measure an aggregate size distribution, but that the Coulter counter is more reliable and much faster, and also provides far better resolution. While absolute measurements of aggregate size differ based on the three evaluation techniques, we show that linear correlations are sufficient to account for these differences ( $R^2 > 0.99$ ). We then demonstrate the utility of the novel Coulter counter methodology by monitoring the dynamics of a batch process and find that the mean aggregate size increases by 55% during the exponential growth phase, but decreases during stationary phase. The results indicate that the Coulter counter method can be routinely used for advanced process characterization, particularly to study the relationship between aggregate size and secondary metabolite production, as well as a source of reliable experimental data for modeling aggregation dynamics in plant cell culture.



## 2.1 Introduction

Plant cell culture is an alternative production technology for complex natural products that cannot be chemically synthesized or extracted in high yields from native sources. In particular, suspension cultures consisting of undifferentiated plant cells are attractive industrially, especially compared to other types of *in vitro* plant systems such as differentiated cultures and transfected hairy root cultures, due to their relative similarity to other microbial cell culture systems (Kolewe et al. 2008). While a number of these suspension cell culture processes have been commercialized, most notably *Taxus* spp. for the production of the anti-cancer agent paclitaxel, more widespread application of this technology has thus far been limited due to some of the unique difficulties associated with plant cell culture, including low metabolite yields, biochemical and genetic instabilities, and difficulties associated with scaleup (Georgiev et al. 2009).

One unique characteristic of plant cell cultures is the tendency of plant cells to grow as aggregates. Plant cells remain connected via shared cell walls after division, and as a result, aggregates ranging from two to several hundred cells exist in culture. Aggregates can reach sizes up to several millimeters, and microenvironments within these larger aggregates are formed due to oxygen and other nutrient diffusion limitations (Naill and Roberts 2004; Hulst et al. 1989). The effect of aggregate size on metabolic activity has been studied extensively, but no definitive trend regarding aggregate size and secondary metabolite production has emerged across species and cell culture systems. Larger aggregates have been shown to have a positive effect on secondary metabolite production (Edahiro and Seki 2006; Hulst et al. 1989), a positive effect up to a critical size (Zhao et al. 2003; Madhusudhan and Ravishankar 1996), or a negative effect (Pepin et al. 1999; Bolta et al. 2003). Additionally, aggregate size has been shown to affect

culture growth rates (Forni et al. 1999), rheological properties of the culture broth (Rodriguez-Monroy et al. 2004), and is the most likely cause of heterogeneity in single cell populations (Naill and Roberts 2005a; Naill and Roberts 2005b).

In order to engineer plant cell cultures by manipulating aggregate size, it is necessary to first have a practical and reliable method to measure the aggregate size distribution. Dry weight is the standard measure of biomass in plant cell culture, and the most common method to measure the aggregate size distribution is similar in nature. A crude biomass distribution is typically obtained by separating a sample of aggregates on a series of filters or sieves with different pore sizes and determining the dry weight of each resulting size fraction (i.e., McDonald et al. 2001; Zhao et al. 2003; Keßler et al. 1999; Madhusudhan and Ravishankar 1996; Mavituna and Park 1987). This method is often employed due to the straightforward nature of the procedure and materials required. However, it is both time-consuming and inefficient, and dry weight results can take greater than 24 hrs to obtain.

Other methods have been investigated to determine plant cell aggregate size, including *ex situ* image analysis and the focused beam reflectance method (FBRM). Image analysis techniques consist of sample dilution and plating, followed by microscopic image acquisition and subsequent analysis using a variety of software programs (Pepin et al. 1999; Rodriguez-Monroy et al. 2004). While this method can allow for better resolution than filtration, the processing steps increase the likelihood of altering the aggregate size due to breakage. Additionally, the throughput is extremely low, resulting in a long and labor intensive procedure to obtain statistically significant results, which is impractical for periodic measurements of multiple cultures. FBRM is an optical technique in which the duration of a particle's reflectance as it passes a laser

corresponds to the particle length. This method was shown to reliably measure total biomass and could also be correlated to aggregate size; however, biomass concentration and aggregate size showed significant cross interaction in FBRM measurements, such that both properties could not always be determined from a single measurement (McDonald et al. 2001).

A common method for determining particle size distributions in a range of applications, including but not limited to biological systems, is the electrical resistance pulse sizing technique, commonly known as the Coulter principle. Particles suspended in a conducting salt solution will cause a voltage pulse when passing through an aperture across which a constant current is applied, due to the displacement of electrolyte by the particle (Graham 2003). The amplitude of the voltage pulse is proportional to the volume of the particle. This technique was first applied to size bacteria over 50 years ago (Kubitschek 1958), and very quickly became a standard method to count and size yeast, bacteria, and mammalian cells (Kubitschek 1969). While several early attempts were made to apply this principle to plant cells, equipment limitations, including a maximum aperture size of 560  $\mu\text{m}$ , necessitated the use of either protoplasts or cultures treated with enzymes (Kubek and Shuler 1978), and was acknowledged to be applicable only to cultures with low levels of aggregation. As electrical resistance pulse sizing technology has matured, no further research has been published to investigate the suitability of this technique to plant cell cultures.

In this study, we characterize plant cell culture aggregates using a specially equipped Coulter counter, and compare results with filtration and image analysis. As a total biomass measurement can be determined from a biomass distribution, we also compare biomass measurements obtained with the Coulter counter to standard dry weight

measurements. We show that the aggregate size distributions obtained are reliable and well resolved, and then apply this method to characterize the evolution of *Taxus* cultures over a batch period. Results demonstrate the usefulness of this method for routine cell culture monitoring, which we ultimately intend to use for a quantitative investigation into aggregation dynamics and their effect on secondary metabolism.

## **2.2 Materials and Methods**

### **2.2.1 Cell Cultures**

*Taxus cuspidata* cell line P991 was provided by the U.S. Plant Soil and Nutrition Laboratory in Ithaca, NY, and maintained in our laboratory. Cells were subcultured every two weeks into fresh medium, consisting of Gamborg B5 basal salts, supplemented with 20 g/L sucrose, 2.7  $\mu$ M naphthalene acetic acid, and 0.1  $\mu$ M benzyladenine, and adjusted to pH 5.5 prior to autoclaving. 150 mg/L citric acid, 150 mg/L ascorbic acid, and 6.0 mM glutamine were filter sterilized and added post-autoclave. Cell cultures were maintained in either 125 mL or 500 mL glass Erlenmeyer flasks with Bellco (Vineland, NJ) foam closures, and incubated in gyratory shakers in constant darkness at 23°C and 125 rpm. Every two weeks, inoculum was subcultured to fresh media at a ratio of 1:4 for a total volume of 50 mL with 2-3 mL packed cell volume in 125 mL flasks or 200 mL in 500 mL flasks. For total biomass measurements, 2 mL samples were taken with a cut-tip pipette, filtered and rinsed with ~5 mL distilled water over Miracloth<sup>®</sup> (Calbiochem, San Diego, CA) and weighed to determine fresh weight. Samples were then dried in an oven at 50°C to a constant weight and recorded as dry weight. Glucose and sucrose were measured from cell culture media samples using a blood glucose analyzer (YSI 2700 Select Biochemistry Analyzer, YSI Life Sciences, Yellow Springs, OH).

### **2.2.2 Filtration**

Filters were made by sealing together nylon mesh sheeting with pore sizes of 2000  $\mu\text{m}$ , 1360  $\mu\text{m}$ , 1000  $\mu\text{m}$ , 710  $\mu\text{m}$ , 500  $\mu\text{m}$ , 300  $\mu\text{m}$ , and 80  $\mu\text{m}$  (Small Parts, Inc., Miramar, FL) or 1680  $\mu\text{m}$  polypropylene mesh sheeting with a contact adhesive (Amazing Goop All Purpose, Eclectic Products, Eugene, OR) to hollowed out polypropylene chemical containers (Sigma-Aldrich Co., product G5893, St. Louis, MO). The surface area of each filter was approximately 635  $\text{cm}^2$ . For each mesh size, after the initial filtration step (divided across several filters if necessary for a maximum amount of ~1 g biomass / filter), the biomass remaining on the filter was gently immersed in a wash solution (Gamborg B5 basal salts), and this wash was repeated at least twice. Smaller meshes generally required more washes than larger meshes, as the percent open area of the mesh decreases with decreasing pore size, causing the formation of a cell cake. The permeate from each filtration step was combined, and this process was repeated for each successively smaller mesh size. The accumulated biomass on each filter was then backflushed with wash solution onto Miracloth for subsequent dry weight measurement, or backflushed with diluent (see next section) into the Multisizer sample beaker for subsequent analysis via the Coulter counter.

### **2.2.3 Electrical Resistance Pulse Amplitude Sizing**

A Multisizer 3 Coulter counter (Beckman Coulter, Inc., Brea, CA) with a 2000  $\mu\text{m}$  aperture, the largest size available, was used for electrical resistance measurements. Diluent consisting of 65:35 Isoton (1% NaCl with preservatives, Beckman Coulter):Glycerol (Fisher Scientific), was vacuum filtered over a series of 3.5  $\mu\text{m}$  and 2.0  $\mu\text{m}$  depth filters prior to use (Whatman, Inc., Piscataway, NJ) and recycled using the same filters following each set of samples. Recycling was required for a larger scale

application of this method due to the substantial quantities of diluent used for sample analysis, and we found that diluent could be reused for an indefinite period of time. Diluent resistivity and baseline noise, determined using diluent only, and 200  $\mu\text{m}$  latex standard spheres (Thermo Scientific, Fremont, CA) were used to check calibration before each run. The amplitude of the voltage pulse associated with each particle passing through the aperture of the Coulter counter was converted to a particle volume based on the calibration coefficient, which is determined using the 200  $\mu\text{m}$  standard beads. A -400 mA current was applied across the aperture. Data were collected and analyzed using Beckman Coulter Multisizer software, or exported and analyzed in MATLAB (Mathworks, Natick, MA). For cell culture analysis, 2 mL samples of well mixed cell culture broth were collected using a cut pipette tip, and diluted into 380 mL diluent. Samples were run for 60 seconds, at a flow rate of 5.1 mL/sec.

#### **2.2.4 Image Acquisition and Analysis**

For experiments comparing measurements between the Coulter counter and image analysis, 0.5 mL samples were collected from the well-mixed residual diluted samples remaining after Coulter counter analysis. Fluorescein diacetate from a stock solution of 5 mg/mL in acetone was added at a final working concentration of 20 mg/L, and cell samples were incubated for at least 15 minutes, resulting in the fluorescence of viable cells and aggregates, which could easily be distinguished from cell debris. 150  $\mu\text{L}$  samples were then spread over a microscope slide using a cut pipette tip, and no coverslip was used to prevent flattening and breakage of aggregates. Images were collected on an Olympus IX71 (Center Valley, PA) inverted epifluorescent microscope using a 4X/.13 Olympus UPlanFl objective for a total magnification of 40X, with 470/40 nm excitation and 495 nm longpass emission filters (Chroma, Rockingham, VT). Between 50-100

pictures were taken and combined into a single mosaic using a moving stage and a custom script with IP Lab Software (BD Bioscience, Rockville, MD) to cover the sample area, which was generally 1-2 cm<sup>2</sup>, depending on how the sample was plated on the slide. Each image was converted to a binary image by adjusting the binary threshold manually, and both the area in pixels of each particle and ellipse fitting parameters were automatically determined using ImageJ (National Institutes of Health, Bethesda, MD). In calculating the aggregate volume, we assumed that the polar axis was the average of the major and minor axes. An equivalent spherical diameter was then calculated from each aggregate volume.

## **2.3 Results and Discussion**

### **2.3.1 Standard Methods to Measure Aggregation**

A crude biomass distribution obtained by filtering (Fig. 2.1a) reveals that the majority of biomass consists of aggregates between 300 µm and 1500 µm. Time course experiments monitoring changes in aggregate profiles over a batch subculture period using this technique proved ineffectual (data not shown), as the poor resolution in the distribution combined with error resulting from flask to flask variability prevented detection and quantification of subtle differences in distribution shapes. This was not unexpected as no significant changes in aggregate distribution were observed in shake flask plant cell cultures using similar techniques (Madhusudhan and Ravishankar 1996; Zhao et al. 2003). The limitations of this method, in addition to procedural inefficiency, are that resulting distributions are coarse and generally imprecise, and it has been suggested that usefulness of filtration is limited in differentiating aggregate sizes (McDonald et al. 2001, Trejo-Tapia et al. 2003). In fact, if the sieving method does not include several wash steps, particularly a gentle immersion in wash solution for a

tangential flow filtration effect as opposed to simply adding wash solution over the cell cake, the resulting separation is extremely poor. Even with careful washing, it is virtually impossible to remove all of the smallest aggregates from each fraction, and the separation efficiency of the filters is still imperfect (see *Validation of Aggregate Size Measurement*).

In addition to the inherent limitations of mechanical separation, the distribution resolution is limited by the number of filters used to separate the aggregates into fractions and the amount of total biomass needed for analysis. Minimization of biomass is important so that aggregate distributions can be monitored over time by periodic sampling of the same cultures as opposed to harvestation of an entire culture to obtain a single distribution point. As the number of fractions increases, reliability is limited due to the lack of biomass in each aggregate size fraction. If the sample size is significantly increased and entire cultures are used to overcome biomass limitations, reliability is limited due to the flask to flask variability inherent in plant cell culture.

The typical size and morphology of *Taxus cuspidata* aggregates can be seen in Fig. 2.1b. Aggregates of more than a few cells are generally non-spherical, and the equivalent spherical diameters range from 50  $\mu\text{m}$  to around 2000  $\mu\text{m}$ . Although there have been reports of single cells in plant culture medium (Bolta et al. 2003), very few single cells were found in our *Taxus* cultures, though many aggregates of only 2-3 cells were present.

Quantifying size distributions using these images is inherently difficult due to the low numbers of large aggregates present in shake flasks and the resulting low probability of collecting these aggregates in the small sample volume used for image analysis. All studies using this technique have reported a mean aggregate size, as opposed to an



aggregate distribution and the mean or expected value of the distribution. A minimum of 1000 counts was needed (Edahrio and Seki 2006) to determine the average aggregate size in strawberry suspension cultures. The counts needed to determine a reliable distribution is much higher, and will depend on the range of aggregate sizes present and the number of bins used in specifying the distribution.

Additionally, the non-spherical nature of the aggregates requires the assumption of a standard orientation of all the aggregates on the slide so that an equivalent aggregate volume can be calculated from the aggregate area, which is directly measured. We assumed that the polar axis was an average of the major and minor axes, which essentially minimizes the irregularity of each aggregate by assuming the roundest shape possible. Several reports have used a similar averaging method (Edahiro and Seki 2006; Keßler et al. 1999), while others have used the minor axis as the polar axis (Pepin et al. 1999). Calculating volume using either the major or minor axis as the polar axis will shift the equivalent spherical diameter size distribution. While the differences based on these assumptions can be accounted for using separate correlations to other measurement techniques (see below) and are irrelevant when comparing between samples, they are indicative of the difficulties in determining an “absolute” aggregate size or volume.

### **2.3.2 Electrical Resistance Pulse Amplitude Sizing**

For this application, the particle volumes are binned and displayed as a volume distribution against particle diameter, where for each distribution bin, the total volume of particles is plotted versus the equivalent spherical diameter (Fig. 2.2). This representation is most practical, as a volume distribution is essentially a biomass distribution, and the particle diameter provides an intuitive visualization of the aggregate size profile. As with the measurement of any population distribution, a higher number of total counts will yield

smoother data; however, one requirement of the method to be developed was that it would allow for multiple samples to be taken from a shake flask over a time course experiment, which limited the sample volume available. Due to this sample size limitation, a trade-off was made in which the sample size (and thus total counts) was minimized, yet still accurately and consistently characterized the size distribution.

Quantifying precision in the measurement of a distribution as opposed to the measurement of a single value requires the comparison of every point along the distribution. Standard statistical comparisons generally provide a confidence level as to whether a distribution fits the shape of a known type of distribution (e.g., normal). In this case, we are interested in directly comparing the precision of measured distributions. To do this, we used a variation of a mean squared error calculation, and compared results of a single distribution measurement to the average of five distributions, the average of two distributions to the average of five distributions, and so on. Reducing the resolution of the distribution by using fewer bins in conjunction with using a moving average significantly improved precision (Fig. 2.2), while still maintaining a relatively fine resolution of 50 bins or size classes. Averaging two distributions compared to using only one further reduced the mean squared error by 44%. While further improvements could be gained by including additional samples in the analysis, improvements were modest (e.g., 22% reduction in error with 50% more biomass) and would require significantly more biomass, hence limiting time course studies. Therefore, two x 2 mL samples at each time point were determined to be sufficient to reliably characterize the aggregate distribution.

A concern about the suitability of this method was whether the stirring action of the impeller in the sample beaker would break apart the aggregates and alter the size

distribution. For all impeller speeds within the typical operating range, the impeller speed had no effect on the distribution measured (data not shown), implying that the aggregates are in fact insensitive to the shear effects of the impeller. This is likely due to a combination of low residence time in the sample beaker and the 40% glycerol diluent, which reduces shear forces on the aggregates as a result of its increased viscosity.

### **2.3.3 Total Biomass Correlation**

The area under the volume distribution curve represents the total volume of particles analyzed from the sample. If the density of aggregates is constant with respect to aggregate size, this volume should linearly correlate to the total biomass of the sample via the biomass density. A series of dilutions was performed for culture samples, and samples from each dilution were collected for both dry weight analysis and particle sizing on the Coulter counter. As both the shape and mean of the size distribution of the same cell line varied significantly over the course of several months and subculture cycles (Fig. 2.3a), this dilution series was repeated to ensure that correlations developed from the distribution data are applicable over the range of aggregate sizes that may be encountered.

The shape of the aggregate distributions showed no significant differences upon dilution (Fig. 2.3b) (representative data from one dilution series), indicating that particle size measurement is not affected by biomass concentration. A concentration dependent effect on particle size measurement in these types of aperture flow systems is known as coincidence. Coincidence refers to the passing of multiple particles through the aperture at the same time, which results in larger particle measurements when uncorrected for. Coincidence is biomass dependent since the probability of two or more particles passing through the aperture simultaneously increases with increasing particle concentration. Much of the earlier work on electronically sizing cellular particles focused on developing

algorithms to determine coincidence (Kubek and Shuler 1978), but the coincidence correction algorithm embedded within the Multisizer software appeared to adequately account for this effect, since no concentration dependent effect on distribution shape was observed (Fig. 2.3b).

Fig. 2.3c shows a linear relationship between dry weight and total particle volume. A single correlation fits all of the data extremely well ( $R^2$  of 0.999), and can thus be used as a general correlation regardless of aggregate morphology. While the density of aggregates of different sizes may vary, the variation is less than the error introduced from sampling alone, and can therefore be neglected. Therefore, the technique presented here provides for a fast and reliable option to measure total biomass in plant cell culture systems. Finding a fast and simple method to determine biomass has long been a subject of investigation in plant cell culture process engineering research, as dry weight measurements are time consuming and require a time lag of at least one day (Ryu et al. 1990). Very few simple methods exist due to the aggregated nature of plant cells, which precludes methods such as cell counting, and the heterogeneity of the cell culture broth, which precludes measurements such as optical density. Fast methods such as packed cell volume have limited reliability, while others such as media conductivity have been shown to have limited applicability across different plant systems (Kwok et al. 1992).

Analysis of the relative error confirms that the Coulter counter is as reliable a measurement method for total biomass as dry weight measurements (Fig. 2.3d). In fact, when comparing these two methods, the precision of the total biomass measurement is dependent on the properties of the sample, rather than on the measurement technique used. This is due in large part to the heterogeneous nature of the broth and difficulties in

obtaining a well mixed sample of aggregates, which tend to settle when not under constant agitation. When the mean aggregate size was larger or total biomass was lower, the precision of distribution measurements decreased (Fig. 2.3e). This could be predicted from basic statistical considerations, as samples with the same total biomass but larger aggregate size, or less total biomass with the same aggregate size, will both consist of fewer total particles. Attempts were not made to alter the sample size to account for differing biomass concentrations during subsequent cell culture monitoring for the sake of procedural consistency.

#### **2.3.4 Validation of Aggregate Size Measurement**

To quantitatively evaluate whether the Coulter counter could differentiate particles of different sizes, fractions of aggregates that had been filtered were analyzed. Fig. 2.4a shows an obvious separation of filtered fractions. The overlap of fractions is likely due in large part to inefficient filtration as discussed earlier, which is a result of inconsistent filtration of non-spherical aggregates and the aggregate breakage that inevitably occurs over the course of several wash steps. The poor efficiency of filtration, especially the presence of smaller particles in the retentate caught on the filter, is apparent on visual inspection of suspended aggregate fractions following filter backflush, as well as in the spread of data in the size distribution of each fraction (Fig. 2.4a). This effect is particularly noticeable in the distributions of the largest fractions, where the spread of the data due to small aggregates is apparent because of the low numbers of large aggregates. While this limited separation efficiency must be taken into account when using filtration as a means to study aggregate size effects, these results demonstrate that filtration can be a reasonably effective means to separate plant cell aggregates given the proper procedural

consideration, and also demonstrate that the Coulter counter can adequately differentiate plant cell aggregates on the basis of size.

It is apparent that the particle volumes measured on the Coulter counter are smaller than the corresponding filter sizes used to separate the fractions. This discrepancy is a result of using latex beads as a calibration standard, which are different than cell aggregates in a number of ways: aggregates have a heterogeneous, highly aqueous composition, resulting in some residual conductivity and less equivalent volume of electrolyte displaced; aggregates may compress slightly due to the pressure drop when crossing the aperture; and diluent may enter the aggregate via open pores or diffusion, which will also result in less displaced electrolyte and a lower measured particle volume. Regardless of the differences in absolute size measured, a linear correlation ( $d_{\text{coulter counter}} = 0.57 d_{\text{filter}}$ ,  $R^2 = 0.998$ ) was developed using the mean of the volume distribution for each fraction versus the equivalent volume weighted midpoint of the filters used to separate the aggregates (Fig. 2.4b). A similar correlation was also developed for the mean particle size collected via image analysis ( $d_{\text{image analysis}} = 1.14 d_{\text{filter}}$ ,  $R^2 = .995$ ). For this correlation, the mean of the particles as opposed to the mean of the distribution was used since only several hundred counts as opposed to several thousand were taken for each fraction. From this analysis, it appears that image analysis gives slightly larger particle sizes than the corresponding filter fractions. This is in part due to the assumption that the polar axis is the average of the major and minor axes. The polar axis may actually be smaller than either the major or minor axis, and the calculations to determine particle size could likely be tuned to reach a closer absolute agreement with the filter values.

A comparison of sizes measured using all three techniques points to the difficulties in determining the absolute size of plant cell aggregates. While the Coulter

counter underestimates particle size compared to the other methods, the relative sizes are consistent as evidenced by linear correlations (Fig. 2.4b), and the calibration coefficient to convert voltage pulses to particle sizes could easily be altered so that measurements match either filtration or microscope measurements. However, filtration is inconsistent due to irregularly shaped aggregates and aggregate breakage, and sizes obtained from microscopic image analysis are dependent on the assumptions used in calculating particle size. For continuing work, relative differences in aggregate size are of more practical value, and the Coulter counter is as accurate and reliable a method as any other to determine these relative differences, and can be utilized at a fraction of the procedural effort.

### **2.3.5 Batch culture process monitoring**

To demonstrate the utility of the aggregate size measurement using the Coulter counter, we monitored the aggregate distribution over a batch subculture period, in conjunction with sucrose and glucose measurements in the culture media. Fig. 2.5a shows the evolution of the aggregate distribution over approximately two weeks. Total biomass accumulation was evaluated using the previously described correlation, and the resulting growth curve showed the expected pattern of exponential growth followed by a stationary phase, which corresponded to the point at which sugar was exhausted in the media (Fig. 2.5b). Over the course of the batch, the aggregate distributions spanned an increasing broader range of sizes, and showed a significant increase in mean aggregate size of 55% (Fig. 2.5c). The increase in aggregate size corresponded with the exponential growth phase of the culture, but this trend reversed once the culture reached stationary phase. This suggests that aggregate growth is associated with cell division and growth as opposed to other possible mechanisms such as agglomeration. Slight variations with

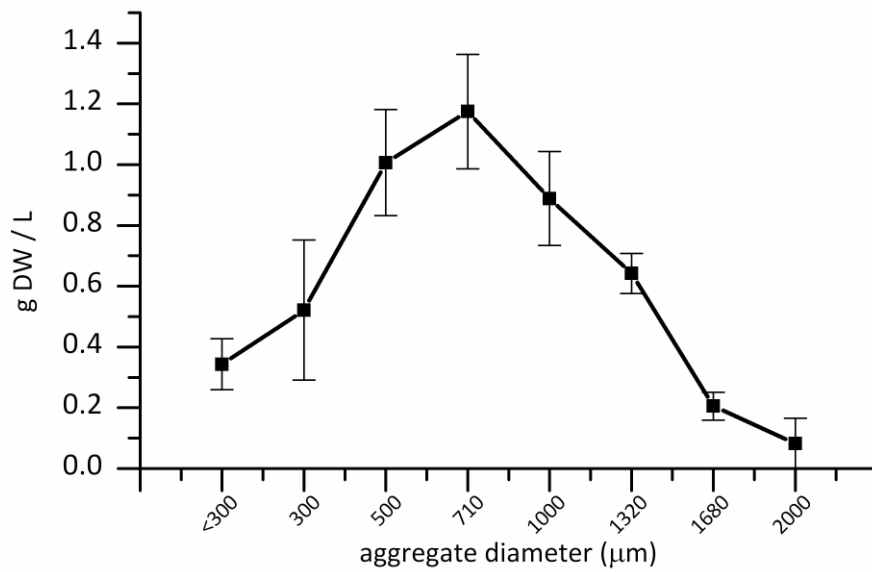
respect to sugar consumption and a batch culture's progression through the growth cycle are likely present over several subculture cycles, which are typically not controlled for as subculturing is determined by time (14 days) as opposed to process measurements (see Methods for details). It is possible that these variations contribute to the periodic size fluctuations observed (Fig. 2.3a), though there are most likely other contributing factors, including underlying metabolic variability as well as epigenetic and circadian variations. Understanding the phenomenological basis of these variations is a goal of continuing work.

## **2.4 Conclusions**

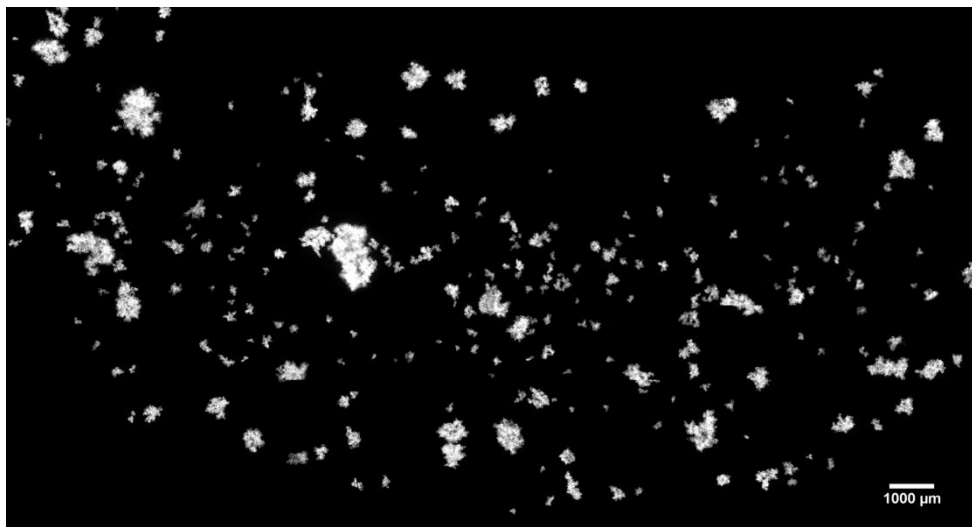
We have evaluated several methods for characterizing plant cell aggregates in tissue suspension culture, including a novel technique utilizing electrical pulse amplitude sizing, or Coulter counter technology. The Coulter counter can quickly measure total biomass as a cumulative total of all aggregates, and provides reliable data compared to the standard method of dry weight. The Coulter counter can also measure aggregate size distributions as well as, if not more accurately, than both filtration and image analysis and can be used for a fraction of the time and procedural effort. The absolute size of aggregates is difficult to quantify, but relative measurements from all three techniques can be linearly correlated. Analysis of changes in the aggregate size distribution over a batch cycle reveals that aggregate size increases during exponential growth but decreases during stationary phase, and demonstrates the utility of this method for future studies into the advanced process characterization of plant cell tissue culture dynamics.



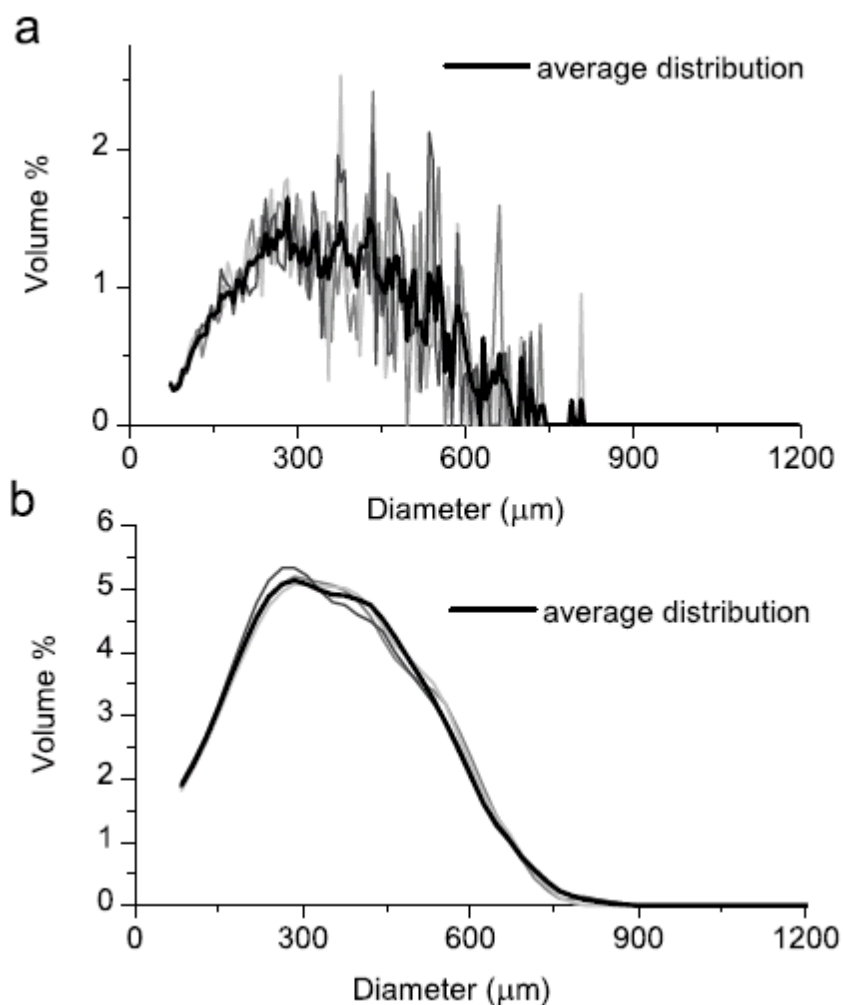
a



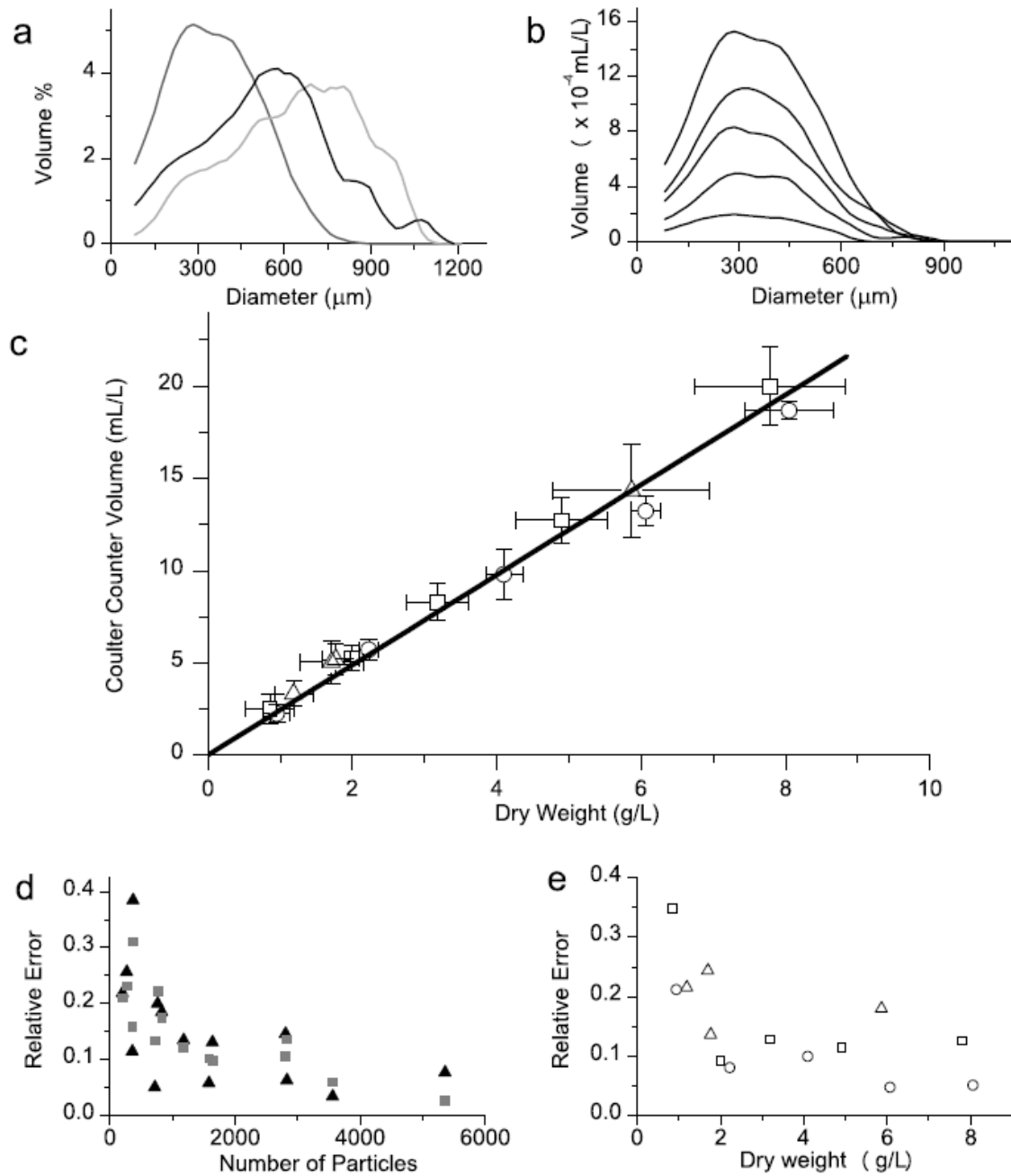
b



**Figure 2.1** Typical size and morphology of aggregates in *Taxus cuspidata* suspension culture. (a) Distribution obtained from filtration of cultures on day 7; data points represent biomass retained on corresponding filter size, error bars represent standard deviations from three replicate flasks. (b) Composite image of aggregates stained with fluorescein diacetate.

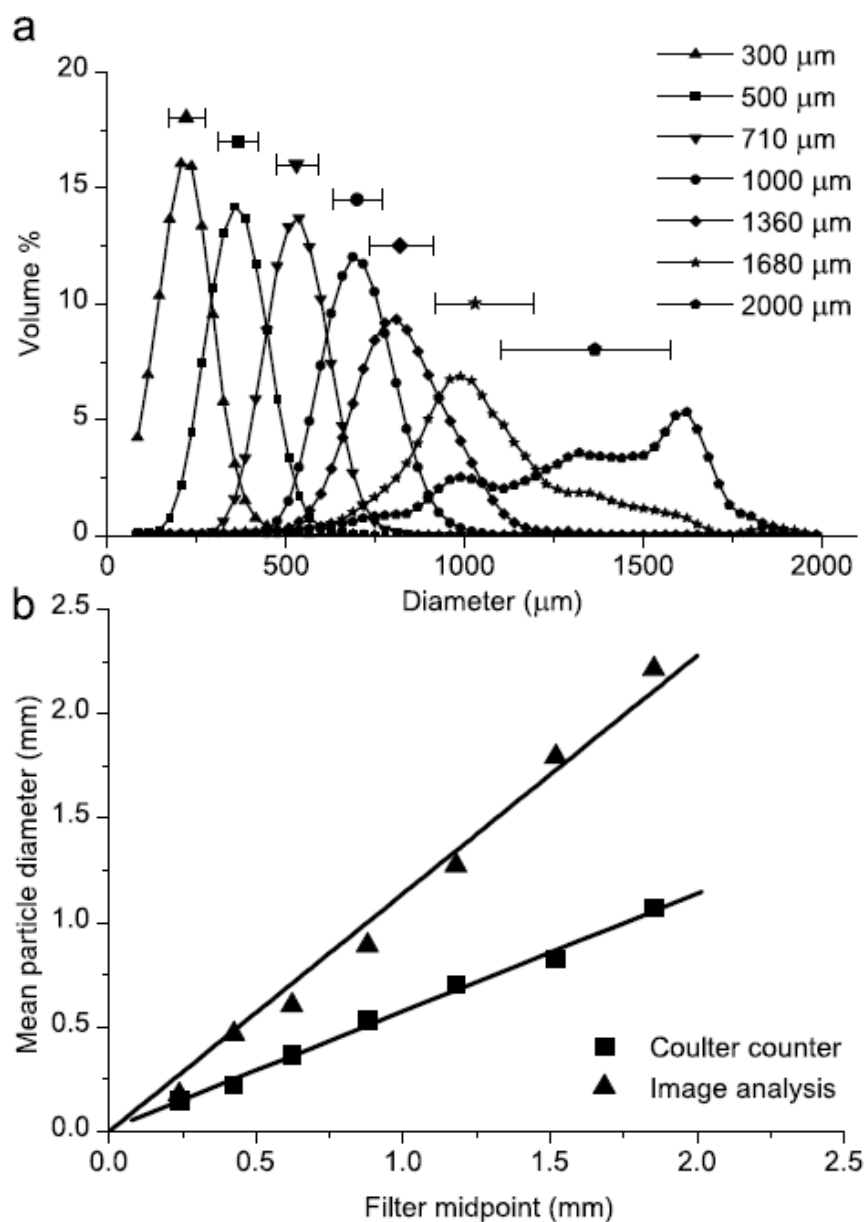


**Figure 2.2** Aggregate size distributions obtained using a Coulter counter. (a) Resolution of 200 size bins from 70 to 1,200  $\mu\text{m}$ , comparing average of five samples to three representative individual samples. (b) Resolution of 50 size bins from 70 to 1,200  $\mu\text{m}$ , smoothed using a weighted moving average, comparing average of five samples to three representative averages of two samples.

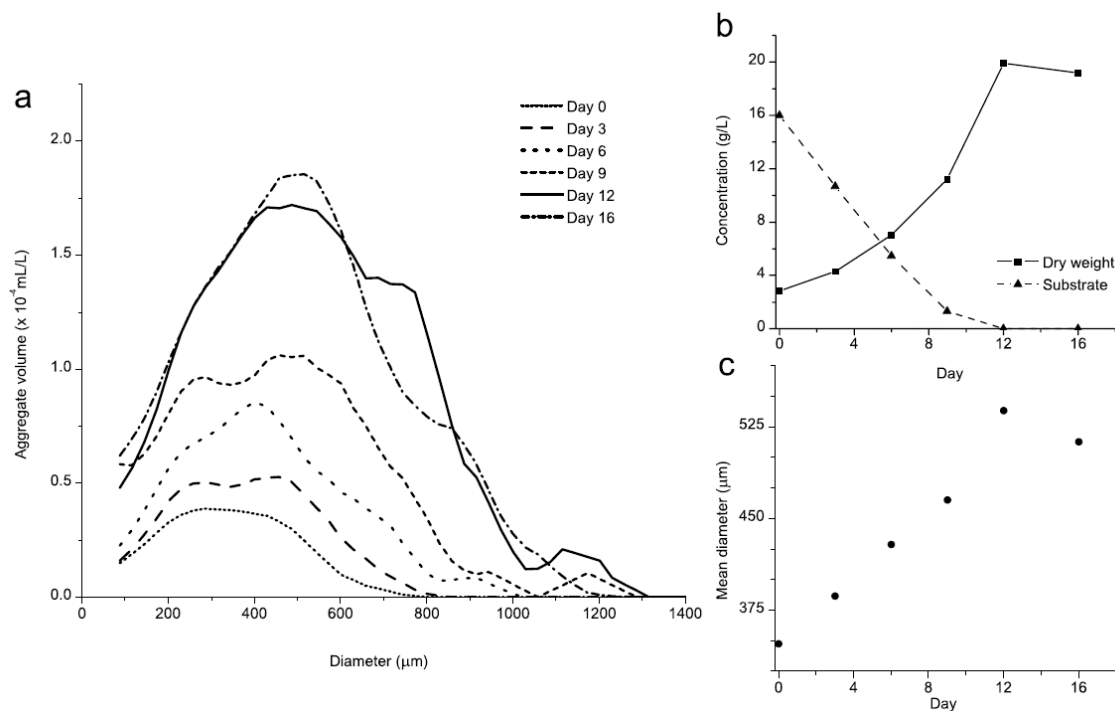


**Figure 2.3** Suitability of the Coulter counter method to measure total biomass. (a) Average aggregate size distributions from mid-exponential phase cultures of *Taxus cuspidata*. (b) Effect of biomass concentration on distribution curves from a dilution series of culture with a mean aggregate size of 365  $\mu\text{m}$ . (c) Correlation between dry weight and total volume measured on Coulter counter for cultures with different

aggregate size distributions (from Fig. 2.3a); Small = mean 365  $\mu\text{m}$ , Medium = mean 537  $\mu\text{m}$ , Large = mean 625  $\mu\text{m}$ , error bars represent standard deviation of 5 measurements. (d) Comparison of relative error (standard deviation divided by mean) for total biomass measurements comparing dry weight and Coulter counter biomass, based on total particle number as determined by the Coulter counter. (e) Comparison of relative error in total biomass measurements (average of Coulter counter and dry weight error) for cultures with different aggregate size profiles (Fig. 2.3a and Fig. 2.3b)



**Figure 2.4** Validation and correlations of Coulter counter size measurements. (a) Size distributions of aggregates separated on a series of filters and analyzed on the Coulter counter; each data point above the corresponding distribution represents the median and error bars represent median 50% of data, labels refer to filter size on which the sample was collected. (b) Correlation between mean size of filtered fractions determined using both the Coulter counter and image analysis against the volume weighted average of filter sizes between which aggregates were separated.



**Figure 2.5** Process monitoring of cell cultures over batch subculture period. (a)

Aggregate distribution data obtained on a Coulter counter over successive days of the batch cycle, each curve represents the average of two 2mL samples. (b) Growth curve obtained from distribution data; biomass correlation (**Fig. 2.3c**) used to calculate dry weight from Coulter counter data, substrate concentration is the sum of sucrose and glucose in the culture medium. (c) Mean diameter of aggregates over batch cycle.

## CHAPTER 3

### ANALYSIS OF AGGREGATE SIZE AS A PROCESS VARIABLE AFFECTING PACLITAXEL ACCUMULATION IN *TAXUS* SUSPENSION CULTURES

Plant cell aggregates have long been implicated in affecting cellular metabolism in suspension culture, yet the rigorous characterization of aggregate size as a process variable and its effect on bioprocess performance has not been demonstrated. Aggregate fractionation and analysis of biomass-associated product is commonly used to assess the effect of aggregation, but we establish that this method is flawed under certain conditions and does not necessarily agree with comprehensive studies of total culture performance. Leveraging recent advances to routinely measure aggregate size distributions, we developed a simple method to manipulate aggregate size and evaluate its effect on the culture as a whole, and found that *Taxus* suspension cultures with smaller aggregates produced significantly more paclitaxel than cultures with larger aggregates in two cell lines over a range of aggregate sizes, and where biomass accumulation was equivalent prior to elicitation with methyl jasmonate. *T. cuspidata* P93AF cultures with mean aggregate sizes of 690  $\mu\text{m}$  and 1100  $\mu\text{m}$  produced 22 mg/L and 11 mg/L paclitaxel, respectively, a 2-fold increase for smaller aggregates, and *T. cuspidata* P991 cultures with mean aggregate sizes of 400  $\mu\text{m}$  and 840  $\mu\text{m}$  produced 6 mg/L and 0.3 mg/L paclitaxel, respectively, an increase of 20-fold for smaller aggregates. These results demonstrate the importance of validating experiments aimed at a specific phenomenon with total process studies, and provide a basis for treating aggregate size as a targeted process variable for rational control strategies.

### 3.1 Introduction

Natural products have traditionally served as a valuable source of pharmaceuticals, particularly chemotherapeutics (Cragg et al. 2009), but the use of natural products as drug leads has been significantly reduced over the past two decades (Koehn and Carter 2005). One major factor contributing to this decline is the uncertainty of drug product supplies, a challenging constraint unique to complex plant derived natural products (McChesney et al. 2007). Plant cell culture technology, particularly dedifferentiated suspension cultures that are amenable to similar bioprocessing technologies used for large scale mammalian cell culture, is a production alternative for natural products which cannot easily be extracted from natural sources or chemically synthesized. Despite the proven, albeit limited, commercial success of plant cell culture processes to supply pharmaceuticals (notably paclitaxel), the widespread application of this technology has been hampered due to several characteristics of plant cells: slow growth rates; low yields of secondary metabolites; aggregation, which induces cellular heterogeneity and creates difficulties for process scale up; and inherent variability in these properties, which is poorly understood (Kolewe et al. 2008; Lee et al. 2010).

Plant cells grow as aggregates in suspension culture. As plant cells remain connected via cell walls following cytokinesis, aggregates of two to a few hundred cells are created after many rounds of cell division. In liquid suspension culture, these aggregates are periodically broken due to the shear forces resulting from agitation, creating a wide distribution of aggregate sizes from less than 100  $\mu\text{m}$  in diameter to greater than 2 mm. Cells within larger aggregates are subject to different microenvironments with respect to nutrient availability, cell to cell signaling, and applied surface shear forces. Aggregates have long been associated with affecting cellular



metabolism and causing differentiation (e.g., Kubek and Shuler 1978; Street et al. 1965), and the heterogeneity observed in plant cell populations has been attributed in part to aggregation (King and Street 1977; Naill and Roberts 2005; Yanpaisan et al. 1998). Despite the demonstrated importance of this property regarding the underlying cell biology of plant cells in culture, relatively little work has focused on the characterization and understanding of aggregate size as a process variable and its effect on bioprocess performance.

Attempts have been made to ascertain the effect of aggregation with regard to metabolite production by analyzing aggregates of different sizes from within a culture. Most often, a fractionation technique in which aggregates are separated on a series of sieves or filters has been used to obtain information about the biomass-associated content of a particular secondary metabolite or group of metabolites. Results from this approach generally suggest that larger aggregates have a positive effect on secondary metabolite production (Eda Hiro and Seki 2006; Franke and Bohm 1982) or a positive effect up to a critical size (Hulst et al. 1989; Madhusudhan and Ravishankar 1996; Zhao et al. 2003), but have also been inconclusive (Kessler et al. 1999). However, none of these studies have validated their results by confirming that cultures with larger aggregate size profiles result in higher product titers. This is critical since fractionation only accounts for biomass-associated metabolites, but not for metabolites released into the extracellular medium, which may be a significant fraction of the total produced (Bourgaud et al. 2001; Hirasuna et al. 1996).

Several other methods have been used to study aggregate size effects. Colored products such as anthocyanins have been measured directly via microscopy, and indicate that smaller aggregates favor higher metabolite production (Pepin et al. 1999). Another

approach is to alter the starting aggregate size distribution, which has shown that smaller aggregates (Hanagata et al. 1993; Kinnersley and Dougall 1980) or cultured single cells (Bolta et al. 2003) result in higher metabolite production. The primary difficulties with this strategy are to ensure that aggregate size is the only variable manipulated and to confirm that cultures maintain disparate aggregate distributions over time, which has traditionally been difficult to achieve due to the lack of a suitable method to routinely measure aggregate size. The inconsistent results across systems may be partly a result of fundamental differences between distinct plant species, as their unique metabolites and metabolic pathways may be differentially controlled under varying conditions. However, as no studies exist where multiple approaches were compared, it is difficult to determine whether conflicting trends are the result of unique characteristics of individual systems or incomplete experimental analysis.

In this work, we explored the effect of aggregation on production of paclitaxel, an important anti-cancer drug, in *Taxus cuspidata* suspension cultures. We evaluated intra-culture trends by measuring biomass-associated paclitaxel in aggregates of different sizes, and monitored changes at several time points after eliciting paclitaxel production with methyl jasmonate. Utilizing a technique to quickly and reliably measure aggregate size distributions (Kolewe et al. 2010), we developed a new methodology to initiate cultures with dissimilar aggregate sizes, and treated aggregate size as a process variable which could be measured and directly assessed as to its effect on total paclitaxel accumulation. These experiments were repeated with two cell lines and a variety of aggregate distributions to establish a relationship between aggregate size and total paclitaxel accumulation. The combination of approaches to consider aggregate size as a process variable and to analyze aggregates of different sizes within a culture provides a more comprehensive understanding of the effects of aggregation on culture performance.

## **3.2 Materials and Methods**

### **3.2.1 Cell cultures**

*Taxus cuspidata* cell lines P991 and P93AF were provided by the U.S. Plant Soil and Nutrition Laboratory in Ithaca, NY, and maintained in our laboratory. Medium was prepared consisting of Gamborg B5 basal salts (Sigma-Aldrich Co., St. Louis, MO), supplemented with 20 g/L sucrose, 2.7  $\mu$ M naphthalene acetic acid, and 0.1  $\mu$ M benzyladenine, adjusted to pH 5.5, and autoclaved. 150 mg/L citric acid, 150 mg/L ascorbic acid, and 6.0 mM glutamine were filter sterilized and added post-autoclave. Cell cultures were maintained in 250 mL or 500 mL glass Erlenmeyer flasks with Bellco (Vineland, NJ) foam closures, and incubated in gyratory shakers in constant darkness at 23°C and 125 rpm. Every 2 weeks, inoculum was subcultured to fresh media at a ratio of 1:4 for a total volume of 200 mL with ~10 mL packed cell volume in 500 mL flasks. To stimulate paclitaxel production, cultures were elicited on day 7 with 150  $\mu$ M methyl jasmonate (Sigma-Aldrich) and covered with aluminum foil.

### **3.2.2 Aggregate Fractionation and Paclitaxel Analysis**

Following elicitation with 150  $\mu$ M methyl jasmonate on day 7, aggregates from an entire flask were filtered as previously described (Kolewe et al. 2010). Aggregates were subsequently transferred to pre-weighed 1.5 mL centrifuge tubes, and evaporated to dryness under vacuum. Sample tubes were weighed again to determine dry weight. Dried biomass was resuspended in 1 mL acidified methanol (0.01% glacial acetic acid), and homogenized for 1 hour in a sonicating bath (VWR Scientific). Samples were centrifuged at 10 g for 25 min., the supernatant was collected and evaporated to dryness under vacuum, and samples were resuspended in 200  $\mu$ L acidified methanol by vortexing followed by sonication for 30 min. Concentrated samples were filtered using 0.22  $\mu$ m

filters (Acrodisk<sup>®</sup>, Sigma-Aldrich) into HPLC sample vials. An isocratic 50:50 water:acetonitrile mobile phase was used on a Waters (Milford, MA) Alliance 2695 HPLC system with full scan photodiode array detector and Taxsil column (Varian Inc, Lake Forest, CA). 10  $\mu$ L were injected for each sample. Taxanes were identified based on spectrum and retention time comparison with authentic standards (Sigma-Aldrich) and quantified using an extracted chromatogram at 228 nm based on a standard curve (1 mg/L to 50 mg/L).

### **3.2.3 Aggregate Size Distribution Manipulation and Total Culture Studies**

Aggregate size distributions were measured using a Coulter counter (Kolewe et al. 2010) on day 14 prior to subculture. The filter whose size most closely corresponded to the mean aggregate size of the upstream cultures was sterilized under UV light for at least 4 hours. Cultures used for inoculation were combined, 80 mL of culture broth were passed over the filter and washed via immersion and gentle swirling in B5 basal salt solution, and the aggregates caught on the filter were backflushed into fresh media prepared for subculturing, resulting in a culture of larger aggregates. The original culture flow through was passed over a filter made of 80  $\mu$ m nylon mesh (Sefar Filtration Inc., Depew, NY) with the permeate set aside. The remaining wash solution containing aggregates which had passed through the initial filtration was also passed over the 80  $\mu$ m filter. The aggregates caught on the filter were backflushed into fresh media, resulting in a culture with smaller aggregates. This process was repeated yielding replicate sets of “small” and “large” aggregate distributions. All of the culture permeate was collected and filtered over a 0.22  $\mu$ m filter (Nalgene, Fisher Scientific) and 30 mL of this conditioned media were added to each culture. Unfiltered cultures were also initiated as a control per standard subculture procedures (section 3.2.1). The cultures initiated were

alike in all initial process parameters, including total biomass, except for the aggregate size distribution. Aggregate distributions were measured using a Coulter counter, and these measurements were used to determine total biomass dry weight and aggregate size based on previously published correlations (Kolewe et al. 2010). All aggregate size and biomass dry weight data are presented based on these correlations. Aggregate distribution samples were taken at day 0, immediately prior to elicitation with 150  $\mu$ M methyl jasmonate on day 7, and day 14. 1 mL samples of well mixed culture broth including cells and media were taken with a cut pipette tip periodically post-elicitation for up to 3 weeks, and samples were extracted and analyzed for paclitaxel via HPLC (see section 3.2.2).

### **3.3 Results**

#### **3.3.1 Biomass-Associated Paclitaxel in Aggregate Fractions**

Aggregates of *T. cuspidata* spanned a wide distribution, ranging from less than 100  $\mu$ m to over 2 mm in diameter (Fig. 3.1). Fractionation experiments indicated that the largest aggregates (greater than 1.7 mm) for *T. cuspidata* cell line P991 showed higher levels of specific paclitaxel accumulation (Fig. 3.2a). Smaller aggregates in these cultures all accumulated about the same amount of paclitaxel, but past a critical size, paclitaxel levels were almost doubled. This trend was consistent over several days corresponding to the peak production times indicated by maximum total paclitaxel concentration (defined as cell-associated and extracellular paclitaxel in a sample of well mixed culture broth). Subsequent experiments with the same cell line, performed with a slight modification where replicate flasks were combined prior to fractionation, showed the same initial trend of higher accumulation in the largest aggregates, but evening out over time (Fig. 3.2b). The aggregate size distributions were variable over multiple subculture cycles, and comparison of crude size distributions determined on the basis of

dry weight for each aggregate size range indicated that cultures in Fig. 3.2a contained a significantly higher percentage of smaller aggregates than the cultures in Fig. 3.2b (Fig. 3.2c). Total paclitaxel concentration in these cultures with smaller aggregates (5.5 mg/L) one week post elicitation was almost double the paclitaxel concentration of the cultures with larger aggregates (2.9 mg/L) one week post elicitation, indicating that differences between cultures may be more significant than trends found within a particular culture. Based on this analysis, fractionation experiments are inconclusive regarding the effect of aggregate size on paclitaxel accumulation, and a better method is needed to evaluate process performance.

### **3.3.2 Aggregate Size Manipulation and Total Paclitaxel Accumulation**

Cultures with dissimilar aggregate distributions were successfully initiated by filtering inoculum using a filter size based on the measured aggregate size distribution (Fig. 3.3). The terminology small aggregate cultures, large aggregate cultures, and unfiltered cultures will be used to refer to cultures initiated from aggregates that passed through the initial filter, cultures initiated from aggregates that were retained on the initial filter, and cultures initiated from unfiltered aggregates, respectively. This methodology produced cultures with different aggregate sizes and the same total biomass. As plant cell aggregates are fragile, particularly when not submersed in liquid, the elimination of excessive handling steps is critical, and this method is preferable to the alternate approach of filtering aggregates and portioning fresh weight prior to inoculation. Though the cultures did have overlap in their size distributions, they were clearly distinct, which is most easily indicated by their disparate mean aggregate sizes (390  $\mu\text{m}$ , 630  $\mu\text{m}$ , and 810  $\mu\text{m}$  for small aggregate cultures, unfiltered cultures, and large aggregate cultures in Fig. 3.3). The  $d_{90}$  is the diameter value below which 90% of biomass was found, and was used

to provide a measure of the maximum aggregate size that contributed significantly to the total biomass. Distributions with a larger mean aggregate size also had a larger  $d_{90}$  (623  $\mu\text{m}$ , 1110  $\mu\text{m}$ , and 1250  $\mu\text{m}$  for small aggregate cultures, unfiltered cultures, and large aggregate cultures in Fig. 3.3), though this value was also dependent on the shape of the distribution and did not simply scale with the mean.

Cultures of *T. cuspidata* P93AF that were initially filtered at 710  $\mu\text{m}$  were monitored in detail for both aggregate distributions and paclitaxel accumulation over time. As aggregate distributions broadened and shifted to the right over several doublings of biomass, the filtered and unfiltered distributions remained distinct both prior to elicitation with methyl jasmonate and through one week post elicitation as growth slowed down (Fig. 3.4 a,b,c). Prior to elicitation the mean aggregate sizes were 690  $\mu\text{m}$ , 940  $\mu\text{m}$ , and 1100  $\mu\text{m}$ , while the  $d_{90}$  values were 1060  $\mu\text{m}$ , 1540  $\mu\text{m}$ , and 1690  $\mu\text{m}$  for small aggregate cultures, unfiltered cultures, and large aggregate cultures, respectively. Total biomass was nearly the same for all three sets of cultures through one week post-elicitation while cultures were at their maximum productivity (Fig. 3.4d). Paclitaxel was detectable in total culture samples starting at five days post elicitation, and small aggregate cultures immediately showed higher productivity (3.0 mg/L/day) than both the unfiltered cultures and large aggregate cultures (both 1.6 mg/L/day). Productivity based on biomass shows the same trend, where small aggregate cultures (0.36 mg/g DW biomass/day) had more than double the productivity of large aggregate cultures (0.16 mg/g DW biomass/day). Productivity declined one and a half weeks post elicitation for all three sets of cultures, and stopped for large aggregate cultures, while paclitaxel accumulation in small aggregate cultures and unfiltered cultures continued at much lower levels compared to their initial rates. Three weeks post elicitation, all cultures showed relatively high levels of paclitaxel accumulation, and small aggregate cultures had 22

mg/L compared to 13 mg/L for unfiltered cultures and 11 mg/L for large aggregate cultures. In this case, the  $d_{90}$  values for unfiltered cultures and large aggregate cultures were much closer than their mean aggregate sizes, indicating that unfiltered cultures did develop some large aggregates. This maximum aggregate size may limit the overall productivity and account for the similar production levels in these two cultures (see section 3.4.2).

In two experiments with separate *T. cuspidata* cell lines (P991 and P93AF) both initially separated with a 500  $\mu\text{m}$  filter, filtered and unfiltered aggregate distributions remained distinct prior to elicitation, and did not show significant differences in total biomass (Fig. 3.5 a,b Table 3.1). For both cell lines, small aggregate cultures produced significantly more paclitaxel than the unfiltered cultures, and large aggregate cultures produced significantly less paclitaxel than the unfiltered cultures (Fig. 3.5 c,d). For the *T. cuspidata* P991 cell line, differences in paclitaxel accumulation were obvious after one week post elicitation, and large aggregate cultures accumulated very low amounts of paclitaxel (0.3 mg/L). In comparison, for the *T. cuspidata* P93AF cell line, large aggregate cultures accumulated moderate levels of paclitaxel by two weeks post elicitation (1.9 mg/L). The difference in *T. cuspidata* P93AF paclitaxel accumulation between cultures with smaller aggregates and unfiltered cultures was only evident after two weeks. Comparing unfiltered cultures from the two cell lines, even though the mean aggregate sizes were within 25  $\mu\text{m}$ , the  $d_{90}$  values were 1290  $\mu\text{m}$  and 1060  $\mu\text{m}$  for *T. cuspidata* P991 and *T. cuspidata* P93AF, respectively, which may help to explain the higher paclitaxel levels in *T. cuspidata* P93AF (see section 3.4.2). Paclitaxel accumulation could not be correlated with mean aggregate size alone as it appeared to be influenced by the presence of even a few large aggregates as described by the  $d_{90}$ , but the



results of these experiments indicate a clear trend in *T. cuspidata* cultures: cultures with smaller aggregates accumulate more paclitaxel.

As aggregate size periodically fluctuated over repeated subculturing, cultures were eventually available with much larger aggregates. For *T. cuspidata* P991, aggregates were initially separated with a 1000 µm filter and propagated (Fig. 3.6, Table 3.2). Differences in growth were evident after one week, as small aggregate cultures increased biomass 2.5-fold, while the unfiltered and large aggregate cultures both increased by just under 2-fold. Post elicitation, all of the cultures began to turn brown and necrotic, and none of the cultures remained viable one week later. While paclitaxel was detected in cultures with smaller aggregates, it was below the limit of quantification using HPLC. No paclitaxel was detected in either the unfiltered cultures or large aggregate cultures, indicating that extremely large aggregates are unable to produce paclitaxel.

### **3.4 Discussion**

#### **3.4.1 Limitations of Aggregate Fractionation**

Fractionation and analysis of biomass-associated paclitaxel in aggregates of different sizes would appear to be the simplest method to discern the effect of aggregate size on secondary metabolite production, but results from this analysis do not agree with results from the more compelling whole culture studies or a comparison of total paclitaxel concentration between the two fractionation experiments. The fractionation method is critically limited in several aspects: many secondary metabolites, including paclitaxel, are excreted into the culture medium, and a significant percentage can be non cell-associated; accumulation of metabolites within aggregates does not necessarily correlate with production of metabolites; and aggregate size distributions are dynamic as aggregates

continuously grow and frequently break apart, so it is impossible to know whether an aggregate of a specific size has recently broken off from a larger aggregate or has grown to this size over time. Aggregate breakage likely helps to explain the lack of a clear trend several days after accumulation levels were initially higher in larger aggregates (Fig. 3.2b), as aggregates in each size class were likely derived in part from aggregates of the largest sizes. Breakage of aggregates has not been explicitly mentioned in previous published studies, and may also help to explain, for instance, why larger aggregates initially had higher levels of ajmalicine, but then decreased levels over time as levels in smaller aggregates increased (Kessler et al. 1999).

In larger aggregates, higher levels of paclitaxel are likely the result of an increased storage capacity rather than increased production. The increased accumulation of secondary metabolites in larger aggregates has been attributed in part to cellular differentiation in several systems (Zhao et al. 2003; Xu et al. 1998; Zhao et al. 2001), as differentiation (Xu et al. 1998; Zhao et al. 2001; Kuboi and Yamada 1978) and hollow spaces (Singh and Curtis 1994) are found in larger aggregates. Differentiation is often thought to be a requirement for secondary metabolite synthesis, as some metabolites and the upregulation of genes necessary for biosynthesis are only found in specialized organs (Leonard et al. 2009; Pasquali et al. 2006). However, as *T. cuspidata* cultures with larger aggregates produce less paclitaxel, any cellular differentiation would be associated with enhanced paclitaxel storage rather than synthesis. In addition, paclitaxel is hydrophobic and has been shown to accumulate predominately in the cell wall (Roberts et al. 2003). In larger aggregates, cell walls that are thicker may accumulate more paclitaxel, and the release of extracellular paclitaxel may be reduced, particularly for cells in the interior of the aggregate.

More importantly, accounting for only cell-associated secondary metabolites on a weight percentage basis is a fundamentally flawed method to analyze metabolite production when significant levels are found in the extracellular medium. In *Taxus* spp., there have been varying reports of cell-associated vs. non cell-associated paclitaxel, from little to no extracellular paclitaxel (Wickremesinhe and Arteca 1994) to over 90% extracellular (Hirasuna et al. 1996) to intermediate levels (Wang et al. 2001). Even if it has been explicitly found in a particular system that a large percentage of product is cell-associated (which is often not stated), this assumption should be used with caution and validated in experiments using total concentration, including cells plus media in the culture broth.

### **3.4.2 Aggregate Size as a Process Variable**

In experiments where the initial aggregate size distribution was modified, cultures with relatively smaller aggregate sizes in the context of each experiment consistently accumulated more paclitaxel. This was seen in different cell lines and over a range of aggregate distributions. This methodology allowed for the measurement of total paclitaxel concentration, accounting for both cell-associated and extracellular product, which is the most critical parameter for process optimization. The relationship between aggregate size and paclitaxel accumulation could not be correlated only on the basis of mean aggregate size, however, as the aggregate distributions also varied in their shape, and the width of these distributions appeared to affect culture performance. Properties associated with the largest aggregates present will affect the entire culture due to both the constant breakage of large aggregates into smaller pieces, as well as the release of cellular material from larger aggregates, including signaling molecules and other important compounds that associate with cell debris. The  $d_{90}$  was shown to provide a reasonable measure to quantify

the size of the largest aggregates present. Such a measure of maximum aggregate size may help to explain why some cultures with similar mean aggregate sizes accumulated different amounts of paclitaxel (Fig 3.5), and also why some cultures with different mean sizes accumulated about the same amount of paclitaxel (Fig 3.4). Aggregation, however, is not the only cause of variability, and in some cases, larger aggregates from one experiment accumulated more paclitaxel than smaller aggregates from another experiment. There are certainly additional genetic, epigenetic, and other environmental conditions that contribute to inherent productivity levels, and some of these same factors likely contribute to the variability in aggregate size which is seen over time (Kolewe et al. 2010). In any case, we have found that as a general rule, cultures with smaller aggregates are superior to cultures with larger aggregates with respect to total paclitaxel accumulation.

It is unclear whether previous studies with different cell systems, which contrast the relationship between aggregate size and secondary metabolite production found here, are the result of inherent differences amongst plant systems, or the result of differing methodologies. The fractionation technique to measure cell-associated metabolites was used in all studies indicating that larger, dedifferentiated aggregates were beneficial (Edahiro and Seki 2006; Franke and Bohm 1982; Hulst et al. 1989; Madhusudhan and Ravishankar 1996; Zhao et al. 2003). By contrast, in the two reports which utilized a process analysis approach by initiating cultures consisting of different aggregate sizes, cultures with smaller aggregates were found to be superior (Hanagata et al. 1993; Kinnersley and Dougall 1980). While no reports exist where the two techniques were compared directly, larger aggregates were found to be slightly beneficial for the production of anthocyanin in *Daucus carota* via the fractionation technique (Madhusudhan and Ravishankar 1996), while smaller aggregates were found to be

superior in the same system via whole culture process analysis (Kinnersley and Dougall 1980). Our results follow this same pattern. Several reports which analyze total process performance and demonstrate that larger aggregates are superior utilize compact callus cultures (Xu et al. 1998; Zhao et al. 2001), which are suspension cultures consisting of large aggregates in which a high degree of differentiation is present by design.

Investigations into the process performance of cultures where large dedifferentiated aggregates are suggested to be beneficial, as well as explicit examinations regarding the effect of microenvironments within aggregates on individual cells, are targets for further study.

The results here suggest a clear process optimization strategy: maintain small aggregates to increase paclitaxel accumulation. The filtration technique described here is not an ideal solution, as large amounts of biomass would be wasted, which cannot be afforded due to the relatively slow growth rates of plant cells. A more practical solution would be to increase the rate at which aggregates break by increasing shear via higher agitation. Shear stress is generally considered to be detrimental to plant cell cultures and many bioreactor configurations have been developed to minimize shear stress (Georgiev et al. 2009; Huang and McDonald 2009), which suggests that increases in agitation will eventually lead to shear stresses that negatively impact cell metabolism. However, it has been demonstrated in another plant system that an optimum shear rate exists which maximizes both cell growth and productivity (Zhong et al. 1994), and a similar point may be found in *Taxus* cultures where the benefits from smaller aggregate size would outweigh the disadvantages of increased shear stress. Ideally, conditions could be determined in which aggregate size can be manipulated by adjusting specific process parameters without negatively impacting cells. A more comprehensive understanding of aggregate dynamics and the underlying phenomena which control changes in the

aggregate distribution that are seen both during each batch and over repeated cycles would be extremely beneficial. To this end, mathematical modeling would be preferable to large experimental arrays as the number of parameters to be evaluated combined with the long batch time for plant cell cultures make such experimental approaches impractical. Population balance equation models can be used to describe cell aggregates as a particulate system (Lin et al. 2008), and provide a suitable framework for a predictive model that we are currently developing.

### **3.5 Conclusion**

We have developed a method to treat aggregate size as a process variable and assess its effect on bioprocess performance via the total accumulation of paclitaxel. Cultures initiated with different aggregate distributions maintained these differences over time, but showed little differences in biomass production. Significant differences were consistently found in paclitaxel accumulation, where small aggregate cultures accumulated from 2-fold up to 20-fold the total paclitaxel of larger aggregate cultures. By contrast, results from aggregate fractionation experiments indicate that larger aggregates may accumulate slightly more paclitaxel than smaller aggregates from within the same culture, and that this method is not suitable for assessing the effect of aggregate size on process performance, especially in systems where significant extracellular product may be present. The results provide a basis for novel control strategies aimed at manipulating aggregate size for process optimization, and in particular indicate that limiting aggregate size in *T. cuspidata* suspension cultures will significantly increase paclitaxel yields.

### 3.6 Additional experiments

A major limitation regarding analysis of the smallest aggregates was uncovered in this filtration approach. There were small aggregates less than 80  $\mu\text{m}$  in culture, which made up an insignificant amount of the total biomass, generally less than one percent of dry weight. It was impossible to separate these aggregates by filtration because there was also a significant amount of debris present in culture, which markedly increased post elicitation as biomass growth slowed. This debris contained large pieces of cell wall from disintegrated aggregates, which could be long and sinewy (Fig 3.7a), and which formed a filter cake on smaller mesh sizes. This problem was exacerbated by the fact that the percentage of open filter area decreased as pore size decreased, as the diameter of the nylon mesh strands was comparable to the diameter of the actual pores. Instead of a nylon mesh filter for the smallest size, we used Miracloth<sup>®</sup>, a depth filter with a nominal 25  $\mu\text{m}$  pore size which is designed to be less prone to clogging. The additional biomass collected on Miracloth<sup>®</sup> compared to the 80  $\mu\text{m}$  mesh was comprised in part of single cells and doublets, but still contained a significant amount of debris, which was confirmed visually (Fig 3.7a). In attempting to analyze these smallest aggregates, we observed that the amount of paclitaxel in the biomass collected on Miracloth<sup>®</sup> markedly increased at later times as more debris was generated. This result suggests that the observed increase in paclitaxel in biomass collected on this small filter (Fig. 3.7b) was likely due to increased accumulation in cellular debris rather than smaller aggregates.

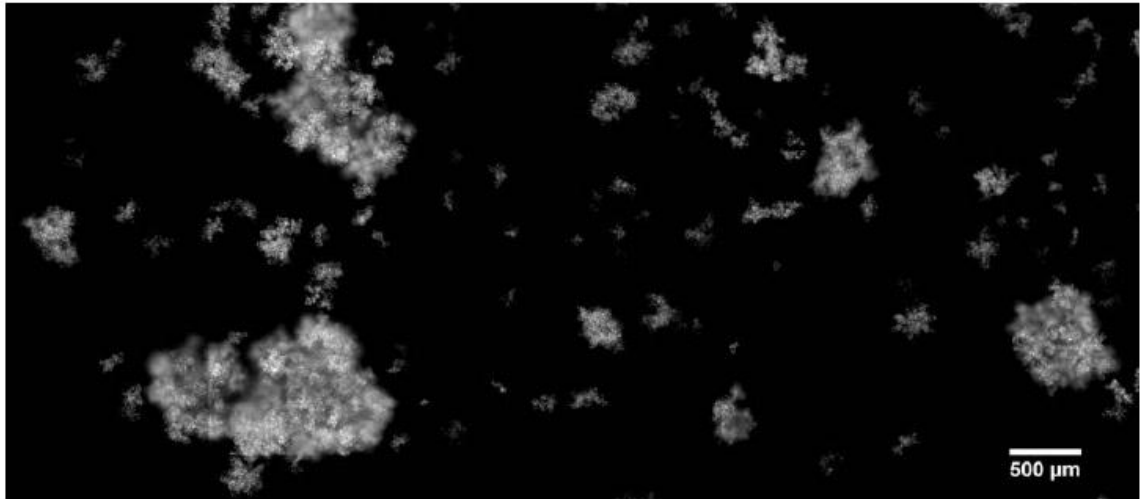
culture designation	<i>T. cuspidata</i> P991C		<i>T. cuspidata</i> P93AF	
	total biomass (g DW/L)	mean aggregate diameter (µm)	total biomass (g DW/L)	mean aggregate diameter (µm)
Small (Initiated < 500 µm)	5.9	400	6.8	520
Unfiltered	6.0	720	6.2	700
Large (Initiated > 500 µm)	5.7	840	6.3	850

**Table 3.1** Aggregate size and biomass prior to elicitation for cultures initiated with different aggregate size distributions.

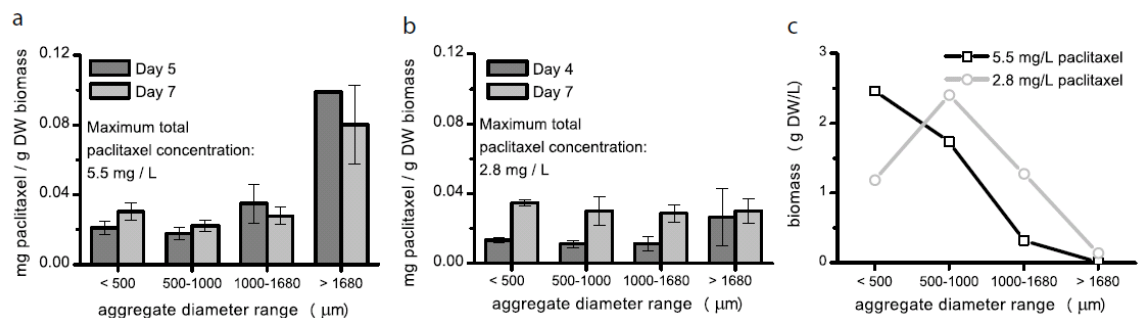
Culture designation	total biomass at culture initiation (g DW/L)	mean aggregate diameter (µm)	paclitaxel accumulation post elicitation
Small (Initiated <1000 µm)	4.6	880	Trace
Unfiltered	4.1	1440	None
Large (Initiated >1000 µm)	4.6	1750	None

**Table 3.2** Aggregate size and paclitaxel accumulation for cultures initiated from large aggregate distribution.



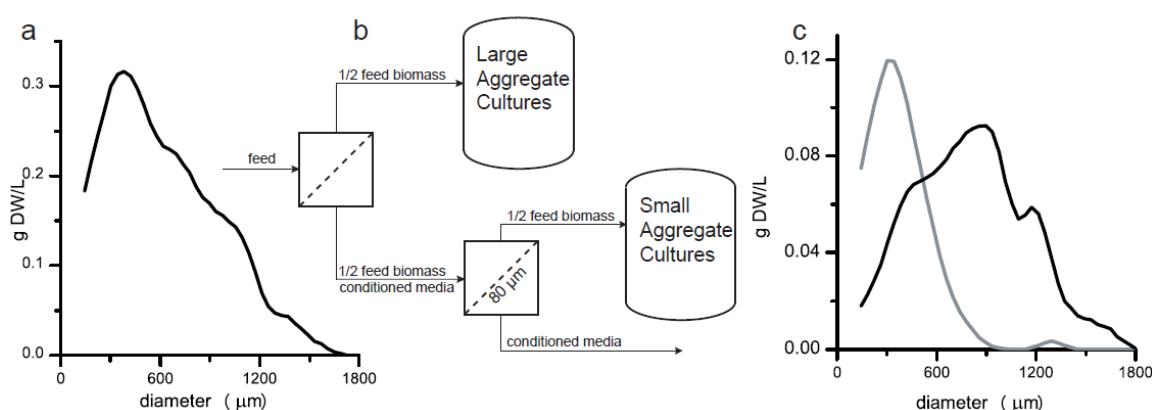


**Figure 3.1** Typical aggregates in *T. cuspidata* suspension culture. Fluorescent images stained with fluorescein diacetate to indicate viability, showing a wide distribution of aggregate sizes, and large irregularly shaped aggregates.

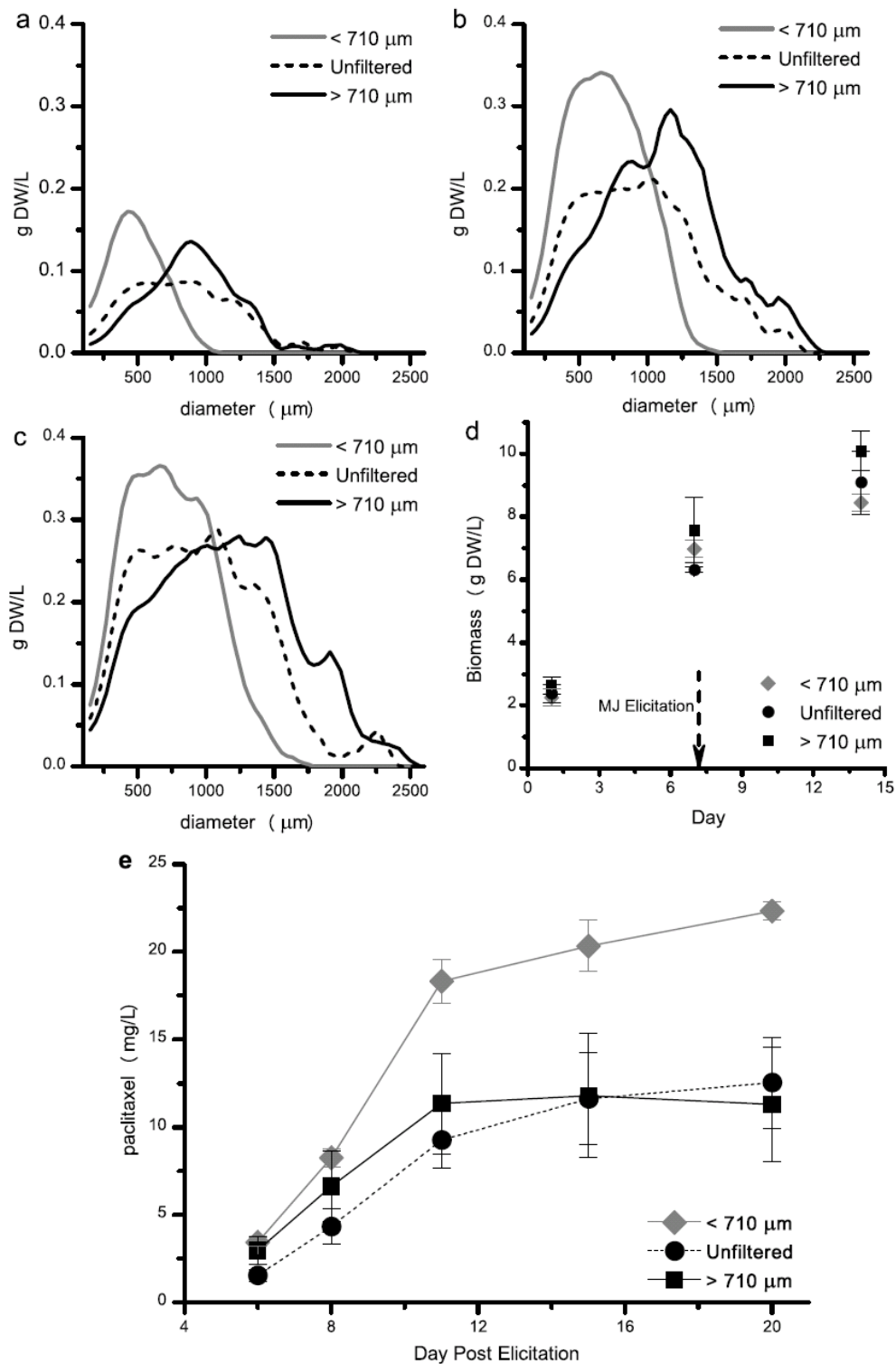


**Figure 3.2** Fractionation and analysis of aggregates of *T. cuspidata* P991 post elicitation with methyl jasmonate in two similar experiments. Filter sizes are indicated on the bottom axis. (a) Fractions collected five and seven days post elicitation, with day seven total paclitaxel concentration as indicated, error bars represent standard error from three replicate flasks. (b) Fractions collected four and seven days post elicitation with day seven total paclitaxel concentration as indicated. In a slightly modified procedure, replicate flasks were combined prior to fractionation and error bars represent standard error of three samples for each aggregate size class. (c) Crude aggregate size

distributions obtained based on total dry weight of aggregates collected in each size class, with black line corresponding to (a) and grey line corresponding to (b).

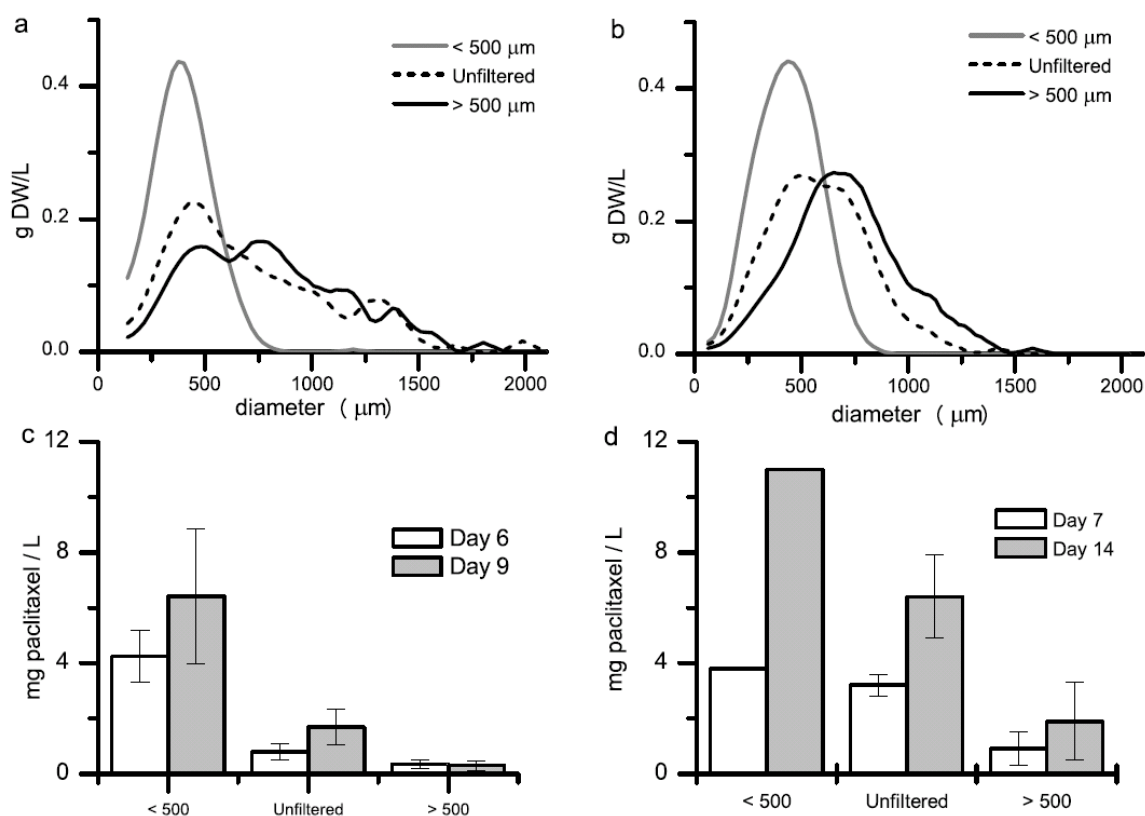


**Figure 3.3** Overview of filtration process to initiate cultures with differing aggregate distributions. (a) Based on the upstream distribution measured using a Coulter counter, a filter was chosen which corresponded as close as possible to the mean of the distribution. (b) Twice the normal culture broth (80 mL) used for subculturing was filtered, with half of the biomass caught on the filter, and half passed through and subsequently caught on an 80 μm filter. (c) Cultures that differed only in the aggregate size distribution and were almost identical in total biomass, defined as large aggregate cultures and small aggregate cultures, were thus initiated.

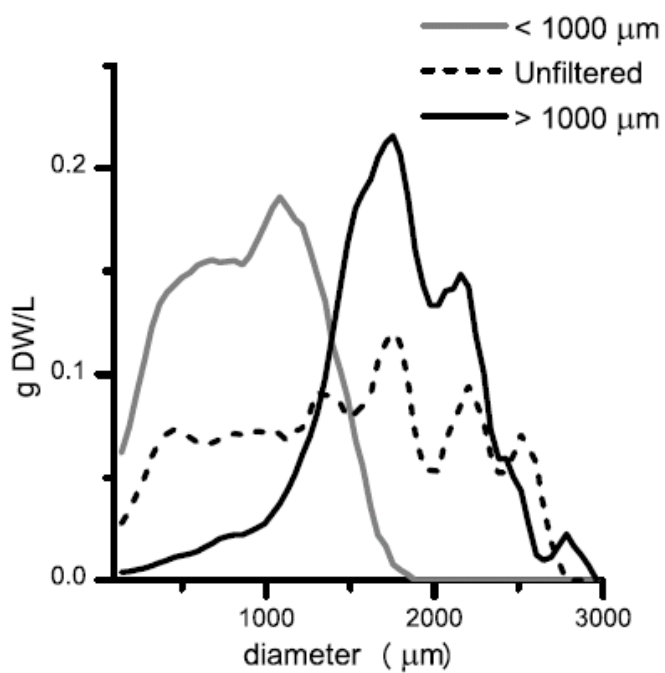


**Figure 3.4** Detailed time course profiles of aggregate size distributions and paclitaxel accumulation in *T. cuspidata* P93AF. (a), (b), and (c) Aggregate size distributions at culture initiation, immediately prior to elicitation, and one week post elicitation, respectively. (d) Total biomass obtained using size distributions and a previously

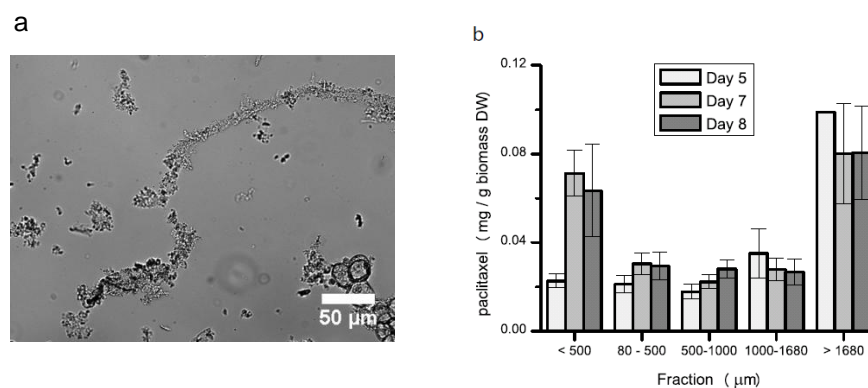
published correlation. (e) Total paclitaxel concentration (cell-associated plus extracellular) for three weeks post elicitation. All data points, including size distributions, are averages of three biological replicate flasks and error bars represent standard error.



**Figure 3.5** Aggregate size distributions of *T. cuspidata* immediately prior to elicitation with methyl jasmonate for (a) cell line P991C and (b) cell line P93AF. Total paclitaxel concentration post-elicitation for (c) cell line P991C and (d) cell line P93AF. Error bars represent standard error of three replicate flasks except for P93AF < 500 which represents duplicate flasks.



**Figure 3.6** Aggregate size distributions measured for *T. cuspidata* P991C after subculture initiation in which aggregates used for inoculum were filtered using a 1000  $\mu\text{m}$  filter. Cultures used to generate inoculum for this experiment had extremely large aggregates relative to typical *T. cuspidata* aggregate sizes.



**Figure 3.7** Cellular debris and its effect on aggregation fractionation experiments. (a) Brightfield microscope image of *T. cuspidata* P991 showing debris which is not visible in fluorescent images. (b) Fractionation experiment presented in Figure 3.2a, but including data representing biomass which was filtered through 500 μm and collected on Miracloth, compared to the second column which represents biomass which was filtered through 500 μm and collected on 80 μm.

## CHAPTER 4

### A POPULATION BALANCE EQUATION MODEL OF AGGREGATION DYNAMICS IN *TAXUS* SUSPENSION CELL CULTURES

The nature of plant cells to grow as multicellular aggregates in suspension culture has profound effects on bioprocess performance. Recent advances in the measurement of plant cell aggregate size allow for routine process monitoring of this property. We have exploited this capability to develop a conceptual model to describe changes in the aggregate size distribution that are observed over the course of a *Taxus* cell suspension batch culture. We utilized the population balance equation framework to describe plant cell aggregates as a particulate system, accounting for the relevant phenomenological processes underlying aggregation, such as growth and breakage. We compared model predictions to experimental data to select appropriate kernel functions, and found that larger aggregates had a higher breakage rate, biomass was partitioned asymmetrically following a breakage event, and aggregates grew exponentially. Our model was then validated against several data sets with different initial aggregate size distributions and was able to quantitatively predict changes in total biomass and mean aggregate size, as well as actual size distributions. We proposed a breakage mechanism where a fraction of biomass was lost upon each breakage event, and demonstrated that even though smaller aggregates have been shown to produce more paclitaxel, an optimum breakage rate was predicted for maximum paclitaxel accumulation. We believe this is the first model to use a segregated, corpuscular approach to describe changes in the size distribution of plant cell aggregates, and represents an important first step in the design of rational strategies to control aggregation and optimize process performance.

## 4.1 Introduction

Plant cell culture is an important technology for the renewable supply of many natural products which are difficult to produce by traditional methods such as natural harvest or chemical synthesis. Though dedifferentiated plant cells can be grown in suspension culture and cultivated on a large scale using bioprocesses similar to other industrial cell cultures, the application of this technology is limited by low yields, slow growth rates, the tendency of plant cells to aggregate, and variability in all of these properties (Huang and McDonald 2009; Kolewe et al. 2008). In particular, plant cells grow as aggregates from two to thousands of cells (50  $\mu\text{m}$  to well over 2000  $\mu\text{m}$  in diameter). Cell aggregation has long been implicated in affecting the metabolism of individual cells (Kubek and Shuler 1978; Street et al. 1965) and many attempts have been made to understand and quantify the effect of aggregates on secondary metabolite production (Eda Hiro and Seki 2006; Hanagata et al. 1993; Hulst et al. 1989; Kessler et al. 1999; Kolewe et al. 2011; Madhusudhan and Ravishankar 1996; Pepin et al. 1999; Zhao et al. 2003).

Despite the importance of aggregation as a fundamental characteristic of plant cell cultures, a rigorous characterization of aggregation dynamics has remained elusive. Detailed information regarding aggregate size distributions has traditionally been difficult to obtain, as the prevalent measurement technique, mechanical sieving (Kessler et al. 1999; Mavituna and Park 1987; Zhao et al. 2003), provides low resolution data. As a result, most studies have tended towards qualitative and semi-quantitative observations. Early studies based on microscopy indicated that an increase in cell aggregate size was correlated with culture growth, and that a decrease in aggregate size was observed once



growth stopped (Street et al. 1965; Wallner and Nevins 1973). Later studies confirmed the same behavior using size distributions obtained from sieves (Mavituna and Park 1987), and the idea of aggregates transitioning into different size classes over the course of batch subculture provided the basis for simple models to describe these changes (Hanagata et al. 1993; Kuboi and Yamada 1978). Various other techniques have been explored to measure aggregate size (McDonald et al. 2001; Pepin et al. 1999), but only recently has a reliable method been developed to enable the routine collection of aggregate size distributions (Kolewe et al. 2010). Aggregate size can now be monitored as any other process variable, enabling both a more comprehensive evaluation of process performance regarding metabolite production (Kolewe et al. 2011), as well as a more detailed mathematical description of aggregation dynamics, which is presented here.

A variety of models have been proposed to describe growth and product formation in plant cell cultures. Significant effort has been made to clarify the impact of nutrients on growth dynamics in plant suspension and hairy root cultures by developing models of increasing complexity (Cloutier et al. 2008; Curtis et al. 1991; Mairet et al. 2010; Vangulik et al. 1992). These models however are unsegregated and treat biomass as a culture-averaged parameter, and do not account for the heterogeneity of biomass induced by aggregation. The segregated models which have been developed typically divide biomass into general classifications (i.e., active and non-active) and use kinetic descriptions to account for growth dynamics (Bailey and Nicholson 1989; Schlatmann et al. 1999; Sirois et al. 2000; Vangulik et al. 1993). While providing some detail regarding the heterogeneity of biomass and indirectly accounting for aggregates of different sizes via the biomass classifications, these models do not explicate the phenomena underlying the transitions amongst classifications. A corpuscular approach has been used based on the cell cycle of individual plant cells (Degunst et al. 1990) but these results are difficult

to compare to experimental data as plant cells do not grow as single cells. In this work we also take a corpuscular approach, but model plant cell aggregates as the basic cellular unit.

Population balance equations (PBEs) are used to model particulate systems in a variety of fields and applications ranging from solid-liquid systems such as crystallization to emulsions in food processing to cellular systems (Ramkrishna and Mahoney 2002; Ramkrishna 2000). PBEs are used to describe how a particle distribution changes over time based on kernel functions representing the relevant phenomena which affect the particles. In applied biology, PBEs have been most often used for systems with single cell particles such as yeast or bacteria (e.g., Henson 2003; Kromenaker and Srienc 1991; Mantzaris et al. 1999; Tsuchiya et al. 1966). They have also been used to describe multicellular systems such as filamentous fungi (Lin et al. 2008; Liu et al. 2005) and plant hairy root cultures (Han et al. 2004). There are no reports to our knowledge of PBEs used to describe aggregates in plant cell suspension culture, a problem which presents unique challenges including the lack of mechanistic detail available regarding plant cell aggregate growth and breakage processes.

In this work, we present a PBE model to describe and predict changes in the size distribution of plant cell aggregates that are observed experimentally. We select kernel functions and fit unknown kernel parameters based on a comparison of model predictions and experimental data. The model is then validated against several non-fitted experimental datasets with different starting distributions. Finally, the model is used to qualitatively evaluate the effect of culture shear environment by predicting paclitaxel accumulation from aggregate distributions corresponding to different breakage rates,

demonstrating potential usefulness of the approach for developing process optimization strategies.

## **4.2 Materials and Methods**

### **4.2.1 Experimental**

*Taxus cuspidata* cell line P991 suspension cell cultures were maintained in our laboratory as previously described (Kolewe et al. 2010), and plant cell aggregate size distributions were measured using a Coulter counter technique (Beckman Coulter, Inc., Brea, CA) (Kolewe et al. 2010). The measured aggregate volume was previously shown to correlate with biomass dry weight with  $\rho = 2.44 \text{ g DW} / \text{mL}_{\text{Coulter Counter volume}}$  (Kolewe et al. 2010), so all distribution data are presented here in terms of dry weight. For batch experiments, 200 mL working volume shake flasks were run in triplicate, and samples were taken every 2-3 days for aggregate size distribution measurements via the Coulter counter method and extracellular sugar measurements via a YSI 2700 Select Biochemistry Analyzer (YSI Life Sciences, Yellow Springs, OH). To initiate cultures with different initial aggregate size distributions from the same inoculum pool, aggregates were passed over a sterile 500  $\mu\text{m}$  nylon mesh filter and the permeate was used to initiate cultures in triplicate with the same initial biomass concentration as unfiltered cultures, as described previously (Kolewe et al. 2011).

### **4.2.2 Model Formulation**

The PBE results from a number balance on particles with respect to a given state variable, in this case aggregate volume,  $v$ . Assuming that plant cell aggregates can both grow and break apart, the governing PBE includes terms for the disappearance of aggregates due to the formation of larger aggregates by growth, the disappearance of

aggregates due to the formation of smaller aggregates by breakage, and the formation of aggregates due to the breakage of larger aggregates, and is given by:

$$\frac{dn(v,t)}{dt} = g(v, S') - \Gamma(v)n(v,t) + \int_0^v p(v, v')\Gamma(v')n(v',t)dv' - \int_v^\infty p(v, v')\Gamma(v')n(v',t)dv' \quad (1)$$

where  $n(v,t)$  is the continuous number density function and  $n(v,t)dv$  is the number of aggregates in size range  $v$  to  $v + dv$  at time  $t$ ,  $g(v, S')$  is the growth rate for aggregates of size  $v$  and effective intracellular concentration of total sugar  $S'$ ,  $\Gamma(v)$  is the breakage frequency for aggregates of size  $v$ , and  $p(v, v')$  is the partitioning function describing the distribution of daughter aggregates of size  $v$  resulting from the breakage of mother aggregates of size  $v'$ , assuming each breakage event results in two daughter aggregates. The partitioning function is subject to the following constraints:

$$\int_0^\infty p(v, v')\Gamma(v')n(v',t)dv' = \int_0^\infty \Gamma(v)n(v,t)dv \quad (2)$$

ensuring the function is a proper probability density function and that the volume of daughter particles is equal to the volume of the mother particle from which the daughters were derived.

It is evident that biomass is lost due to cell death and biomass degradation once a plant cell suspension culture passes through its stationary phase (Bailey and Nicholson 1989; Sirois et al. 2000). As the PBE (1) is formulated to conserve volume, an additional sink term must be added to account for the biomass loss which is observed experimentally. The breakage of multicellular aggregates is not a natural process, but instead results from the shear stress that is part of the artificial *in vitro* suspension culture

environment. Breakage then would not be expected to occur cleanly along cell-cell boundaries and instead results in cell lysis and the generation of cellular debris, both of which contribute to a total reduction in biomass. This phenomenon was described by adding an additional term to (1), which accounted for the disappearance of aggregates associated with partitioning via a parameter,  $b$ , representing the fraction of biomass which does not partition into daughter particles upon a breakage event. This parameter was assumed to be independent of aggregate size, so all breakage events result in the same percentage of biomass loss. The resulting PBE is similar to (1) but includes an additional parameter to account for the loss in biomass associated with the aggregate birth term:

$$\frac{dN}{dt} = \dots \quad (3)$$

where  $b = 0$  corresponds to no biomass loss, in which case (3) reduces to (1), and  $b = 1$  corresponds to complete biomass loss. To capture time course changes in a batch culture, (3) was coupled to a substrate balance which accounted for substrate depletion as a result of cell growth:

$$\frac{dS}{dt} = \dots \quad (4)$$

where  $S$  is the total extracellular sugar concentration and  $Y$  is a constant yield coefficient. The intracellular substrate concentration was calculated from the extracellular substrate concentration using a first-order filter:

$$\frac{dS_i}{dt} = \dots \quad (5)$$

This phenomenological equation represents a number of physiological processes including substrate uptake and conversion to usable sugars, where  $\alpha$  is the rate constant for these lumped processes and describes how quickly cells respond to environmental changes (Zhu et al. 2000).

The determination of appropriate forms for the kernel functions  $\Gamma(v)$ ,  $p(v, v')$ , and  $g(v, S')$  was emphasized since one major objective of this study was to gain insights into growth and breakage phenomenon underlying the formation of plant cell aggregate distributions. Consequently, different functional dependencies were evaluated. The breakage frequency,  $\Gamma(v)$ , was considered to be either size independent or size dependent as follows:

$$\Gamma(v) = a, \Gamma(v) = av^{1/3}, \Gamma(v) = av, \quad (6)$$

where  $a$  is an adjustable parameter. Size independent breakage is often used for qualitative predictions (Vanni 2000) and was used to describe breakage of filamentous fungi (Lin et al. 2008). A power law dependence on particle diameter has also been used in a number of systems for better quantitative predictions (Vanni 2000) or where breakage is a result of shear (Barthelmes et al. 2003). For this case we limited the breakage frequency to be linearly dependent on diameter or linearly dependent on volume, as shown in (6). A generalized partitioning function, the extended Hill-Ng binary power-law product distribution (Diemer and Olson 2002), was used:

$$\frac{B(v, v')}{B(v)B(v')} = \frac{1}{1 + q} \quad (7)$$

where  $B$  is the beta distribution, and  $q$  is a parameter greater than zero. This function covers a broad range of distributions from equal partitioning where both daughter

aggregates are the same size ( $q \rightarrow \infty$ ), to a random or uniform distribution ( $q = 1$ ), to an asymmetric U-shaped distribution in which there is a higher probability for one small and large aggregate ( $0 < q < 1$ ), and to an erosion-type distribution, which describes the stripping of an infinitesimally small aggregate, ( $q \rightarrow 0$ ) (Fig. 4.1). The growth kernel,  $g(v, S')$ , was dependent on both aggregate size and effective substrate concentration,  $g(v, S') = r(v)m(S')$ . Several forms of size dependence growth were considered:

$$r(v) = \mu_{max}, r(v) = \mu_{max}v^{2/3}, r(v) = \mu_{max}v \quad (8)$$

where  $\mu_{max}$  is an adjustable parameter. The size independent expression,  $r(v) = \mu_{max}$ , has been used to describe the growth of single cell organisms (Nielson and Villadsen 1994; Zhu et al. 2000). The function where the rate of increase of the radius is constant,  $r(v) = \mu_{max}v^{2/3}$ , has been used to describe the growth of filamentous fungi (Nielson and Villadsen 1994), and corresponds to preferential growth of cells on the outer surface of an aggregate. The exponential growth expression,  $r(v) = \mu_{max}v$ , which corresponds to the uniform growth of all cells in the aggregate, has also been used to describe filamentous fungi (Lin et al. 2008). Monod saturation kinetics was used for substrate dependence of the growth rate:

$$\text{-----} \quad (9)$$

where  $K_m$  is an adjustable parameter.

### *Numerical Solution*

The PBE was solved by discretizing the number distribution and employing a fixed-pivot technique (Kumar and Ramkrishna 1996). The spatial derivatives were

approximated according to a method presented by David et al. (1991), and the resulting ODEs were solved using the MATLAB integration code ode45 (Mathworks, Natick, MA). This discretization method ensured that both total aggregate number and volume were conserved, which was deemed particularly important for this work as the total volume, or biomass, is a critical property. The accuracy of the numerical method was assessed using test cases of breakage only and growth only to confirm that total aggregate number and volume were in fact conserved.

To facilitate comparisons to model predictions, Coulter counter measurements were smoothed in MATLAB using a locally weighted scatterplot smoothing regression combined with a moving average. This histogram was then converted to a continuous number density, interpolated, and re-binned to produce a discretized distribution with variable spacing  $\propto v^3$  (uniform spacing in diameter) on a grid of 750 points, which was determined to be required for numerical solution convergence. As the minimum aggregate size which could be measured was larger than the smallest aggregates known to exist, the measured distribution was interpolated using cubic splines to  $n(v_{min}, t) = 0$ , where  $v_{min}$  is the minimum size of an aggregate, taken in this case to be a single cell with a diameter of 30  $\mu\text{m}$ . To directly compare model predictions to experimental data, simulated distributions were re-binned according to the original histogram grid spacing, and a residual error,  $\Psi$ , was calculated based on discretized the volume distribution  $N_v = v N(v, t)$ , as follows:

(13)

---



where  $N_v(v_i, j)$  and  $\hat{N}_v(v_i, j)$  are the measured and predicted, respectively, values of the discretized aggregate distribution at aggregate size  $v_i$  and experimental time point  $j$ , and the error is summed over  $N+1$  distribution points and  $M$  experimental time points. This residual allowed for a direct comparison of goodness-of-fit between distributions which differed significantly in their magnitudes, and values of  $\Psi$  were used to judge the quality of the model predictions.

### 4.3 Results

A representative dataset covering the range of aggregate sizes normally found in culture was used to structure and fit the model. Distributions shifted to larger aggregate sizes as the area under the aggregate curve increased, indicating that aggregate size increased concurrent with growth (Fig. 4.2a). Taking the first moment of the continuous number distribution (or equivalently the cumulative volume of the discretized volume distribution)

(14)

and converting to biomass, a growth curve providing an overview of the entire batch process was constructed (Fig. 4.2b). The growth curve could be divided into three general phases: an initial growth phase where substrate limitations are not present; a substrate-limited growth phase; and a stationary/death phase where growth is negligible and biomass is lost. To systematically identify appropriate kernel functions and parameters, these phases of the growth curve were simulated individually in an attempt to isolate the individual kernels, and then these pieces were integrated to simulate the entire growth curve.

#### 4.3.1 Stationary phase: breakage frequency and partitioning kernels

After sugar was depleted and growth stopped, breakage was the only phenomena which needed to be considered. Therefore, this data was used to identify the breakage and partitioning kernel functions independent of growth. The aggregate size distributions shifted to smaller aggregate sizes, and the area under the aggregate curve decreased (Fig. 4.3). A simplified breakage-only model, where the growth term was removed from (3), was used to discriminate (6) and parameterize (7), starting with Day 12 as the initial distribution and Day 16 as the final distribution. The best fit for each form of the breakage kernel was determined, where  $a$ , the breakage rate constant,  $q$ , the parameter determining the daughter distribution function, and  $b$ , the parameter accounting for the loss of biomass upon breakage, were chosen by trial-and-error to give the minimum  $\Psi$  value. Two combinations of breakage and partitioning fit the experimental data well: size-independent breakage combined with the partitioning most resembling an equal distribution ( $q = 50$ ,  $b = 0.3$ ) and  $v^{1/3}$  dependent breakage combined with a steep U-shaped partitioning function ( $q = 0.1$ ,  $b = 0.15$ ) (Fig. 4.3). Neither combination could be definitively selected based on this analysis, but breakage proportional to  $v$  was clearly worse than the other two combinations as indicated by both the residual error and a qualitative comparison. Therefore, this combination was not considered for subsequent analysis.

#### 4.3.2 Initial growth phase: growth rate kernel

Keeping the breakage and partitioning kernel functions and parameters fixed for the two best combinations from the death phase analysis, the growth rate functional dependency was evaluated using data from the initial growth phase, Day 0 to Day 6. This analysis clearly showed that an exponential growth kernel provided the best fit to the

experimental data (Fig. 4.4). While the kernel corresponding to growth of cells only on the aggregate surface yielded better results than size-independent growth for both breakage-partitioning combinations, this kernel did not show an increase in mean aggregate size as observed experimentally. As the exponential growth function was the only kernel which qualitatively fit the experimental data (Fig. 4.4c,f), the other growth kernels were not considered for further analysis. Even though the exponential growth kernel in combination with the size-dependent breakage kernel gave a slightly better quantitative fit than with the size-independent breakage kernel, the improvement was small and not significant enough to specify the breakage kernel.

To further differentiate between the two breakage and partitioning kernel combinations we incorporated the intracellular substrate concentration,  $S'$ , using (5) to account for the lag phase before growth, ran the simulation for an additional two days to allow comparison with another experimental data point, and simultaneously refit parameters for each of the kernel functions. The size-dependent breakage combination resulted in approximately 20% improvement over the size-independent combination (Fig. 4.5). The size-independent breakage kernel significantly overestimated the contribution of large aggregates, and this error was more pronounced at later time points (Fig. 4.5b). While the size-dependent breakage combination underestimated the contribution of small aggregates, particularly at the later time points (Fig. 4.5a), this combination fit the experimental data reasonably well and was used for further simulations.

### **4.3.3 Entire batch: substrate-dependent growth**

To simulate the time course of the entire batch culture, the additional parameters  $Y$  and  $K_m$  in (4) and (9) were estimated from data over the entire 16 day experiment, and Table I lists the final model parameters and functions used for these simulations. Figure 6

shows comparisons of measured and simulated results for: aggregate distributions from Day 0 to Day 12, corresponding to points where biomass continued to increase (Fig. 4.6a); the biomass growth curve (Fig. 4.6b); the mean aggregate diameter (Fig. 4.6c); the total number of aggregates (Fig. 4.6d); and the amount of sugar in the extracellular media (Fig. 4.6e). While the simulated distributions began to diverge from the measured distributions in the latter stages of the growth curve, the calculated results for total biomass and mean aggregate size fit the experimental data extremely well (Fig. 4.6b,c). The comparison of the total number of aggregates indicates that the model underpredicted the total number as culture time progresses (Fig 4.6d). This is due to an underestimation of small aggregates, which make up a much larger percentage of the total aggregate number compared to their contribution to the total biomass. Extracellular sugar was described reasonably well by the model, although the initial rate of depletion was underestimated (Fig. 4.6e), which was likely due to over-simplification of the uptake and storage of sugars as a first order process.

As the model was fit to the experimental data presented in Figure 4.6, it could be expected to agree reasonably well based on the number of adjustable parameters available. To test whether the model was sufficiently extensible to be used in a predictive capacity, we simulated several other data sets using the same kernel functions and model parameters as used in Figure 4.6. Aggregates from the same inoculum pool as used to generate Figure 4.6 were filtered to obtain a smaller initial distribution. The results indicate that our model can quantitatively predict the dynamic behavior of aggregate distributions that are significantly different from those used to fit the model (Fig. 4.7). In fact, the fit obtained in Figure 4.7 was slightly better than that in Figure 4.6 according to the  $\Psi$  measure, even though some qualitative behavior, such as a continuation of growth for several days past that observed experimentally (Fig. 4.7b), was slightly worse. To test

whether the model was sufficiently robust to describe cultures which may differ in underlying properties other than the initial aggregate distribution, we used data from batch experiments that were carried out one year previously. Again, the model provided reasonable predictions, particularly during the growth phase for the most critical properties of total biomass and mean aggregate size (Fig. 4.8), indicating the broad extensibility of the model over the range of aggregate sizes encountered under normal processing conditions.

#### 4.3.4 Predicted effect of breakage rate on paclitaxel accumulation

To demonstrate the potential usefulness of the model and illustrate how it could be used in the development of bioreactor operating strategies, we performed simulations to quantitatively predict paclitaxel accumulation under different breakage rates, which could correspond to different shear environments induced by agitation. Though the relationship between paclitaxel accumulation and aggregate size is not simple (Kolewe et al. 2011), we modeled it using a simple sigmoidal curve, where smaller aggregates produce more paclitaxel, and paclitaxel production decreases to the limit zero as aggregate size increases (Fig. 4.9a). An additional ODE was used to couple paclitaxel accumulation to the PBE:

$$\frac{dT}{dt} = q(v) - \mu T \quad (15)$$

where  $T$  is the paclitaxel concentration and  $q(v)$  is the paclitaxel production rate. As smaller aggregates produce more paclitaxel (Kolewe et al. 2011), an obvious strategy to enhance paclitaxel accumulation would be to increase the number of small aggregates by increasing the breakage rate. However, an increase in breakage will also result in a

greater loss of biomass according to our model. We performed a series of simulations starting from an experimentally determined aggregate distribution on Day 8, which is when cultures are normally elicited to stimulate paclitaxel production (Kolewe et al. 2011). The breakage rate constant,  $a$ , was varied to alter the effective breakage rate, and each simulation was run for two weeks post elicitation. The relative paclitaxel concentration is shown for a series of breakage rate constants in Fig. 4.9b. These results indicate that there is an optimum breakage rate to maximize paclitaxel accumulation, which occurred due to the balance of higher production in smaller aggregates with the loss of biomass at higher breakage rates.

## 4.4 Discussion

### 4.4.1 Kernel functions provide qualitative insight into aggregation phenomena

We have developed a population balance equation (PBE) model to predict the evolution of *Taxus* plant cell aggregate size distributions based on a phenomenological description of events which affect the aggregates. Rather than assume specific functional dependencies for the kernels representing the relevant phenomena from the outset, we evaluated a number of kernel functions that represented a range of realistic physiological possibilities, and selected the functions which best fit measured aggregate distributions from batch culture experiments. These phenomena can be difficult to measure independently, as breakage and partitioning, for instance, are inexorably linked, so this approach helps to provide insight into the qualitative nature of these processes. A size-independent breakage function has been previously used in cell-aggregating systems, though it was suggested that a size-dependent breakage function may be more appropriate (Lin et al. 2008). Though we did not account for shear effects explicitly, shear-dependent breakage has been modeled in other cellular systems using a size-dependence of  $v^{1/3}$

(Barthelmes et al. 2003). This description of breakage, while highly simplified from a mechanistic perspective, fit our data better than the size-independent function.

We found that the partitioning of aggregates after a breakage event was most accurately modeled with a slightly U-shaped distribution, where the most likely outcome was one small and one large aggregate. While more computationally demanding than a random distribution or a perfectly equisized distribution, the U-shaped function best fit the experimental data and was deemed to be the most physiologically plausible scenario. It was previously reported that cultures initiated with only large aggregates quickly developed a population of very small aggregates in tobacco culture (Kuboi and Yamada 1978), indicating that smaller pieces broke off the larger aggregates, rather than an alternative scenario of all aggregates breaking in half. We incorporated a loss of biomass associated with each breakage event to account for the biomass decrease observed in the stationary phase of batch culture. The nominal value for the biomass loss, corresponding to the parameter  $b$ , was found to be 20%. The effects of increased shear are most often associated with an impact on cellular metabolism (Gong et al. 2006), but these results suggest that an increased breakage rate (indirectly associated with increased shear in this study) would also lead to a direct loss of biomass, which may have a greater impact on culture performance.

By evaluating three different growth rate functions, we determined that the growth rate of individual aggregates appeared to be an exponential function of aggregate volume. This function implies that all cells within an aggregate are actively dividing, as opposed to the alternative growth functions considered such as size-independent growth or growth just on the aggregate surface. Exponential growth is probably not entirely mechanistically realistic, as diffusion limitations associated with larger aggregates (Curtis

and Tuerk, 2006; Hulst et al. 1989) will cause metabolic changes for cells in the interior. However, the PBE framework presented here is general enough that a more detailed description of aggregate metabolism and growth could be incorporated into the model. While the model fits based on these empirically determined kernel functions were qualitatively correct, higher quality fits than those presented here have been achieved with PBE models of other particulate systems. We considered this study to be a preliminary effort that demonstrates the utility of PBE modeling of plant cell aggregation and suggest that incorporation of additional mechanistic detail into the kernel functions will likely improve model fits and predictions.

#### **4.4.2 Utilization of the model as a predictive tool to guide operating strategies**

The PBE model presented here can both reproduce experimental data from batch cell cultures, and predict the behavior of batch experiments for which the model was not fitted. Importantly, we presented model predictions of the aggregate distributions, as opposed to only lumped descriptions such as total biomass and substrate concentration that are presented for PBE models in other aggregating systems such as plant hairy roots (Han et al. 2004) or fungal hyphae (Liu et al. 2005). This distinction is critical as our purpose for creating a segregated model was to describe the distribution of biomass, which has been shown to impact critical process parameters such as paclitaxel accumulation (Kolewe et al 2011). Furthermore, as we have shown that paclitaxel accumulation is affected not only by the mean aggregate size but also by the shape of the distribution, the ability to predict the full aggregate distribution is an important feature of this model. In contrast to a PBE model based on more detailed mechanistic descriptions of aggregation and growth where only one set of experimental data was described (Lin et al. 2008), we developed a model based on a more simplified depiction of the relevant



phenomena and demonstrated that it agreed well with non-fitted data sets. Thus, the model is appropriate for predicting process performance and running simulations to guide experimental investigations.

An important result obtained from simulation of different breakage rates was that there is an optimum breakage rate for the production of paclitaxel. While our analysis did not account for the potential negative impact of shear on cellular metabolism, this optimum was the result of a balance between the higher productivity of smaller aggregates and the loss of biomass associated with a higher breakage rate. These results point to the need for more sophisticated operating strategies aimed at managing aggregate size, as simply increasing aggregate breakage is not a viable approach. To this end, incorporating the effect of other process operating parameters, including shear and dissolved oxygen, as well as eventually including a more detailed structured description of nutrient utilization, would be highly desirable. Especially for plant cell cultures, where growth rates are very slow and culture performance is highly variable, simulations of culture conditions to optimize specific properties is highly preferable to large experimental arrays, which would be more suited to validation of the model predictions. This work represents an important step towards the development of strategies to control aggregate size, and the PBE model provides a basis to add additional details regarding both environmental conditions affected by reactor operating parameters as well as the state of individual cells within the aggregates.

#### **4.5 Additional numerical details**

The aggregate size distribution,  $n(v,t)$  was discretized such that

Utilizing a fixed-pivot technique where the particle population is represented by  $x_i$  in each size range  $v_i < x_i < v_{i+1}$ , the discretized form of the PBE is:

$$\frac{1}{N_i} \frac{dN_i}{dt} = \sum_{j=1}^M \left( \frac{1}{N_j} \frac{dN_j}{dt} \right) \frac{N_j}{N_i} \quad (16)$$

$$\frac{1}{N_i} \frac{dN_i}{dt} = \sum_{j=1}^M \left( \frac{1}{N_j} \frac{dN_j}{dt} \right) \frac{N_j}{N_i} \quad (17)$$

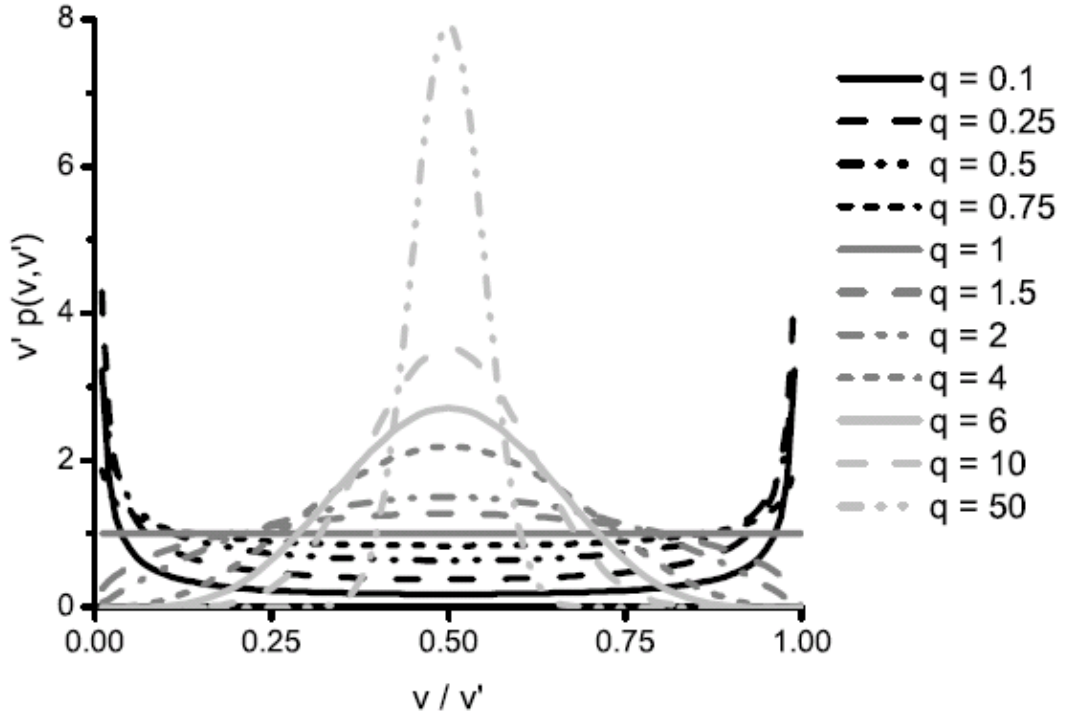
where  $p_{i,k}$  represents the contribution to the population of the  $i$ th size class from breakage of a particle size  $x_k$  (Kumar and Ramkrishna 1996). This method ensures that both total number and volume are conserved, which is particularly relevant for this work as the total volume, or biomass, is a critical property. Based on this approach,  $p_{i,k}$  is uncoupled from the dynamic behavior of the system, depending only on  $p(v,v')$  and the selection of grid points, and these integrals were calculated using an integral function in MATLAB (quadgk). As  $N_i$  are defined only at  $x_i$ ,  $n(v_i)$  were approximated:

$$\frac{1}{N_i} \frac{dN_i}{dt} = \sum_{j=1}^M \left( \frac{1}{N_j} \frac{dN_j}{dt} \right) \frac{N_j}{N_i} \quad (18)$$

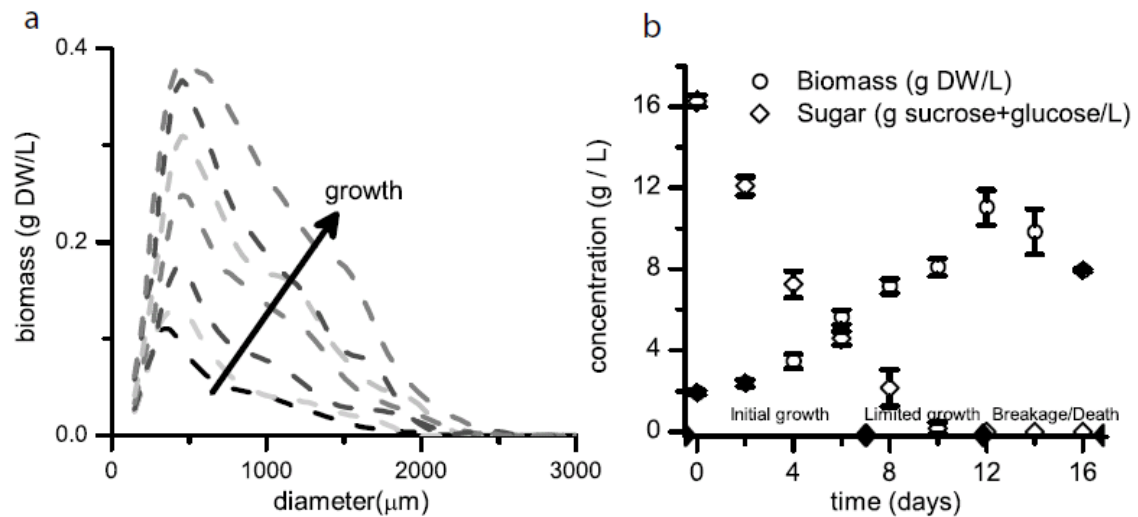
according to (David et al. 1991).

Function	Value
$\Gamma(v)$	$av^{1/3}$
$r(v)$	$\mu_{max}v$
Parameters	Value
$a$	0.00024
$b$	0.2
$q$	0.75
$\mu_{max}$	0.205 /day
$K_m$	1 g/L
$Y$	0.45
$\alpha$	0.75

**Table 4.1** Nominal functions and parameter values used for Figures 4.6-4.9.

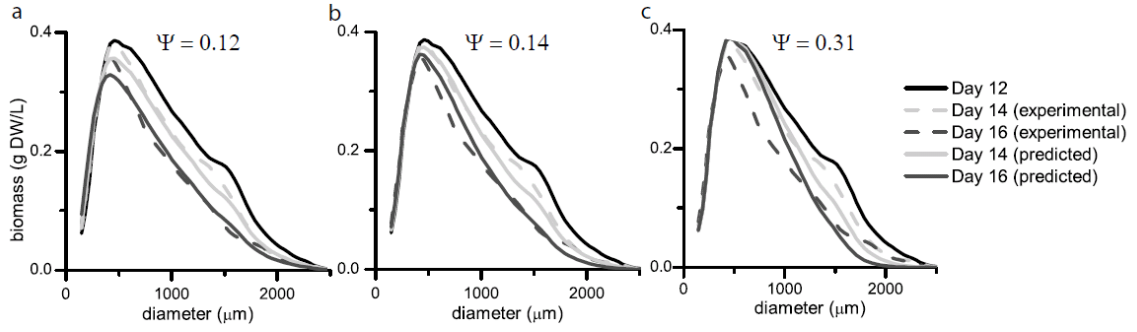


**Figure 4.1** Extended Hill-Ng binary power-law product partitioning function, where the parameter  $q$  determines the shape of the distribution. The daughter aggregate distribution resulting from the breakage of an aggregate in three limiting cases are:  $q = 0.1$ , a sharp U-shaped distribution with the highest probability of one small and one large aggregate;  $q = 1$ , a uniform distribution with all possibilities equally likely; and  $q = 50$ , with the highest probability of two equal sized aggregates.

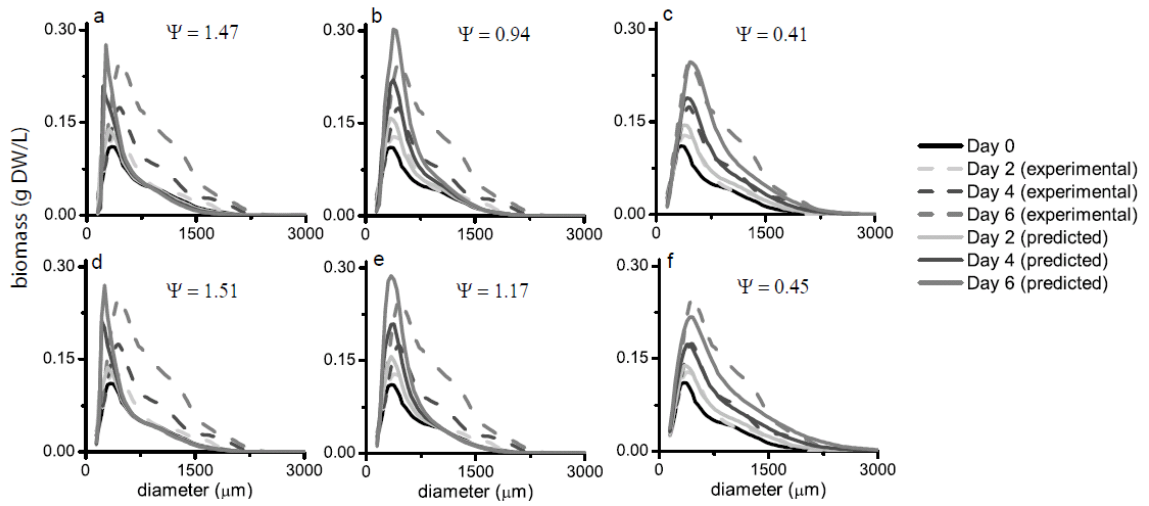


**Figure 4.2** Experimental data used to structure and parameterize the model. (a)

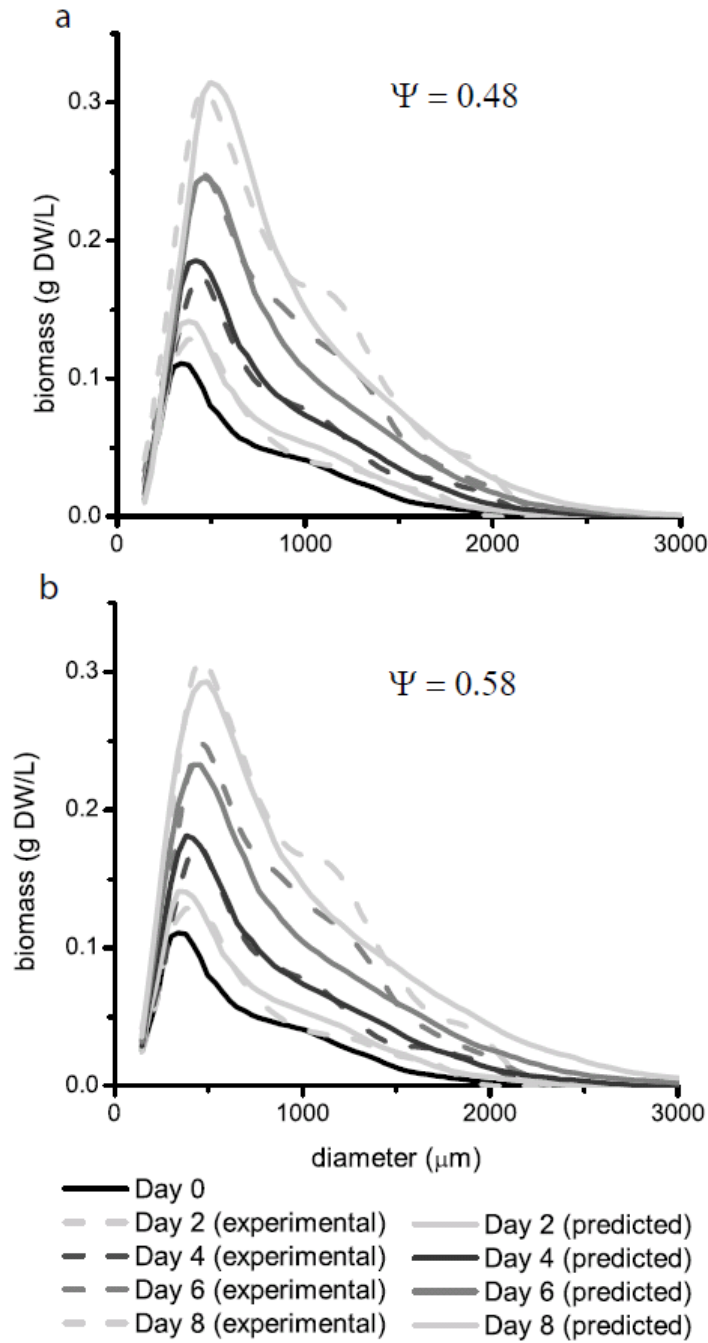
Aggregate size distributions for Day 0 through Day 12, the period over which total biomass increased. (b) Biomass growth curve, illustrating phases of initial growth which was not substrate limited, substrate-limited growth where substrate was being depleted, and a stationary/death phase once sugar has been depleted. Error bars represent standard deviation of three replicate flasks.



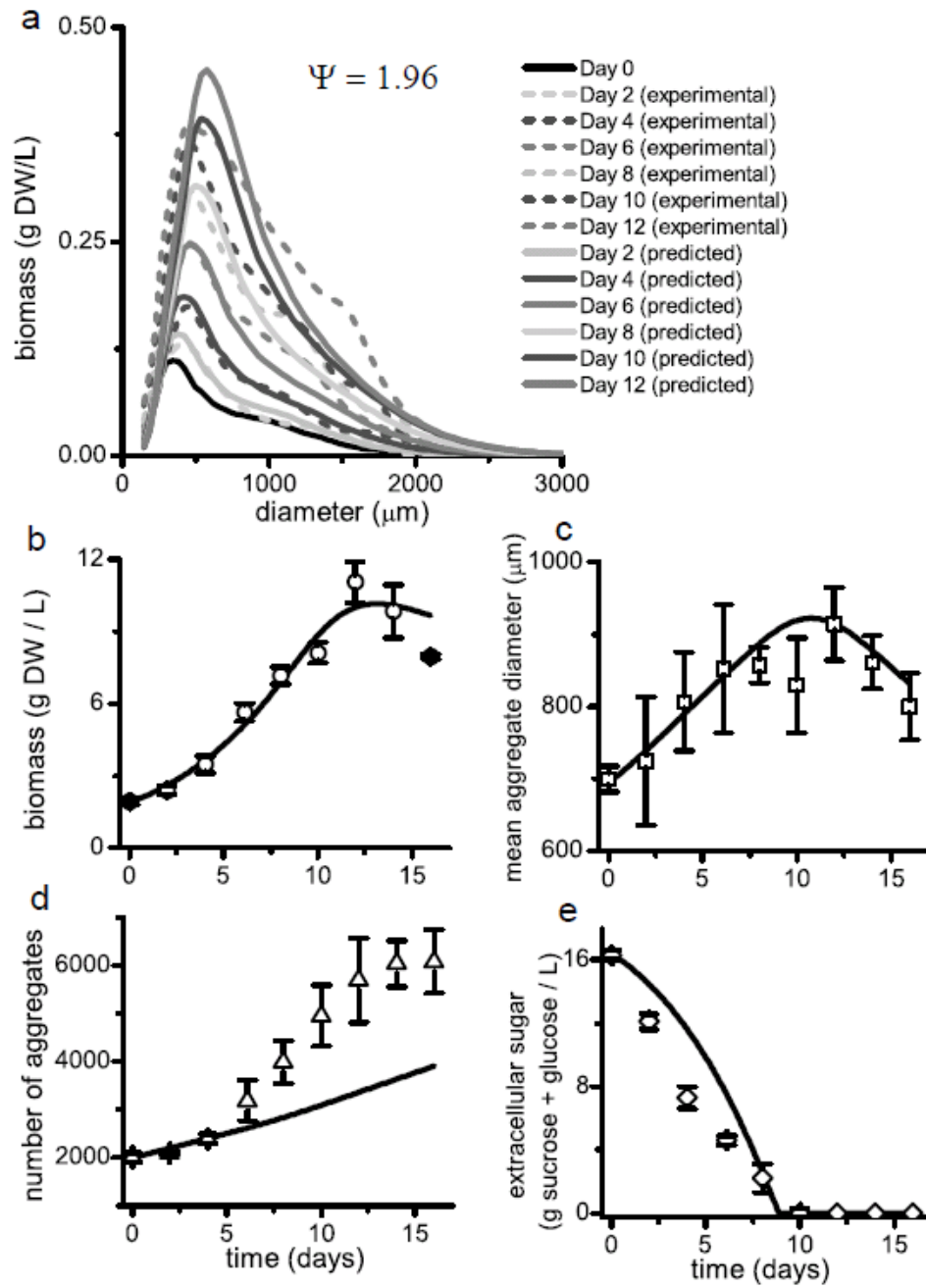
**Figure 4.3** Effect of the breakage kernel function on predicted distributions for a breakage only model, where model input was the Day 12 experimental distribution. (a)  $\Gamma = a$ , (b)  $\Gamma = av^{1/3}$ , and (c)  $\Gamma = av$ . In each case, the breakage rate constant, the partitioning function constant, and the breakage-associated biomass loss fraction were selected to minimize the residual error,  $\Psi$ .



**Figure 4.4** Effect of growth kernel size dependence on predicted distributions from the initial growth phase, Day 0 to Day 6, using the breakage combinations from the breakage-only model and fitting the specific growth rate,  $\mu_{max}$ , to minimize the residual error,  $\Psi$ . (a)  $\Gamma = av^{1/3}$ ,  $m = \mu_{max}$  (b)  $\Gamma = av^{1/3}$ ,  $m = \mu_{max} v^{2/3}$  (c)  $\Gamma = av^{1/3}$ ,  $m = \mu_{max} v$  (d)  $\Gamma = a$ ,  $m = \mu_{max}$  (e)  $\Gamma = a$ ,  $m = \mu_{max} v^{2/3}$  and (f)  $\Gamma = a$ ,  $m = \mu_{max} v$ .

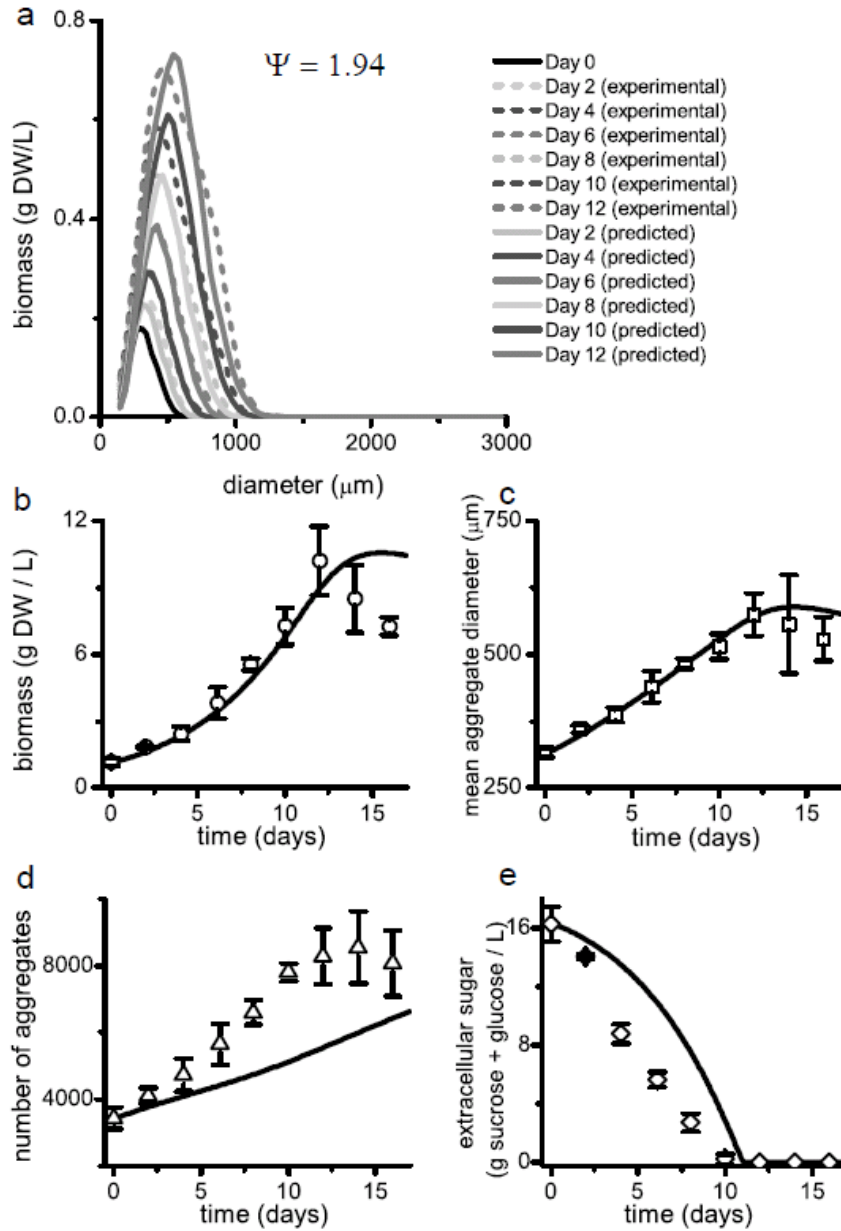


**Figure 4.5** Simulation of growth from Day 0 through Day 8. A linear, first-order differential equation for the intracellular substrate concentration was added to account for the slight lag phase, and all kernel functions were simultaneously refit to minimize the residual error,  $\Psi$ . (a)  $\Gamma = av^{1/3}$ ,  $m = \mu_{max} v$  and (b)  $\Gamma = a$ ,  $m = \mu_{max} v$ .

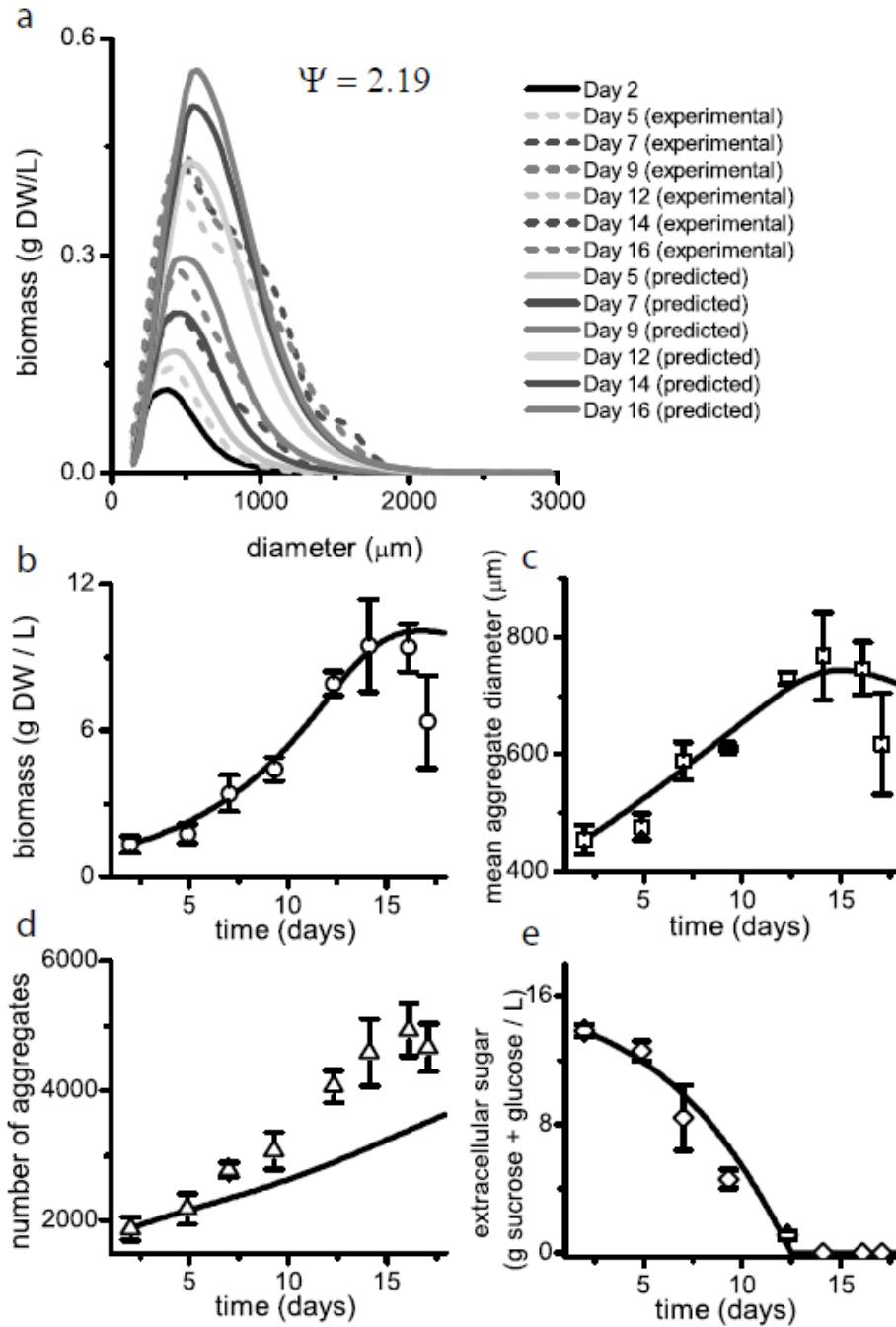


**Figure 4.6** Comparison of experimental data and model predictions for the entire batch process. (a) Aggregate distributions corresponding to the culture growth period, Day 0 to Day 12. (b) Total biomass growth curve. (c) Mean aggregate size. (d) Total number of aggregates. (e) Total extracellular sugar concentration. Error bars represent the standard deviation of three replicate flasks.

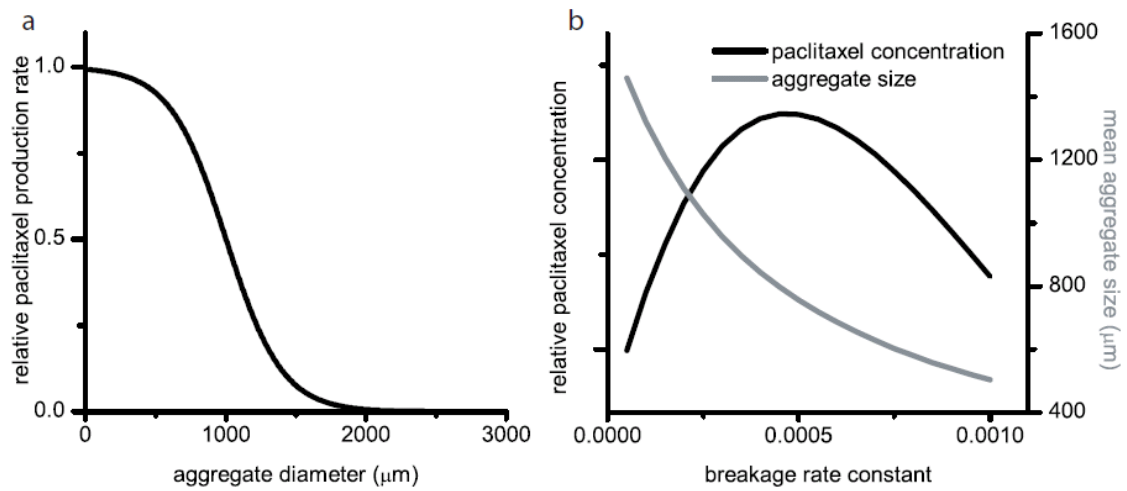




**Figure 4.7** Comparison of experimental data and model predictions for the entire batch process. The model is compared to a non-fitted dataset with a significantly smaller initial aggregate distribution than that used for parameter estimation (Fig. 6). (a) Aggregate distributions corresponding to the culture growth period, Day 0 to Day 12. (b) Total biomass growth curve. (c) Mean aggregate size. (d) Total number of aggregates. (e) Total extracellular sugar concentration. Error bars represent the standard deviation of three replicate flasks.



**Figure 4.8** Comparison of experimental data and model predictions for the entire batch process. The model is compared to a non-fitted dataset obtained one year earlier than that used for parameter estimation (Fig. 6). (a) Aggregate distributions corresponding to the culture growth period, Day 2 to Day 16. (b) Total biomass growth curve. (c) Mean aggregate size. (d) Total number of aggregates. (e) Total extracellular sugar concentration. Error bars represent the standard deviation of three replicate flasks.



**Figure 4.9** Simulated paclitaxel accumulation under different shear conditions. (a) Sigmoidal representation of the dependence of paclitaxel production on aggregate size. (b) Total paclitaxel accumulation and mean aggregate size after two weeks in batch cultures where different shear environments were simulated by adjusting the breakage frequency constant  $a$ .

## CHAPTER 5

### ON THE RELATIONSHIP OF SINGLE CELL HETEROGENEITY TO AGGREGATION

The nature of plant cells to grow as aggregates in suspension culture has important implications on the metabolism and function of the individual cells that make up the aggregates. Microenvironments within these aggregates are created due to nutrient limitations, cell-cell signaling environments, and applied shear forces arising from the fluid dynamics of the agitated *in vitro* culture environment. Though aggregation is generally assumed to be linked to the heterogeneity of cell populations which has been previously observed, no explicit, quantitative connection between aggregation and heterogeneity has been demonstrated. In this study, we isolated cell populations from aggregates of different sizes, and assessed these cells using flow cytometry for both primary metabolic activity, via cell cycle participation, and secondary metabolic activity, via paclitaxel accumulation. Our results indicated that cells isolated from smaller aggregates contained higher levels of paclitaxel, while cells isolated from larger aggregates were more actively participating in cell division. These results demonstrate a direct link between aggregate size and cellular heterogeneity, and also suggest an inverse relationship between primary metabolism and secondary metabolism in individual cells.

#### 5.1 Introduction

Plant cell culture technology is a renewable and scalable production alternative for important plant-derived natural product pharmaceuticals when traditional commercial supply routes such as natural harvest or chemical synthesis are unfeasible due to environmental scarcity or chemical complexity. Bioprocesses consisting of dedifferentiated plant cells in suspension culture can be scaled up using similar

approaches to industrial-scale microbial and mammalian cell culture processes, and have demonstrated worldwide commercial success to supply several important products, most notably the anti-cancer drug paclitaxel (Exposito et al. 2009). However, in addition to slower growth rates and lower yields compared to other cell culture systems, the more widespread application of plant cell culture technology is also limited by unique difficulties including the nature of plant cells to grow as aggregates, cell population heterogeneity, and lack of complete genetic information for most medicinal plant species (Kolewe et al. 2008; Lee et al. 2010). In particular, plant cells remain connected after cell division, and form aggregates of up to thousands of cells which can reach sizes of several millimeters. Aggregation has long been recognized as a critical property of plant cell cultures, and significant effort had been made to understand the effect of aggregate size on culture properties such as secondary metabolite accumulation (Edahiro and Seki 2006; Hanagata et al. 1993; Hulst et al. 1989; Kessler et al. 1999; Kinnersley and Dougall 1980; Kolewe et al. 2011; Madhusudhan and Ravishankar 1996; Pepin et al. 1999; Zhao et al. 2003) and growth (Capataz-Tafur et al. 2011; Dubuis et al. 1995; King et al. 1973; Watts et al. 1984; Yang et al. 1994), as a means to develop process improvement strategies.

In larger aggregates, cells are subject to different microenvironments with respect to nutrient availability, cell-cell signaling, and applied fluid shear forces. Explicit measurements of nutrients or other chemicals within aggregates are very difficult, and as a result, most information available regarding the nature of these microenvironments is based on theoretical predictions. Significant effort has been made to model oxygen diffusion in aggregates as nutrient limitations are generally assumed to be the predominant effect of aggregate size (Curtis and Tuerk 2006; Hulst et al. 1989; Pepin et al. 1999), and the predictions of transport limitations have been qualitatively validated by studies showing a lower consumption of oxygen by larger aggregates (Ananta et al. 1995;

Dubuis et al. 1995; Zhong et al. 2002). In many cases, however, the typical range of aggregate sizes found in culture is less than the critical size where nutrient limitations are predicted to be dominant (Kessler et al. 1999). In these cases, shear exposure and cell-cell interactions would be the dominant environmental variations affecting cells in aggregates of different sizes. Regardless of the particular condition primarily responsible for changes in cell metabolism and/or function, the effect of microenvironments on individual cells is generally inferred on the basis of properties of the entire culture or lumped measurements of aggregates in a particular size class. Most observations regarding changes in cell function have been based on the analysis of microscope images, which has been very useful in identifying cells which begin to differentiate in very large aggregates (Ellis et al. 1996; Hoekstra et al. 1990; Kuboi and Yamada 1978; Xu et al. 1998), and has also been used to quantitatively identify cells which accumulate colored metabolites such as anthocyanin (Pepin et al. 1999). Unfortunately, many critical properties regarding both primary and secondary metabolism cannot be identified on the basis of imaging alone.

To study individual cells and characterize heterogeneous cell populations, flow cytometry is most often used as it can provide quantitative information regarding specific properties of single cells (Shapiro 2003, Dolezel et al. 2007). For plant cells growing as aggregates, this presents a challenge as single cell particles rather than aggregates must be used for analysis. Depending on the particular property of interest different types of particles can be used. Analysis of cell cycle activity and participation can be used assess cell growth and primary metabolic activity, and only requires isolated nuclei. Nuclear DNA content is typically used to differentiate cells in active phases of the cell cycle, while the detection of thymidine analogs which have been incorporated into nuclear DNA can be used to differentiate between actively cycling and non-cycling cells. Previously, the incorporation

of BrdU (bromodeoxyuridine) into DNA could only be detected via immunofluorescence (Gratzner 1982; Yanpaisan et al. 1998; Naill and Roberts 2005a), but more recently, a simpler method has been developed based on the click chemistry detection of EdU (5-ethynyl-2'-deoxyuridine) (Salic and Mitchison 2008), and has been further demonstrated in plants (Kotogany et al. 2010). Analysis of non-pigmented secondary metabolites requires immunofluorescent methods, and in the case of *Taxus cuspidata* cells, also requires single cell particles with intact cell walls (Naill and Roberts 2004), as a large percentage of paclitaxel is found in the cell wall (Roberts et al. 2003). Flow cytometric approaches have been used to identify heterogeneous populations in a variety of systems with respect to cell cycle participation (Lucretti et al., 1999; Yanpaisan et al. 1998; Naill and Roberts 2005a) as well as secondary metabolism (Naill and Roberts 2005b), but this heterogeneity has only been putatively linked to aggregation, and has not been explicitly demonstrated.

In this work, we explored the effects of differential microenvironments associated with aggregate size on the properties of cell populations isolated from these aggregates. Specifically, we fractionated aggregates by size, and isolated either nuclei or intact single cells from these size fractions. To evaluate the primary metabolic activity of the cell populations, we measured nuclear DNA content to determine the distribution of cells in different cell cycle phases, and also identified those nuclei which had incorporated EdU as a measure of cell cycle participation. To evaluate secondary metabolic activity, we stained cells with a paclitaxel antibody and compared the relative fluorescence between samples. The results help to explain the source of heterogeneity which has previously been observed in plant cell culture systems, provide insight into the effect of aggregate microenvironments on the behavior of individual cells, and also suggest what the critical characteristics of these microenvironments may be.

## **5.2 Materials and Methods**

### **5.2.1 Cell Culture Maintenance**

*Taxus cuspidata* P93AF and *Taxus canadensis* C093D cell lines were provided by the United States Plant Soil and Nutrition Laboratory (Ithaca, NY) and used in all the experiments. Every 2 weeks, cells were subcultured into fresh medium which consisted of Gamborg's B5 basal salts (3.2g/L) with 20g/L of sucrose, supplemented with growth regulators 2.7  $\mu$ M naphthalene acetic acid (NAA) and 0.1  $\mu$ M benzylaminopurine (BAP), and adjusted to a pH of 5.5 prior to autoclaving. 150 mg/L citric acid, 150 mg/L ascorbic acid, and 900 mg/L glutamine were filter sterilized, and added post-autoclave. Suspensions were maintained in 500 ml Erlenmeyer flasks capped with Bellco (Vineland, NJ) foam closures at 23 °C and 125 RPM in gyratory shakers in the dark. Subculture transfers were done with 40 ml of inoculum (corresponding to a packed cell volume of 8-9 mL) originating from a 14-day old suspension culture in 160ml of fresh medium.

### **5.2.2 Analysis of Single Cell Paclitaxel Accumulation**

For analysis of secondary metabolic activity, cultures were elicited with 150  $\mu$ M methyl jasmonate on day 7 post-transfer. Five days post-elicitation, cultures were partitioned into fractions based on aggregate size by sieving through nylon mesh (80, 500, 1000, 1680 and 2000  $\mu$ m pore size), and washed via immersion and gentle swirling in 100 mL B5 basal salt solution. The isolated aggregate fractions were enzymatically digested with pectolyase Y-23 (MP Biomedicals, Solon, OH) (0.5% w/v) and cellulase (Sigma Aldrich, St. Louis, MO) (0.04% w/v) to obtain single cells, as described previously (Naill and Roberts, 2004). Single cells isolated from all the aggregate fractions were counted using a hemacytometer, and resuspended to achieve a density of approximately  $10^6$  cells/ml, followed by fixation with 1% (w/v) paraformaldehyde at 4 °C for 1 hour. Post-



fixation, 1mL of cells from each digest were stained for paclitaxel content with the primary monoclonal anti-paclitaxel antibody (Cardax Pharmaceuticals, HI) at a ratio of 1:200 in BPT buffer (0.25% BSA, 0.05% Triton-X, 0.02% sodium azide in 10 mM PBS) for 1 hr at room temperature, periodically inverting sample containers to ensure adequate mixing, and washed three times with 1mL BPT. Primary antibody incubation was followed by incubation with the secondary PE-conjugated antibody (Southern Biotech, AL) at a ratio of 1:200 at BPT for 1 hr at room temperature and washed three times in 1 mL BPT before resuspending in 0.5 mL BPT for analysis on the flow cytometer. As a control, cells were stained with the secondary PE-conjugated antibody only (Gaurav et al., 2010).

For single cell analysis, a Becton Dickinson (San Jose, CA) LSRII analytical flow cytometer equipped with an argon laser tuned to 488 nm was used. The cytometer setup, quality control procedure and optimization of laser and fluidics performance were performed according to the instructions in the BD LSR II User's Guide. For each measurement, a minimum of 10000 events were collected. The control (secondary PE-conjugate only) was run at the same settings as the sample and was used to set the level of background fluorescence. Mean PE-fluorescence was used as an indicator of paclitaxel content in each of the fractions.

### **5.2.3 Analysis of Cell Cycle Participation**

In order to initiate cultures which had different aggregate size distributions but were alike in all other properties, aggregates were filtered prior to inoculation as previously described (Kolewe et al., 2011). Two sets of biological replicate cultures were initiated, in separate experiments, with both *T. cuspidata* and *T. canadensis* cell cultures, where each set of replicates had dissimilar aggregate size distributions and will be

referred to as large aggregate cultures and small aggregate cultures. A Multisizer 3 Coulter counter equipped with a 2,000  $\mu\text{m}$  aperture (Beckman Coulter, Brea, CA) was used to measure the aggregate size distribution of the cultures. For analysis of cell cycle participation, EdU (5-ethynyl-2'-deoxyuridine) (Invitrogen, Carlsbad, CA) a terminal alkyne containing a nucleoside analog of thymidine was used. Individual cultures were incubated with 10  $\mu\text{M}$  EdU for approximately one length of the active cell cycle (~ 40 hours) (Naill and Roberts 2005), prior to the isolation of nuclei. For each experiment, aggregates from a specific culture were fractionated to compare the growth of cells originating from different aggregate sizes. For both experiments, three aggregate fractions were chosen to divide the aggregate distribution into three equal fractions by biomass. Small aggregate cultures were divided into fractions of < 300  $\mu\text{m}$ , 300 – 710  $\mu\text{m}$ , and > 710  $\mu\text{m}$ , and large aggregate fractions were divided into fractions of < 710  $\mu\text{m}$ , 710 – 1320  $\mu\text{m}$ , and > 1320  $\mu\text{m}$ .

Approximately 0.5 g of biomass from flasks that had been incubated with EdU were obtained from either fractionated aggregates (see section 5.2.2) or a well-mixed culture sample. 1 mL of Galbraith buffer (45 mM  $\text{MgCl}_2$ , 30 mM sodium citrate, 20 mM 3-(N-morpholino)-propanesulfonic acid (MOPS), 1% (v/v) Triton X-100, pH 7.0) at 4 °C was added to the biomass sample in a petri dish, and the aggregates were chopped ~ 500 times with a sharp razor to disrupt cell walls and allow nuclei to be released. An additional 2 mL of Galbraith buffer was added to resuspend the chopped biomass, and this suspension was successively filtered through 80  $\mu\text{m}$  and 22  $\mu\text{m}$  nylon mesh to remove tissue fragments and whole cells. To differentiate cell cycle phases, 0.75 mL of the filtered solution was aliquoted, 1 mg/mL RNase was added, followed by 1 mg/mL propidium iodide. The samples were stained for 30-60 minutes before analysis on the flow cytometer, where forward scatter, side scatter, and PI fluorescence data were

collected. A minimum of 5000 events were collected in the gated region of a forward scatter and side scatter plot, corresponding to the isolated nuclei. DNA histograms (Coefficient of variation < 5%) obtained by PI staining were used to differentiate between cell-cycle phases. The percentage of cells in the different cell-cycle phases was calculated using the Watson Pragmatic Model of FlowJo (v7.6) software (Tree Star, Inc., San Carlos, CA).

The remaining nuclei solution (approximately 2.25 mL) for each sample was centrifuged at 700 g and 4 °C for 3.5 minutes, supernatants were removed, and the nuclei were incubated with 250 µl EdU detection cocktail (Invitrogen, Click-iT EdU Alexa Fluor 488 Flow Cytometry Assay kit, cat no: C35002) at room temperature for 30 minutes. For one sample reaction (250 µl), the following amounts of kit components were used: 219 µl of 1X Click-iT Reaction buffer (Component G), 5 µl Copper (II) sulfate solution (Component H, 100 mM aqueous CuSO<sub>4</sub>), 1.25 µl Alexa Fluor 488 azide (Component B) and 25 µl 1X buffer additive (component I) 2 mL of Galbraith's buffer was added to the samples as a wash, samples were centrifuged at 700 g and 4 °C for 3.5 minutes, and resuspended in 0.5 mL Galbraith's buffer for subsequent staining and analysis. 1 mg/mL of RNase was added followed by 1 mg/mL 7-AAD, and samples were incubated for 30-60 minutes at room temperature before analysis on the flow cytometer. Forward scatter and side scatter were used to differentiate nuclei from debris, and a logarithmic scale histogram of Alexa-488 was used to assess EdU incorporation. A sample of nuclei isolated from a flask that had not been incubated with EdU was used to set the background fluorescence, and the percent positive population was identified using the Overton cumulative histogram subtraction of FlowJo (v7.6) software, which is an algorithm that essentially subtracts histograms on a channel-by-channel basis to provide a percent of positive cells.

## 5.3 Results

### 5.3.1 Heterogeneity in Paclitaxel Accumulation

To evaluate the effect of aggregate size on the paclitaxel accumulation patterns of individual cells in *Taxus* cell culture, cultures were elicited with methyl jasmonate, and aggregate fractions were isolated using a series of sieves. Fractionation with sieves is a common technique used to explore the effect of aggregation on secondary metabolite accumulation, and here we extend this approach to analyze individual cells isolated from aggregates of different sizes, rather than the lumped biomass of each aggregate size fraction. These aggregate fractions of different sizes were then enzymatically digested to form single cells, and the single cell yield, defined as the percentage of single cells compared to total number of aggregates after digestion, was found to be similar from aggregates of different sizes. This result was expected as aggregates are typically broken into pieces of successively smaller size due to the combination of enzymes and agitation, as opposed to having single cells peeled off in layers. Single cells were identified from samples run on the flow cytometer based on forward and side scatter properties, and gated to eliminate cell debris (Fig. 5.1a). The relative PE-fluorescence was then used to compare the paclitaxel content of single cells from these different populations (Fig. 5.1b,c), where an increase in PE-fluorescence indicated higher paclitaxel levels, as all samples were stained with the same concentration of primary and secondary antibodies at the same cell density (Gaurav et al., 2010). Single cells isolated from smaller aggregate fractions had higher mean paclitaxel levels, while single cells isolated from a sample of unfiltered aggregates had mean paclitaxel levels in the middle (Fig. 5.1c), as would be expected. Single cells isolated from the smallest aggregates exhibited the broadest range of paclitaxel accumulation levels (Fig 5.1d), indicating that while the highest producing cells originated from the smallest aggregates, not all cells isolated from these aggregates

would be classified as high-producers. This result can be attributed in part to aggregate breakage, where aggregates of a specific size class, particularly the smallest aggregates, may contain a percentage of aggregates that result from larger aggregates dissociating. The breakage of aggregates into smaller aggregates has been observed in several reports (Kuboi and Yamada 1978; Hanagata et al. 1993) and a more detailed understanding of this phenomenon is one aim of the population balance equation model of aggregation dynamics that we have developed (see Chapter 4).

This analysis indicates the presence of intra-cultural heterogeneity within a culture with respect to paclitaxel production, which agrees with previous studies (Naill and Roberts 2005b), and suggests that aggregation is one of the key parameters responsible for this variation. Flow cytometric analysis of individual cells directly assesses cell-associated paclitaxel, and is a better indicator of secondary metabolic activity in aggregates of different sizes than analysis of biomass-associated paclitaxel of the entire aggregate, which may include noncell-associated paclitaxel stored in the apoplast or hollow spaces. These data also agree with previous results where cultures with smaller aggregates accumulated higher levels of paclitaxel than cultures with larger aggregates (Kolewe et al., 2011). Though examination of secondary metabolite accumulation from the cell population perspective does help to explain the process performance profiles of these cultures and also provides direct evidence regarding the source of population heterogeneity within a particular culture, it does not clarify the differences in metabolic variation responsible for this heterogeneity or provide a more fundamental explanation of how aggregate size affects individual cell metabolism. An analysis of cell cycle activity would provide a more complete description of cell metabolism than the analysis of secondary metabolite accumulation alone.

### 5.3.2 Cell cycle heterogeneity

To assess the effect of aggregate size on cell cycle activity, cultures with different aggregate size distributions were initiated, which allowed for comparisons between different cultures as well as for different sized aggregates within individual cultures. These experiments were carried out with two separate cell lines, and the dissimilar aggregate distributions which were initiated (Fig. 5.2) provided a wide range of aggregate sizes to evaluate. An overview of these intra-culture trends is shown for both *T. cuspidata* (Fig. 5.3) and *T. canadensis* (Fig. 5.4) at several time points. DNA content analysis can differentiate between  $G_0/G_1$ , S, and  $G_2$  nuclei, and here we present a combined percentage of S/ $G_2$  as an indicator of cells actively undergoing cell division. The incorporation of EdU can be used to differentiate between non-cycling ( $G_0$ ) and cycling ( $G_1/S/G_2$ ) cells as only cells which have replicated their DNA during the incubation period would incorporate EdU into their DNA. For both cell lines and in both small aggregate cultures and large aggregate cultures, at later time points, primary metabolism was reduced as fewer cells were actively dividing based on the analysis of DNA content and fewer cells were participating in the cell cycle based on EdU incorporation. For instance, on Day 12 compared to Day 7 in the small aggregate *T. cuspidata* cultures, there was an average of 45% fewer S/ $G_2$  nuclei and 58% fewer EdU positive nuclei in the three aggregate fractions, and similar decreases were evident in all of the cultures. This result is expected as growth slows down as a batch culture progresses, and it has been previously observed that the percentages of S and  $G_2$  cells were higher in the early exponential stages of batch culture (Naill and Roberts 2005a). These results confirm that both staining methods were suitable to compare cell cycle activity amongst different samples.

The general trend comparing aggregates from within the same cultures indicated that larger aggregates contained more actively dividing cells than smaller aggregates (Fig. 5.3, 5.4). In several cases, these trends were clearly evident from a qualitative perspective. For example, in large aggregate cultures of *T. cuspidata* (Fig 5.3b) and small aggregate cultures of *T. canadensis* (Fig 5.4a) data indicated that larger aggregates had a much higher percentage of cells classified as S/G<sub>2</sub>, at all time points. To quantify these results, we compared the largest fraction (> 1320 µm) to the smallest fraction (< 300 µm) for each cell line at each time point (Table 5.1). The largest aggregates contained from 11% to 220% more G<sub>2</sub>/M nuclei, and from 3% to 62% more EdU positive nuclei, and these relative differences tended to be much greater at later time points. Another measure of the increased primary metabolism in larger aggregates is the percent increase between every two neighboring fractions (i.e. 300 – 710 µm compared to < 300 µm; > 710 µm compared to 300 – 710 µm, and so on), averaged over every time point and every culture. In total, larger aggregates of *T. cuspidata* had 23% more G<sub>2</sub>/M nuclei and 9% more EdU positive nuclei than smaller aggregates, while larger aggregates of *T. canadensis* had 15% more G<sub>2</sub>/M nuclei and 5% more EdU positive nuclei than smaller aggregates. Taken as a whole, the general pattern indicated that larger aggregates contained more actively dividing cells than smaller aggregates.

A well-mixed, representative sample from both the large aggregate culture and small aggregate culture for each cell line was also compared at each time point, to assess whether the patterns that were observed within a culture amongst aggregate sizes correlated with results observed between the bulk cultures. Analysis of DNA content indicated that large aggregate cultures contained more actively dividing cells than small aggregate cultures at most time points (Fig. 5.5a,b), though *T. canadensis* cultures contained very few S/G<sub>2</sub> cells on day 12 (less than 10% total) so the difference was not

noticeable at that particular point. Large aggregate cultures of *T. cuspidata* also exhibited a similar trend with EdU incorporation, where large aggregate cultures contained ~ 10% more EdU positive cells than small aggregate cultures (Fig. 5.5d). A comparison of *T. canadensis* cultures revealed no differences in the percentage of EdU positive cells between large aggregate cultures and small aggregate cultures (Fig. 5.5c). These results follow a similar trend to the aggregate fractionation experiments. Typically, large aggregate cultures contained a higher percentage of cells actively participating in the cell cycle than small aggregate cultures, though these differences varied in their magnitude.

## 5.4 Discussion

These results suggest that negative effects due to nutrient limitations are not apparent at the typical aggregate sizes present in these *Taxus* cell cultures, and that other aggregate-related effects are more important influences on cellular metabolism. In particular, both cell-cell signaling and protection from shear may be beneficial to cells in the interior of larger aggregates with regard to primary metabolism. In smaller aggregates, cells likely experience relatively more stress due to physical forces, including: surface shear effects, as a higher percentage of cells in a smaller aggregate are on the surface and directly exposed to the environment; and the severe forces arising from the rapid movement of aggregates in turbulent eddies, to which smaller aggregates are more susceptible based on an analysis of the response time of aggregates of different sizes in these turbulent eddies (Kessler et al. 1999). Shear stress has been shown to be detrimental to plant cells (Dunlop and Namdev 1994; Kieran et al. 1997) and the minimization of shear forces is an important design criteria for plant cell bioreactors (Georgiev et al. 2009; Huang and McDonald 2010), but some amount of shear stress may be beneficial for secondary metabolism. Secondary metabolic activity can be induced



with various forms of stress, including MJ elicitation, which was utilized in this study. In fact, an optimum shear rate was identified that maximized both growth and secondary metabolite production (Zhong et al. 1994), indicating that shear can also be advantageous to an extent. Additionally, in the interior of aggregates, cells are surrounded by other cells, encouraging cell-cell communication via plasmodesmata and other signaling mechanisms (Lucas and Lee 2004). The lack of access to other cells and the direct exposure to other elements of the exterior environment, such as cell debris and many undefined components of the conditioned extracellular medium, may negatively impact the primary metabolism of cells at and near the surface of aggregates.

Regardless of the mechanistic link to aggregation, these results demonstrate an inverse relationship between primary and secondary metabolism, which is associated with aggregate size. The connection between primary and secondary metabolism has been previously observed at the process scale, and the potential for a two-stage culture is a well-known process design criteria (Bourgaud et al. 2001; Payne et al. 1991), but this connection has yet to be explicitly demonstrated at the cellular level. While the simultaneous, multiparameter detection of cell cycle and paclitaxel accumulation remains a target of ongoing work, these results do link these properties at the cellular level via aggregation. The causative relationship between primary and secondary metabolism is also unknown. For instance, it is unclear whether the environmental factors described above predominantly affect either primary or secondary metabolism, which in turn affect the other, or whether it is possible for both primary and secondary metabolism to be active simultaneously. The further investigation of cellular properties in specific regions of the aggregate would help to answer these questions. In another plant cell culture system where the accumulation of secondary metabolites could be directly observed by microscopy, it was found that only cells on the periphery of aggregates in *Vaccinium*

*pahalae* accumulated anthocyanin (Pepin et al. 1999). For properties which cannot be visually observed, an approach to selectively dissociate cells from specific regions of the aggregate prior to analysis would be very useful, similar to the work which led to the identification of quiescent cells in the interior of tumor spheroids (Freyer and Sutherland 1980)

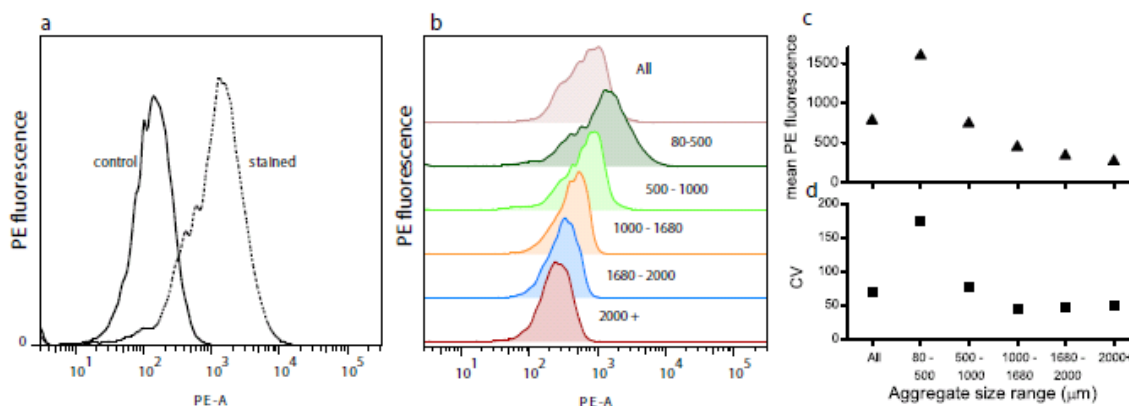
Another possible explanation for suppressed secondary metabolism in larger aggregates is that MJ elicitation is not uniformly effective in larger aggregates, and MJ may not penetrate into the interior of all aggregates. These effects may be amplified as MJ is produced endogenously by cells in response to external stimuli (Gundlach et al. 1992), and the biosynthesis of jasmonic acid is regulated by a positive feedback loop (Wasternack 2007). Growth did appear to be slightly less suppressed in larger aggregates (Fig. 3.3d) than smaller aggregates post elicitation, which would be expected if not all cells within a larger aggregate were stimulated by MJ. In systems where elicitation is not needed to stimulate secondary metabolism (Edahiro and Seki 2006; Hulst et al. 1989; Kessler et al. 1999), this limitation would not be observed. Gene expression analysis of cultures with different aggregate sizes indicates that in some cases, mRNA expression of paclitaxel biosynthetic pathway genes is the same even though significant differences in bulk paclitaxel accumulation are evident (Patil et al. 2011). This would indicate that the genes are likely upregulated at similar rates and that the effectiveness of MJ elicitation alone is not responsible for the difference observed in every case. The further identification and assessment of genes associated with specific cellular responses such as the response to shear stress or hypoxia would also help to clarify the dominant features of the aggregate micronenvironment responsible for the changes in cellular metabolism which have been reported here.

## 5.5 Conclusion

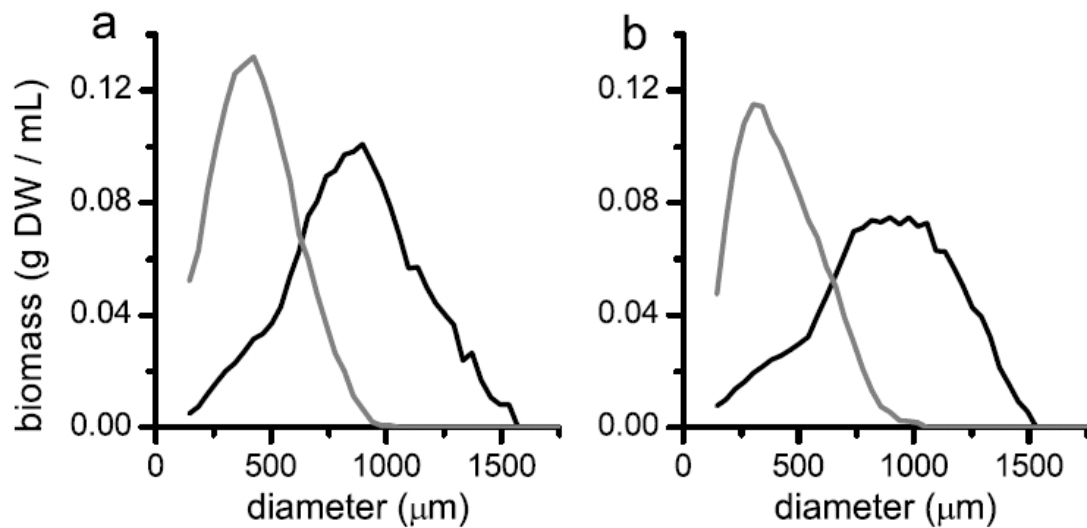
Flow cytometric analysis of single cells isolated from different sized aggregates in *Taxus* suspension culture indicated that aggregation significantly contributed to heterogeneity in secondary metabolite accumulation, and that cells isolated from smaller aggregates contained higher levels of paclitaxel. Cell cycle activity, analyzed by both nuclear DNA content as well as EdU incorporation, indicated that larger aggregates contained more actively dividing cells than smaller aggregates. These results suggest that primary and secondary metabolism are likely inversely related, and that other environmental factors such as applied shear stress and cell-cell signaling are potentially more important than nutrient limitations in the typical aggregate sizes encountered in *Taxus* suspension cultures.

<i>Taxus cuspidata</i> P93AF			<i>Taxus canadensis</i> C093D		
Day	% increase of G <sub>2</sub> /M	% increase of EdU positive	Day	% increase of G <sub>2</sub> /M	% increase of EdU positive
7	60	62	5	11	4
12	220	30	8	70	3
			12	146	30

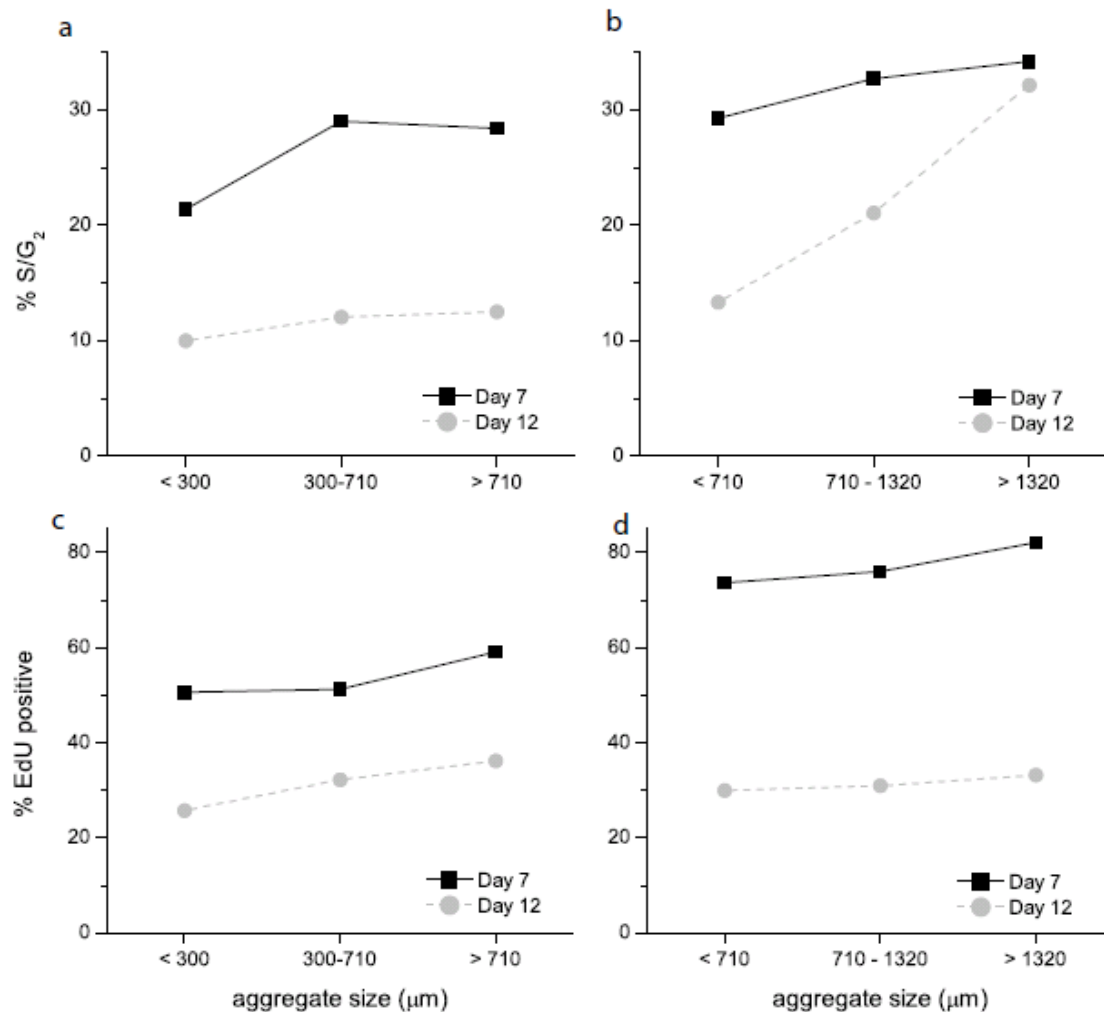
**Table 5.1** Comparison of cell cycle activity measured by both DNA content and EdU incorporation in terms of percent increase in largest aggregates ( > 1320  $\mu$ m) compared to smallest aggregates ( < 310  $\mu$ m).



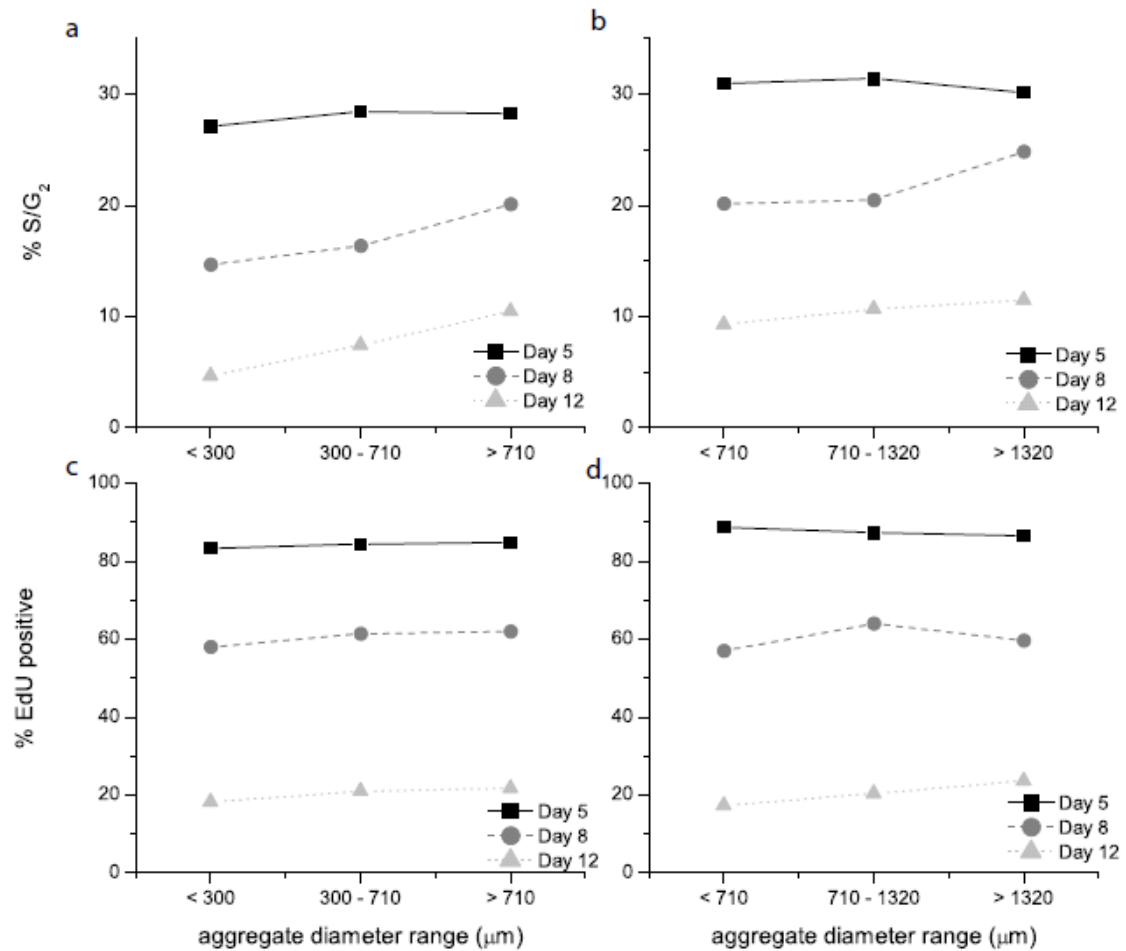
**Figure 5.1** Analysis of paclitaxel content in single cells isolated from different sized aggregates within a culture. (a) Comparison of single cells stained with secondary antibody only (control) and single cells stained with both primary and secondary antibody (stained), where the relative PE fluorescence indicates paclitaxel level. (b) Comparison of PE fluorescence in single cells isolated from different sized aggregates, which were isolated from *T. cuspidata* P991 cells 5 days following elicitation with 150  $\mu\text{M}$  methyl jasmonate. The size range of each aggregate fraction is listed in ( $\mu\text{m}$ ) next to the corresponding histogram, and “All” indicates a sample of well-mixed biomass which includes aggregates of all sizes.



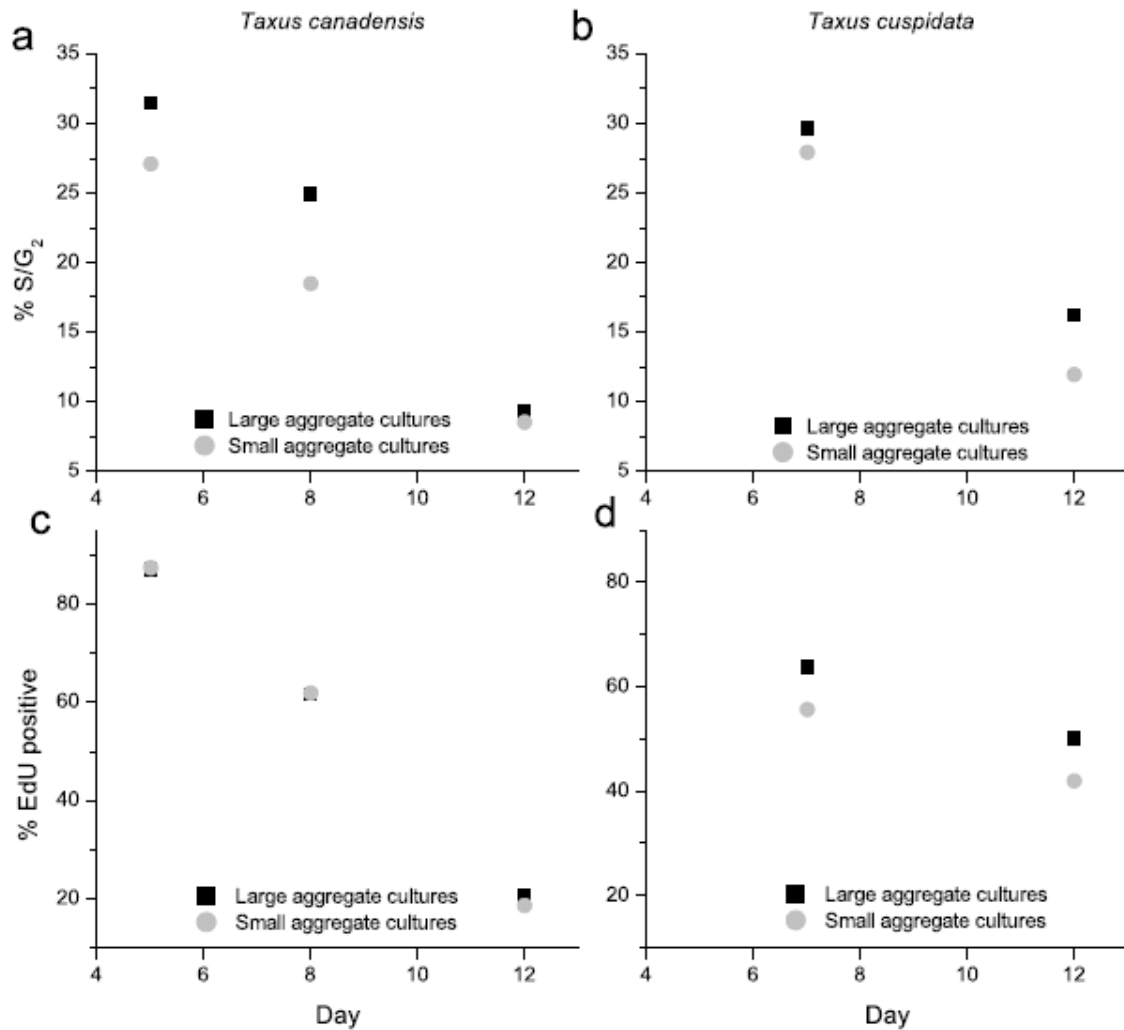
**Figure 5.2** Initial distributions for small aggregate cultures and large aggregate cultures filtered at 710  $\mu\text{m}$ . (a) *T. canadensis* CO93D and (b) *T. cuspidata* P93AF cultures used for aggregate fractionation and analysis of cell cycle activity. Each distribution curve represents the average distribution measured using a Coulter counter of three replicate flasks.



**Figure 5.3** Cell cycle activity of aggregate fractions isolated from *T. cuspidata* P93AF cultures at several time points. Two sets of cultures with different aggregate size profiles were fractionated in order to provide a wide range of aggregate sizes. (a) and (c) Fractions isolated from small aggregate cultures. (b) and (d) Fractions isolated from large aggregate cultures. (a) and (b) Percentage of S/G<sub>2</sub> cells classified by DNA content analysis of nuclei which were stained with PI. (c) and (d) Percentage cells which had incorporated EdU into their DNA after incubation with EdU for the length of the cell cycle (~ 40 hours). Positive nuclei were determined based on a comparison to a control that was not incubated with EdU.



**Figure 5.4** Cell cycle activity of aggregate fractions isolated from *T. canadensis* C093D cultures at several time points. Two sets of cultures with different aggregate size profiles were fractionated in order to provide a wide range of aggregate sizes. (a) and (c) Fractions isolated from small aggregate cultures. (b) and (d) Fractions isolated from large aggregate cultures. (a) and (b) Percentage of S/G<sub>2</sub> cells classified by DNA content analysis of nuclei which were stained with PI. (c) and (d) Percentage cells which had incorporated EdU into their DNA after incubation with EdU for the length of the cell cycle (~ 40 hours). Positive nuclei were determined based on a comparison to a control that was not incubated with EdU.



**Figure 5.5** Comparison of cell cycle activity between small aggregate cultures and large aggregate cultures at several time points for (a) and (c) *T. canadensis* C093D (b) and (d) *T. cuspidata* P93AF at several time points. Data points represent a well mixed sample containing aggregates of all different sizes representative of the aggregate distribution for that particular flask. (a) and (b) Percentage of S/G<sub>2</sub> cells classified by DNA content analysis of nuclei which were stained with PI. (c) and (d) Percentage cells which had incorporated EdU into their DNA after incubation with EdU for the length of the cell cycle (~ 40 hours). Positive nuclei were determined based on a comparison to a control that was not incubated with EdU.



## CHAPTER 6

### IMPACT AND FUTURE WORK

#### 6.1. Impact

This thesis focused on the characterization and analysis of plant cell aggregation, a critical characteristic of plant cell cultures that has been relatively ignored from a quantitative engineering perspective, despite its generally accepted importance. The work in this thesis has a broad impact for the *Taxus* system for production of paclitaxel, to plant cell culture systems in general, and to the fields of biochemical and bioprocess engineering analysis. The impact of this work is due to both the specific results obtained, but more importantly due to the methods that were developed and the multi-scale engineering approach that was applied. The specific results demonstrating the benefits of small aggregates for paclitaxel production are of immediate impact to commercial processes used to produce paclitaxel, and the characterization of aggregation dynamics and the changes in the aggregate size distribution provide a starting point for the development of control strategies aimed at increasing paclitaxel yields.

The development of a rapid and reliable method to measure aggregate size is also of immediate impact to *Taxus* cell culture systems, but is also generally applicable to any plant cell culture system. This technique allowed for a rigorous engineering analysis of aggregation, and was the key enabling technology for all of the subsequent work in this thesis and its continuation in the Roberts Laboratory. Coulter counter technology is quite mature and is used in many cell culture systems, but has not been demonstrated for routine use in plant cell culture systems or in measuring and quantifying aggregates. The barriers to applying this technology to plant cell culture were not challenging from a

technical perspective, but from a practical perspective: the methodology needed to be simple enough that junior researchers could be easily trained, minimize use of consumables so that it was not prohibitively expensive, fast enough that it could be routinely used for process monitoring, and accurate enough with minimal sample volume so that sampling would not affect batch culture performance in experiments with multiple time points. Regarding the last point, a tradeoff was reached where the same sample volume (2 x 2 mL) would be used for every flask for the sake of consistency, even though we demonstrated that the precision of the measurement depended on a number of factors, most importantly the number of particles, which depended on both aggregate size and biomass concentration. In some cases, the accuracy of the aggregate size measurement could have been increased by adjusting the sample volume for a specific condition (e.g., analyze more samples for large aggregate or low biomass cultures); however, a consistent, simple procedure was more important for the overall success of the project than a modest increase in accuracy for a limited number of samples. The method developed was higher resolution, more accurate, more precise, and faster than any other method available to measure aggregate size; while it could likely have been optimized further to strengthen one particular aspect of analysis, the balance was most important, and resulted in a practical, usable technique that was the basis for the rest of the work in this thesis.

The analysis of the effect of aggregate size on paclitaxel accumulation demonstrated a significant result for *Taxus* cultures, but more importantly demonstrated the necessity of a process engineering approach. The vast majority of previous studies that evaluated aggregate size in plant cell culture systems assessed the effect of aggregation via fractionation and biomass-associated metabolite accumulation in aggregates of different sizes. This method was the starting point for these studies, and

consistently gave results that indicated that larger aggregates accumulated higher levels of paclitaxel. However, rather than suggesting that a process with larger aggregates may be beneficial, I wanted to directly confirm this strategy, and thus developed a method to initiate cultures with aggregates of different sizes. By filtering the inoculum pool and initiating “large aggregate cultures” and “small aggregate cultures” in which all other process variables were the same, the effect of aggregate size as a process variable could be directly assessed. This approach clearly and unmistakably contrasted the results from the fractionation approach. A similar contrast was found in the literature, where in two separate studies on the same system, *Daucus carota*, a fractionation approach indicated larger aggregates were beneficial (Madhusudhan and Ravishankar 1996), but a whole culture approach demonstrated that cultures with smaller aggregates had higher levels of product accumulation (Kinnersley and Dougall 1980). The contrast results from the two methods exposed the limitations of the fractionation approach, in that the analysis of biomass-associated metabolites is a fundamentally flawed method to analyze metabolite accumulation when a significant proportion of metabolites are found in the extracellular media. More than the specific result obtained where smaller aggregates resulted in superior process performance with respect to paclitaxel accumulation, this work was important because it demonstrated both the criticality of confirming investigations aimed at a specific property of cell cultures with total process studies, and also showed that connecting a specific property with the total process performance was possible with the proper experimental approach.

The development of a population balance equation (PBE) model of aggregation dynamics was important for a number of reasons. First, the original modeling objective of “modeling paclitaxel variability” was the motivation behind rigorously characterizing the aggregate size distribution and developing the Coulter counter method. In this sense,

the model had an impact for guiding what particular property of the system needed to be characterized, and the rigor at which these data needed to be collected, so that the experimental data would be useful for modeling. Previous reports aimed at characterizing aggregate size demonstrated neither the resolution nor precision of our method; without the motivation of a model to describe the experimental data, I likely would not have developed such a rigorous method to characterize aggregate size, and would not have obtained many of the critical observations that resulted. The development of the model was thus important for guiding how we viewed the cell culture system, and this approach would be applicable to the development of a model in any biological system for which the underlying cause of a specific output (in this case, paclitaxel variability) is poorly understood. The development of a mathematical model, consisting of equations which quantitatively describe specific relationships, demands a much more thorough understanding of the biological system than the description of such a system from a purely qualitative or theoretical perspective. I believe that the demonstration of this approach is perhaps the most important aspect of the theoretical part of this thesis. The engineering approach to both develop and apply a mathematical model to the *Taxus* system resulted in a thorough characterization and analysis of an important system property, which had previously been only qualitatively, and often incorrectly, understood.

The PBE model itself was an important step towards the rational control of aggregate size, and also provided insights into the phenomenological processes underlying changes in the aggregate distribution. This model was the first application of population balances to address plant cell aggregate distributions, and described changes in plant cell aggregate size in both much more detail and from a much more sophisticated theoretical perspective than the few previously published models describing aggregation in other plant cell culture systems. Compared to PBE models describing other

aggregating systems, this is also the first model to our knowledge to explicitly account for biomass loss upon breakage, which appears to be a crucial property and should be considered in other systems as well. As a tool, the model can be used as a basic guide to developing control strategies, such as determining optimal breakage rate, as well as in suggesting process strategies based on manipulating conditions such as the time of batch subculture or elicitation. A more favorable aggregate size could be obtained based simply on varying the culture age as aggregate size changes with time, and these changes can be predicted by the model as it is been currently developed and validated. We consider this model to be a preliminary study which forms the basis for a much more detailed description of *Taxus* cultures, which could provide further insight into properties which may be difficult to determine experimentally, and which could also serve as a tool to develop more advanced process control strategies. This model provides a framework to incorporate further detail from a number of levels: a more detailed description of the underlying cell biology and metabolism, additional media components, transport/uptake reactions, and effect of reactor and/or environmental conditions such as agitation and aeration.

The investigation into the relationship between aggregation and cellular heterogeneity had perhaps the highest impact from the scientific perspective. Cellular heterogeneity has been observed in *Taxus* systems (in this laboratory) as well as in many other plant cell culture systems, and while aggregation was often assumed to be related to this heterogeneity, no quantitative evidence existed in support of this assumption. The analysis of single cell paclitaxel levels from different size aggregates demonstrated that aggregation was in fact a major, contributing source of heterogeneity in secondary metabolic activity. While the analysis of cell cycle participation did not support aggregation as the primary cause of subpopulations with respect to cycling or noncycling

cells, it demonstrated that aggregation contributes to heterogeneity of this property, as different aggregate sizes had different proportions of active, cycling cells. This analysis also indirectly provided a link between primary metabolism and secondary metabolism in *Taxus* aggregates by showing that cells isolated from larger aggregates had higher primary metabolic activity but lower secondary metabolic activity, while cells isolated from smaller aggregates had the opposite trends. Previously, many hypotheses had emerged regarding aggregation and its relationship to primary and secondary metabolism in plant cell culture. The prevailing view to this point, and what had initially been the hypothesis before this work in our laboratory, was that larger aggregates may contain nutrient-deficient regions that may cause cells to be less active in growth. It was thought that these cells in nutrient-deficient regions had the potential to differentiate or be specialized for secondary metabolism. The results from the analysis in this thesis demonstrated otherwise – that cells in larger aggregates were actually more active in growth and that those in smaller aggregates were more active in secondary metabolism. So the long proposed link between primary and secondary metabolism was supported by these results; however the relationship of aggregation to these properties was shown to be the opposite of what had been widely assumed.

## **6.2. Future Work**

The work in this thesis will allow for continuation of certain research thrusts that have already begun, and also motivate new investigations that can utilize some of the techniques that were developed. From a process engineering perspective, the identification of aggregate size as a key process variable leads to the obvious question of how to control aggregate size. Shake flasks are limited in terms of the operating conditions available to be manipulated, so for a more detailed investigation of conditions

affecting aggregation, cultures should be run in controlled bioreactors. The obvious place to begin is to vary the shear rate via agitation, and to confirm whether there is in fact an optimum agitation rate for the production of paclitaxel, as predicted by the PBE model. Care should be taken, however, to consider aggregate size and paclitaxel accumulation as separate process outputs; the manipulation of a specific operating condition will always affect additional culture properties besides aggregate size, and the basic relationship between aggregate size and paclitaxel accumulation will not necessarily hold as other variables are modified. Aside from agitation, manipulation of aeration or dissolved oxygen may also affect aggregate size. Besides operating conditions, aggregate size can also be manipulated via process conditions, such as time of inoculation, biomass concentration upon inoculation, and potentially even fed-batch strategies. For instance, even if the total amount of sucrose is the same, if it is fed in a two-step or three-step scheme, the growth rate may be slower, while the breakage rate is maintained, resulting in a smaller aggregate distribution and higher paclitaxel productivity.

For these types of process engineering investigations, the PBE model should be utilized and expanded. Any operating condition can theoretically be integrated into the PBE framework; however, much the same as specifying the functional dependencies and forms of the kernel functions with respect to aggregate size, the functional dependencies and forms of the kernel functions with respect to specific operating conditions must be investigated in a targeted and systematic manner. For example, shear can be incorporated into the division intensity function, and targeted experiments can be run with various shear rates. Shear is a common operating condition that has been incorporated into many PBE models in various systems, so this work should be relatively straightforward, provided there is a reliable bioreactor system where the explicit shear rate can be accurately determined. Dissolved oxygen should be investigated in much the same way,

although it is unclear from the outset what this variable will affect. It will likely influence the growth kernel, but may also affect the breakage kernels, due to its potential effect on other fundamental properties of aggregates as described below.

Additional complexity can also be added to the PBE model by incorporating more information about the underlying biology. The starting point should be a model of an individual aggregate – how cells within an aggregate grow, and how cells within an aggregate produce and accumulate paclitaxel. Based on the results in Chapter 5, it should be possible to develop this model using experimental data that have already been obtained. The experimental evidence suggests that in the range of aggregate sizes typically encountered, nutrient limitations are not the dominant factor, so the model should be developed from a qualitative starting point that cells on the outer regions of aggregates have reduced primary metabolism due to either shear or exposure to the extracellular media, that cell-cell contact is beneficial for primary metabolism, and that primary and secondary metabolism have an inverse relationship. Though the specific mechanisms underlying these effects are not known, they can be modeled using a process variable representing that particular property which is subject to either cell signaling mechanisms or diffusion. An additional level of complexity that should eventually be accounted for is that not all aggregates of a specific size are necessarily the same. For instance, an aggregate which has recently broken off a larger aggregate may be different than an aggregate which has grown to the same size over time. These aggregates likely differ in terms of their secondary metabolic capacity, as a cell in the interior of an aggregate which is now exposed to the environment may have a substantial time delay before secondary metabolite synthesis becomes fully active. There may also be specific properties that can be accounted for explicitly. For instance, the composition of cell walls may differ in aggregates of different sizes. If larger aggregates do in fact develop thicker



cell walls, this would likely affect their breakage probability, and these factors would need to be taken into account in the basic kernel functions.

To this end, a more detailed, fundamental understanding of plant cell aggregates is needed. This should be achieved along three general areas: genetic, cell population, and aggregate-specific. As we develop molecular biology tools to understand system-wide gene expression, these tools should be applied to understand not just differences in paclitaxel accumulation, but differences in the aggregate size distribution as well. For instance, in addition to finding differences in gene expression which are directly related to paclitaxel synthesis by comparing high and low producing cultures, system-wide gene expression in large aggregate and small aggregate cultures can be compared without elicitation and independent of their paclitaxel accumulating capabilities. Beyond system-wide gene expression, we should look for targeted genes that may be involved in aggregation, potentially those involving cell wall synthesis, and these genes should be considered as targets for metabolic engineering. In fact, genes that are shown to be involved in aggregation would be a target for gene silencing approaches if those techniques could be applied and developed to *Taxus* cultures. From a cell population perspective, achieving spatial resolution of cell populations within an aggregate would be extremely beneficial in understanding the effect of aggregation on cell metabolism. A modification of the single cell digestion procedure can likely be used to isolate cells from specific regions of the aggregates (e.g., inner and outer) and these cells could be analyzed utilizing the same approaches as in Chapter 5. Varying the enzyme concentration, digestion times, and agitation rates would be the first steps in developing this type of procedure. As laser micro-dissection becomes more widely available and cost-effective, this technology may also be applied to physically separate different regions of the aggregates.

In addition to applying specific techniques such as flow cytometry and gene expression analysis to answer additional questions about aggregation, there are other fundamental aspects of aggregation that may require the development new techniques. The assessment of cell walls, both in aggregates as a whole as well as in different regions of aggregates, might provide enormous insight into how aggregates develop, and why aggregation profiles are variable. Both an evaluation of the total cell wall mass, as well as the different components of the cell wall such as cellulose and pectin, may be challenging to quantify and reliably measure, but critical in terms of more fully understanding the aggregation phenomenon. As cell walls and their composition are critical properties of many types of biomass that are currently being engineered for biofuels production, metabolic engineering strategies might be applied from these systems to manipulate the cell wall composition and thus aggregation profiles in *Taxus* cultures.

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