Analysis of Aggregate Size as a Process Variable Affecting Paclitaxel Accumulation in Taxus Suspension Cultures

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Analysis of aggregate size as a process variable affecting paclitaxel accumulation in *Taxus* suspension cultures

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Abstract

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 μm and 1100 μm 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 μm and 840 μm 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.

Keywords

Plant cell culture; cell aggregation; paclitaxel; Taxus; bioprocess engineering

1 Introduction

Natural products have traditionally served as a valuable source of pharmaceuticals, particularly chemotherapeutics, but the use of natural products as drug leads has been significantly reduced over the past two decades. One major factor contributing to this decline is the uncertainty of drug product supplies, a challenging constraint unique to complex plant derived natural products. 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

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difficulties for process scale up; and inherent variability in these properties, which is poorly understood\textsuperscript{4,5}.

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 $\mu$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.,\textsuperscript{6,7}), and the heterogeneity observed in plant cell populations has been attributed in part to aggregation\textsuperscript{8–10}. 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\textsuperscript{11,12} or a positive effect up to a critical size\textsuperscript{13–15}, but have also been inconclusive\textsuperscript{16}. 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\textsuperscript{17,18}.

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\textsuperscript{19}. Another approach is to alter the starting aggregate size distribution, which has shown that smaller aggregates\textsuperscript{20,21} or cultured single cells\textsuperscript{22} 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 \textit{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\textsuperscript{23}, 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.

2 Materials and Methods

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 μM naphthalene acetic acid, and 0.1 μM 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 μM methyl jasmonate (Sigma-Aldrich) and covered with aluminum foil.

2.2 Aggregate Fractionation and Paclitaxel Analysis

Following elicitation with 150 μM methyl jasmonate on day 7, aggregates from an entire flask were filtered as previously described. 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 μL acidified methanol by vortexing followed by sonication for 30 min. Concentrated samples were filtered using 0.22 μm filters (Acrodisk®, 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 μL 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).

2.3 Aggregate Size Distribution Manipulation and Total Culture Studies

Aggregate size distributions were measured using a Coulter counter 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 μm 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 μm 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 μm 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 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 correlations23. 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 μM 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 2.2).

3 Results

3.1 Biomass-Associated Paclitaxel in Aggregate Fractions

Aggregates of T. cuspidata spanned a wide distribution, ranging from less than 100 μm to over 2 mm in diameter (Fig. 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. 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. 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. 2a contained a significantly higher percentage of smaller aggregates than the cultures in Fig. 2b (Fig. 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.

3.2 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). 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 μm, 630 μm, and 810 μm for small aggregate cultures, unfiltered cultures, and large aggregate cultures in Fig. 3). The d90 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 d90 (623 μm, 1110 μm, and 1250 μm for small aggregate...
cultures, unfiltered cultures, and large aggregate cultures in Fig. 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 μm 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. 4a, b, c). Prior to elicitation the mean aggregate sizes were 690 μm, 940 μm, and 1100 μm, while the d90 values were 1060 μm, 1540 μm, and 1690 μm 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. 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 d90 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 4.2).

In two experiments with separate *T. cuspidata* cell lines (P991 and P93AF) both initially separated with a 500 μm filter, filtered and unfiltered aggregate distributions remained distinct prior to elicitation, and did not show significant differences in total biomass (Fig. 5a, b Table 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. 5c, 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 μm, the d90 values were 1290 μm and 1060 μm for *T. cuspidata* P991 and *T. cuspidata* P93AF, respectively, which may help to explain the higher paclitaxel levels in *T. cuspidata* P93AF (see section 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 d90, 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. 6, Table 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.

4 Discussion

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. 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.

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, as differentiation and hollow spaces 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.

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. 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 to over 90% extracellular to intermediate levels. 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.
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 5), and also why some cultures with different mean sizes accumulated about the same amount of paclitaxel (Fig 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. 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. 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. 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, while smaller aggregates were found to be superior in the same system via whole culture process analysis. Our results follow this same pattern. Several reports which analyze total process performance and demonstrate that larger aggregates are superior utilize compact callus cultures, 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, 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\textsuperscript{35}, 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\textsuperscript{36}, and provide a suitable framework for a predictive model that we are currently developing.

**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.

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**References**


Figure 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 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.
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 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 (22). (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 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 6.
Aggregate size distributions measured for *Taxus cuspidata* P991C after subculture initiation in which aggregates used for inoculum were filtered using a 1000 μm filter. Cultures used to generate inoculum for this experiment had extremely large aggregates relative to typical *T. cuspidata* aggregate sizes.
**Table 1**

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

<table>
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<th>culture designation</th>
<th>total biomass (g DW/L)</th>
<th>mean aggregate diameter (µm)</th>
<th>total biomass (g DW/L)</th>
<th>mean aggregate diameter (µm)</th>
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Table 2
Aggregate size and paclitaxel accumulation for cultures initiated from large aggregate distribution

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<th>mean aggregate diameter (μm)</th>
<th>paclitaxel accumulation post elicitation</th>
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