

Ultra Scale-Down Characterization of the Impact of Conditioning Methods for Harvested Cell Broths on Clarification by Continuous Centrifugation—Recovery of Domain Antibodies from rec *E. coli*

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ABSTRACT: The processing of harvested *E. coli* cell broths is examined where the expressed protein product has been released into the extracellular space. Pre-treatment methods such as freeze–thaw, flocculation, and homogenization are studied. The resultant suspensions are characterized in terms of the particle size distribution, sensitivity to shear stress, rheology and solids volume fraction, and, using ultra scale-down methods, the predicted ability to clarify the material using industrial scale continuous flow centrifugation. A key finding was the potential of flocculation methods both to aid the recovery of the particles and to cause the selective precipitation of soluble contaminants. While the flocculated material is severely affected by process shear stress, the impact on the very fine end of the size distribution is relatively minor and hence the predicted performance was only diminished to a small extent, for example, from 99.9% to 99.7% clarification compared with 95% for autolysate and 65% for homogenate at equivalent centrifugation conditions. The lumped properties as represented by ultra scale-down centrifugation results were correlated with the basic properties affecting sedimentation including particle size distribution, suspension viscosity, and solids volume fraction. Grade efficiency relationships were used to allow for the particle and flow dynamics affecting capture in the centrifuge. The size distribution below a critical diameter dependant on the broth pre-treatment type was shown to be the main determining factor affecting the clarification achieved.

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Introduction

The preparation of antibodies for therapy is largely based on the use of platform processes producing monoclonal antibodies in mammalian cell systems. Antibody fragments, such as Fabs (~57 kDa) comprising two chains each of a variable and constant domain, or Fvs (~27 kDa) comprising two variable domains as two chains or as an engineered single chain (scFv), retain similar antigen binding capacity as the whole antibody and are suitable for expression in microbial cells such as *E. coli*; they are now entering late stage clinical testing (Andersen and Reilly, 2004; Holt et al., 2003). Domain antibodies (dAbs, ~12–15 kDa) are based on a single variable domain from the heavy and light chain. These also exhibit high binding affinity and specificity despite lacking most of the constitutive part of a full antibody (Jespersen et al., 2004; Saerens et al., 2012). The opportunity now exists to establish platform processes for the production of various antibody fragments using different cell hosts. Such fragments are likely to behave very differently in a process environment; for example, they tend to be more hydrophobic (Ewert et al., 2003; Nieba et al., 1997) and of reduced mass solubility (Famm et al., 2008; Tanha et al., 2006).

The use of ultra scale-down techniques for the evaluation of process options has been discussed in literature: for centrifugation clarification (e.g., Boychyn et al., 2000, 2001, 2004), for centrifuge dewatering and sediment discharge (Chan et al., 2006; Tustian et al., 2007), for membranes (Ma et al., 2010), for pumps (Zhang et al., 2007), and for filters (Reynolds et al., 2003). Large scale centrifugation performance based on its clarification efficiency can be evaluated at the bench scale through the use of the Σ theory, which allows for comparison between centrifuges of different sizes and, using appropriate calibration factors, different centrifuge types (Ambler, 1959).

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The ultra scale-down (USD) technique mimicking centrifugation is based on the principle that significant hydrodynamic stress will be encountered by the process material in the centrifuge before it enters the settling region. The level of the shear stress depends on the type of machine used (Boychyn et al., 2004). Shear stress is known to disrupt mammalian cells (Hutchinson et al., 2006; Kamaraju et al., 2010; Tait et al., 2009; Zaman et al., 2009), flocs (Berrill et al., 2008), and precipitates (Bell et al., 1982; Byrne et al., 2002; Hoare et al., 1982). Maybury et al. (2000) observed that there could be a 10–58% error in predicting the capacity of a continuous centrifuge if hydrodynamic stress equivalent to the conditions which prevail in the feed zone was not applied to the process material prior to centrifugation. Rotating disc or capillary devices (Boychyn et al., 2001; Chan et al., 2006; Ma et al., 2002; McCoy et al., 2009; Tait et al., 2009) to mimic the effect of process shear stress may be used. Computational fluid dynamics have been used to help establish correlations between the device and the process shear stress (e.g., Boychyn et al., 2001, 2004), but verification is also achieved experimentally (Hutchinson et al., 2006). The use of flocculating agents to aid the separation of cellular material from fermentation broths is particularly affected by pH, cell concentration, flocculant type (or chemistry), and fluid mechanics (Gasner and Wang, 1970; Wang et al., 1970). *E. coli* aggregation using cationic polymers occurs through charge redistribution on the surface of originally negatively charged *E. coli* debris (the charge-mosaic model); here the polymer has to be of sufficient length to ensure positively charged areas are created to induce aggregation (Trewick and Morgan, 1977).

The use of flocculants to enhance the recovery of cells and cell debris has been demonstrated for centrifugation (Milburn et al., 1990; Salt et al., 1995) and for filtration (Aspelund et al., 2008). In addition, a high proportion of nucleic acids, lipids, and colloidal particles are shown to be selectively precipitated from soluble proteins. An ultra scale-down approach was used to optimize the flocculation of *E. coli* heat lysed cell broth extract using cationic polymer followed by clarification by centrifugation (Berrill et al., 2008) with successful verification at scale using a disc stack centrifuge.

In this paper, the physical characterization of cell broths conditioned by a range of methods including flocculation, are correlated with ultra scale-down methods. This is to help gain a better understanding of their processing.

Materials and Methods

All chemicals were provided by Sigma–Aldrich (Dorset, England) unless otherwise stated. *E. coli* cells (strain W3110) expressing a V_H domain antibody (13.1 kDa) were cultured in a complex medium (Biopharm Process Research, GSK, Stevenage, UK) containing yeast extract (1 L SR1000DLL bioreactor, dia 100 mm, fitted with triple Rushton impellers, dia 46 mm, $N=1,200$ rpm, DASGIP AG, Jülich, Germany). The cells were grown to OD 40 (dry cell weight 13 g L^{-1}) with a post-induction time of 45 h leading to 80% cell autolysis at harvest as measured by release of nucleic acid (fluorescence Qubit[®]

assay; Life Technologies Ltd., Paisley, UK). Cell broth was used within 3 h of harvest for bioprocessing studies or for freezing (-80°C) in 50 mL aliquots for subsequent studies. This latter approach was to allow reproducible studies irrespective of variations in the fermentation broth.

Cell Broth Processing

Homogenization Studies

A high-pressure batch homogenizer (Gaulin Micron Lab40; APV Gaulin, Lubeck, Germany) operated at 500 bar, 4°C for two passes was used to achieve complete cell disruption.

Flocculation Studies

A 12.5% w/v polyethyleneimine (PEI, $(\text{C}_2\text{H}_5\text{N})_m$, $M_W=50,000\text{--}100,000$, Sigma–Aldrich) water based solution was aged for 0.5 h. The solution, 2 mL, was added to cell broth, 50 mL, at 6 mL min^{-1} at the tip of the impeller in a 100 mL baffled reactor fitted with an impeller (six bladed Rushton turbine, dia 20 mm, 1,150 rpm, $Re=2000$, $\bar{G}=(P/V\mu)^{0.5}=800\text{ s}^{-1}$) to reach a final PEI concentration of 0.5% w/v. The solution was left to mix for $t=1$ h at 21°C before processing ($\bar{G}t\sim 3\times 10^6\text{ gg}10^5$ required for full floc strength to be gained (Bell and Dunnill, 1982)).

Ultra Scale-Down Studies

Samples were exposed to shear stress for 20 s in a rotary disc device (20 mL stainless steel chamber of 50 mm internal diameter and 10 mm height, fitted with a stainless steel rotating disc of 40 mm diameter and 0.1 mm thickness with disc speed 0–20,000 rpm) controlled by a custom designed power pack (UCL mechanical workshop). The disc speed was related to maximum energy dissipation rates, ε (W kg^{-1}), using a computation fluid dynamic derived empirical correlation ($\varepsilon=1.7\times 10^{-3} N^{3.71}$ where N is disc speed, revs s^{-1} , $33<N<250$; Zhang et al., to be published). Two conditions were used, $N=6,000$ rpm, $\varepsilon=0.045\times 10^6\text{ W kg}^{-1}$ and $N=12,000$ rpm, $\varepsilon=0.53\times 10^6\text{ W kg}^{-1}$ as equivalent of conditions experienced in the feed zone of hydro-hermetic and non-hermetic disc stack centrifuges respectively (to note previously reported values for the same disc speed conditions of $\varepsilon=0.019\times 10^6\text{ W kg}^{-1}$ and $0.37\times 10^6\text{ W kg}^{-1}$ (e.g., Lau et al., 2013; Tait et al., 2009) are for a computation fluid dynamic analysis of the whole disc outer edge rather than the corner at the disc edge). Each combination of flocculation and shear preparation was carried out in duplicate and tested for sedimentation and size distribution properties as described below ($n=2$).

The sedimentation properties of sheared and non-sheared samples were characterized using a test tube centrifuge in terms of equivalent settling area (Σ_T):

$$\Sigma_T = \frac{V_{\text{lab}}\omega^2}{2g \ln(2R_o/(R_o + R_i))} \quad (1)$$

where V_{lab} is the volume of process material in the centrifuge tube, ω is the radial speed, g is the acceleration due to gravity, R_i is the inner radius (the distance between the centre of rotation and the top of the liquid), and R_o is the outer radius (the distance between the center of rotation and the bottom of the tube). A multi-well centrifuge was used (Centrifuge 5810R; Eppendorf, Hamburg, Germany, equipped with a 2 mL deep square well plate format (Abgene, Epsom, UK)) using a method previously described by Tait et al. (2009). The microwell plate was filled with sample volumes (V_{lab}) of either 500, 1,000, or 1,500 μL in selected plate locations and spun for times (t) of 5, 10, 25, or 50 min and speeds of 3,000 or 4,000 rpm. The top 40% of the resulting supernatant was recovered from each well taking care not to disturb the sediment. Well-clarified supernatant, that is, baseline value for clarification, was prepared by centrifugation for 0.5 h at maximum RCF of 16,000 (Centrifuge 5415R; Eppendorf, 12,300 rpm). All operations were carried out at 21°C. The centrifugation conditions were recorded in terms of values of $V_{\text{lab}}/(t\Sigma_T)$.

Analytical Methods

Clarification and Solids Weight Fraction

The solids content of the sample and feed supernatants was estimated by OD at 600 nm. The solids remaining (S) was characterized by:

$$S = \frac{\text{OD}_S - \text{OD}_O}{\text{OD}_F - \text{OD}_O} \times 100 \quad (2)$$

where OD_S is the optical density of the supernatant of the centrifugation sample under test, OD_O is the optical density of the well-clarified supernatant (i.e., the baseline sample produced by extended centrifugation—see previous section), and OD_F is the optical density of the sample prior to centrifugation. Solids weight fraction in a sample was measured by weight difference prior to and after centrifugation. A 1.5 mL Eppendorf tube spun for 2 min at 10,000 RCF was used for this purpose. All experiments were carried out in triplicate.

Particle Size Distribution

Processed cell broth samples were evaluated using blue and red (laser) light diffraction through a sample flow cell (Mastersizer 2000, equipped with a Small Volume Dispersion Unit, Malvern Instruments Ltd., Worcestershire, UK) working in the particle size detection range of 0.01–2,000 μm . Refractive and absorption indices used were 1.59 and 0.000, respectively, that is, as for latex particles and all reported results are as for equivalent latex particle diameter (the indices are unknown for many materials studied here but changes in their value to reflect these possible differences give only small variations in resulting size distributions). Results were measured in triplicate ($n = 3$) and then recorded as volume percentage, V , against particle size interval, W and mean size of

interval, d_i . Representative size distributions are presented from the duplicate preparations ($n = 6$). The variance in key parameters of size distributions (e.g., volume fraction of fine particles) is less than $\pm 10\%$.

Protein Analysis

The concentration of product in samples under investigation was analyzed using protein A chromatography (HPLC Agilent 1200, Agilent Technologies UK Ltd., West Lothian, UK, fitted with a 1 mL HiTrap MabSelect[®] Xtra, GE Healthcare Life Sciences, Buckinghamshire, UK). Loading and equilibration were performed using a 0.1 M PBS buffer at pH 7.3. Samples were diluted in a defined fashion in equilibration buffer to a concentration of ~ 0.1 mg/mL, filtered using 0.22 μm PVDF syringe filter and then placed on a cooled auto-sampler (4°C) for the duration of the analysis cycle. Elution was performed using a 13 mM HCl buffer at pH 1.9 with product eluted recorded at 280 nm. Calibration was performed using standard solutions of pure domain antibody at a concentration of 1.08 mg mL⁻¹ (GSK). Protein aggregation was quantified using size exclusion chromatography (HPLC Agilent 1200; Agilent Technologies UK Ltd., fitted with a Tosoh TSK-Gel[®] G2000SW_{XL} column, 5 μm particle size, Tosoh Bioscience GmbH, Stuttgart, Germany).

Rheology

A cup-and-bob rheometer was used (Brookfield DV-2+ viscometer fitted with spindle CP40, Brookfield Engineering Laboratories, MA), exposing 0.5 mL of treated cell broth samples to shear rates of 37.5–1,500 s⁻¹ in seven increments with 30 s hold at each increment for increasing and decreasing shear sweeps.

Results and Discussion

Cell broths are highly complex suspensions. Three of the fundamental properties which impact centrifuge performance and which are relatively accessible to measurement are the particle size distribution, the suspension solids volume fraction and the suspension rheology. For flocs and aggregates these properties may be affected by exposure to hydrodynamic stress. The following results firstly explore the effects of cell broth conditioning on these properties. The measurement of density (or difference in density between the sedimenting species and the suspension) is challenging for a material comprising flocculated and precipitated material and entrapped liquor. As the size of precipitates increases it is observed that the proportion that is entrapped liquor increases (Bell et al., 1982). Attempts to separate the settling species from the surrounding liquor (e.g., centrifugation or filtration) can impact the material composition itself making density measurement difficult. The shape factor is difficult to characterize due to the irregular shape. The combined effects of all the above properties may be measured by ultra scale-down centrifugation.

Particle Size Distributions

Particle size distribution analysis is used to compare and contrast the effect of different methods of cell broth conditioning. Since there are six orders of magnitude of particle size to be dealt with in the same distribution, two methods of presentation are used. Firstly, a logarithmic size scale allows all features of the size distribution to be represented. Also, the focus is on volume rather than number distribution as it is the volume fraction remaining, for example after centrifugation, which is a key performance factor affecting subsequent stages, such as filtration. However, a logarithmic distribution visually misrepresents the amounts of different particle species present. To overcome this, fingerprints of the volume frequency distributions are also presented in such a way to allow direct comparison of the amount of the different species present and of the effects of processing, for example, how one species might break-up into a second. These volume frequencies are adjusted to reflect the different amounts of solid phase in the samples to either allow for loss of volume fraction (e.g., due to floc or cell break-up releasing entrapped liquor), or to allow for gain in volume fraction (e.g., due to precipitation of soluble material or to liquid entrapment in growing flocs).

Figure 1 shows the analysis of fresh cell broth as obtained directly from the fermenter. Also shown is the effect of shear stress as might be experienced in the feed zone of a non-hermetic (high shear stress feed zone) disc stack centrifuge which might be used to clarify this material. Three peaks are identified for both non-sheared and sheared material. For the non-sheared material about 1% (all percentages will be quoted on a volume basis only) of the material is in the 0.8–4 μm range (Fig. 1b), this size range probably being representative of whole cells and cell ghosts. There is little evidence of debris, that is, particles occurring in the sub 0.8 μm range. A further 1% is in the 10–50 μm range (Fig. 1c) and the final 98% in the 50–800 μm range (Fig. 1d). Both of these are evidently aggregates of the cell species. The effect of shear stress is to disrupt the largest aggregates reducing their volume to 88% (from 98%) and yielding significant increases in both the first two peaks but yet again no evidence of sub 0.8 μm material which would be typical of cell debris.

Figure 2 shows the same analysis but for freeze-thawed cell broth. The same three peaks are observed in addition to a fourth peak in the sub 0.8 μm range, that is, debris-like material. In addition the volume fractions of the 0.8–4 μm (Fig. 2b and c) and the 10–50 μm peaks (Fig. 2c) are considerably higher and more akin to the sheared material in Figure 1. This new fine material may either be the result of cell break-up or of macromolecular precipitation both of which may occur using during ice crystal formation. The freeze-thawed cell broth appears to be less sensitive than fresh material to shear stress, the only significant effect being the break-up of some aggregates in the >200 μm range to form aggregates within the same peak. Hence, it is not expected that clarification of this material will be affected by shear

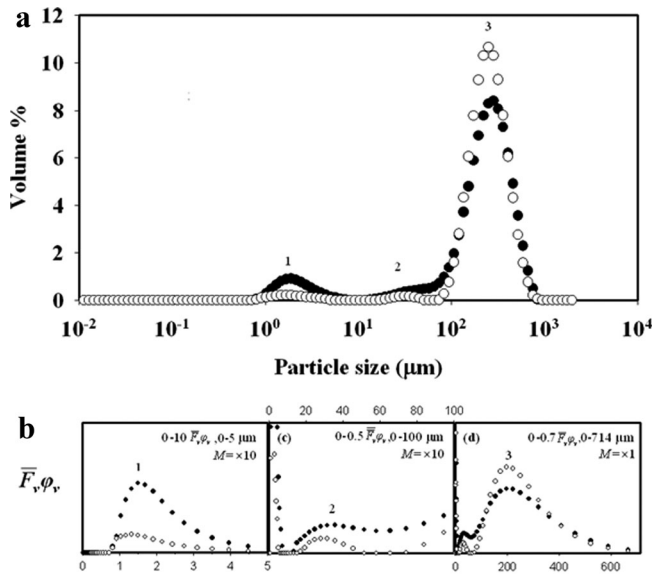


Figure 1. Properties and effect of shear stress for fresh cell broth. Volume size distributions are presented for (○) fresh cell broth, and for (●) cell broth sheared at an equivalent maximum power dissipation, ϵ , of $0.53 \times 10^6 \text{ W kg}^{-1}$. The relative solids fraction, ϕ_v , is 0.11 for sheared and non-sheared material. Volume ratios of peaks 1, 2, 3, are 2:1:97 for non-sheared and 8:4:88 for sheared autolysed cell broth. The size distributions are presented as (a) the whole distribution on logarithmic size scale, and the volume frequency distributions emphasizing (b) small, (c) medium, and (d) large sized particles. In order to rationalize the data sets, size distributions were converted to volume frequency distribution, that is, for particle channel i , $F_{v,i} = V_i/W_s$, where i is the size range of the channel. For such plots the volume fraction of particles in size range d_1 to d_2 is given by $(\int_{d_1}^{d_2} F_{v,i} dd) / \int_0^{\infty} F_{v,i} dd$, where the integrated area for a size range is directly proportional to the volume of particles. Axis scales for $F_v \phi_v$, and d and the relative magnification, M , of the figures are given in inserts. In all cases representative size distributions are presented where the variance in key size parameters (e.g., volume fraction of fine particles) is less than 10% ($n=2$).

stress in a centrifuge feed zone even for a non-hermetic (high shear stress) feed zone.

Separate studies, not shown here, indicate considerable variability in the size distribution of the fresh cell broth, with greater or lesser extent of fine particle formation. Even though it appears to be less sensitive to shear stress, freeze-thawed cell broth was characterized as the most challenging material with respect to clarification by centrifugation and hence this material was taken forward to subsequent studies. Similar feed solids volume fractions and domain antibody supernatant concentrations were noted for fresh and thawed cell broth. The use of a thawed cell broth offers consistency of starting material. A final study with fresh cell broth will be used to help verify the flocculation and centrifugation studies.

Figure 3 shows the effect of homogenization on thawed cell broth. Homogenization at 500 bar for two passes is used as it reflects a typical process for complete cell disruption and total product release (Bailey and Maegher, 1997). As expected, there is a considerable shift towards smaller particle sizes with all aggregates above $\sim 10 \mu\text{m}$ (Fig. 3c and d) being disrupted resulting in just 9% of the solids remaining in the 1–10 μm range, these probably being whole cell ghosts and their

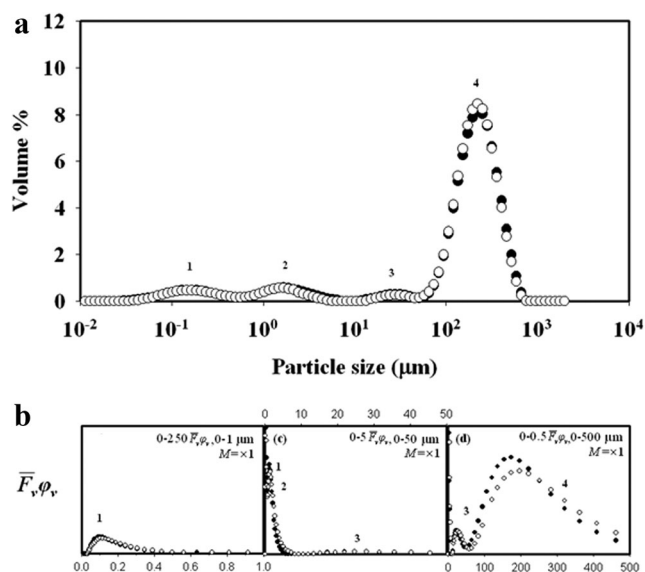


Figure 2. Properties and effect of shear stress for freeze-thawed cell broth. Volume size distributions are presented for (○) thawed cell broth, and for (●) thawed cell broth sheared at equivalent ε of $0.53 \times 10^6 \text{ W kg}^{-1}$. See Figure 1 for explanation of the size distributions. The relative solids fraction, φ_v is 0.11 w/v for non-sheared and sheared material. Volume ratio of peaks 1, 2, 3, 4 are 5:7:4:84 for both materials.

aggregates. The remaining 91% of the particles are submicron with a 10-fold increase in the volume of particles in the 0.08–0.30 μm range (Fig. 3a). The concentration and amount of product in the supernatant remained the same as the fraction

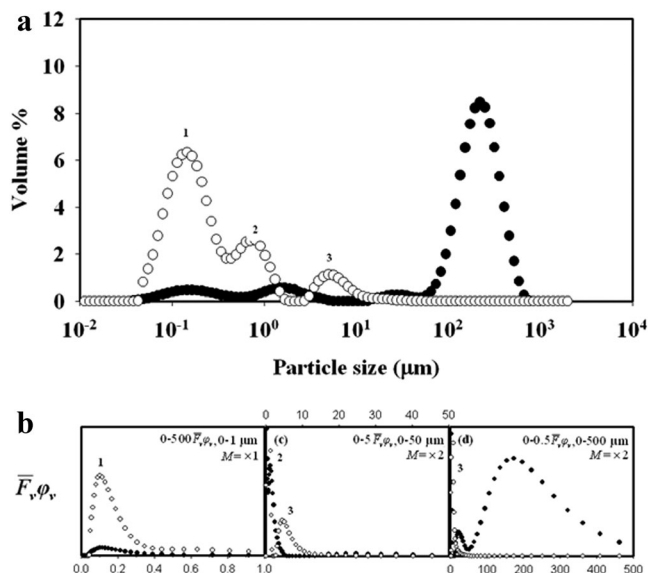


Figure 3. Homogenization, 500 bar for two passes, of freeze-thawed cell broth. Volume size distributions are presented for (●) sheared thawed cell broth and for (○) homogenate. See Figure 1 for explanation of size distributions. The relative solids fractions, φ_v are 0.11 w/v for thawed cell broth and 0.078 w/v for homogenate. The volume ratio of peaks 1, 2, 3 is: 72:19:9.

of solids decreased from 0.11 to 0.078 w/v. This implies a small but probably insignificant release of intracellular product ($\sim 3\%$) upon homogenization. As expected, exposure of the homogenate to the highest stress levels found in a centrifuge feed zone resulted in no further changes of particle size distribution or solids fraction (results not shown here).

Since there is little product yield benefit of homogenization, the subsequent focus of the cell broth processing was on the use of flocculation of cell broth to enhance recovery by centrifugation. Here the important features are the aggregation into larger flocs of particles less than $\sim 5 \mu\text{m}$ (Fig. 4) and the increase in solids fraction from 0.11 to 0.15 w/v. Two species of flocs appear to be formed. Solids in the range of 5–50 μm make up 20% of the distribution and, interestingly, the overall solids fraction of particles in the 0–50 μm range has increased from ~ 0.025 to 0.030 w/v. The difference is possibly due to precipitate formation of soluble or colloidal material (Salt et al., 1995). The volume of aggregates $> 50 \mu\text{m}$ appears to increase from 0.090 w/v for thawed cell broth to 0.120 w/v, this again being possibly due to precipitation as well as liquid entrapment. The apparent loss of particles in the range 150–500 μm is only partly accounted for by the increase above 500 μm and it is possible that the preparation of PEI flocs using mixing for 1 h at relatively high speed led to some break-up of larger aggregates over time. Such an effect is

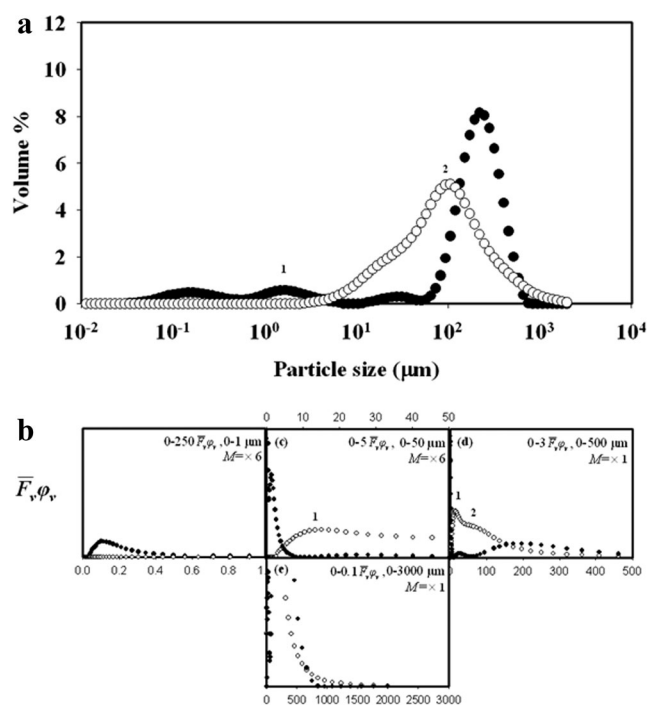


Figure 4. PEI flocculation of freeze-thawed cell broth. Volume size distributions are presented for (●) non-sheared thawed cell broth (see Fig. 2 for description) and for (○) PEI-flocculated thawed cell broth. See Figure 1 for explanation of size distributions. The relative solids fractions, φ_v are 0.11 w/v for thawed autolysate and 0.15 w/v for PEI flocculated material (φ_v values quoted are corrected for dilution factor with PEI solution). The volume ratio of peaks 1 and 2 is $\sim 20:80$.

also noted in Figure 2 with large aggregates ($>200\ \mu\text{m}$) being broken-up by shear stress (a phenomenon also reported in previous work; for example, see Gasner and Wang (1970)).

The effect of hydrodynamic stress on the flocs at levels equivalent to that found either in the low or high shear stress feed zone industrial-scale centrifuge is studied in Figure 5. The majority of the large aggregates are disrupted to form aggregates in the $10\text{--}50\ \mu\text{m}$ size range (Fig. 5b–d). However, there is no evidence of the reappearance of the sub $2\ \mu\text{m}$ particles present in thawed cell broth (Fig. 5b). Microscope images (not shown here) help confirm the changes observed, especially of the effect of shear on the large aggregates present in the flocculated material. While the size distribution of the non-sheared fresh material was considerably different to that of thawed material, the final overall size distributions of sheared material were similar especially after exposure to high hydrodynamic stress (Fig. 6a). Again, no particles were evident below $\sim 2\ \mu\text{m}$ (see Fig. 6b) although compared with flocs of thawed material the flocculated fresh material does contain a higher proportion of fine particles (Figs. 5b and 6b) which, however, appear to be less sensitive to shear stress. These are all factors which should impact the ability to process these flocculated materials by centrifugation at scale.

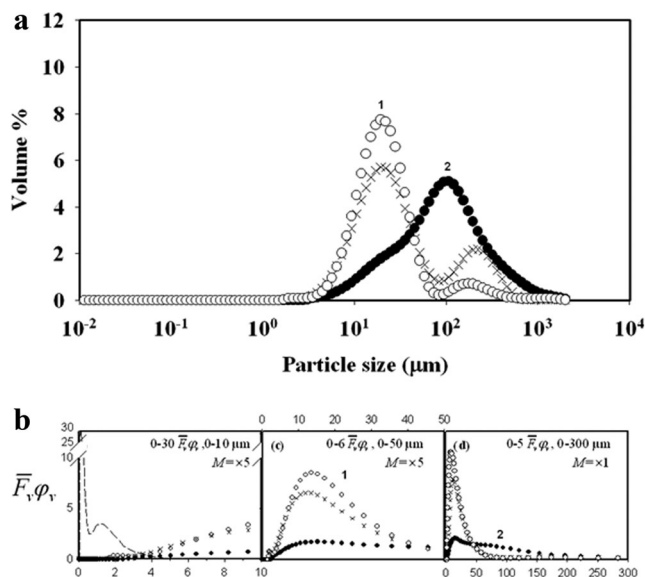


Figure 5. Effect of shear stress on PEI flocculated freeze-thawed cell broth. Volume size distributions are presented for (●) PEI flocculated cell broth and for PEI flocculated cell broth sheared at equivalent ε of (×) $0.045 \times 10^6\ \text{W kg}^{-1}$ and (○) $0.53 \times 10^6\ \text{W kg}^{-1}$. The relative solids fractions, φ_v , are $0.13\ \text{w/v}$ for PEI flocculated thawed cell broth sheared at $0.045 \times 10^6\ \text{W kg}^{-1}$ and $0.12\ \text{w/v}$ for PEI flocculated thawed cell broth at $0.53 \times 10^6\ \text{W kg}^{-1}$ (φ_v values quoted are corrected for dilution factor with PEI solution). The volume ratios of peaks 1 and 2 are for (●) 20:80, (×) 78:22, (○) 93:7. Data for thawed cell broth (Fig. 2) is shown for comparison in Figure 5b (---).

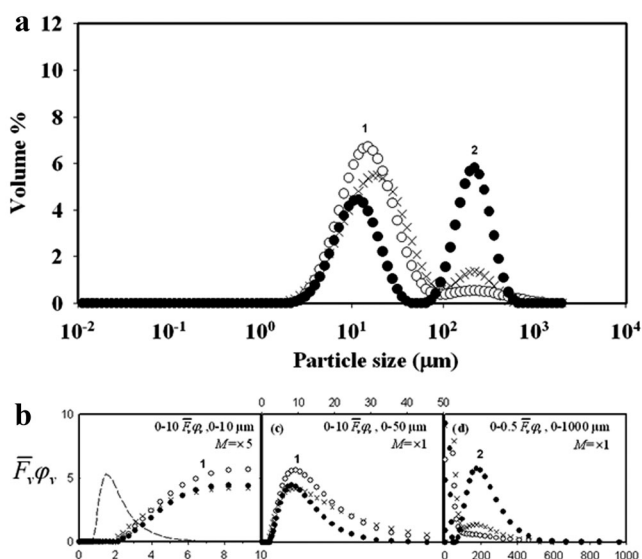


Figure 6. Effect of shear stress on PEI flocculated fresh cell broth. Volume size distributions are presented for (●) PEI flocculated fresh cell broth, and for PEI flocculated fresh cell broth sheared at equivalent ε of (×) $0.045 \times 10^6\ \text{W kg}^{-1}$ and (○) $0.53 \times 10^6\ \text{W kg}^{-1}$. The relative solids fractions of the non-sheared and sheared material are as for flocculated fresh cell broth (see Fig. 5). The volume ratios of peaks 1 and 2 are (●) 50:50, (×) 87:13, (○) 93:7. Data for fresh cell broth (Fig. 1) is shown for comparison in Figure 6b (---).

Suspension Rheological Properties

Some examples of flow properties of the various materials studied under laminar flow conditions are given in Figure 7 in terms of the apparent viscosity as a function of shear rate during exposure to extended cycles of shear. All materials exhibited moderate shear thinning characteristics (n values range from 0.75 to 0.95) with both time dependent behavior and some irreversible loss of structure. This overall rheo-destructive behavior makes it necessary to relate rheological measurements under defined laminar flow conditions to the flow conditions which prevail during ultra scale-down test tube centrifugation and, for the purposes of prediction of full-scale operation, the conditions which apply during continuous flow centrifugation. Some materials, especially non-sheared PEI flocculated suspensions, exhibited considerable slip at the rheometer walls which led essentially to measurement of just the continuous phase. In these cases, the rheological properties of closely related material are used (e.g., PEI flocculated material exposed to preconditioning by low hydrodynamic stress in the ultra scale-down shear device).

Ultra Scale-Down Centrifugation

The range of centrifugation conditions studied is representative of those commonly used in industrial scale centrifugation, $(Q/\Sigma_D) = V_{\text{lab}}/(t\Sigma_T) > \sim 3.0 \times 10^{-9}\ \text{m s}^{-1}$, where Q is the flow-rate and Σ_D is the Sigma value for a full scale

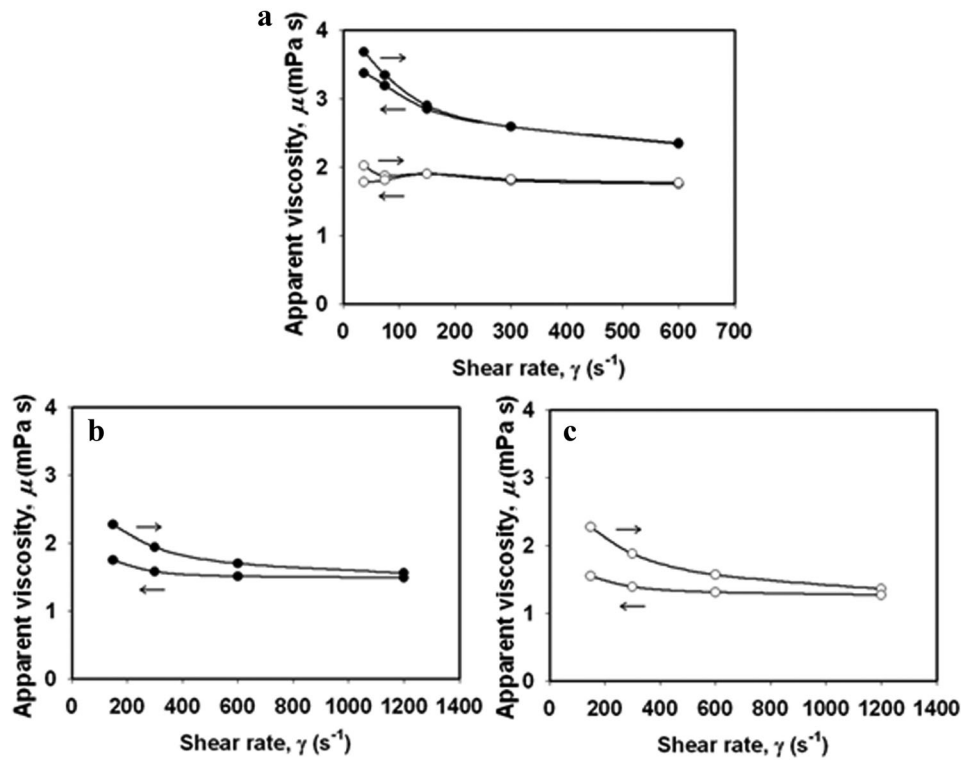


Figure 7. Rheological characterization of processed cell broth. Data presented:

Broth pre-treatment	Increasing shear		Decreasing shear	
	n	k ($\text{N s}^n \text{m}^{-2}$)	n	k ($\text{N s}^n \text{m}^{-2}$)
(a) Thawed cell broth (●)	0.83	6.76×10^{-3}	0.83	6.76×10^{-3}
(a) Homogenate (○)	0.95	2.34×10^{-3}	0.95	2.34×10^{-3}
(b) PEI flocs, $\varepsilon = 0.045 \times 10^6 \text{ W kg}^{-1}$	0.82	5.50×10^{-3}	0.92	2.51×10^{-3}
(c) PEI flocs, $\varepsilon = 0.53 \times 10^6 \text{ W kg}^{-1}$	0.75	7.76×10^{-3}	0.90	2.46×10^{-3}

The rheology of non-sheared flocs was not recordable, most likely due to slip at the disc surface in the presence of large flocs. Temperature was maintained at 23°C in the viscometer using a cooling water circuit.

centrifuge (the value includes a calibration factor to allow for non-ideal flow (e.g. Hutchinson et al. (2006)). For the recovery of solids from fresh cell broth (Fig. 8a) and thawed cell broth (Fig. 8b), similar properties are obtained with ~13% solids remaining at the highest centrifuge throughputs and little evidence of the performance being affected by exposure of the material to even the higher hydrodynamic stress levels studied. At low centrifuge throughputs there are slightly higher levels of solids carry over for the thawed as compared with the fresh cell broth, this probably reflecting the differences in size distributions at the fine end with the former containing peaks in the submicron range (compare Figs. 1 and 2). The sensitivity to shear stress of the fresh cell broth (Fig. 1) is not reflected by changes in the clarification achieved (Fig. 8a); the small extent of change expected is within the range of uncertainty of the analysis. Extremely low

equivalent centrifuge throughputs are required to achieve say 10% level of solids remaining in the supernatant for homogenate (Fig. 8c). It was also observed that homogenization did not contribute to any extra release of product (data not shown). As might be expected the centrifuge performance is not affected by shear stresses imposed in advance.

For PEI flocculated cell broth (Fig. 8d and e) high levels of clarification are achieved reflecting the major changes in the particle size distribution especially in the sub-5 μm range (Figs. 5b and 6b) and the reduced viscosity. It is also evident that the flocculated material is sensitive to shear stress with up to a doubling of solids carry over when comparing no to high shear stress pre-processing. The fourfold increase in solids carry over when processing flocculated fresh rather than flocculated thawed cell broth (Fig. 8e compared with 8d) matches with the respective size distributions at the fine end

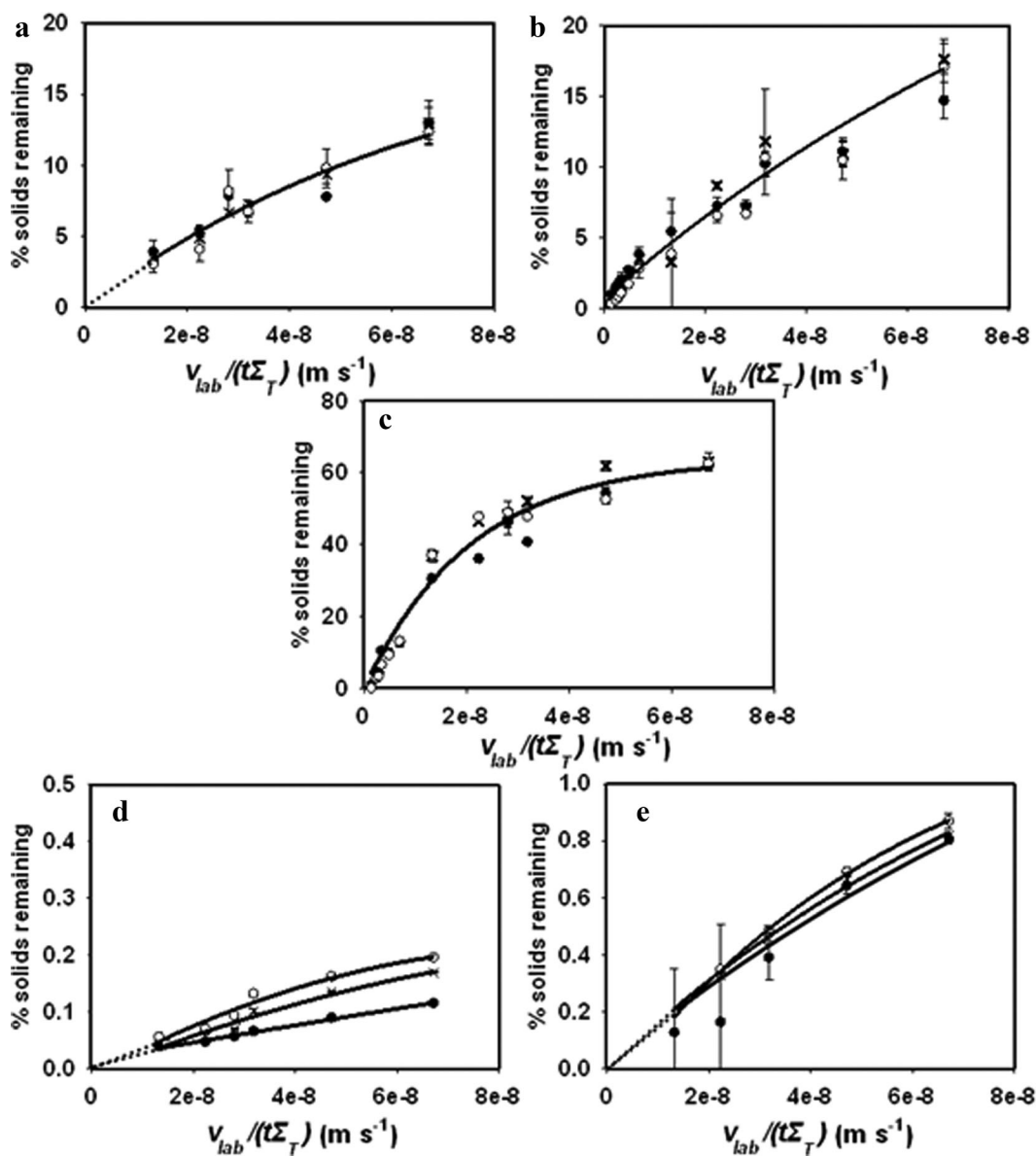


Figure 8. Ultra scale-down centrifugation analysis of processed cell broth: (a) fresh cell broth, (b) freeze-thawed cell broth, (c) homogenized thawed cell broth, (d) PEI flocculated thawed cell broth, and (e) PEI flocculated fresh cell broth. The different suspensions were all exposed to conditions of: (●) no shear; (×) $\varepsilon = 0.045 \times 10^6 \text{ W kg}^{-1}$; (○) $\varepsilon = 0.53 \times 10^6 \text{ W kg}^{-1}$ and then processed by ultra scale-down centrifugation. Data presented as mean \pm average ($n=2$); lines are best least squares fit using 3rd order polynomials. For graphs (a–c) single correlations are given as there is no consistent trend with increasing shear rate. In all cases the correlations are fitted through the origin which provides the control.

(Figs. 5b and 6b). Also matched is the impact of shear stress with over a doubling in solids carry over in going from no to high shear stress for flocs from a thawed cell broth compared with those from a fresh cell broth where there is $\sim 20\%$ increase in solids carry over.

Figure 9 explores the effect of the stresses involved during continuous-flow centrifuges on the recovery of domain antibodies. No change in the concentrations of the domain antibodies or their molecular variants (e.g., dimers) was observed in the ultra scale-down shear stress tests, even under excessive (1 h) exposure to shear stress. These results are

consistent with previous observations on antibody fragments, for example, fAbs (Harrison et al., 1997) and monoclonal antibodies, for example, mAbs (Reid et al., 2010) where the impurities present in the broth probably serve to act as surface-active protectants of the proteins even if there are denaturing (e.g., air/liquid) interfaces present. No significant difference was observed in the yield of domain antibody or in the profile of monomer and dimers for any of the cell broth conditioning methods studied in this paper, that is, freeze–thawing, homogenization or PEI flocculation (data not shown here). As discussed earlier there may be a

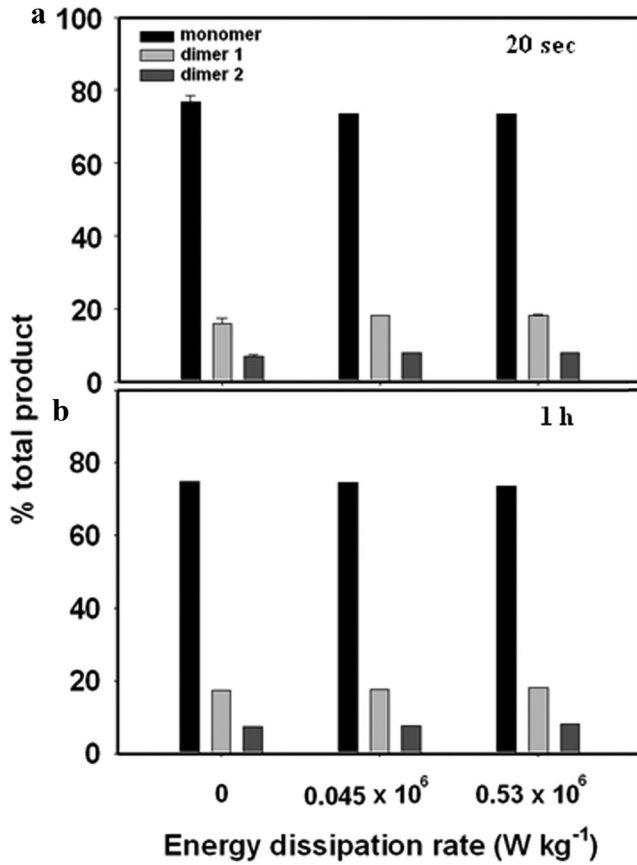


Figure 9. Product stability during shear stress of cell broth. The product purity profile in terms of monomer and two dimer forms after exposure to different shear stress levels for (a) 20 s (i.e., the same overall equivalent exposure to stress as in a centrifuge feed zone) and (b) for 1 h. In all cases thawed cell broth was used as a starting material. Data presented as mean \pm SD ($n=3$).

small increase due to the lower solids volume fraction after homogenization. Studies on the effect of broth conditioning on sediment dewatering will be a key issue in the future if more intensive high solids concentration processing is of interest (Tustian et al., 2007).

Correlating Suspension Properties and Centrifuge Performance

From the definition of the settling capacity of a centrifuge, $V_{lab}/t\Sigma_T$ we have (Ambler, 1959; Maybury et al., 1998):

$$d_c = \left(\frac{18\mu}{\Delta\rho g} \right) \left(\frac{V_{lab}}{t\Sigma_T} \right) (f(\phi_v))^{0.5} \quad (3)$$

where d_c is the critical particle size above which all particles are recovered and $f(\phi_v)$ is a correction factor to allow for the volume fraction of solids. Various expressions exist for $f(\phi_v)$ depending on the suspension type; in this study $f(\phi_v) = 1/(1 - \phi_v)$ is assumed (Richardson et al., 2002), this being based on the theoretical reduction in area available for upward flow

(other correlations were considered but the hindered settling conditions which exist in the flow between disc spaces are insufficiently well-characterized to justify the use of any one correlation). For the purpose of this work $V_{lab}/t\Sigma_T = 3.50 \times 10^{-8} \text{ m s}^{-1}$ was chosen; this is equivalent to flowrates ranging from 10^2 L h^{-1} for small pilot scale centrifuges to 10^5 L h^{-1} for the larger industrial scale machines used for broth processing. Viscosity values, μ , used in the correlation were taken from Figure 7 at $\gamma = 1 \text{ s}^{-1}$ as an approximation of low shear condition in a centrifuge tube. The predicted percentage of solids in the supernatant after centrifugation, S_{pred} , is given by:

$$S_{pred} = \sum_{N=0}^{\infty} V(d_i) \times (1 - T(d_i)) \quad (4)$$

where $T(d) = (d/d_c)^2$ for $d < d_c$,

$$T(d) = 1 \text{ for } d \geq d_c$$

where $V(d_i)$ is the percentage volume of particles in a channel i of average size d_i . Calculated values of d_c are given in Figure 10 legend. The comparison for predicted and actual solids remaining is presented in Figure 10, this showing good correlation but with some offset at the very low levels of solids remaining achieved using flocculation. This may be for a variety of reasons including differences in the densities of the different sized particles being recovered, the approximations made for the rheological properties which prevail during ultra scale-down centrifugation and the uncertainty in

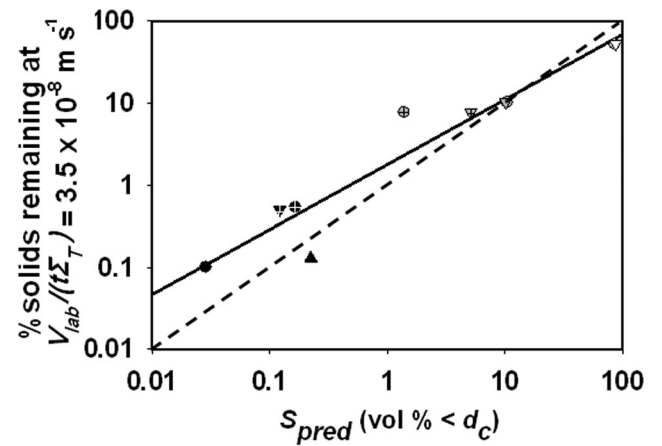


Figure 10. Correlation between the particle volume fraction $d < d_c$ and the volume fraction in suspension after centrifugation at $V_{lab}/(t\Sigma_T) = 3.50 \times 10^{-8} \text{ m s}^{-1}$; data presented for fresh cell broth (\oplus , no shear; ∇ , high shear) and freeze-thawed cell broth (\bullet , no shear; ∇ , high shear), $\mu = 6.76 \times 10^{-3} \text{ N s m}^{-2}$, $d_c = 2.79 \text{ }\mu\text{m}$; homogenate (\circ , no shear; ∇ , high shear), $\mu = 2.34 \times 10^{-3} \text{ N s m}^{-2}$, $d_c = 1.61 \text{ }\mu\text{m}$; PEI flocculated cell broth (\oplus , \bullet , no shear; \blacktriangle , \triangle , low shear; \oplus , \blacktriangle , fresh; \bullet , \blacktriangle , thawed) $\mu = 5.50 \times 10^{-3} \text{ N s m}^{-2}$, $d_c = 2.54 \text{ }\mu\text{m}$; PEI flocculated cell broth high shear (∇ , fresh; ∇ , thawed) $\mu = 7.76 \times 10^{-3} \text{ N s m}^{-2}$, $d_c = 3.01 \text{ }\mu\text{m}$; Viscosity values taken from Figure 7; measurements for fresh autolysate, PEI flocculated cell broth at low shear and PEI flocculated fresh cell broth were all affected by slip at the rotating bob; viscosity values were taken from nearest neighbour material. Assumed $\Delta\rho = 75 \text{ kg m}^{-3}$ (Baldwin et al., 1995). Low shear stress, $\varepsilon = 0.045 \times 10^6 \text{ W kg}^{-1}$; high shear stress $\varepsilon = 0.53 \times 10^6 \text{ W kg}^{-1}$; — best least square fit for all data points; - - - parity line.

particle size distributions especially at the fine end of the distribution. The key value of this ability to correlate the predicted and experimentally observed extents of solids remaining is that it helps verify the observations made especially of changes in the particle size distribution as a function of the different conditioning procedures used.

Prediction of Large Scale Centrifugation From USD Results

The critical aspect here is the translation of the ultra scale-down results achieved using either low or high shear stress pre-treatment. The rheological properties which relate to the settling zone of a continuous flow centrifuge will relate to those after exposure to extensive shear cycles with for example a threefold reduction of apparent viscosity being possible (Fig. 7). There are then two methods to translate the ultra scale-down data:

(a) via the use of equivalent capacity/settling areas, that is, $Q/\Sigma_D = V_{lab}/t\Sigma_B$ where a calibration factor is used in the evaluation of Σ_D to account for non-ideal flow patterns.

(b) use of the particle size distributions as in this paper but with an alternative expression for the grade efficiency as a function of d_i relative to d_c (Mannweiler and Hoare, 1992):

$$T(d_i) = 1 - \exp(-0.865(d_i/d_c)^{2.08}) \quad (5)$$

Again this expression allows for non-ideal flow patterns in the settling zone of the full scale centrifuge. However it does require the Σ_D value for the full-scale centrifuge to be based on the assumption of ideal streamline flow patterns, that is, without any calibration factor used. The value of this approach is that it takes into account the larger part of the size distribution at the fine end which determines the extent of solids carry over (i.e., $T(d_i) < 1$ for $d_i/d_c < 2.5$).

Conclusions and Future Work

Ultra scale-down centrifugation provides insight of centrifuge performance as may occur at full scale. Alternatively the suspension physical properties may be combined to help predict centrifuge performance. The good correlation achieved in this study between the two methods for characterization of centrifuge performance allows the importance to be assessed of various suspension properties such as the volume fraction at the fine end of a size distribution, for example, $< \sim 3 \mu\text{m}$ depending on suspension viscosity and solids volume fraction. For autolysates the presence of a significant proportion by volume of particles at the fine end of the distribution leads to the prediction of substantial solids carry over during industrial scale centrifugation. However this material is not unduly affected by the levels of shear stress which prevail in the feed zones of continuous centrifuges. The effect of homogenization is to increase substantially the amount of material at the fine end and there is over fourfold increase in solids carry over during centrifugation. Flocculation of the cell broth with PEI

removes nearly all material at the fine end. This, combined with a reduction in viscosity, leads to a major 20- to 50-fold reduction in supernatant solids carry over in the centrifuge as compared with non-flocculated cell broth. However, the PEI flocculated material is sensitive to shear stress and this can lead to ~ 20 – 100% increase in solids carry over when using a centrifuge equipped with a high shear stress non-hermetic feed zone (reduced to 5–50% increase when using a low shear stress hydro-hermetic feed zone). The effects of such process improvements on clarification now needs to be assessed in terms of the impact on the whole bioprocess sequence, for example, on subsequent filtration stages. This will help better relate the control of physical properties needed in processing of challenging materials such as concentrated autolysed microbial cell broths.

Nomenclature

d	particle diameter (m)
d_c	critical particle diameter (m)
d_i	impeller diameter (m)
$F_{V,i}$	volume frequency distribution where i is the channel number
g	gravitational acceleration (m s^{-2})
\bar{G}	mean velocity gradient (s^{-1})
k	flow consistency index ($\text{N s}^n \text{m}^{-2}$)
n	flow behavior index
N	disc speed (rps)
N_S	stirrer speed (rps)
OD_S	optical density of supernatant at 600 nm
OD_O	optical density of supernatant after extended centrifugation at 600 nm
OD_F	optical density of feed at 600 nm
P	mixing vessel power input ($= P_o \rho N S^3 d_i^5$) (W)
Q	flow-rate through a disc stack centrifuge ($\text{m}^3 \text{s}^{-1}$)
P_o	power number
r_e	the effective radius of the centrifuge (m)
R_i	inner radius of a centrifuge tube (m)
R_o	outer radius of a centrifuge tube (m)
RCF	relative centrifugal force (g)
$T(d)$	fraction of particles of diameter $\leq d$ that will sediment
v_s	settling velocity in a centrifugal field (m s^{-1})
V_{lab}	volume of process material in a test tube centrifuge (m^3)
$V(d_i)$	percentage volume of particles in a channel i of average size d_i
W	size-interval width in particle size distribution (μm)
ε	maximum power dissipation (W kg^{-1})
ρ	fluid density (kg m^{-3})
$\Delta\rho$	density difference between solid and liquid phase (kg m^{-3})
θ	half-disc angle (rad)
γ	shear rate (s^{-1})
μ	dynamic viscosity (Pa s)
Σ_D	equivalent settling area of a disc stack centrifuge (m^2)

Σ_T equivalent settling area of a test tube centrifuge (m^2)
 φ_v relative solids fraction of a cell suspension (w/v)
 ω angular velocity of a centrifuge (rad s^{-1})

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