ARTICLE

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Integration of Host Strain Bioengineering and Bioprocess Development Using Ultra-Scale Down Studies to Select the Optimum Combination: An Antibody Fragment Primary Recovery Case Study

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ABSTRACT: An ultra scale-down primary recovery sequence was established for a platform *E. coli* Fab production process. It was used to evaluate the process robustness of various bioengineered strains. Centrifugal discharge in the initial dewatering stage was determined to be the major cause of cell breakage. The ability of cells to resist breakage was dependant on a combination of factors including host strain, vector, and fermentation strategy. Periplasmic extraction studies were conducted in shake flasks and it was demonstrated that key performance parameters such as Fab titre and nucleic acid concentrations were mimicked. The shake flask system also captured particle aggregation effects seen in a large scale stirred vessel, reproducing the fine particle size distribution that impacts the final centrifugal clarification stage. The use of scale-down primary recovery process sequences can be used to screen a larger number of engineered strains. This can lead to closer integration with and better feedback between strain development, fermentation development, and primary recovery studies.

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KEYWORDS: ultra scale-down; primary recovery; antibody fragment; strain development

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Introduction

Selection of production strains is a pivotal decision point early in process development that can potentially affect the whole bioprocess. Successful strain development integrated with expression vector development and screening, can result in increased expression levels of target protein and in significant gains in process productivity.

The expression of soluble heterologous proteins is known to be a challenge in microbial systems, potentially necessitating the introduction of additional purification steps (Singh and Panda, 2005). A number of cellular mechanisms (Baneyx and Mujacic, 2004; Glick, 1995) gene sequence properties (e.g., de Smit and van Duin, 1990; Mita et al., 1988; Wen et al., 2008) and product properties (e.g., Mayer et al., 2007; Roodveldt et al., 2005) can contribute to the formation of misfolded proteins. This rapidly leads to a large experimental space where the search for higher yields can only be achieved by using high-throughput technology. The ease with which recombinant DNA technology can create strains shifts the bottleneck towards the characterization studies, particularly those related to bioprocess development.

Candidate strains developed and identified during the metabolic and genetic optimization stage are subsequently characterized more extensively during process optimization studies. Typically microwell, shake flask, or small bioreactor studies are conducted in combination with multifactorial experimental design or search algorithms to optimize biomass, growth rate, and feeding strategies (Islam et al., 2007; Weuster-Botz, 2000). Growth rates can significantly affect cellular properties, for example there is a dynamic trade-off between ribosome concentration and plasmid copy

number at different growth rates and protein production rates (Hoffmann et al., 2004). More particularly, for the Fab expressing *E. coli* system investigated here, it has been demonstrated that the leakage of expressed protein from the periplasm is dependent on the specific growth rate (Bäcklund et al., 2008).

With the product located in the periplasm, the primary recovery step involves a centrifugal solid–liquid separation step and the discharged heavy-phase is retained for processing. Cell damage as a result of feed zone shear or discharge impaction increase the intracellular contaminants released and introduced to the purification stage (Gray et al., 1972). As such, unexpected biological property changes e.g. reduced viability or increased shear sensitivity of newly introduced strains may become apparent during the primary recovery stage investigations. Such dynamic system behavior requires that strain selection, fermentation, and primary recovery studies be investigated together. Scaled-down primary recovery studies can be useful to help inform the strain selection process. High cell-density fed-batch fermentation studies conducted in miniaturized bioreactors can already deliver process relevant material to evaluate some aspects of primary recovery such centrifugal clarification through low volume studies (Ali et al., 2012).

In this study a scaled-down primary recovery sequence of a platform-based Fab production process is implemented (Spitali, 2008). Pilot scale dewatering centrifugation, periplasmic extraction, and clarification centrifugation stages are mimicked with several ultra-scaled down (USD) devices and protocols. USD clarification techniques were integrated (Boychyn et al., 2004; Maybury et al., 2000; Tustian et al., 2007) and combined with the USD centrifugal discharge mimic (Chan et al., 2006) to characterize feed stream behavior during centrifugation. A method was implemented to conduct periplasmic extraction at low volume in shake flasks. A scale-down comparison was performed using several novel cell strains.

Materials and Methods

Reagents

All reagents were purchased from BDH Chemicals (Merck Ltd., Lutterworth, UK), except where otherwise stated, and were of the highest grade available.

Sample Analysis

Calculation of Cell Breakage

The relative strength of strains was determined indirectly based on the amount of product or dsDNA released normalized as a percentage of the total amount available. The total available product and dsDNA were determined from samples homogenized with a single pass at 2 kbar (30 kpsi) using a Constant TS Benchtop high-pressure homogenizer (Score Group plc, Peterhead, UK). The release was calculated using the equation:

$$\text{Release} = \frac{\left([\text{Analyte}]_{\text{s}} - [\text{Analyte}]_{\text{ref}}\right) \times \left(1 - f_{\text{wcw}}\right)}{[\text{Analyte}]_{hom} \times \left(1 - f_{hom}\right)} \times 100\% \quad (1)$$

where the analyte concentrations with subscripts s, ref, and hom represent the test sample, baseline reference and homogenized material respectively. f_{wcw} and f_{hom} are the volume fractions of solids in test samples and homogenate, respectively. Release in heavy phase samples were calculated after correcting for higher cell and total available analyte concentrations.

dsDNA Analysis

Total dsDNA analysis was done with the Quant-iT PicoGreen kit (Invitrogen, Paisley, UK). A standard curve was prepared in the range of 0.1–2.0 μg/mL from a 100 μg/mL lambda DNA standard and samples diluted in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5) for analysis. Measurements were made in flat-bottomed microtiter plates (BD Biosciences, Oxford, UK) with a FLUOstar Optima (BMG LABTECH Ltd, Aylesbury, UK).

Cell Viability FACS Analysis

The viability of cells in the harvested broth was measured with flow cytometry (BD FACSCalibur, BD Biosciences) using Bis(1,3-dibarbituric acid)-trimethine oxanol (BOX) (Sigma–Aldrich, Gillingham, UK) and propidium iodine (PI) (Sigma–Aldrich). The broth was diluted with physiological saline to an optical density of 0.005 AU followed by addition of BOX and PI to give final concentrations of 0.5 and 5 μg/mL, respectively. Prepared samples were incubated for 10 min before FACS analysis.

Protein-G HPLC Analysis

Antibody fragment (Fab) product concentration was determined by HPLC analysis using an Agilent 1200 series HPLC (Agilent Technologies UK Ltd., Wokingham, UK). Analysis was conducted according to the method reported by Bäcklund et al. (2008).

Particle Size Analysis

Size analysis of suspended solids from initial and conditioned extract was done by laser light diffraction (Mastersizer 2000S connected to a Hydro SM wet dispersion unit, Malvern Instruments Ltd., Malvern, UK). Samples were diluted hundred fold in physiological saline to prevent further aggregation and measured in triplicate. The percentage

undersize d_5 and d_{50} characteristic descriptors of particle size distribution were used for comparing data from different studies.

Fermentation

E. coli Host Strains, Vectors, and Fermentation Processes

The parent *E. coli* wild-type host strain (K12 derivative, W3110) was modified to produce a number of different host strains. Deletions and mutations in periplasmic proteolytic enzymes Tsp (Hara et al., 1991; Silber et al., 1992) and Spr (Aramini et al., 2008) were performed, resulting in strains A2 (*spr* mutation) and A1 (*tsp* deletion and *spr* mutation). These modified strains were assessed alongside the wild-type W3110 in this work.

A number of expression vectors consisting of the same backbone but some engineered to co-express an *E. coli* "helper protein" to increase protein refolding and yields were tested. The vectors tested were for the expression of Fab #1 (CDP870) and Fab #2 (CDP7657) with and without co-expression of factor D (DsbC) (Chen et al., 1999; Missiakas et al., 1994; Zapun et al., 1995).

Fermentations were carried out at 2 L scale (Biostat B Plus, Sartorius, Goettingen, Germany) and at 20 L scale (Biostat C Plus and CDCU, Sartorius, Germany). Three different fermentation protocols were assessed. The FP1 fermentation process consisted of a batch growth phase on glycerol, followed by a switch to lactose in order to induce the cells. Post-induction growth was phosphate-limited and lactose concentration was maintained in excess up until harvest, reaching a DCW of approximately 40 g/L. The FP2 and FP3 fermentation processes replaced lactose induction with IPTG induction and growth rates pre and post induction were controlled with a glycerol feed. The FP3 process incorporated an exponential feeding stage and higher induction temperature allowing higher biomass concentrations to be reached prior to induction. Improvements to growth media were also incorporated into the FP3 process and the number of additions required during the fermentation was reduced. Increased biomass levels were achieved with the FP2 and FP3 processes, reaching approximately 45 g/L (DCW) and 60 g/L (DCW), respectively.

USD Studies

USD feed zone studies were conducted with a rotating disc device (RDD) depicted in Figure 1A (Boychyn et al., 2001). Fermentation broth was loaded into the 4°C pre-chilled chamber of the RDD and the disc rotated at preset speeds for 20 s. Shear treatment was performed at nine rotation speeds in the range of 4,000–21,000 rpm corresponding to 1.7×10^3–$8.2 \times 10^3\ s^{-1}$ maximum shear rates. Samples were centrifuged at 13,000 rpm for 5 min in a Hettich Mikro 200 microfuge (Tuttlingen, Germany) and the supernatant retained for analysis.

Centrifugation clarification scale-down to laboratory scale was based on Sigma theory (Ambler, 1959) where a constant ratio of the feed flow rate (Q) to the equivalent settling area (Σ) was maintained (Boychyn et al., 2004; Maybury et al., 2000). Hindered settling due to high solids concentration was corrected for using the methodology described by (Tustian et al., 2007). USD centrifugation studies were conducted with a Hettich Rotanta 460R bench top centrifuge (Tuttlingen, Germany) using 15 mL Falcon™ tubes (BD Biosciences). Using the RDD, the harvest feed was shear treated at 2×10^4 W/kg for 20 s before centrifugation and diluted with well-clarified broth prepared by the

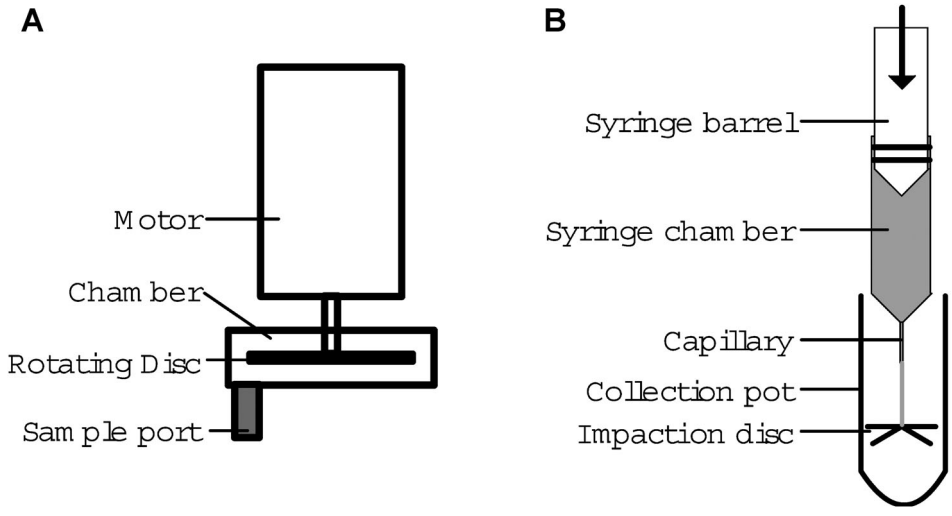

Figure 1. Schematic representations of the USD devices. **A**: The rotating disc subjects test material housed in the chamber to shear treatment. **B**: The capillary discharge device accelerates test material housed in a syringe chamber through a capillary onto an impaction disc.

centrifugation of fermentation broth at 11,000*g* for 30 min in a Beckman Coulter Avanti J-26 XPI (Beckman Coulter, High Wycombe, UK). Clarification performance was determined over the Q/cΣ range of 2×10^{-9}–2×10^{-7} m/s.

The optical densities of clarified supernatant, feed material, and well-clarified broth samples were measured at 600 nm using a Genesys 10 UV spectrophotometer (Thermo Scientific, Leicestershire, UK). Where required, samples were diluted with physiological saline (0.85% (w/v) NaCl in deionized water). The clarification levels were calculated using the equation:

$$\text{Solids remaining} = \frac{\text{OD}_{\text{feed}} - \text{OD}_{\text{sup}}}{\text{OD}_{\text{feed}} - \text{OD}_{\text{ref}}} \times 100\% \qquad (2)$$

where OD_{feed} refers to the feed stream before centrifugation, OD_{sup} refers to the supernatant obtained after the centrifugation test condition, and OD_{ref} was taken to be equivalent to 100% clarification achieved in an extensively centrifuged sample.

USD discharge was mimicked with a capillary discharge device (CDD), depicted in Figure 1B, which was designed and constructed in-house. It comprised a cylindrical barrel chamber with an internal diameter of 36 mm and working volume of 24 mL, fitted with a capillary at the exit. Chan et al. (2006) demonstrated the use of such a CDD to study impaction damage of *E. coli*. Two key findings from this study were incorporated into the equipment and experimental design. The same approach was used for calculating the jet break-up point (X_b) where maximum damage occurs (see also Shavlovsky, 1972) and the residence time in the capillary was kept below 0.004 s to minimize breakage of cells inside the capillary. All studies were conducted using a capillary with a diameter of 0.25 mm and length of 25 mm. Discharge material impinged onto an impaction plate positioned 75 mm below the capillary exit nozzle.

Shear treated broth was centrifuged and supernatant equal to two-thirds of the total volume was removed before the pellet was resuspended in the remaining supernatant to obtain USD heavy phase with a dewatering level of 66.7% (v/v) using equation:

$$\text{Dewatering} = \left(1 - \frac{m_{\text{HP}}}{m_{\text{F}}}\right) \times 100\% \qquad (3)$$

where m_{HP} and m_{F} represent the mass or volume of the heavy phase and feed streams, respectively. The USD heavy phase was loaded into the syringe chamber and aliquots of 6 mL were discharged at 10, 20, 30, and 40 m/s, respectively. Discharged heavy phase was clarified as described before and the supernatant retained for analysis.

Pilot Centrifugation

A Westfalia Pathfinder SC 5 continuous disc stack centrifuge (GEA Westfalia Separator Group, Oelde, Germany) was operated with bowl speeds between 8,000 and 12,500 rpm and flow rates between 50 and 700 L/h. The dewatering and clarification runs were typically conducted at 50–70 L/h with a bowl speed of 10,800 rpm giving a Q/Σ of 9×10^{-9} m/s. The heavy phase was discharged from the solids holding space approximately every 4 min at a bowl speed of 9,800 rpm. Dewatering of the pilot scale heavy phase was typical in the range of 61–72% (w/w) as determined with Equation (3).

The velocity of the discharge was estimated via equation (Letki et al., 1997):

$$v_{\text{N}} = C\left(2p_{\text{N}}/\delta_{\text{N}}\right)^{1/2} \qquad (4)$$

where p_{N} is the discharge pressure (Pa), δ_{N} is the heavy phase mass density (1,100 kg/m^3), and C a friction coefficient taken to be 1 for a split bowl discharge mechanism. The discharge pressure was estimated from the equation:

$$p_{\text{N}} = \frac{\delta\omega^2}{2}\left(r_{\text{N}}^2 - r^2\right) \qquad (5)$$

where δ is the intermediate mass density (1,050 kg/m^3), ω (rad/s) is the angular velocity, r_{N} the radius of free liquid surface (m), and r the radius of discharge (m).

Periplasmic Extraction

Equal volumes of heavy phase, stock extraction buffer (300 mM Tris–HCl, 30 mM EDTA, pH 7.4) and deionized water were combined to form the extract solution. Large scale extraction was done either at 5 or 20 L scale in stirred tank reactors at a volumetric power consumption of 2 W/kg and a temperature of 60°C. USD extraction was performed in duplicate in sealed 250 mL baffled shake flasks. A volume of 50 mL extract was agitated at 300 rpm in an Innova 42 shake incubator (New Brunswick Scientific, Edison, NJ) programmed to mimic the temperature profile of the pilot scale system. The initial extracts were conditioned with 30% (v/v) acetic acid at 18°C and 300 rpm for at least 30 min before performing USD clarification. The initial and conditioned extracts were sampled for analysis.

Results and Discussion

Pilot and USD Primary Recovery of Antibody Fragment (Fab)

The Fab primary recovery sequence involved four steps (Spitali, 2008). In the first step harvested fermentation broth was dewatered by continuous disc stack centrifugation. The discharged heavy phase solids process stream was resuspended and initial periplasmic extraction performed overnight in a stirred vessel. The heat-treatment step is effective in removing misfolded Fab species through denaturation and aggregation while retaining host cell proteins inside the intact cytoplasms of the *E. coli* spheroplasts. The extract was then

Table I. List of feed streams investigated.

Feed stream	Strain	Product	Factor D	Fermentation protocol	Harvest OD (AU 600 nm)	Cell viability (%)
Feed 1	W3110	Fab #1	No	FP1	82.4	81.9
Feed 2	A1	Fab #1	Yes	FP3	133.6	—
Feed 3	A1	Fab #2	Yes	FP3	119.6	91.7
Feed 4	A2	Fab #2	Yes	FP3	142	94.3
Feed 5	W3110	Fab #1	No	FP1	90.4	80.7
Feed 6	W3110	Fab #2	No	FP3	110	70.4
Feed 7	W3110	Fab #1	No	FP3	130.4	95.7
Feed 8	W3110	Fab #2	No	FP2	100.4	74.2
Feed 9	A2	Fab #1	Yes	FP3	154.4	—

conditioned with acetic acid and the final light phase process stream retained after clarification again by continuous disc stack centrifugation. The pH adjustment is performed in order to condition the feed material in preparation of the first chromatography step. Depending on the feed stream a reduction in pH may have the added benefit of separating additional impurities into the solid phase. The pilot scale dewatering step was mimicked with four USD steps: (i) treatment in RDD to mimic the feed zone shear, (ii) bench scale centrifugation to mimic clarification, (iii) removal of fixed volume supernatant to mimic dewatering, and (iv) treatment with CDD to mimic the discharge event. The mimic of the light phase process stream from the final clarification step comprised two separate USD steps. Only the feed zone and clarification mimics were required since the discharged solids process stream was not of interest in this study.

Feed material used for the evaluation of the scale-down mimics constitute of combinations of 3 host strains (W3110, A1, and A2), two products each expressed in two different vectors (Fab #1 and Fab #2) and produced using three fermentation protocols (FP1, FP2, and FP3). Representativeness of the USD mimics was assessed in parallel by using the pilot scale feed stream generated at each step of the process sequence. The feed stream properties are summarized in Table I.

Evaluation of USD Mimics

Centrifuge Feed Zone Damage

Typical results for product and dsDNA release after treatment in the RDD are shown as a function of maximum energy dissipation rate (ε_{max}) in Figure 2A. Measurable damage was only observed when the ε_{max} exceeded 10^5 W/kg. This is in agreement with results previously reported where *E. coli* loses viability in capillary shear studies when the shear stress exceeds the threshold of 1,250 N/m^2 (Lange et al., 2001). A shear stress of 1,250 N/m^2 is approximately equivalent to energy dissipation rates of 2×10^5–5×10^5 W/kg for typical *E. coli* broth viscosities in the range of 3×10^{-3}–9×10^{-3} Pa s. With the shear treatment limited to 20 s at ε_{max} conditions of 10^6 W/kg it was possible to determine differences in cell breakage rates and cell strengths in comparative studies. For example in Figure 2A the W3110 wild type strain (Feed 7) withstood shear damage better than the genetically modified higher yielding A2 strain (Feed 9). For both strains the cultivation protocol and product expressed were the same.

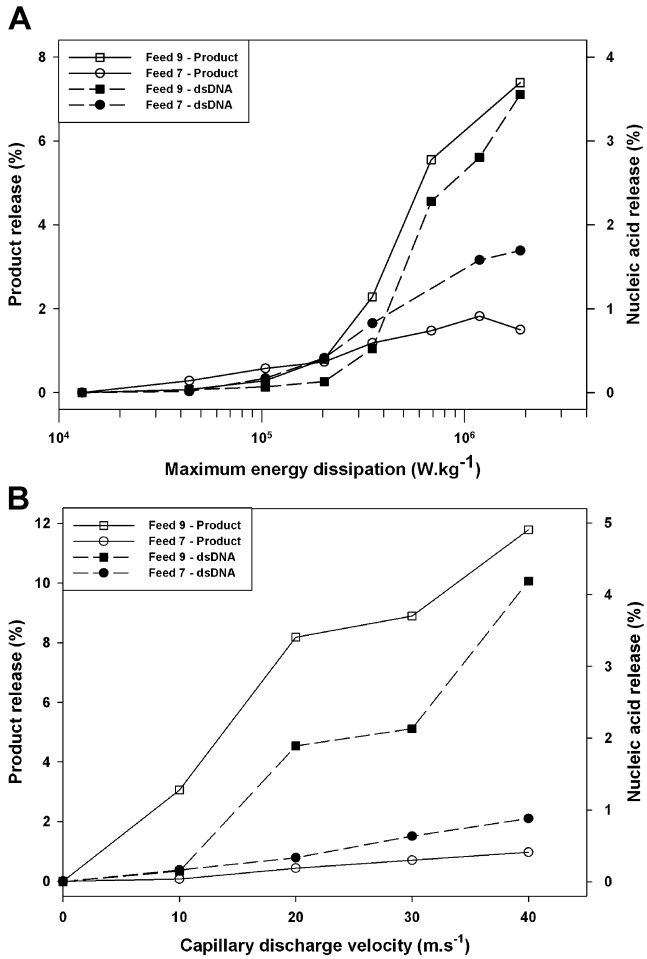

Figure 2. **A**: Cell breakage was measured as the release of product and nucleic acid after treatment in the RDD. Results are shown for two feed streams: (□,○) Feed 9 is sensitive to shear and (■,●) Feed 7 is more resistant to shear damage. **B**: Cell breakage was measured as the release of product and nucleic acid after treatment in the CDD. Results are shown for two feed streams: (□,■) Feed 9 is sensitive to impaction and (○,●) Feed 7 is more resistant to impact damage.

Several experimental studies reported in literature have investigated cellular damage inflicted by feed zones of pilot scale disc stack centrifuges. Based on CFD analysis and measurements of the extent of cell breakage (Boychyn et al., 2001; Hutchinson et al., 2006), an attempt was made to estimate the ε_{max} for two industrial centrifuges designed with either a hydro-hermetically sealed (Alfa Laval BTPX 305H) or standard non-flooded (Alfa Laval BTPX 205) feed zone. The ε_{max} values were estimated to be 2×10^4 W/kg and 3.6×10^5 W/kg for a hydro-hermetically sealed or standard non-flooded feed zone, respectively. Using a similar approach (Westoby et al., 2011) estimated a feed zone ε_{max} value of 3×10^4 W/kg for another centrifuge with a hydro-hermetically sealed feed zone (Westfalia SCS 6). The Alfa Laval BTPX 305H and Westfalia SCS 6 were both pilot scale systems with bowl volumes of 3.1 and 1.8 L, respectively.

Here the feed zone ε_{max} of the disc stack centrifuge was estimated by measuring the release of product located in the periplasmic space due to membrane shear damage. USD studies were conducted at ε_{max} values of 2×10^4, 4×10^5, and 2×10^6 W/kg. Cell damage due to feed zone shear was monitored by determining the difference in the product concentrations of the supernatant of the broth entering the centrifuge and the supernatant of the light phase exiting the centrifuge. The results for both USD and pilot centrifugation studies are listed in Table II for a number of feed streams. As expected, the product release increased with ε_{max} in all three USD studies, but overall very little release was observed. No product release was detected in broth after passing through the disc stack centrifuge feed zone. The ε_{max} of the feed zone with a bowl speed of 9,800 rpm was estimated to be lower than 2×10^4 W/kg.

Clarification and Dewatering

In the disc stack centrifuge there is bulk liquid flow between the discs which disturbs the sedimentation process and requires an efficiency factor to compensate for the reduction in the level of clarification. Empirically the correction factor has been determined to be in the range of 0.45–0.73 for disc stack centrifuges (Ambler, 1959; Axelsson, 1999; Morris, 1966). Using a dilute cell suspension (0.5% w/v), a correlation between the clarification efficiency factors of the disc stack centrifuge and USD clarification mimic was established (data not shown). The efficiency factor for the disc stack centrifuge was estimated to be 0.45 (efficiency factor for USD mimic is unity). At higher solid concentrations, typically when >10% (v/v), the particles in suspension hinder the settling process (Richardson and Zaki, 1954). The fraction of the solids in suspension was taken into account when determining the settling rate correction factor using dry cell weight (w/v) measurements of feed streams and the methodology described by Tustian et al. (2007).

Pilot scale dewatering (Fig. 3A) and extract clarification (Fig. 3B) were performed for harvested material from several different feed streams and the USD clarification prediction studies conducted in parallel. For both centrifugation steps the USD predictions provided a good estimate of the actual clarification levels obtained in the pilot centrifuge. The USD dewatering level was fixed at 66.7% by removing supernatant equal to two-thirds of total volume before resuspension.

Table II. Estimation of disc stack feed zone shear using USD shear mimic.

	USD centrate			Pilot centrate
Feed zone type	Hydro-hermetic seal	Standard	USD maximum	Disc stack
$\varepsilon_{max} \times 10^{-4}$ (W/kg)	2	4	200	—
Feed stream	Product released in centrate (g L^{-1})			
Feed 6	n.d.	0.01	0.03	n.d.
Feed 9	n.d.	0.01	0.04	n.d.
Feed 2	0.01	0.05	0.10	n.d.
Feed 8	0.01	0.07	0.15	—

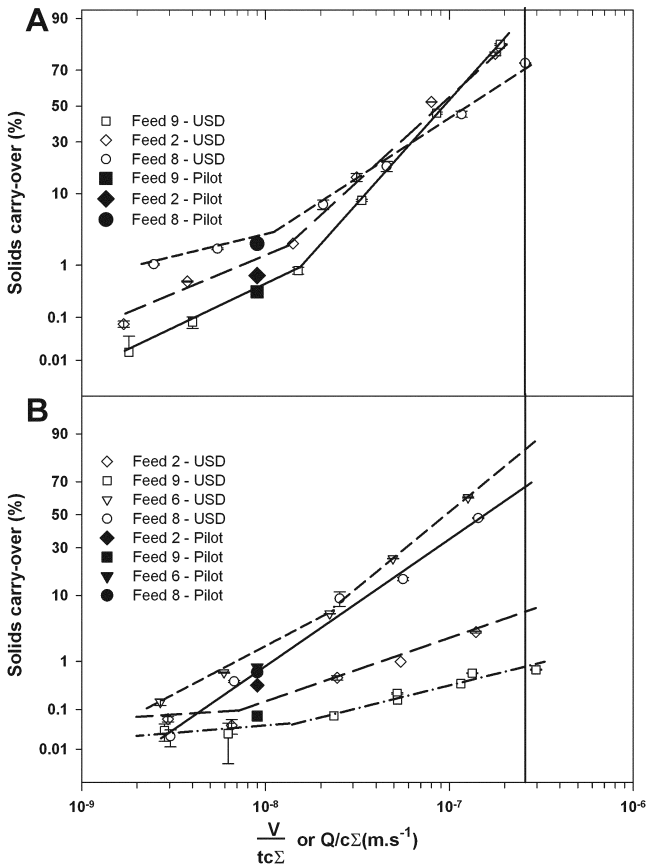

Figure 3. The USD centrifugation mimic was used to predict clarification levels for centrifugation operations and compared to pilot scale results. **A**: Dewatering clarification studies of harvest broth from three fermentation processes compare well for pilot and USD scales. The feed streams were: Feed 9 USD (□) and pilot scale (■), Feed 2 USD (◇) and pilot scale (◆) and Feed 8 USD (○) and pilot scale (●). **B**: Clarification studies of conditioned extract compare well for USD and pilot scales. The feed streams were Feed 2 USD (◇) and pilot scale (◆), Feed 9 USD (□) and pilot scale (■), Feed 6 USD (▽) and pilot scale (▼) and Feed 8 USD (○) and pilot scale (●).

Discharge Damage

Concentrated cells containing periplasmically located Fab, was discharged at high velocity as the heavy phase process stream. Previous studies suggested that discharge from disc stack centrifuges can result in between 10% and 20% disruption of *E. coli* (Gray et al., 1972). From mass balance analysis it was observed that most of the intracellular content release occurs during the dewatering step. The extent of release varied with the feed stream studied. The discharge step was considered critical since it could potentially impact significantly on subsequent purification steps.

Typical results from a USD discharge study are shown in Figure 2B. The extent of damage increased in a linear manner with discharge velocity over the range investigated. Chan et al. (2006) observed similar linear breakage behavior with increase in discharge velocity. At discharge velocities of 40 m/s clear differences in the strength properties of feed streams were observed. The W3110 wild type strain (Feed 7) was less susceptible to damage than the genetically modified high yielding A2 strain (Feed 9). These results were consistent to those obtained from the feed zone study.

The release of product and dsDNA from pilot scale and USD derived heavy phase are compared for several feed streams in Figure 4. The results show that the USD mimic is able to predict the ranking of different feed streams based on susceptibility to damage as measured by either product or dsDNA release. On average two to three times more content was released from the pilot scale discharge than with the USD mimic. The discharge velocity of the disc stack centrifuge at 9,800 rpm was estimated to be 94 m/s with Equations (3) and (4). This suggests that the relative differences in damage between scales is likely due to the differences in discharge velocities.

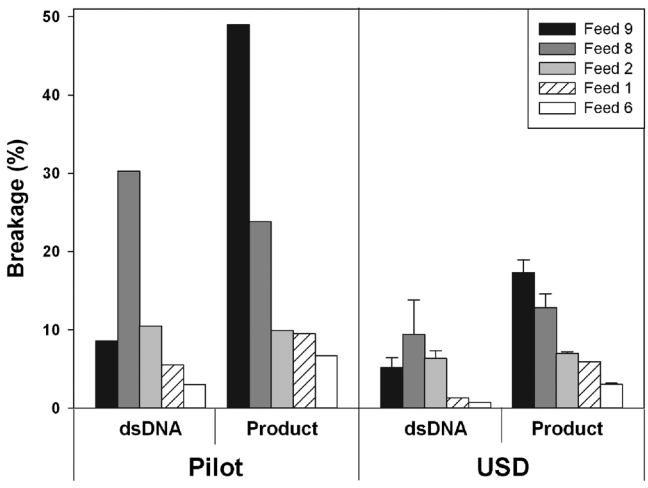

Figure 4. Pilot centrifuge and USD capillary discharge results for five feed streams. Feed streams are ranked according to their propensity to break based on product release. Results from CDD can predict relative level of breakage for feed streams evaluated. Error bars represent the standard deviation of results from duplicate USD experiments.

Periplasmic Extraction

A process relevant USD mimic of the extraction step should provide information on the chemical composition, particle size distribution (PSD) and particle strength of feed obtained with the pilot stirred vessel.

It is well established that mixing intensity impacts the equilibrium particle size of aggregated solids. At present there is no theoretical basis for matching mixing intensities in stirred vessels and flasks agitated by shaking. From experimental studies it was determined that a shaking speed of 300 rpm produced extract PSDs matching those of a stirred vessel operated at 2 W/kg ungassed power input most closely (data not shown). This setup was used for all the studies and proved to be reliable at producing similar fine particle populations for the feed streams evaluated.

In screening studies it is more convenient to report a single particle size descriptor, rather than a complete size distribution, for the purpose of comparing feed properties. The d_5 population descriptor, representing the undersize cut-off for the smallest 5% of particles by volume, was deemed to be the most relevant size indicator. For three reasons it is useful for comparing the properties and behaviors of the various feed streams: (i) the finest particles have largest influence on clarification performance; (ii) it reports values between 0.1 and 5 μm with the typical size of intact *E. coli* spheroplasts approximately in the middle of the log-scaled range; and (iii) it reports aggregation propensity of the smallest debris particles. From Table III it can be seen that the d_5 values are very similar for five feed streams in either pilot scale or shake flask studies.

The d_5 population descriptor also captures aggregation effects occurring after conditioning for Feed 2, Feed 8, and Feed 9 which are absent in the Feed 1 and Feed 5 feed streams. The mean particle diameter, d_{50}, showed larger differences when comparing the pilot and shake flask feed material, making it less useful for detecting aggregation effects.

Shear studies were conducted on several feed streams to evaluate particle strength. Due to the extensive conditioning period the feed streams were not susceptible to shear. Either no or very small changes in the d_5 population descriptor occurred (data not shown).

Based on the comprehensive experimental volumetric power consumption study (Peter et al., 2006b) and values reported for a similar configuration (300 mL nominal volume shake flask with 48 mL fill volume and shake diameter of 25 mm) it is estimated that the shake flask extraction study was performed at approximately 8–9 W/kg volumetric power consumption. Differences in geometry and mode of mixing lead to differences in the magnitudes of energy dissipation ranges and ε_{max} values for the two mixing systems (Peter et al., 2006a). The difference in hydrodynamic conditions is likely the reason that no direct comparison between

Table III. Summary of particle size for the extraction steps from pilot and shake flask studies.

		Extraction					
		Initial			Conditioned		
Feed stream	Scale	d_5	d_{50}	d_{90}	d_5	d_{50}	d_{90}
Feed 9	Pilot	0.1	17	67	3.6	9	19
	USD	0.1	1	59	4.0	10	17
Feed 2	Pilot	2.0	30	117	2.7	27	112
	USD	2.2	57	377	3.0	21	154
Feed 8	Pilot	0.1	6	87	2.2	9	51
	USD	1.8	128	441	2.0	88	427
Feed 1	Pilot	0.2	10	69	0.15	7	61
	USD	0.1	3	26	0.08	2	23
Feed 5	Pilot	0.1	5	64	0.11	6	67
	USD	0.1	2	31	0.09	3	26

volumetric power consumption and particle size can be obtained for the two mixing systems.

The product and dsDNA concentrations obtained at pilot and USD scales are shown in Figure 5A and B. The ranking of the feed streams based on product concentration was similar for both shake flask and pilot scale studies. A small product loss was observed upon conditioning at both scales. The dsDNA concentration of the initial extract of feed streams spanned a wide range (3–600 mg/L) and both the shake flask and extractions ranked combinations similarly. The range of soluble dsDNA in the conditioned extract was much lower and spanned a narrower range (0–6 mg/L) which made the ranking less reliable.

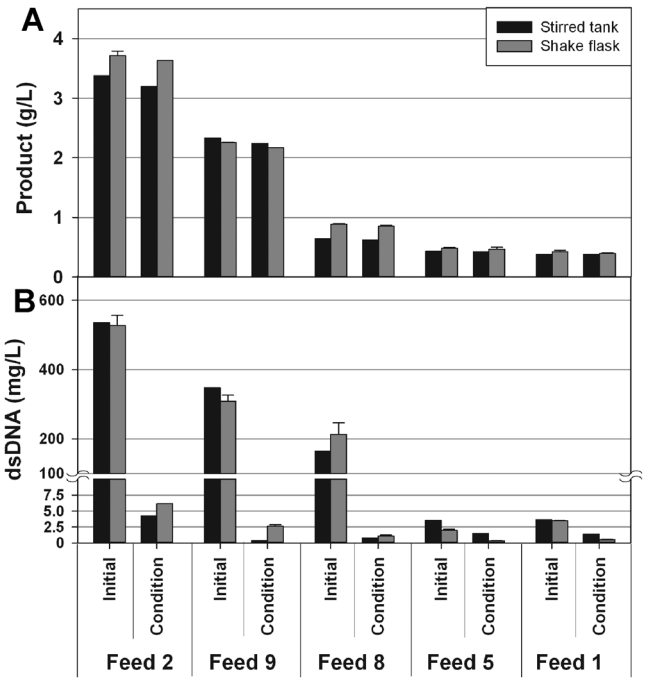

Figure 5. Pilot and USD scale periplasmic extraction for five feed streams. **A**: Feed streams are ranked according to product yield. **B**: Feed streams are ranked according to dsDNA concentration in initial extract. Product and dsDNA concentrations are very similar. Error bars represent the standard deviation of results from duplicate USD experiments.

Complete USD Primary Recovery

A study was conducted evaluating the performance of various host strain/vector/process combinations with the USD primary recovery sequence. Four harvest feed streams were processed using only USD devices and protocols and compared on the basis of key feed properties with pilot scale results (Table IV). Overall feed property values were very similar across scales for a given feed stream. As expected the dsDNA concentration of the USD heavy phase stream was significantly lower compared to the pilot scale process stream. Interestingly the dsDNA levels in most feed streams were very similar after the initial extraction step. The increased dsDNA levels in the shake flask studies suggests that the spheroplast released some intracellular content under the conditions prevailing in the shake flasks (Fig. 5A). Sufficient data is available in literature demonstrating that the shake flask mixing conditions will not result in breakage of *E. coli* during regular cell cultivation. However, no data is available in literature on the shear sensitivity of *E. coli* spheroplasts during bioprocessing operations. In the stirred vessel care is taken to control the pH, as well as the redox potential by means of a nitrogen gas overlay. These controls are not possible in the shake flasks. With time the pH of the extract tends to become more acidic and oxidative effects may further compromise membrane integrity. In addition the membrane fluidity increases at 60°C which will further increase the shear sensitivity of the spheroplasts. Further studies will be required to better understand the effects of pH and oxidative stress on the strength properties of *E. coli* spheroplasts and how to counter these effects during the course of shake flask studies.

Insights into Biomaterial Properties

As part of the feed stream selection process, candidates that displayed high levels of resistance to shear damage in feed zone and discharge mimics were sought. The physical

Table IV. Summary of key process variable for primary recovery at pilot and USD scale.

		Dewatering					Extraction process						Clarification
			Light phase		Heavy phase		Initial extraction			pH adjustment			
Feed stream	Scale	Solids carry-over (%)	dsDNA (mg/L)	Product (g/L)	dsDNA (mg/L)[a]	Product (g/L)[a]	dsDNA (mg/L)[a]	Product (g/L)[a]	d_5 (μm)	dsDNA (mg/L)[a]	Product (g/L)	d_5 (μm)	Solids carry-over (%)[a]
Feed 9	Pilot	0.3	6	0.03	346	1.7	164	2.3	0.1	0.3	2.2	3.6	0.07
	USD	0.4	5	0.04	205 ± 49	0.6 ± 0.1	143 ± 82	2.2 ± 0.1	0.08	3 ± 0.3	2.1	2.2	5E-03
Feed 2	Pilot	0.7	48	0.28	561	2.9	535	3.3	2.0	4	3.2	2.7	0.3
	USD	1.1	84	0.29	469 ± 62	2.8 ± 0.1	527 ± 29	3.7 ± 0.1	2.3	6	3.6	2.4	0.01
Feed 6	Pilot	2.0	7	0.15	175	0.7	174	1.0	0.09	3	0.9	2.9	0.8
	USD	—	6	0.15	39 ± 1	0.6	349 ± 3	1.3	0.08	2	1.2	1.7	0.5
Feed 8	Pilot	2.2	—	—	611	1.7	348	0.7	0.09	1	0.6	2.2	0.6
	USD	2.4	177	0.32	309 ± 65	1.1 ± 0.1	308 ± 18	0.9	0.09	1 ± 0.2	0.9	1.8	0.7

[a]USD extraction experiments were performed in duplicate and variation is reported as standard deviation of measurements.

features of the feed stream are a result of a combination of factors including strain, vector (product and factor D expression), fermentation protocol, and process conditions.

Several feed streams were subjected to discharge damage at 40 m/s and results are reported in Figure 6A and B. Results are shown for the expression of two different products in three different strains, all using fermentation protocol FP3 (Fig. 6A). It is evident that strains behave differently depending on the product expressed. Strains expressing Fab #2 typically yielded 10–20% lower cell concentrations than those expressing Fab #1. Fab #2 may be more demanding on the cellular expression machinery or exert a mild cytotoxicity effect resulting in a lower overall growth rate.

Both strains A1 and A2 expressed lower levels of Fab #2 than of Fab #1. Factor D was co-expressed in all four compared feed streams. The higher level of Fab #1 expression in strains A1 and A2 appears to correlate with reduced resistance to discharge damage. Strain W3110 again behaves differently with the two different products, yielding a higher Fab #2 titre. This may be due to lack of factor D co-expression in this feed stream. The higher level of Fab expression also correlates to a reduction in the resistance to discharge damage with the W3110 strain. From this study it is not clear what the cause of the reduced membrane robustness is. It is possible that the high level of over-expression diverts energy away from general cellular maintenance leading to weaker membrane structures. Alternatively the high level of protein translocation across the inner membrane alters the structures and strength properties of the inner membrane. Further studies need to be performed to separate the effects of over-expression and translocation to determine the most likely mechanistic driver for decreased cell strength.

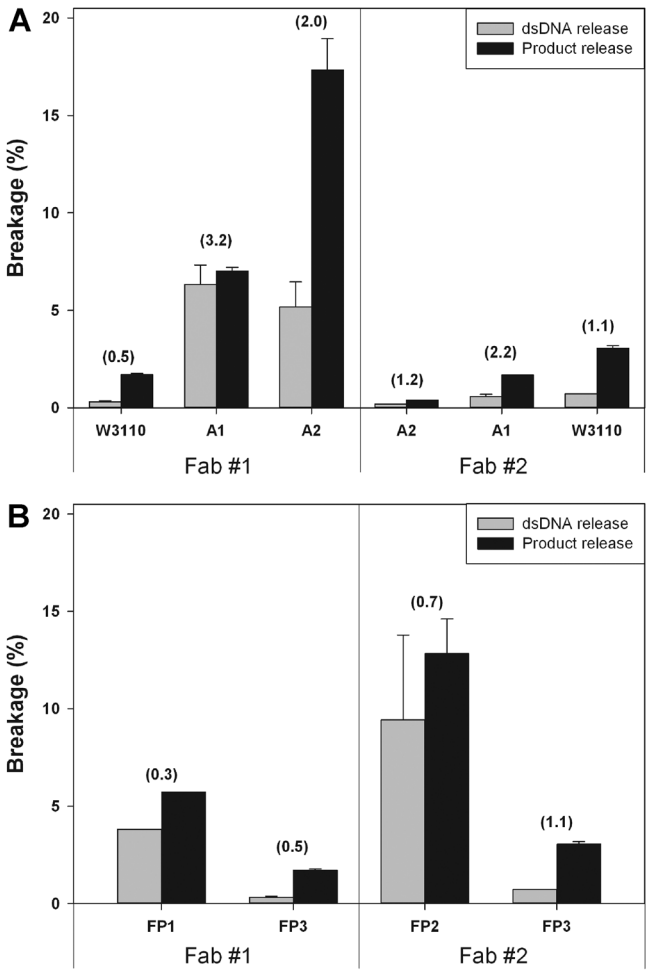

Figure 6. **A**: Comparison of the resistance of feed streams to discharge damage. Strains W3110, A1, and A2 expressed Fab #1 and Fab #2, respectively using the same fermentation protocol (FP3). Product yield is noted in brackets for each feed stream. Cell strength varied depending on the strain/product pairing and final yield (g/L). Error bars represent the standard deviation of results from duplicate USD experiments. **B**: Comparison of the resistance of feed streams obtained with different fermentation protocols to discharge damage. Product yield (g/L) is noted in brackets for each feed stream. Protocol FP3 designed to improve membrane integrity and reduce periplasmic content leakage produced cells that are more resistant to discharge damage than other protocols. Error bars represent the standard deviation of results from duplicate USD experiments.

Figure 6B shows the effect of fermentation protocol on the cell strength. The FP3 fermentation protocol was developed to improve cell viability (Table I), as well as product yield through the maintenance of a healthier cell population throughout induction and harvest. Figure 6B compares the strength of W3110 cells grown using the FP3 protocol to W3110 cells grown using the FP1 and FP2 fermentation protocols for products Fab #1 and Fab #2, respectively. The figure shows that for both Fab products the FP3 protocol produced cells that were more robust and less susceptible to discharge damage, suggesting that cell viability may be an important fermentation output parameter in the screening process.

Conclusions

The feasibility of linking several primary recovery bioprocess units in a scaled-down bioprocess train was investigated. The prediction of clarification was possible with the centrifugation mimic and feed streams could be accurately ranked based on the clarification levels. The CDD was capable of ranking various feed streams based on their susceptibility to impaction damage. Low operating discharge velocities limited the level of damage induced at small scale resulting in lower overall breakage compared to heavy-phase from pilot scale studies. A shake flask agitation system was operated in a manner to match pilot scale feed properties with regards to fine particle size populations, product, and nucleic acid concentrations. With improvements in the USD discharge design it is feasible that a complete bench scale primary recovery system can be linked to a 2 L fermentation system for bioprocess development studies. The limited number of studies typically conducted at pilot scale poses a challenge when characterizing the behavior of newly developed strains. A scaled-down bioprocess sequence capable of mimicking the actual pilot scale process can be valuable for conduct screening or process characterization studies.

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