Use of Operating Windows in the Assessment of Integrated Robotic Systems for the Measurement of Bioprocess Kinetics

Anthony J. Nealon,[†] Karen E. Willson,[†] Samuel C. R. Pickering,[†] Tim M. Clayton,[‡] Ronan D. O'Kennedy,[‡] Nigel J. Titchener-Hooker,[†] and Gary J. Lye*,[†]

The Advanced Centre for Biochemical Engineering, Department of Biochemical Engineering, University College London, Torrington Place, London WC1E 7JE, United Kingdom, and GlaxoSmithKline PLC, Langley Court, South Eden Court Road, Beckenham, Kent BR3 3BS, United Kingdom

This study examines the utility of an automated liquid handling robot integrated with a microwell plate reader to enable the rapid acquisition of bioprocess kinetic data. The relationship between the key parameters for liquid handling accuracy and precision and the sample detection period has been characterized for typical low-viscosity (<2.0 mPa·s) aqueous and organic phases and for a high-viscosity aqueous phase (60 mPa·s), all exhibiting Newtonian rheology. The use of a simple graphical method enables the suitability of a given automation platform to be assessed once the user has determined the minimum sample detection period and the minimum accurate and precise dispense volume. This provides for a reduction in the duration of any experiment by maximizing well usage within each microwell plate. The suitability of employing an integrated automation platform to gather kinetic data for systems typical of those encountered in bioprocessing is analyzed via a series of case studies. Application to alkaline cell lysis, where disruption is complete within 120 s, showed that the range of available dispense volumes and the number of wells that can be utilized is limited. In contrast, analysis of a system exhibiting slow process kinetics, the fermentation of *Escherichia coli* TOP10 pQR239 in microwell plates, demonstrated that, for a typical sample detection period of 30 min, the only restrictions on the degree of well utilization are the liquid handling accuracy and precision and the volume capacity of the liquid handling robot. Finally, liquid−liquid extraction, an example of a kinetically independent operation, was also examined. In this case, only a single equilibrium measurement is required, which means that the only restrictions to the utilization of the integrated devices are the liquid handling accuracy and precision. Integrated automation platforms represent a powerful process development tool over traditional experimental methods used for bioprocess development. Smaller volumes of reagent and sample can be used to achieve greater throughput, while high levels of reproducibility and sensitivity are maintained.

1. Introduction

The cost of drug development has increased rapidly in recent years (*1*). Furthermore, high-throughput screening (HTS) and advances in combinatorial chemistry have lead to a large increase in the number of potential drug candidates and active ingredients that have been identified. This increase in the number of compounds and the ever-increasing pressure to shorten development cycles and control costs have each contributed to the bottleneck in the production of new medicines moving to process development (*2*).

The above trends now mean that new ways of reducing the amount of time and money invested in process development must be found without reducing the quantity and quality of the data generated. The greatest pressure is currently on the first stage of process development, route scouting (*3*), where a series of alternative process options must be identified and evaluated prior to final process selection. Route scouting involves the use of a large number of small-scale experiments to assess the effect of different operations, reagents and conditions. Commonly this involves simple yes/no or better than/worse than answers. The process identified does not necessarily have to be optimal but must show the capability for future optimization during later scale-up.

A number of guidelines have been produced as aids to speed up process development and to avoid common pitfalls (*4*, *5*). The ultimate goal is to construct a model of each unit operation, allowing the a priori prediction of process performance with a given set of conditions. However, many individual parameters may affect the yield and purity of product recovery, and initial route scouting experimentation should look at the relative importance of many important parameters. Small-scale trials (1−10 mL) are also time-consuming and many experimental approaches now used routinely for route

* To whom correspondence should be addressed. Tel: +44 (0) 20 7679 7942. Fax: +44 (0) 20 7209 0703. E-mail g.lye@ucl.ac.uk.
[†] University College London.
[‡] GlaxoSmithKline.

10.1021/bp049868+ CCC: $30.25
Published on Web 12/03/2004

Table 1. Physical Properties of the Liquid Phases Used in This Study[a]

phase	liquid	temp (°C)	viscosity (mPa·s)	density (kg/m^3)	surface tension (mN/m)
low-viscosity aqueous	RO water	20	1.0019	998.0	72.75
low-viscosity organic	DMSO	25	1.987	1101.4	42.92
high-viscosity aqueous	80% (w/w) glycerol	20	59.78	1208.5	66.00

[a] Data were compiled from ref 34.

scouting do not lend themselves to easy automation and thus limit the rate of experimentation.

Recently we have started to examine the application of microwell plate-based methods for this purpose (*6*, *7*), which have some clear potential advantages. The standardized microwell geometry allows easy automation since the technology is commonly used already in HTS (*8*) and laboratory assays (*9*, *10*). Though microwell plates are routinely used in screening, their potential for process experimentation has hardly been investigated. Common first steps in such an approach would be the use of automated liquid handling robots and fast analytical devices, such as microwell plate readers. Microwell plate readers were initially developed for analytical applications in blood chemistry (*11*), food science (*12*), and microbiology (*13*). Though a number of groups have looked at the measurement of process kinetics within microwell plates (*14*, *15*) there are currently very few kinetic studies that use liquid handling robots and microwell plates (*12*, *16*). Therefore, we have taken the gathering of kinetic data, over the course of a bioprocess operation, as the first test of the utility of using integrated automation platforms in bioprocess development.

For such a microscale processing approach to be useful it must display the following features: (1) be able to reproducibly sample wells with a set frequency, (2) exhibit the flexibility to vary the sample detection period independently of the trial system, and (3) maximize productivity within each microwell plate by a priori prediction of the degree of well utilization. When the combination of a liquid handling robot and a microwell plate reader is used, the minimum sample detection period is inversely proportional to the speed of liquid handling, the speed of detection by the microwell plate reader, and the number of wells utilized on the plate. The relative importance of these three factors on the sample detection period will be investigated in this study. To graphically represent these interactions, the data will be displayed by use of operating windows (*17*), which allow the simple visualization of multivariate and complex design data. They also provide an insight into a unit operation and can be applied to determine operational conditions or provide data for process design when scaling an operation.

2. Materials and Methods

2.1. Materials. Generally accepted models of a low-viscosity aqueous phase, RO water, and a low-viscosity organic phase, dimethyl sulfoxide (DMSO) (Sigma, Poole, Dorset, U.K.), were used to determine liquid handling accuracy and precision (*18*, *19*). To model a Newtonian, high-viscosity liquid, 80% (w/w) glycerol (Sigma, Poole, Dorset, U.K.) was used (*20*). The physical properties of the solvents are given in Table 1. These can have a large influence on the liquid handling ability and therefore must be taken into consideration during the operation of any liquid handling robot.

2.2. Automation Platform and Operation. Automated liquid handling was performed by a four-tip Multiprobe II robot (Perkin-Elmer Inc., Beaconsfield, Bucks., U.K.), while a Spectracount (Perkin-Elmer Inc.) was used as the plate reader (wavelength range 340−670 nm) (*6*). Liquid was dispensed directly into the wells of a standard shallow, 96-well microwell plate (Sarstedt Inc., Newton, NC) situated on the automated stage of the plate reader. Software integration was completed with Macro Express (Insight Software, Kaysville, UT), allowing the automatic take in and reading of the plate after reagent addition.

Two types of pipet tips are available for use on the robot, fixed and disposable. When a single dispense per aspirate is performed, use of both of these tip types is time-consuming. An alternative approach is to use a multiple-dispense function with fixed tips. This involves the aspiration of the total desired volume of a reagent, for all wells to be utilized, in a single step. This is followed by a series of dispenses of the required volume into each well without the need to refill or change tips. This greatly reduces the number of robotic operations required and, hence, the time taken to complete liquid handling.

To allow accurate and precise handling of liquids with different physical properties, the robot was set up with two different performance files. A range of variables such as dispense and aspiration speed (1−1866 μL/s), air gaps (0−10 μL), and waste volumes (30−100%) make up each performance file. The performance files used here were as per the manufacturer's recommendations (J. Wrigley, Perkin-Elmer Inc., personal communication) and are summarized in Tables 2 (low-viscosity phase) and 3 (high-viscosity phase).

The total volume that can be dispensed into each well by the multiple-dispense function will be dependent on the volume capacity of the robot, the number of wells to be utilized, and the influence of the performance file configuration. Components of the performance file, such as the inclusion of air gaps and waste volumes, ensure accurate and precise liquid dispension but will also reduce the maximum dispense volume and increase the dispense time. The performance files used here (Tables 2 and 3) resulted in a maximum dispense volume of 2089.5 μL.

2.3. Dispense Accuracy and Precision. To determine the accuracy and precision of liquid handling, the mean and coefficient of variance (% CV) of each dispense step were calculated. The difference between the measured mean volume and the target volume was used to calculate the precision, while the variance (σ) around the measured mean volume was used to calculate the accuracy (*18*−*20*):

$$\text{precision, \% CV} = [(\text{mean} - \text{target})/\text{mean}] \times 100 \quad (1)$$

$$\text{accuracy, \% CV} = (\sigma/\text{mean}) \times 100 \quad (2)$$

The acceptable lower limit for an accurate and precise dispense was defined as one where both % CV values were below 5% (20).

Initially a comparison of the mean and % CV for each of the four pipet tips was completed for the three types of solvent described in Table 1. Solvent (100 μL) was dispensed into dry, preweighed high-performance liquid

Table 2. Details of Performance Files Used for the Handling of Low-Viscosity Aqueous (RO Water) and Organic (DMSO) Phases

dispense volume (μL)	aspirate speed (μL/s)	aspirate delay (s $\times$ 10^{-3})	dispense speed (μL/s)	dispense delay (s $\times$ 10^{-3})	waste volume (μL)	waste vol (% of aspirate)	transport air gap (μL)	system air gap (μL)
5	10	200	400	200	5	100	3.0	5.0
30	25	200	400	200	10	30	3.0	5.0
50	50	200	400	200	15	30	3.0	5.0
100	75	200	400	200	30	30	3.0	5.0
250	125	200	400	200	75	30	3.0	5.0

Table 3. Details of the Performance File Used for the Handling of the High-Viscosity Phase [80% (w/w) Glycerol]

dispense volume (μL)	aspirate speed (μL/s)	aspirate delay (s $\times$ 10^{-3})	dispense speed (μL/s)	dispense delay (s $\times$ 10^{-3})	waste volume (μL)	waste vol (% of aspirate)	transport air gap (μL)	system air gap (μL)
5	10	500	150	500	5	100	0.0	5.0
30	10	500	150	500	10	30	0.0	5.0
50	10	500	150	500	15	30	0.0	5.0
100	10	500	150	500	30	30	0.0	5.0
250	10	500	150	500	75	30	0.0	5.0

chromatography (HPLC) vials by use of the multiple-dispense function and six dispenses per aspirate, as recommended by the manufacturer for the greatest dispense precision and accuracy (J. Wrigley, Perkin-Elmer Inc., personal communication). After the dispenses were completed, the vials were immediately capped and reweighed. The process was then repeated at the upper limit of 24 dispenses per aspirate. A wider range of volumes (5−200 μL) and number of dispenses per aspiration (3−24) were then studied for each of the model liquids. For these later experiments the liquid handling accuracy and precision measurements were carried out with a single pipet tip.

2.4. Acquisition of Bioprocess Data. To gather bioprocess kinetic data from a given system it is necessary to regularly monitor each well utilized in a plate. This can be achieved by use of a single well but is highly time-consuming and inefficient. To increase the productivity of an automation platform, multiple wells run in parallel can be used, but for this to be achieved the wells need to be coordinated such that they are initiated and monitored as a block rather than individually. To maximize the degree of well utilization, the majority of reagents are added to each well and then a final initiating reagent is added to all wells in one dispense step (e.g., the addition of a substrate to an enzymatic bioconversion). The sample detection period, the time between each monitoring of a specific well on a plate, would then be determined by the number of data points required and the limitations imposed by the integrated robot and plate reader. The minimum sample detection period is dependent on which of the plate setup and plate monitoring time is rate-determining, where

$$\text{plate setup time} = \text{dispense time} + \text{reader initialization time} \quad (3)$$

$$\text{plate monitoring time} = \text{plate reading time} + \text{reader reset time} \quad (4)$$

The dispense time is defined here as the time taken by the robot to dispense a known solvent volume into a specified number of wells. The degree of well utilization (12.5−100%), the dispense volume, and the performance file (Tables 2 and 3) each contribute to the dispense time. The utilization of fewer than 12 wells (12.5%) was not investigated since there would be little or no gain in the operation time over manual liquid handling. For each level of plate utilization, the robot was programmed to perform one aspiration and consequently the number of dispenses per aspirate necessarily increased with well utilization. The Multiprobe II robot used here is fitted with 1 mL syringes. To be able to dispense total volumes greater than 1 mL it is necessary to empty the syringe contents into the robot's system tubing (volume capacity = 3 mL), displacing, but not mixing with, the system fluid (water). Once this is complete, additional reagent can be aspirated into the syringes; this process can then be repeated a number of times. Once the reagent is dispensed, the procedure is put into reverse: the initial dispense is from the syringe, the syringes are reloaded from the system tubing, and dispensing continues. The dispense time was measured for a range of dispense volumes (5−200 μL).

The reader initialization period was defined as the time elapsed from the final dispense step to the initial reading of the first well. The plate monitoring time was defined as the time between reading the first well on the first scan of the plate and reading the first well on the subsequent scan.

2.5. Bioprocess Operating Windows. To display the effects of dispense volume and the minimum sample detection period on the degree of well utilization, the timings and liquid handling data were combined into bioprocess operating windows. The degree of well utilization is represented by the number of bioprocess conditions that can be studied. Each bioprocess condition was performed in quadruplicate to improve the statistical accuracy of the results obtained (100% well utilization for a 96-well plate thus corresponds to 24 quadruplicate measurements of different bioprocess conditions). The operating windows show the lower limits of liquid handling accuracy and precision over the range of dispenses per aspirate studied as well as displaying the effects of dispense volume on the minimum sample detection period for each combination of well utilization and dispense volume. The maximum working volume of the system tubing of the robot is 2089.5 mL and this sets the upper limit on the degree of well utilization that can be achieved for each dispense volume.

3. Results and Discussion

3.1. Accuracy and Precision of Liquid Handling. Underlying the creation of microscale processing technologies (*6*) is the requirement for accurate and precise dispensing of a range of fluids into various microwell plate formats. The initial study of tip dispensing behavior with a fixed target volume of 100 μL showed that, for the aqueous, organic, and high-viscosity aqueous phases studied, the liquid handling capability of each of the four pipet tips on the robot were accurate and precise (Figure

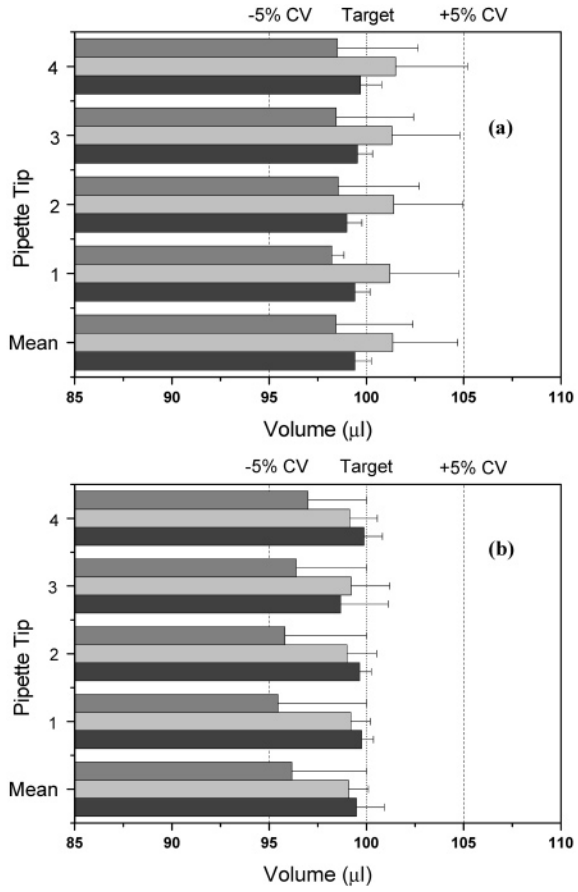

Figure 1. (a) Pipetting accuracy and precision based on six dispenses and (b) pipetting accuracy based on 24 dispenses per aspiration for water, DMSO, and 80% (w/w) glycerol solutions. Precision (blocks) and accuracy (error bars) of each pipet tip in dispensing a programmed volume of 100 μL are shown. The acceptable bandwidth of 5% CV is also shown. Water, black bars; DMSO, light gray bars; 80% (w/w) glycerol, dark gray bars.

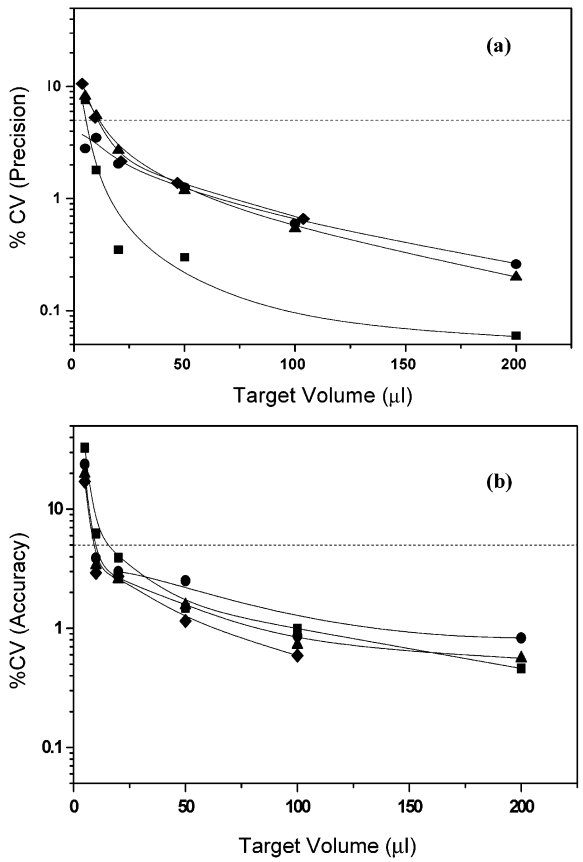

Figure 2. (a) Multiple dispense precision and (b) multiple dispense accuracy with a low-viscosity aqueous phase (RO water). The measured mean dispense volumes are shown for a range of programmed volumes and number of dispenses per aspiration. The cutoff for precise and accurate liquid handling, 5% CV, is indicated by the dashed line. (■) Three dispenses per aspiration; (●) six dispenses per aspiration; (▲) 12 dispenses per aspiration; (◆)24 dispenses per aspiration.

1), that is, below the required 5% CV limit specified in section 2.3 (*18*−*20*). Each pipet tip, therefore, performed in a consistent and reproducible manner. This meant that it was therefore possible to complete the remaining liquid handling experiments with any one of the pipet tips, rather than all four, thus reducing the total number of measurements needed.

Results on the accuracy and precision of the robot over a wide range of operating conditions are shown in Figures 2 (low-viscosity aqueous), 3 (low-viscosity organic), and 4 (high-viscosity aqueous). For all three solvents the liquid handling precision decreased as the number of dispenses per aspirate increased. At the lowest dispense volume of 5 μL, the liquid handling precision was poor (>5% CV) for all numbers of dispenses per aspirate. For all numbers of dispenses per aspirate, the precision increased with dispensed volume. Both of the low-viscosity phase systems showed high precision at all volumes for six dispenses per aspirate (the manufacturer's recommendation for optimum liquid handling accuracy and precision). Above a dispense volume of 10 μL, the high-viscosity aqueous system had a consistent precision around 4% CV.

In contrast, the results for liquid handling accuracy are more complex. The accuracy for the low-viscosity aqueous system increases with the number of dispenses per aspirate and with increasing dispense volume. In the low-viscosity organic system it is harder to find clear trends. The accuracy again increases with increasing dispenses per aspirate, but for dispensing volumes of 50 and 100 μL the accuracy is lower than that obtained for dispensing at smaller volumes. For the high-viscosity aqueous system, the results follow the same trends as the low-viscosity aqueous system. However, in all cases the accuracy is much closer to the specified acceptable limit of 5% CV and does not vary significantly with dispense volumes above 10 μL.

Interestingly, it can be seen that there is a trend from the high-viscosity aqueous phase through the low-viscosity organic phase to the low-viscosity aqueous phase of an increasing degree of accuracy and precision of liquid handling. This is despite modifications in the performance files (Tables 2 and 3) that should enable optimum performance allowing for changes in the physical properties of the fluids.

Table 4 summarizes the liquid handling performance data for each of the liquid phases. This stipulates the minimum volume that can be dispensed in each case with better than 5% CV accuracy and precision. Data collection from bioprocess operations relying on the dispensing of liquid volumes lower than those specified in Table 4 would be inaccurate and only qualitative in nature.

3.2. Kinetics of Data Acquisition. As described in section 2.4, the minimum sample detection period available to acquire data on the kinetics of a given bioprocess operation is determined by the time taken to dispense the initiating reagent into a specified number of wells and the time taken to take a spectral measurement with

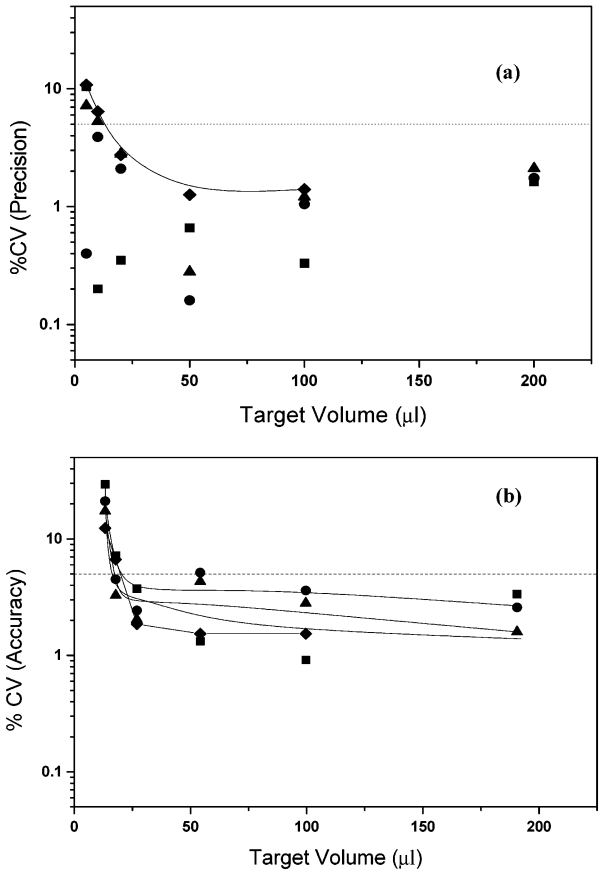

Figure 3. (a): Multiple dispense precision and (b) multiple dispense accuracy with a low-viscosity organic phase (DMSO). The measured mean dispense volumes are shown for a range of programmed volumes and number of dispenses per aspiration. The cutoff for precise and accurate liquid handling, 5% CV, is indicated by the dashed line. (■) Three dispenses per aspiration; (●) six dispenses per aspiration; (▲) 12 dispenses per aspiration; (◆)24 dispenses per aspiration.

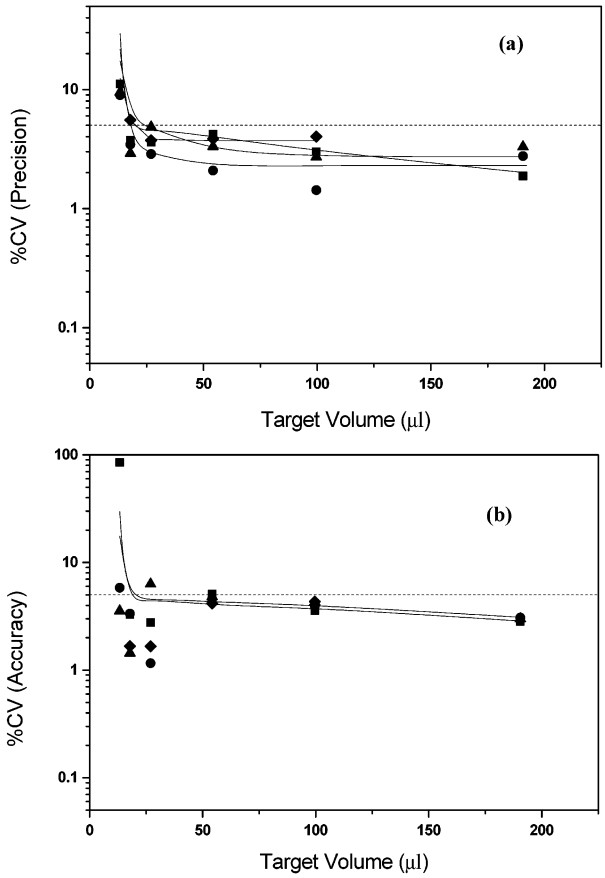

Figure 4. (a) Multiple dispense precision and (b) multiple dispense accuracy with a high-viscosity phase [80% (w/w) glycerol]. The measured mean dispense volumes are shown for a range of programmed volumes and number of dispenses per aspiration. The cutoff for precise and accurate liquid handling, 5% CV, is indicated by the dashed line. (■) Three dispenses per aspiration; (●) six dispenses per aspiration; (▲) 12 dispenses per aspiration; (◆) 24 dispenses per aspiration.

the integrated plate reader. To evaluate the relative significance of the plate setup time and the plate monitoring time, the two values need to be determined. The plate setup time consists of the dispense time and the reader initialization time. The reader initialization period is fixed and was measured at 19 s.

The dispense times vary as a function of dispense volume and the number of dispense per aspiration and are shown in Figure 5. The results for both the low- and high-viscosity systems exhibit sharp vertical steps in the dispense time at regular intervals. This is due to the robot loading the reagent back into the syringes from the system tubing as described in section 2.4. The three main factors affecting the dispense time are the degree of well utilization, the characteristics of the performance file used for a particular solvent, and the dispense volume. From Figure 5 it can be seen that the degree of well utilization is the most important of these factors: (to change from 12.5% to 100% well utilization, for 10 μL additions and a low-viscosity phase, leads to an increase of 80 s in the operation time, while to change from 10 μL to 200 μL additions, at 100% well utilization and a low-viscosity phase, leads to a 30 s increase in operation time, and a change from a low- to a high-viscosity phase, at 100% well utilization and a 10 μL addition, gives an increase of only 20 s). The performance file and dispense volume do have a small effect on the dispense time but this varies with the degree of well utilization. The greater the degree of well utilization, the greater the effect of the performance file and dispense volume. The performance file affects the dispense time because of changes made to the dispense delay and the dispense speed (Tables 2 and 3) to ensure accurate and precise liquid handling, especially for more viscous liquids.

Table 4. Minimum Precise and Accurate Dispense Volumes[a] for the Various Phases Used in This Study

	minimum dispense volume (μL)		
no. of dispenses	RO water	DMSO	80% (w/w) glycerol
3	20	20	10
6	10	10	10
12	20	20	10
24	20	20	20

[a] Variation below 5% CV in both cases.

Finally, to be able to calculate the plate monitoring time, the plate reading time and the reader reset time are needed. The plate reading time was found to be a simple linear function of the degree of well utilization (data not shown), while the reader reset time was found to be constant at 7 s. A comparison between the plate setup time and the plate monitoring time showed that the plate setup time is by far the most important and is thus rate-limiting. This leads to the conclusion that the degree of well utilization is the most important factor affecting the minimum sample detection period.

3.3. Operating Windows for Platform Assessment. To graphically represent the relationship between the

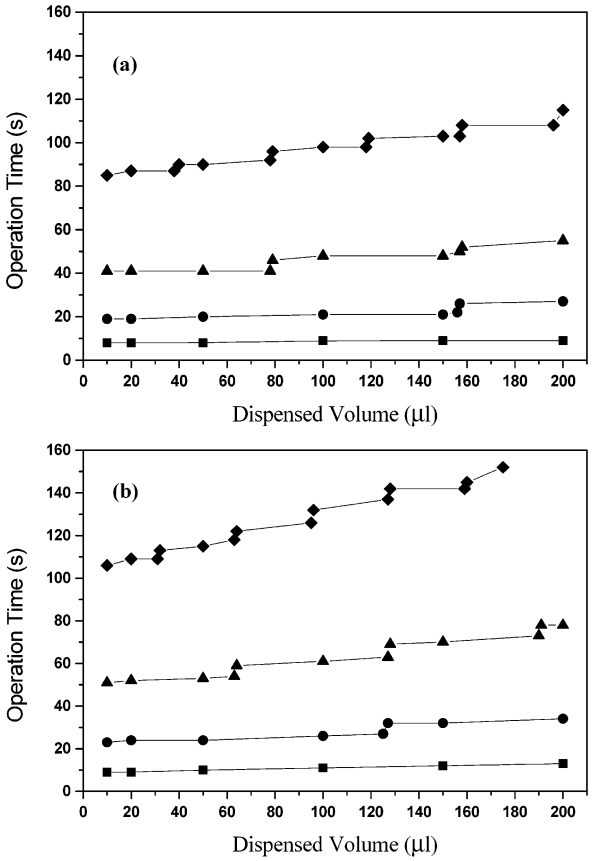

Figure 5. (a) Liquid dispense times for low-viscosity aqueous (RO water) and organic phases (DMSO) and (b) liquid dispense times for a high-viscosity phase [80% (w/w) glycerol]. The figures show the time taken for a single tip to dispense a range of volumes into a number of wells by use of the multiple dispense function. (■) Three dispenses per aspiration; (●) six dispenses per aspiration; (▲) 12 dispenses per aspiration; (◆) 24 dispenses per aspiration.

minimum sample detection period, the dispense volume, and the degree of well utilization, windows of operation (*17*) were constructed for the low-viscosity (Figure 6) and high-viscosity (Figure 7) phases. These were constructed on the basis of the data for pipetting precision and accuracy (Figures 2−4) and the liquid dispense times (Figure 5). Both windows show that the minimum sample detection period increases with increasing well utilization and dispense volume for all three phase systems studied. The boundaries between the utilization of 12−24 wells (i.e., the investigation of 3−6 separate bioprocess conditions, each in quadruplicate), 24−48 wells (i.e., 6−12 bioprocess conditions), and 48−96 wells (i.e., 12 and 24 bioprocess conditions) show step increases in the sample frequency. This is a direct result of the need to load the reagent from the syringes into the system tubing as described in section 2.4.

A comparison of the operating window for both low-viscosity phases (Figure 6) with that for the high-viscosity phase (Figure 7) shows that the boundaries between the various degrees of well utilization have shifted to the right for the high-viscosity system. The changes in the performance files (Tables 2 and 3), dictated by the physical properties of the fluid, lead directly to an increase in the dispense time for the high-viscosity system (Figure 5b) and hence an increase in the minimum sample detection period for an equivalent degree of well utilization. Finally, it should be noted that the sample detection periods shown in Figures 6 and 7 are specific to the experimental setup used here. The use of

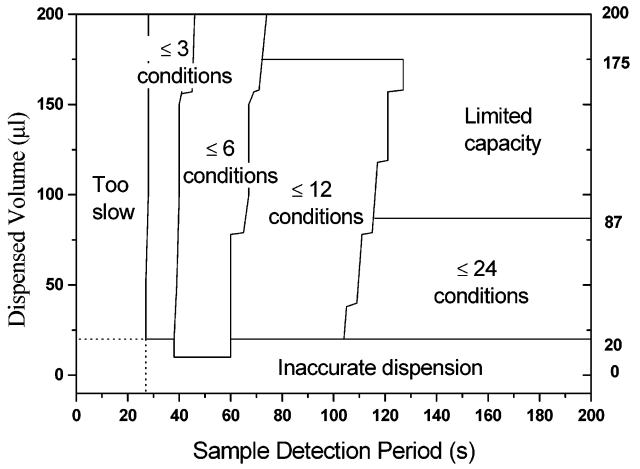

Figure 6. Bioprocess operating window for low-viscosity aqueous (RO water) and organic (DMSO) phases on a standard 96-well plate. The figure shows the dependence of the number of bioprocess conditions (each completed in quadruplicate) that can be studied within a single microwell plate on the accuracy and precision of liquid handling and the time between sampling. Sample detection periods that cannot utilize three or more bioprocess conditions are designated as being too slow. The upper capacity is based on the volume of the system tubing. Note that the indicated values are specific for the four pipet tip Multiprobe II and Spectracount combination used in this study.

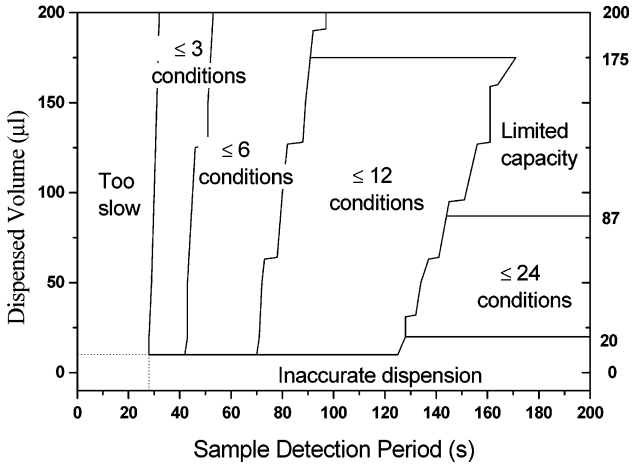

Figure 7. Bioprocess operating window for a high viscosity phase [80% (w/w) glycerol]. The figure shows the dependence of the number of bioprocess conditions (each completed in quadruplicate) that can be studied within a single microwell plate on the accuracy and precision of liquid handling and the time between sampling. Sample detection periods that cannot utilize three or more bioprocess conditions are designated as being too slow. The upper capacity is based on the volume of the system tubing. Note that the indicated values are specific for the four pipet tip Multiprobe II and Spectracount combination used in this study.

another liquid handling robot with a different syringe size, number of pipet tips, or operating speed would result in a change in the sample detection period for each degree of plate utilization. The same would be true if a different microplate reader were to be considered. However, the approach to the construction of the windows of operation is generic and would give valuable insight into the utility and productivity of a given automation setup.

3.4. Other Liquid Handling Issues: Contamination and Liquid Mixing. The use of automated liquid handling platforms as described here has the potential to greatly reduce the time taken to complete route scouting experiments and increase the throughput of new target compounds through the process development

pathway. However, inherent to their use is the need to understand the general limitations of liquid handling equipment and those of integrated detection devices. The most common liquid handling difficulties are tip carryover contamination (*18*), speed of liquid handling, liquid handling accuracy and precision (*19*, *20*), and the speed of the detection device. Speed of liquid handling, liquid handling accuracy and precision, and the speed of the detection device have all been addressed in this work. Tip carryover contamination can be avoided by dispensing above the liquid level in a well with fixed tips. The use of disposable tips also overcomes this problem but increases the minimum sample detection period by a factor of 5 (in the case of the Multiprobe II used here; data not shown), thus greatly reducing the degree of well utilization.

For additions made from each type of tip, it has been assumed throughout this study that when the initiating reagent is added to the bulk fluid within the well, it will be rapidly mixed. This is a reasonable assumption when a small volume of reagent is dispensed within the bulk of a low-viscosity fluid and then shaken (*19*, *22*, *23*). At present there are no published data as to whether mixing in high-viscosity phases in microwells will influence the measurement of bioprocess kinetics. For plates that remain static following sample addition there are no published data on mixing times. An investigation of jet mixing in static wells is therefore currently underway within our laboratory.

The use of integrated detection devices greatly increases the throughput of bioprocess development but can be restrictive on the type of microwell plate that can be utilized. The currently available microplate readers are only compatible with standard shallow-well ($h = 13$ mm) plates. From an analytical and HTS viewpoint this does not present a problem, but in many bioprocess development cases deep-well ($h = 44$ mm) plates have been found to be more suitable (*6*, *7*, *16*, *22*). Currently, sampling from deep-well formats and the transfer of samples into a standard shallow-well plate must therefore take place before analysis can be completed, adding greatly to the sample detection period.

4. Bioprocess Case Studies

In this section the importance of the results displayed in Figures 6 and 7 in relation to some typical bioprocess operations that can be studied by microscale processing techniques is considered (*6*). These relate to parallel investigations underway in our laboratory and elsewhere, which exhibit a range of process kinetics and which will determine the applicability of integrated automated platforms for the collection of microscale process data:

1. Alkaline lysis of *Escherichia coli* cells for the release of plasmid DNA gene therapy vectors and DNA vaccines (*26*). This process is characterized by an initial rapid chemical lysis of the cell membrane in 0−30 s, which reaches completion after approximately 120 s. Cell disruption is followed by a further chemical degradation of released chromosomal DNA until a pseudo-steady state is reached after 200−400 s (*27*). The process is characterized by a marked increase in the viscosity of the broth from 3 to 35 mPa·s during cell lysis, due to the release of the chromosomal DNA, and then a steady decrease to 20 mPa·s as the chromosomal DNA is degraded (*27*). In this example the collection of data for the initial, rapid process of cell disruption is examined.

2. Aerobic fermentation of *E. coli* for the production of an enzyme used in a Baeyer−Villiger bioconversion (*24*). Fermentation processes are characterized by relatively slow kinetics with growth and product formation occurring over a period of 6−8 h or longer. This process has a typical sample detection period of 30 min, and for growth on soluble substrates, the broth viscosity is unlikely to increase above 10 mPa·s.

3. Product recovery by liquid−liquid extraction or solid−liquid adsorption (*25*, *28*). Equilibrium stage separations such as these are kinetically independent systems where the key design parameters, distribution coefficients and adsorption constants, are based on single measurements made once the system has come to equilibrium. The viscosities of the liquid phases will depend on the particular solvents selected but will generally be below 2 mPa·s.

The lysis of bacterial cells by the addition of 0.2 M NaOH containing 1% (w/v) sodium dodecyl sulfate (SDS) is characterized by rapid kinetics (*22*). To be able to use an operating window for the automation platform to predict well utilization, the required sample detection period and final dispense volume need to be specified. The final dispense volume (of the NaOH/SDS solution) for microscale alkaline lysis can realistically be fixed at 25 μL. The quantity of kinetic data that needs to be generated next needs to be established. The quality of data required will have a significant influence on the well utilization. If we take two examples, for the collection of 10 and three equally spaced data points over a period of 120 s, this significance can be demonstrated. If 10 data points are required, then the sample detection time is 12 s; if only three are required, then the sample detection period is 40 s. The well utilizations for these two sample detection periods vary greatly (Figure 6 for a low-viscosity addition). By use of this window, it can be seen that the platform is too slow for a sample detection period of 12 s. In this situation the liquid handling function can be completed accurately and precisely but the integrated robot and plate reader are simply not fast enough to set up and take readings from enough wells to make automation worthwhile. If this is compared to the situation when the sample detection period is 40 s, then the operating window predicts a degree of well utilization (up to 25%) allowing up to six bioprocess conditions to be varied simultaneously (each in quadruplicate). The use of these windows of operation thus not only allows the researcher to predict the degree of well utilization that can be attained with the integrated robot but also allows the productivity of the platform to be tailored to the quality of the data required. This tradeoff will always be necessary for processes exhibiting rapid kinetics.

The aerobic fermentation of microorganisms in microwell plates is a good example of a process characterized by slow kinetics (*6*, *29*−*32*). Cell density and the rate of product formation can both be typically monitored by use of a microwell plate reader (*33*) in order to quantify cell growth kinetics (29) or the optimum time for the introduction of enzyme suppression (*24*). The sample detection period for fermentations is typically long, in the range of 30 min, since the overall process will take many hours. In this case, it can be seen that for both low-viscosity additions, for example, NaOH for pH control (Figure 6), and high-viscosity additions, for example, soya oil during fed-batch operation, (Figure 7) the sample detection period is too large to influence the degree of well utilization. The suitability of the automation platform is now determined solely by the liquid handling functionality: a lower limit determined by accuracy and precision and an upper limit set by the capacity of the robot. The ability to perform automated and parallel microwell

fermentations represents a rapid approach to the establishment of process operating conditions such as medium composition, pH (*7*), and temperature. Current work on the quantification of oxygen transfer rates (*6*, *22*, *23*, *30*) will enable the performance of aerobic microwell fermentations to be related to larger scales of operation.

Finally, in the case of kinetically independent systems, such as liquid−liquid extraction and solid−liquid adsorption (*25*, *28*), the key process measurements are obtained only after the system has reached equilibrium. No intermediate measurements are generally necessary unless it is required to have in-process samples to ensure a true equilibrium is reached. This will free the automation platform from the constraint of the sample detection period, allowing 100% plate usage in all instances. In such cases the only factors influencing the operation of the system would be the liquid handling accuracy and precision.

5. Conclusions

It has been shown in this work that a liquid handling robot and a microwell plate reader can be effectively integrated and may be used to gather bioprocess kinetic data under a range of conditions. The approach has been shown to be limited by two key constraints, namely, the accuracy and precision of liquid handling and the sample detection period. Furthermore, the sample detection period is the major influence on the degree of well utilization within a plate. As the frequency of measurement increases, the number of wells that can be utilized within a plate decreases until a point is reached where the integrated robot and plate reader offer little advantage over manual liquid handling. The necessity of reducing the time of liquid handling requires also that multiple dispenses per pipet tip are used. The viscosity of the process fluid has also been shown to have an effect on the degree of well utilization. The necessity of maintaining liquid handling accuracy and precision leads to a reduction in the degree of well utilization for a given sample detection period as the viscosity of the final reagent increases. It has also been demonstrated that the quality of data needed for a system with rapid kinetics is linked with the productivity per throughput of the experiment. A decrease in the number of data points required leads to a greater degree of well utilization and hence greater productivity.

The integration of a liquid handling robot and a plate reader represents a powerful platform for automated route scouting. When compared to traditional methods of route scouting, it allows small volumes of reagent and sample to be used with a greater throughput, while high reproducibility and sensitivity are maintained. We have shown that the operating window concept can be applied successfully to a number of types of bioprocess steps with varying process characteristics when the two key variables of dispense volume and sample detection period are known. This approach may be used to visualize the tradeoffs implicit in determining the suitability of a given process step and to reduce the length of any experiment by maximizing well usage within each plate, prior to carrying out any practical work.

Acknowledgment

We thank the U.K. Joint Infrastructure Fund (JIF), the Science Research Investment Fund (SRIF), and the Gatsby Charitable Foundation for funds to establish the UCL Centre for Micro Biochemical Engineering. Financial support from the U.K. Engineering and Physical Sciences Research Council (EPSRC), in the form of an engineering doctorate (Eng.D.) studentship for A.J.N., and from the U.K. Biotechnology and Biological Sciences Research Council (BBSRC), in the form of Ph.D. studentships for K.E.W. and S.C.R.P., is also acknowledged.

References and Notes

(1) Littlehales, C. The price of a pill. *Mod. Drug Discovery* **1999**, *2*, 21.
(2) *2*) Weinmann, H.; Harre, M.; Tilstam, U. Breaking the new bottleneck: Automated synthesis in chemical process research and development. *Org. Process Res. Dev.* **1999**, *3*, 304−318.
(3) Pollard, M. Process development automation: An evolutionary approach. *Org. Process Res. Dev.* **2001**, *5*, 273−282.
(4) Mukesh, D. Succeed at process development. *Chem. Eng. Prog.* **1999**, *95*, 24−26.
(5) Ager, D. GMP and the future of pharma process development. *Chim. Oggi* **2001**, *19*, 8.
(6) Lye, G. J.; Ayazi-Shamlou, P.; Baganz, F.; Dalby, P. A.; Woodley, J. M. Accelerated design of bioconversion processes using automated microscale processing techniques. *Trends Biotechnol*. **2003**, *21*, 29−37.
(7) Elmahdi, I.; Baganz, F.; Dixon, K.; Harrop, T.; Sugden, D.; Lye, G. J. pH control in microwell fermentations of *S. erythraea* CA340: influence on biomass growth kinetics and erythromycin biosynthesis. *Biochem. Eng. J.* **2003**, *16*, 299−310.
(8) Berg, M.; Undisz, K.; Thiericke, R.; Zimmerman, P.; Moore, T.; Posten, C. Evaluation of liquid handling conditions in microplates. *J. Biomol. Screen.* **2001**, *6*, 47−56.
(9) Zheng, Y.; Wozniak, C. A. Adaptation of a β-1,3-Glucanase assay to microplate format. *Biotechniques* **1997**, *22*, 922−926.
(10) Kitchen, C. J.; Wang, A. Q.; Musson, D. G.; Yang, A. Y.; Fisher, A. L. A semi-automated 96-well protein precipitation method for the determination of montelukast in human plasma using high performance liquid chromatography/fluorescence detection. *J. Pharm. Biomed. Anal.* **2003**, *31*, 647−654.
(11) Bednar, B.; Condra, C.; Gould, R. J.; Connolly, T. M. Platelet aggregation monitored in a 96-well microplate reader is a useful evaluation of platelet agonists and antagonists. *Thromb. Res.* **1995**, *77*, 453−463.
(12) Cameron, R. G.; Buslig, B. S.; Shaw, P. E. Adaptation of a spectrophotometer assay for pectinmethylase to a kinetic microplate reader. *J. Food Sci.* **1992**, *57*, 1006−1008.
(13) Metzger, Z.; Featherstone, L. G.; Ambrose, W. W.; Trope, M.; Arnold, R. R. Kinetics of co-aggregation of *Porphyromonas gingivalis* with *Fusobacterium nucleatum* using an automated microtiter plate assay. *Oral Microbiol. Immunol.* **2001**, *16*, 163−169.
(14) John, G. T.; Heinzle, E. Quantitative screening method for hydrolases in microplates using pH indicators: Determination of kinetic parameters by dynamic pH modelling. *Biotechnol. Bioeng.* **2001**, *72*, 620−627.
(15) Grant, K. B.; Pattabhi, S. Use of a fluorescence microplate reader for the detection and characterisation of metal assisted peptide hydrolysis. *Anal. Biochem.* **2001**, *289*, 196−210.
(16) Doig, S. D.; Pickering, S. C. R.; Lye, G. J.; Woodley, J. M. The use of microscale processing technologies for quantification of biocatalytic Baeyer−Villiger oxidation kinetics. *Biotechnol. Bioeng.* **2002**, *80*, 42−49.
(17) Woodley, J. M.; Titchener-Hooker, N. J. The use of windows of operation as a bioprocess design tool. *Bioprocess Eng.* **1996**, *14*, 263−268.
(18) Astle, T. W.; Akowitz, A. Accuracy and tip carry over contamination in 96-well pipetting. *J. Biomol. Screen*. **1996**, *1*, 211−216.
(19) Stevens, M. E.; Bouchard, P. J.; Kariv, I.; Chung, T. D. Y.; Oldenburg, K. R. Comparison of automation equipment in high throughput screening. *J. Biomol. Screen.* **1998**, *3*, 305−311.
(20) Olsen, K. K. A quantitative study of factors governing automatic pipetting performance. *Am. Lab*. **2000**, *32*, 49−51.

(21) Weiss, S.; John, G. T.; Klimat, I.; Heinzle, E. Modelling of mixing in 96-well microplates observed with fluorescence indicators. *Biotechnol. Prog.* **2002**, *18*, 821−830.
(22) Duetz, W. A.; Witholt, B. Effectiveness of orbital shaking for the aeration of suspended bacterial cultures in square-deepwell microtiter plates. *Biochem. Eng. J.* **2001**, *7*, 113−115.
(23) Pickering, S. C. R. Microscale approaches to the design of bioconversion processes. Ph.D. Thesis, University of London, 2004.
(24) Doig, S. D.; O'Sullivan, L. M.; Patel, S.; Ward, J. M.; Woodley, J. M. Large scale production of cyclohexanone from *Escherichia coli* TOP10 pQR239. *Enzyme Microb. Technol.* **2001**, *28*, 265−274.
(25) Willson, K. E. Microscale approaches to the design of equilibrium stage separation processes. Ph.D. Thesis, University of London, 2004.
(26) Levy, M. S.; O'Kennedy, R. D.; Shamlou, P. A.; Dunnill, P. Biochemical engineering approaches to the challenges of producing pure plasmid DNA. *Trends Biotechnol.* **2000**, *18*, 296−305.
(27) Ciccolini, L. A. S.; Shamlou, P. A.; Titchener-Hooker, N. J.; Ward, J. M.; Dunnill, P. Time course of SDS-alkaline lysis of recombinant bacterial cells for plasmid release. *Biotechnol. Bioeng.* **1998**, *60*, 768−770.
(28) Welch, C. J.; Shaimi, M.; Biba, M.; Chilenski, J. R.; Szumigala, R. H., Jr.; Dolling, U.; Mathre, D. J.; Reider, P. J. Microplate evaluation of process adsorbents. *J. Sep. Sci.* **2002**, *25*, 847−850.
(29) Minas, W.; Bailey, J. E.; Duetz, W. A. Streptomycetes in micro-cultures: Growth, production of secondary metabolites and storage and retrieval in the 96-well format. *Antonie van Leeuwenhoek* **2000**, *78*, 297−305.
(30) John, G. T.; Klimant, I.; Wittmann, C.; Heinzle, E. Integrated optical sensing of dissolved oxygen in microtiter plates: A novel tool for microbial cultivation. *Biotechnol. Bioeng.* **2003**, *81*, 829−836.
(31) Maharbiz, M. M.; Holtz, W. J.; Howe, R. T.; Keasling, J. D. Microbioreactor arrays with parametric control for high throughput experimentation. *Biotechnol. Bioeng.* **2004**, *85*, 376−381.
(32) Kostov, Y.; Harms, P.; Randers-Eichhorn, L.; Rao, G. Low cost micro bioreactor for high throughput bioprocessing. *Biotechnol. Bioeng.* **2001**, *72*, 346−352.
(33) Duetz, W. A.; Rüedi, L.; Hermann, R. O'Connor, K.; Büchs, J.; Witholt, B. Methods for intense aeration, growth, storage and replication of bacterial strains in microtiter plates. *Appl. Environ. Microb.* **2000**, *66*, 2641−2646.
(34) *CRC Handbook of Chemistry and Physics*, 83rd ed.; CRC Press: Boca Raton, FL, 2002−2003.

Accepted for publication October 1, 2004.

BP049868+