

This article was developed from the winning student poster presentation at the 2006 ISPE Annual Meeting.

# Small-Scale Bioreactor Platform for Bioprocess Optimization

by Marisha Ben-Tchavtchavadze, Michel Perrier, and Mario Jolicoeur

## Introduction

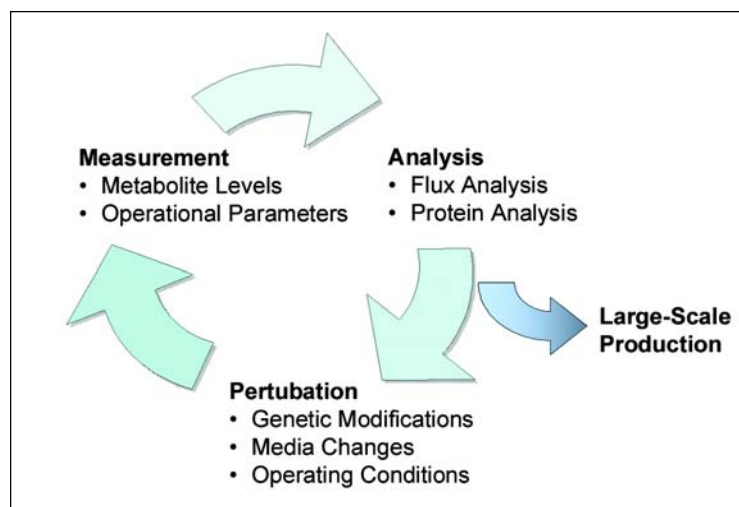
The discovery of recombinant DNA technology has changed the face of the pharmaceutical industry in the last 20 years due to the introduction of the large-scale bioprocess production of therapeutic proteins. It has become such an invaluable technique that the use of microorganisms and mammalian cells to produce therapeutic metabolites and proteins is estimated to increase by approximately 50% over the next five years, making it into a \$53 billion industry by 2010.<sup>1,2</sup> This trend is far from surprising due to the opportunity to discover and produce new therapeutic targets with tools such as metabolic engineering. This dedication to increase biopharmaceutical production also has become critical to assure that the demand of needy patients does not surpass availability and is not only of important commercial value, but also of great social value.

Recombinant protein production is accomplished by the use of several different expression systems such as bacteria, yeasts, plant, insect, and mammalian cells. Although bacte-

ria and other prokaryotic microorganisms have the advantage of producing high protein yields and production costs are relatively low, they do not possess the cellular machinery to perform post-translational modifications, such as glycosylation, essential for the production of many biomedically active proteins. Therefore, eukaryotic cells such as plant or mammalian cells are preferred when glycosylation is critical for bioactivity. While the large-scale production of human proteins by plant cells is increasing in interest,<sup>3</sup> most biopharmaceutical processes in the industry employ free-suspension mammalian cells due to their lack of cell walls which makes recovery and purification simpler.

Bioprocesses aim to manipulate and control cell lines to attain the maximum product yield and productivity at the lowest cost and in the most efficient way. Even though bioprocesses using mammalian cells have progressively achieved increased production yields at reduced costs, there are still many hurdles to surpass to fully optimize their recombinant protein production. Mammalian cell cultures are still reporting lower cell densities than microbial cultures for example.<sup>4</sup> Consequently, to control and predict the behavior of the cells to achieve better productivity and product yield, information concerning the cells physiological and metabolic states throughout the culture is necessary. Unfortunately, reliable and comprehensive process data from dynamic systems on living cells are not available for most cellular hosts including mammalian cells.<sup>5</sup> How-

Figure 1. The iterative cycle of metabolic engineering.



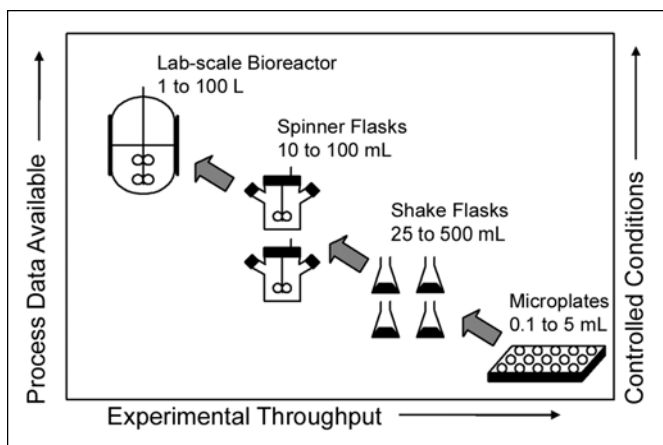


Figure 2. Illustration of the typical relationship between bioreactor monitoring capabilities and high-throughput experimentation.

ever, state-of-the-art non-invasive analytical technologies have been developed in the last few decades, which may help to provide the missing data, making it possible to model cellular processes and choose optimal cell culture operating conditions. This characterization of the bioprocess is typically performed during the development phase and tends to be a very lengthy affair, requiring important investment costs. A large set of experiments testing media components, cell lines, and environmental conditions must be assessed to determine optimal operation parameters in regard to productivity. Since so many conditions need to be evaluated, the commercial viability of using conventional bench-top bioreactors during the initial development is significantly reduced. For this reason, the industry is already looking to small-scale bioreactors that provide a well-defined environment and adequately monitor and control the culture, while providing accurate, complete, and useful data to reduce development process time. The range of data potentially acquired from small-scale bioreactors includes cell physiological and metabolic states as well as operational parameters. Furthermore, the small-scale bioreactor will significantly decrease development costs owing to its high-throughput qualities and the reduced use of raw materials, which are particularly expensive for mammalian cells. In the wake of Process Analytical Technology (PAT) tools, industry is now more than ready to utilize bioprocess data to model cellular performance to enhance bioproduction.

For accurate quantitative metabolic data, studies should be performed on intact living cells as opposed to *ex vivo* or *in vitro* experiments, such as metabolite extractions from cell sampling which exhibit low reproducibility in the literature.<sup>5</sup> Hence, Nuclear Magnetic Resonance (NMR) is one of the few technologies that permits the monitoring of metabolite concentrations and compartmentalization as well as intracellular pH *in vivo* in a non-destructive and non-invasive manner.<sup>6</sup> Combining the small-scale bioreactor with NMR has the added benefit of acquiring crucial metabolic data simultaneously without the necessity of sampling in a defined and controlled environment. Several small-scale bioreactors have been developed to be coupled with the NMR for *in vivo*

measurements of yeast, plant, and animal cells. However, none of the configurations were designed for free suspension mammalian cells which, as stated above, are the most commonly used cellular host in the biopharmaceutical industry and are the main focus of this study.

## Objectives

The primary objective of this project was to design a small-scale bioreactor perfusion system for mammalian cells free-suspension cultures. The design was adapted from a previously successfully developed bioreactor configured for plant cells in our laboratory.<sup>7</sup>

More specifically, the following conditions were analyzed:

- determine fluidization parameters allowing for adequate nutrient and oxygen mass transfer for maintaining high cell density culture
- characterize hydrodynamic profile of the bioreactor
- study and qualify mixing in the bioreactor
- demonstrate the bioreactor efficiency by performing on-line <sup>31</sup>P-NMR *in vivo* measurements

## A Platform for Process Optimization

The production of a protein-of-interest depends on a combination of factors: genetic (e.g., expression levels of key enzymes), physiological (e.g., carbon fluxes, energetic state), and environmental (e.g., O<sub>2</sub>, CO<sub>2</sub>, temperature, and pH). Each of these conditions must be studied and monitored during cell cultivation for process optimization.<sup>8</sup> However, to date, most on-line monitoring tools only measure extracellular components, such as cell density and oxygen consumption to indirectly assess the physiological and metabolic state of the cells. Without intracellular measurements, available process variables are insufficient to fully characterize the process. Therefore, new on-line technology must be selected to acquire as much process data as possible, including both intracellular and extracellular measurements. More sophisticated models will then be developed to mathematically express the cellular activities with the intention of optimizing:

- cellular growth
- product yield
- growth medium composition
- operating parameters including pH, temperature, dissolved oxygen

In the past, cellular processes were lumped into a black box model, which were empirical by nature and gave no information of the underlying mechanisms of the cell metabolism. Metabolic engineering, on the other hand, is an evolved approach that has been refined to fill this gap by adequately measuring, analyzing, and designing bioprocesses optimally.<sup>9,10</sup>

## Metabolic Engineering: A Useful Tool

Metabolic engineering adopts genetic engineering strategies such as recombinant DNA technology for strain improvement

by enhancing or creating new pathways by which to increase product yield. The main difference and advantage of this systematic approach is that it mirrors the age-old adage that “the whole is greater than the sum of its parts.” In other words, while some cellular genetic strategies focus only on a particular pathway and a few of their key enzymes to increase product yield, metabolic engineering recognizes that the living cell is made up of thousands of complex and intertwined metabolic pathways that respond differently to changing environmental conditions. Therefore, a genetic change in one pathway may unknowingly have undesirable effects in others due to the interdependent nature of the metabolic network. Thus, it is insufficient to arbitrarily amplify specific enzymatic genes in a cellular host in the hopes of obtaining maximal protein production with no comprehension of the impact on the entire metabolic network. The amplification may have little or no effect on product yield; therefore, time and money would have been wasted in vain. Blind cellular mutations require long screening processes and give no understanding on the improvement itself. Consequently, the aim of metabolic engineering is to successfully direct metabolic flux toward valuable product formation by understanding the effects of genetic manipulations on the network or the “whole,” making intelligent and directed genetic modifications.

In practical terms, metabolic engineering follows an iterative approach for the continuous improvement of the targeted phenotype based on a sequential series of experiments - *Figure 1*. The knowledge gained in each step leads to the next series of experiments. Where to start in the cycle depends on the objective of the process as well as the initial knowledge on the cells metabolic control. Often, a perturbation is first applied to the cellular host such as a genetic modification to impart improved qualities to the strain. Typically, several cultures will be conducted to obtain a metabolic characterization of the cells, including intracellular and extracellular metabolites levels, bioprocess conditions (pH, pO<sub>2</sub>, and temperature), RNA expression, etc. Metabolic flux and flux control analysis will then give rise to models to better target the next modification. The more accurate and complete the data used to perform whole metabolic analysis, the less the number of iterative cycles will be necessary as desired results will be achieved faster reducing development time and cost. Once targeted results are confirmed by analysis, the bioprocess can be considered for large-scale production.

This data-driven approach can be used for many different applications for bioprocess optimization such as:

- improvement of production yield: directing carbon flow toward specific pathways or increasing enzyme activity
- recombinant protein production: cloning all necessary pathways into host to produce active molecule
- bioprospecting: identifying new cell-lines that exhibit therapeutic compounds or novel enzyme activity
- screening of drug candidates
- elimination or reduction of by-product formation
- strain improvement: more robust in terms of viability
- identifying pathways for cell line enhancement

- media design
- extension of substrate range: to use less costly raw materials or higher viability in previously toxic environment
- extension of cellular physiological conditions: increase tolerance to low oxygen concentration
- screening cell library for enhanced metabolite production

This list is far from exhaustive.<sup>8,9</sup> Any potential improvement to the strain and to environmental conditions, as well as novel discoveries can be verified and further enhanced with this approach making predictive changes a possibility. Up to now, metabolic engineering has been mostly employed to tailor specific traits of cellular hosts such as increased production of ethanol,<sup>12</sup> lactic acid,<sup>13</sup> lysine,<sup>14</sup> propane diol,<sup>15</sup> and therapeutic proteins.<sup>16</sup> All of these examples are industrially relevant processes and demonstrate the range of applicability of this approach. In more detail, Takiguchi and his colleagues<sup>14</sup> were able to increase lysine production molar yields from 7.5% to 30.6% by changing operational parameters based on molar flux distributions. These analyses were derived from metabolic reaction models developed with on-line measurements.

The shortcoming of metabolic engineering is that to use this approach efficiently and in a timely manner, specific tools must be on-hand. As defined earlier, this iterative approach requires many series of tests. Several cultivations are required under traditional methods to attain the large sets of data needed to properly characterize the metabolic network and identify control strategies. To significantly reduce costs, many researchers have begun to use small-scale bioreactors for this phase in the development process.<sup>17,18,19</sup> By using small-scale bioreactors that have high-throughput qualities, many metabolites may be monitored in parallel and the reduced volumes of the vessels save on expensive raw materials.

### Small-Scale Bioreactors

To reduce material and labor costs and accelerate the development phase, the use of small-scale bioreactors is indispensable. In this body of work, a bioreactor is considered “small-scale” or “mini” if its volume is inferior to 100 mL with particular attention to culture tendencies in vessels below 10 mL, such as test-tubes and microtiter plates due to the ease of parallelization and automatization of these systems.<sup>20</sup>

It is important to recognize that regardless the volume of the bioreactor, it is essential that it provides a well-defined environment which can be monitored and controlled to obtain detailed strain characterization and process condition data. An advantage of using small-scale bioreactors is that they permit high-throughput approaches. However, in the past, the level and quality of monitoring and control of the cell culture was proportional to the bioreactor volume as seen in *Figure 2*. This trade-off was mostly due to the lack of appropriate analytical tools such as pH and dissolved oxygen probes whose relative size were not amenable for smaller vessels. However, the use of microtiter plates has become more interesting in the last several years due to the recent availability of innovative integrated miniature sensors helping to close the information

Nucleus	Isotopic Abundance	Biological Applications
<sup>3</sup> H	0	Receptor-ligand interactions
<sup>1</sup> H	0.9998	Metabolites, pH, redox
<sup>19</sup> F	1.0	Cations, O <sub>2</sub> , metabolites
<sup>31</sup> P	1.0	Energy, cations, metabolites
<sup>7</sup> Li	1.0	Transport
<sup>23</sup> Na	1.0	Intracellular Na <sup>+</sup>
<sup>13</sup> C	0.011	Metabolites
<sup>2</sup> H	1.5 x 10 <sup>-4</sup>	Membrane structure
<sup>17</sup> O	3.7 x 10 <sup>-4</sup>	Water structure
<sup>15</sup> N	3.7 x 10 <sup>-2</sup>	Metabolites
<sup>35</sup> Cl	0.755	Intracellular Cl <sup>-</sup>
<sup>14</sup> N	0.996	Metabolites
<sup>39</sup> K	0.931	Intracellular K <sup>+</sup>
<sup>41</sup> K	0.069	Intracellular K <sup>+</sup>

Table A. NMR nuclei and their relative natural abundance and biological applications (adapted from Gillies et al., 1989).

gap between small-scale and lab-scale bioreactors.<sup>21</sup> There are still some limitations to the use of microtiter plates as bioreactors such as high evaporation rates and the relatively high risk of cross-contamination caused by aerosol formation.<sup>20</sup> Furthermore, the benefits of parallelization and automatization in a microtiter platform are counter-balanced by its inefficiency to precisely control operational parameters such as O<sub>2</sub> levels and to acquire *in vivo* metabolic data. Additionally, many believe that culture volumes are too small to adequately characterize the entire process and the cell line.<sup>8,18</sup> Therefore, other small-scale systems are developed and used to overcome these shortcomings.

While several miniaturized systems now exist on the market, it is difficult to find one that can fulfill monitoring and control requirements to simultaneously examine nutrient concentrations, metabolite levels, pH, oxygen, and temperature. The difficulty is not only in choosing the appropriate type of bioreactor for a specific bioprocess application, but combining the bioreactor with the proper analytical tools for extracellular and intracellular measurements and subsequent metabolic characterization.

## Advantages of Non-Invasive Analytical Technology

Metabolic engineering approaches are highly dependent on the tools that are used to measure metabolite levels. Without analytical tools it would be very difficult if not impossible to verify or validate the perturbations or genetic changes applied to the cell line. Therefore, the accuracy of the models defined by metabolic analyses will be proportional to the level of sophistication of the analytical tools chosen. In other words, the choice of technology used to quantify metabolites will directly influence the quality of the results and determine the number of iterative cycles needed to obtain the targeted-outcome (i.e., increased production yield and cell

robustness).

Two categories of analytical technologies can be distinguished: *in vitro* or invasive methods and *in vivo* or non-invasive methods. Invasive methods require sampling for *in vitro* analysis by such instruments as HPLC,<sup>22</sup> MS,<sup>23</sup> spectrophotometer,<sup>24</sup> and nuclear magnetic resonance or NMR.<sup>25</sup> These analytical techniques will provide a global snap-shot of the metabolic state of the cell and will require several series of samples to readily follow metabolism as a function of time. There is some debate on the reliability of sampling methods since metabolites have proven to be unstable. Several studies have shown that inconsistencies in the literature concerning metabolite levels are due to sampling and that these methods may not adequately represent the true metabolic state of the cells.<sup>5,26</sup>

On the other hand, non-intrusive methods allow for on-line *in vivo* measurements of metabolites over time and other important cell culture parameters such as pH, dissolved oxygen, and temperature, while eliminating the necessity of sampling and reducing contamination risks. IR, Raman, fluorescence, confocal optical imagery,<sup>6</sup> and NMR spectroscopy<sup>27</sup> are all examples of this category. NMR is unique as it can distinguish metabolite concentrations as a function of space (i.e., between intracellular and extracellular space) and measure compartmentalization in the cell providing key parameters for subsequent process modeling.<sup>28</sup>

## NMR Spectroscopy of Living Cells

Physicists developed NMR spectroscopy in the 1940s, but it was only in the early 1970s that it was used for the first time for *in vivo* measurements of intact red blood cell suspensions albeit in non-viable conditions.<sup>29</sup> Since then, multiple applications in the biotechnology field have been reported and continue to grow.<sup>30,31</sup> The popularity of this instrument is owing to its non-invasive and non-destructive nature as well as its capacity to measure metabolite levels in complex mixtures without the need for specific assays. The metabolic data elucidated from NMR spectra is used to observe and measure intracellular pH, flux analysis, metabolite quantification, and biochemical kinetic reactions<sup>32</sup> and is of great importance for such disciplines as metabolic engineering.

The basic principle of NMR spectroscopy is that certain nuclei possess intrinsic magnetic moments which are sensitive to magnetic fields. When these nuclei are submitted to strong magnetic fields, their magnetic moments align themselves either parallel or antiparallel to the field, creating a net magnetization. The difference in energy, "E," of the state or the direction of the magnetic moment of each nucleus depends on the strength of the applied magnetic field and the gyromagnetic ratio of the particular nucleus. Resonance is produced when transition between the two energy states occurs due to the application of bursts or pulses of electromagnetic energy at a specific radiofrequency during the NMR experiment. The measured resonance signal is specific to the nucleus and its environmental conditions (i.e., the position of nuclei in a molecule or the solution pH) as well as being proportional to the number of nuclei present. Therefore, NMR also can be used as a quantification tool.<sup>32</sup> The various

nuclei used in NMR are listed in Table A with their respective relative natural abundance and biological applications.

While there are several different nuclei to choose from when using *in vivo* NMR, one of the most commonly studied is  $^{31}\text{P}$  due to its high natural abundance and the importance of phosphorus in essential metabolic compounds such as nucleosides phosphates (ATP, ADP, AMP, NADPH), sugar phosphates (glucose-6P and fructose-6P) and inorganic phosphate (Pi).<sup>32</sup> The majority of metabolic pathways employ ATP and this molecule plays an important role in metabolic reactions and control. Furthermore, ATP levels are considered to characterize the energetic state of the cell. Intracellular flux analysis performed by Henry et al,<sup>34</sup> correlated ATP levels to cell productivity demonstrating the relevance of following the phosphate isotope using NMR spectra. Additionally,  $^{31}\text{P}$ -NMR allows for the monitoring of intracellular pH due to the sensitivity of the chemical shift of the inorganic phosphates to pH intermolecular effects.<sup>28</sup>

Though *in vivo* NMR is a powerful analytical tool providing on-line environmental and metabolic measurements of the cell culture, this technique has a few limitations. The most important is its lack of sensitivity. For a metabolite to be properly identified using NMR, its total concentration in the cell culture must be above 0.1 mM. However, many critical metabolites are only found in low concentrations (less

than 0.1 mM) in the cell. The most common way to circumvent this problem is by increasing cell density until high quality spectra are achieved. Typically, NMR studies of suspended cells call for cell densities of approximately  $10^7$ - $10^{11}$  cells  $\text{mL}^{-1}$ , depending on cell type and size.<sup>32</sup> Therefore, certain process or operation requirements must be met allowing for a viable high density cell culture:<sup>29</sup>

1. Cell suspension must be homogeneous and within the NMR reading zone.
2. Cell suspension must be adequately perfused for proper nutrient delivery and waste removal.
3. Cell suspension must be adequately oxygenated.

To meet all of these constraints, the small-scale bioreactor is an invaluable tool.

### Small-Scale Bioreactor Combined with NMR

High cell density increases sensitivity and permits shorter acquisition times for NMR spectra, providing real-time monitoring. However, this condition also will necessitate a higher degree of control to provide adequate nutrient levels, oxygen supply, waste removal, and other environmental parameters. Therefore, this control can be provided by small-scale bioreactors housed in a standard NMR tube (10-20 mm

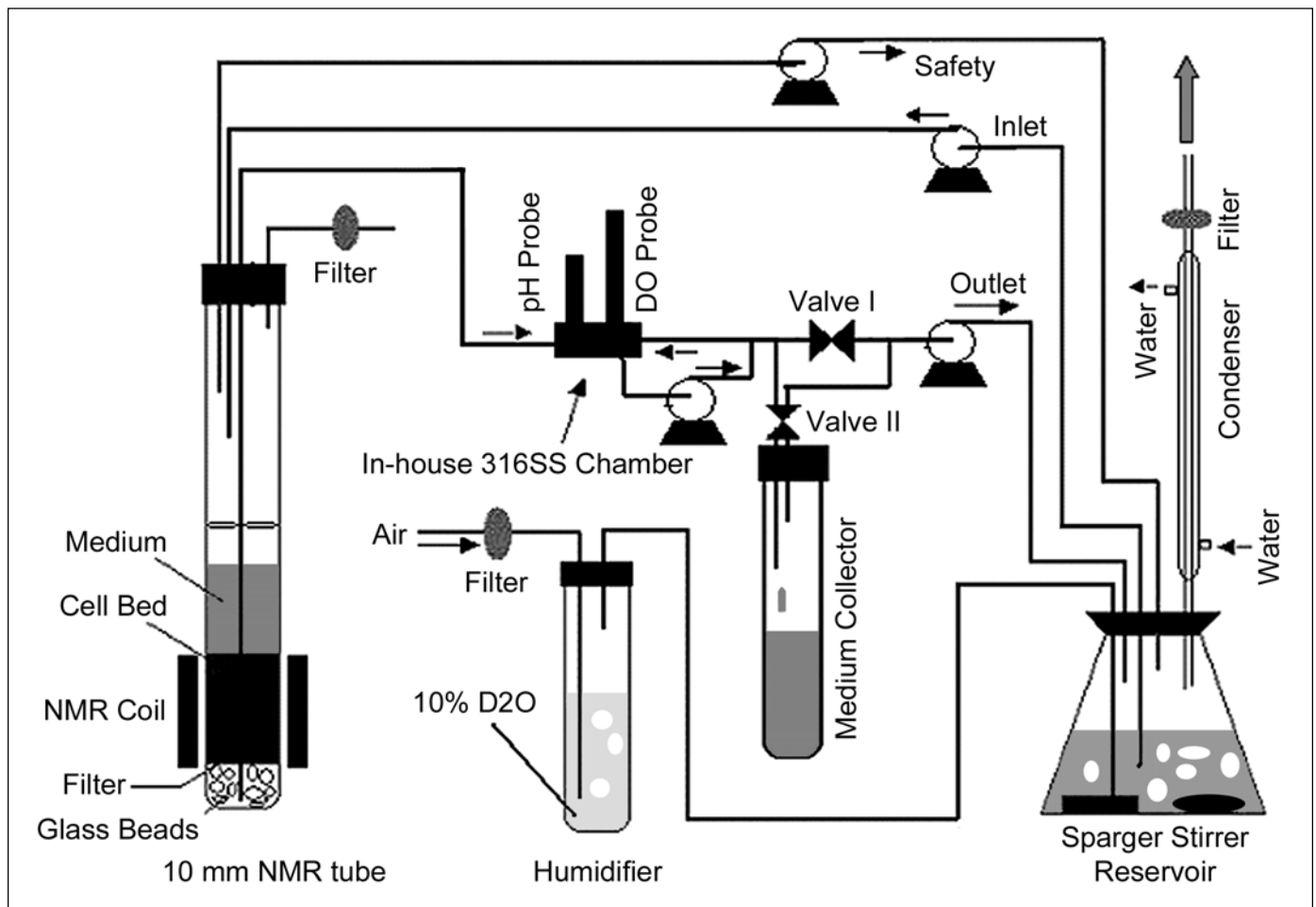


Figure 3. Small-scale bioreactor platform for plant cells.

diameter, 3-20 mL culture volume). Many configurations have been designed and tested using several types of cells, including airlift, microcarrier, hollow-fiber, and compact bed bioreactors.

The airlift small-scale bioreactor has the advantage of being relatively simple and has been used to study bacteria and yeast.<sup>35,36</sup> Oxygenation is provided by bubbling gas in the central tube of the bioreactor as is done in conventional sized air-lift vessels. However, the presence of bubbles in the NMR detection region can disrupt the homogeneity of the magnetic field and broaden the resonance. To perform long-term studies, this configuration is not adequate since it does not bestow suitable conditions for waste removal (i.e., lactate and CO<sub>2</sub>) over time.

Perfusion systems for the small-scale bioreactor platform have the advantage of providing constant nutrient and oxygen supply, while simultaneously removing waste products which allows for long lasting high cell density cultures.<sup>37</sup> However, sophisticated techniques need to be adopted to maintain the cell culture in the NMR reading zone. Many have chosen to make use of microcarrier beads to immobilize the cells in the bioreactor and prevent cell wash-out. For example, Shankland and colleagues<sup>38</sup> immobilized A549 mammalian cells to macroporous gelatine carriers in a perfusion reactor for <sup>31</sup>P and <sup>13</sup>C NMR study. Another configuration for anchorage-dependent cells is the Hollow-Fiber System (HFBR). Gillies et al.,<sup>39</sup> have worked extensively with the HFBR since up to 70% of the reactor volume can be occupied by the cells with this technology and cell densities have been reported to reach above 10<sup>8</sup> cells mL<sup>-1</sup>. These conditions provide ideal circumstances for NMR spectroscopy as seen by real-time <sup>31</sup>P NMR studies (180s acquisition time) of hybridomas, CHO cells as well as C-6 rat glioma. However, adherent cells are not as commonly used in industrial bioprocesses, due to the difficulty of maximizing

surface to volume ratio in large-scale bioreactors and cell transfer through the bioreactor chain. Therefore, there is concern that metabolic data obtained from adherent cells may not be representative of the true nature of free-suspension cell cultures. Consequently, research groups are designing small-scale bioreactors that better reflect their large-scale counterparts such as Gmati et al.<sup>7</sup>

The long-term *in vivo* NMR study of high density plant cell cultures was successfully accomplished by our laboratory by developing a small-scale perfusion bioreactor that sequestered the plant cells in a packed-bed, while providing a homogeneous external environment - *Figure 3*.<sup>7,40</sup> Significant characterization of the hydrodynamic and mass transfer profiles of the bioreactor demonstrated a perfectly mixed system as well as providing control of the perfusion parameters such as oxygen and nutrient supply, pH, and temperature. Inspired by this body of work, a small-scale perfusion bioreactor for free-suspension mammalian cells is developed.

## Design Constraints for Free-Suspension Mammalian Cells

To our knowledge, no other configuration has been successfully designed for *in vivo* NMR measurement of free-suspension mammalian cells. This is invariably due to the specific design constraints imposed by the necessity for high cell density sequestered in the NMR reading zone. Therefore, oxygen becomes a limiting factor in the system. Low solubility of oxygen in media entails that gas diffusion only will not adequately provide for the metabolic needs of the cell. Furthermore, mammalian cells negatively react to high oxygen concentrations and it is generally recommended that pO<sub>2</sub> is maintained between 25 to 50%.<sup>41</sup> Increasing perfusion rate is the simplest solution to assure that oxygen consumption rate of the cells is satisfied. However, increasing flow rate to

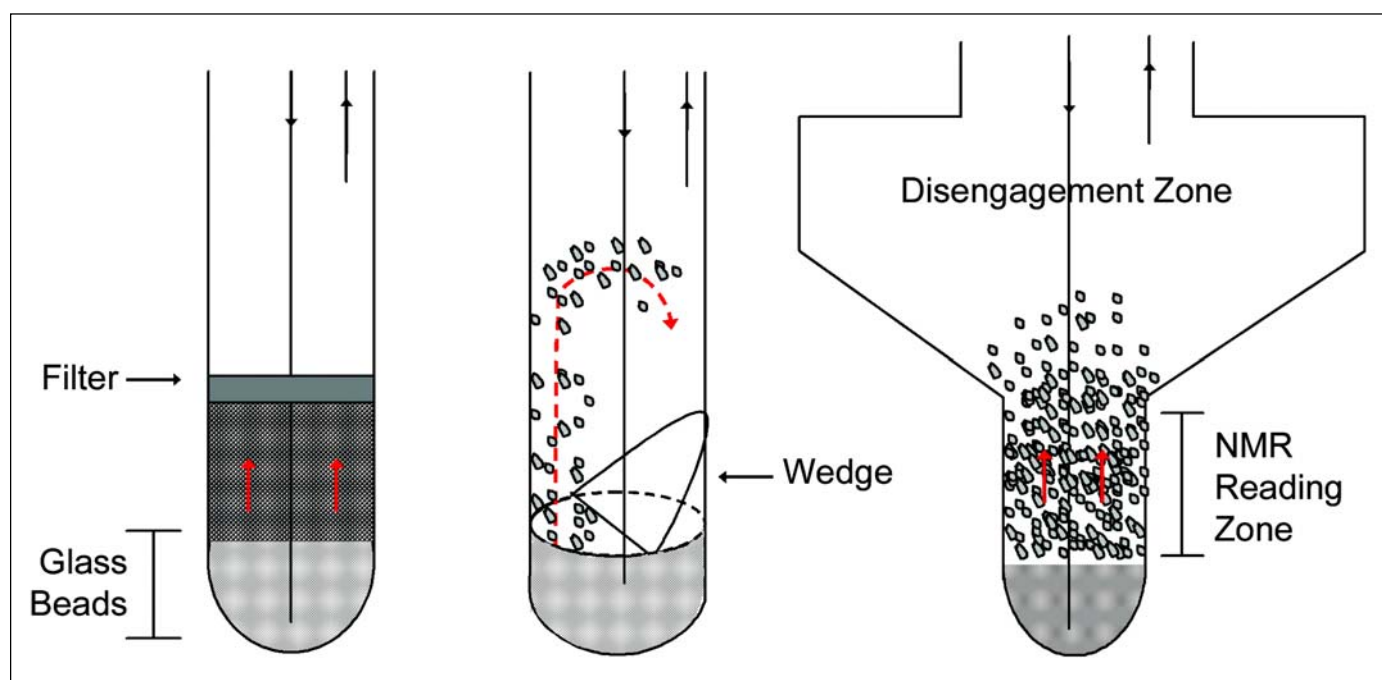


Figure 4. Potential small-scale bioreactor configurations for free-suspension mammalian cells in a 10 mm diameter NMR tube.

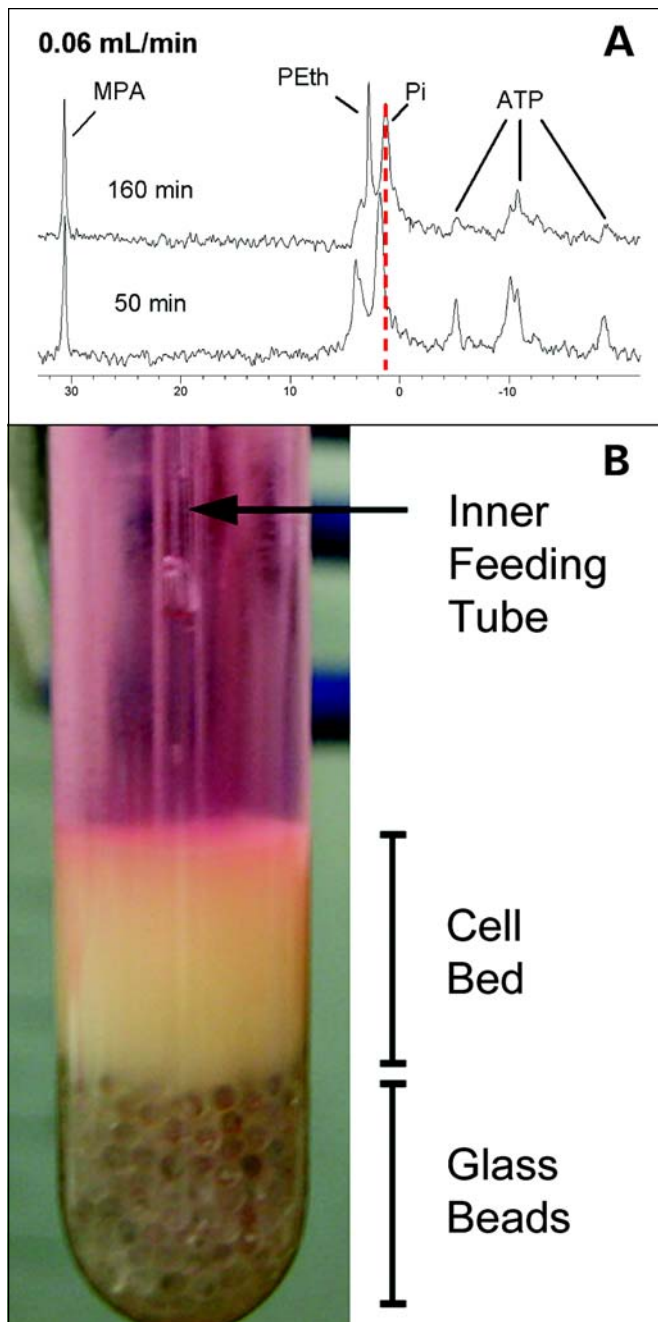


Figure 5. A. Proton-decoupled  $^{31}\text{P}$  NMR spectrum of *in vivo* analysis of 3-d-old CHO (Chinese hamster ovary) cells perfused at  $0.06 \text{ mL min}^{-1}$ . B. Perfused CHO cell bed ( $1.44 \times 10^8$  cells) at  $0.06 \text{ mL min}^{-1}$  in small-scale bioreactor.

suitable levels will most definitely cause cell wash-out from the bioreactor, due to low cell density. The majority of the work for this study will require determining and constructing the appropriate configuration that will allow for high flow rates, while maintaining free-suspension mammalian cells in the NMR reading zone.

Figure 4 presents the various small-scale bioreactor configurations studied. All of these designs have the potential to fulfill the elementary requirements for *in vivo* NMR measurements. The packed-bed configuration (A) has the advantage of being relatively simple to construct and could satisfy

requirements for high flow rate and sequestered cells. But mammalian cells are fairly sensitive to shear stress and the packed-bed may create unviable conditions for the cells. For example, the lack of cell walls and the plasticity of the cell membranes may foster a very tight compact bed which could produce preferential media currents in the bed and heterogeneous conditions. On the other hand, the whirling motion bed (B), initially developed for drying applications,<sup>42</sup> should be compatible with the small-scale bioreactor, because the circulating motion allows for better mixing as well as higher flow rates, while maintaining the cells in the NMR reading zone. The purpose of the wedge is to disrupt the flow and create a preferential current which is the driving force of the whirling motion. The superficial velocity of the fluid will decrease as it goes up the wedge directing the cells back to the bottom of the tube where they will be carried upward again by the entering media. The whirling motion provides an effective method to properly oxygenate the cells, but may be difficult to apply. Optimal wedge configurations and flow rates will have to be evaluated to assure satisfactory operating conditions. Another promising design is the fluidized-bed with the disengagement zone (C). The top section of the fluidized bed is expanded to abruptly reduce the superficial velocity of the media below the minimum fluidization velocity, which serves to return the cells to the narrow part of the NMR tube preventing cell wash-out and assuring high cell density. The advantage of the disengagement zone is that it allows for higher flow rates of the fluidized liquid, which supplies additional oxygen levels to the cells. However, this configuration requires a custom designed NMR tube and probe. Nevertheless, all of the presented configurations merit further investigation to determine the most efficient design.

As stated above, there is one significant limiting factor of the design: oxygen availability. The ability to satisfy mammalian cell respiration is at the base of the successful design and operation of the small-scale bioreactor. Consequently, one of the first steps is to identify the minimal perfusion flow rate required for sufficient oxygen delivery and to subsequently monitor the dissolved oxygen concentration throughout the cultivation. Using a lab-built respirometer, the oxygen specific uptake rate,  $qO_2$ , can be calculated by plotting a time profile of dissolved oxygen and the oxygen consumption of the cells in culture.<sup>44</sup> The  $qO_2$  is then used to determine minimal perfusion flow rate ( $Q$ ) for a given cell density ( $n$ ) and dissolved oxygen concentration ( $DO$ ) using the following relation:

$$Q = \frac{qO_2 \cdot n}{DO}$$

This estimate provides a crucial starting point for the fluidization assays to follow regardless of the small-scale configuration studied.

Mixing of the cells also requires specific attention owing to the fact that it will ensure cell suspension and provide homogeneous nutrient and oxygen concentrations. To characterize the quality of the mixing, Residence Time Distribu-

tion (RTD) experiments will be performed by measuring the evolution of a saline pulse with an electrical conductivity detector. The tracer concentration will then be evaluated in terms of the dimensionless Péclet (Pe) number.<sup>45</sup> When Pe is close to zero, axial dispersion is large and when Pe tends to infinity, axial dispersion is low. By uncovering and comparing the specific mixing dynamics of each small-scale bioreactor, the most appropriate configuration can be chosen for the design constraints imposed by *in vivo* NMR measurements.

Once fluidization and operational parameters have been discerned, *in vivo* NMR measurements of free suspension mammalian cells can start. The spectra will give additional information concerning cell viability, appropriate cell density, and/or dissolved oxygen concentration and further improvements can be applied to the small-scale bioreactor platform to achieve long-term monitoring and control of the cell culture. Preliminary NMR studies of the packed bed design demonstrate that *in vivo* measurements of free-suspension mammalian cells are possible. However, as speculated, the viability of the cells decreases as a function of time and does not allow for long-term *in vivo* measurements as seen in Figure 5 by the chemical shift of the inorganic phosphate (Pi) peak. A chemical shift to the left is indicative of an imposed stress to the cells such as a lack of oxygen. Additionally, the peak intensities decrease as a function of time demonstrating cell death. The packed bed appears to create preferential currents which will be verified by RTD. The other bioreactor configurations will be tested in the meantime and evaluated on the quality of the spectra as well.

## Conclusion

Enhancing cell lines, media, and bioprocesses for greater bioactive protein delivery and cell robustness is the aim of all biopharmaceutical manufacturing industries. To fulfill this goal in an efficient and timely manner, metabolic engineering is progressively becoming an invaluable tool. This approach will lead research and development to better control strategies by targeting the right genes, pathways, and proteins for bioprocess expansion. The small-scale bioreactor combined with NMR technology for *in vivo* measurement provides accurate, relevant, and real-time physiological, operational, and metabolic data necessary for comprehensive metabolic analysis. The development of this bioreactor platform for free-suspension mammalian cells is critical owing to the importance of this particular cellular host in the industry. Preliminary tests have shown that the packed-bed configuration enables *in vivo* NMR measurements for CHO cells. However, optimal operating conditions and bioreactor design still need to be determined. The whirling-motion bed offers particular promise in satisfying all design constraints, providing necessary metabolic data for process optimization. Regardless of the final configuration, the small-scale bioreactor will be able to host numerous types of suspension cells as well as adherent cells and make full use of long-term *in vivo* NMR measurements.

## References

1. Warnock, J.N., and Al-Rubeai, M., "Bioreactor Systems for the Production of Biopharmaceuticals from Animal Cells," *Biotechnology and Applied Biochemistry*, Vol. 45, 2006, pp. 1-12.
2. Pavlou, A.K., and Reichert, J.M., "Recombinant Protein Therapeutics - Success Rates, Market Trends and Values to 2010," *Nature Biotechnology*, Vol. 22, No. 12, 2004, pp. 1513-1519.
3. Stoger, Eva, Ma, Julien K-C, Fischer, Rainer, and Christou, Paul, "Sowing the Seeds of Success: Pharmaceutical Proteins from Plants," *Current Opinion in Biotechnology*, Vol. 16, 2005, pp. 167-173.
4. Butler, M., "Animal Cell Cultures: Recent Achievements and Perspectives in the Production of Biopharmaceuticals," *Applied Microbiology and Biotechnology*, Vol. 68, 2005, pp. 283-291.
5. Gonzalez, B., de Graaf, A., Renaud, M., and Sahm, H., "Dynamic *in vivo* <sup>31</sup>P Nuclear Magnetic Resonance Study of *Saccharomyces Cerevisiae* in Glucose-Limited Chemostat Culture During the Aerobic-Anaerobic Shift," *Yeast*, Vol. 16, 2000, pp. 483-497.
6. Ulber R., Frerichs, J.G., and Beutel, S., "Optical Sensor Systems for Bioprocess Monitoring," *Analytical and Bioanalytical Chemistry*, Vol. 376, No. 3, 2003, pp. 342-348.
7. Gmati, D., Chen, J., and Jolicoeur, M., "Development of a Small-Scale Bioreactor: Application to *in vivo* NMR Measurement," *Biotechnology and Bioengineering*, Vol. 89, No. 2, 2005, pp. 138-147.
8. Ratledge, C. and Kristiansen, B., *Basic Biotechnology*, 3rd ed., Cambridge: Cambridge University Press, 2006.
9. Nielsen, J., "Metabolic Engineering," *Applied Microbiology and Biotechnology*, Vol. 55, 2001, pp. 263-283.
10. Stephanopoulos, G.N., Aristidou, A.A., and Nielsen, J., "Metabolic Engineering - Principles and Methodologies," 1998, San Diego, Academic Press.
11. Raab, R.M., Tyo, K., and Stephanopoulos, G., "Metabolic Engineering," *Advances in Biochemical Engineering Biotechnology*, Vol. 100, 2005, pp. 1-17.
12. Nissen, T.L., Kielland-Brandt, M.C., Nielsen, J., and Villadsen, J., "Optimization of Ethanol Production in *Saccharomyces Cerevisiae* by Metabolic Engineering of the Ammonium Assimilation," *Metabolic Engineering*, Vol. 2, No. 1, 2000, pp. 69-77.
13. van Maris, Antonius J.A., Konings, W.N., Dijken, J.P. van, and Pronk, J.T., "Microbial Export of Lactic and 3-hydroxypropanoic acid: Implications for Industrial Fermentation Processes," *Metabolic Engineering*, Vol. 6, No. 4, 2004, pp. 245-255.
14. Takiguchi, N., Shimizu, H., and Shioya, S., "An On-line Physiological State Recognition System for the Lysine Fermentation Process Based on a Metabolic Reaction Model," *Biotechnology and Bioengineering*, Vol. 55, No. 1, 1997, pp. 170-181.
15. Gonzalez-Pajuelo, M., Meynial-Salles, I., Mendes, F., Andrade, J.C., Vasconcelos, I., and Soucaille, P., "Meta-

- bolic Engineering of *Clostridium Acetobutylicum* for the Industrial Production of 1,3-Propanediol from Glycerol," *Metabolic Engineering*, Vol. 7, No. 5-6, 2005, pp. 329-336.
16. Provost, A. and Bastin, G., "Dynamic Metabolic Modeling Under the Balanced Growth Condition: Dynamics, Monitoring, Control, and Optimization of Biological Systems," *Journal of Process Control*, Vol. 14, No. 7, 2004, pp. 717-728.
  17. Chapple, Susan D.J., Crofts, A.M., Shadbolt, S.P., McCafferty, J., and Dyson, M.R., "Multiplexed Expression and Screening for Recombinant Protein Production in Mammalian Cells," *BMC Biotechnology*, Vol. 6, No. 49, 2006, pp. 1-15.
  18. Betts, J.I. and Baganz, F., "Miniature Bioreactors: Current Practices and Future Opportunities," *Microbial Cell Factories*, Vol. 5, No. 21, 2006.
  19. Kumar, S., Wittmann, C., and Heinzle, E., "Mini-bioreactors," *Biotechnology Letters*, Vol. 26, 2004.
  20. Duetz, W.A., Rüedi, L., Hermann, R., O'Connor, K., Büchs, J., and Witholt, B., "Methods for Intense Aeration, Growth, Storage, and Replication of Bacterial Strains in Microtiter Plates," *Applied and Environmental Microbiology*, Vol. 66, No. 6, 2000, pp. 2641-2646.
  21. Weiss, S., Gernot, T.J., Klimant, I., and Heinzle, E., "Modeling of Mixing in 96-well Microplates Observed with Fluorescence Indicators," *Biotechnology Progress*, Vol. 18, 2002, pp. 821-830.
  22. Henry, O., Kamen, A., and Perrier, M., "Monitoring the Physiological State of Mammalian Cell Perfusion Processes by On-line Estimation of Intracellular Fluxes," *Journal of Process control*, Vol. 17, 2007, pp. 241-251.
  23. Korke, R., Gatti, Marcela de Leon, Lau, Ally Lei Yin, Lim, Justin Wee Eng, Seow, Teck Keong, Chung, Maxey Ching Ming, and Hu, Wei-Shou, "Large Scale Gene Expression Profiling of Metabolic Shift of Mammalian Cells in Culture," *Journal of Biotechnology*, Vol. 107, No. 1, 2004, pp. 1-17.
  24. Balcarcel, R. Robert and Clark, Lindsey M., "Metabolic Screening of Mammalian Cell Cultures Using Well-plates," *Biotechnology Progress*, Vol. 19, No. 1, 2003, pp. 98-108.
  25. Zupke, C. and Stephanopoulos, G., "Intracellular Flux Analysis in Hybridomas Using Mass Balances and in vitro  $^{13}\text{C}$  NMR," *Biotechnology and Bioengineering*, Vol. 45, 1995, pp. 292-303.
  26. Saez, M.J. and Lagunas, R., "Determination of Intermediary Metabolites in Yeast. Critical Examination of the Effect of Sampling Conditions and Recommendations for Obtaining True Levels," *Molecular and Cellular Biochemistry*, Vol. 3, No. 2, 1976, pp. 73-78.
  27. Meehan, A.J., Eskey, C.J., Koretsky, A.P., and Domach, M.M., "Cultivator for NMR Studies of Suspended Cell Cultures," *Biotechnology and Bioengineering*, Vol. 40, 1992, pp. 1359-1366.
  28. Hesse, S.J.A, Ruijter, G.J.G., Dijkema, C., and Visser, J., "Measurement of Intracellular (Compartmental) pH by  $^{31}\text{P}$  NMR in *Aspergillus niger*," *Journal of Biotechnology*, Vol. 77, 2000, pp. 5-15.
  29. Morris, P.G., "NMR Spectroscopy in Living Systems," *Annual Reports on NMR Spectroscopy*, Vol. 20, 1988, pp. 1-60.
  30. Cohen, J.S., Motiei, M., Carmi, S., Shiperto, D., Yefet, O., and Ringel, I., "Determination of Intracellular pH and Compartmentation Using Diffusion-weighted NMR Spectroscopy with pH-sensitive Indicators," *Magnetic Resonance in Medicine*, Vol. 51, 2004, pp. 900-903.
  31. Ratcliffe, R.G, Roscher, A., and Shachar-Hill, Y., "Plant NMR Spectroscopy," *Progress in Nuclear Magnetic Resonance Spectroscopy*, Vol. 39, No. 4, 2001, pp. 267-300.
  32. Fernandez, E.J. and Clark, D.S., "N.M.R. Spectroscopy: a Non-invasive Tool for Studying Intracellular Processes," *Enzyme and Microbial Technology*, Vol. 9, 1987, pp. 259-271.
  33. Gillies, R.J., MacKenzie, N.E., and Dale, B.E., "Analyses of Bioreactor Performance by Nuclear Magnetic Resonance Spectroscopy," *Biotechnology*, Vol. 7, 1989, pp. 50-54.
  34. Henry, O., Perrier, M., and Kamen, A., "Metabolic Flux Analysis of HEK-293 Cells in Perfusion Cultures for the Production of Adenoviral Vectors," *Metabolic Engineering*, Vol. 7, 2005, pp. 467-476.
  35. Kramer, H.W. and Bailey, J.E., "Mass Transfer Characterization of an Airlift Probe for Oxygenating and Mixing cell Suspensions in an NMR Spectrometer," *Biotechnology and Bioengineering*, Vol. 37, 1991.
  36. Melvin, B.K. and Shanks, J.V., "Influence of Aeration on Cytoplasmic pH of Yeast in an NMR Airlift Bioreactor," *Biotechnology Progress*, Vol. 12, 1996, pp. 257-265.
  37. Szwegold, B.S., "NMR Spectroscopy of Cells," *Annual Review of Physiology*, Vol. 54, 1992.
  38. Shankland, E.G., Livesey, J.C., Wiseman, R.W., and Krohn, K.A., "Multinuclear NMR Studies of an Actively Dividing Artificial Tumor," *Physiological Research*, Vol. 51, 2002, pp. 49-58.
  39. Gillies, R.J., Galons, J.P., McGovern, K.A., Scherer, P.G., Lien, Y.H., Job, C., Ratcliff, R., Chapa, F., Cerdan, S., and Dale, B.E., "Design and Application of NMR-compatible Bioreactor Circuits for Extended Perfusion of High-density Mammalian Cell Cultures," *NMR in Biomedicine*, Vol. 6, 1993, pp. 95-104.
  40. Chen, J., In vivo  $^{31}\text{P}$  NMR Study of Phosphate Metabolism for *Eschscholtzia Californica* Using a Perfused Small-scale Bioreactor, Montréal: École Polytechnique de Montréal, 2004.
  41. Restelli, V., Wang, M.D., Huzel, N., Ethier, M., Perreault, H., and Butler, M., "The Effect of Dissolved Oxygen on the Production and the Glycosylation Profile of Recombinant Human Erythropoietin Produced from CHO Cells," *Biotechnology and Bioengineering*, Vol. 94, No. 3, 2006, pp. 481-494.
  42. Yates, J.G., *Fundamentals of Fluidized-bed Chemical Processes - Butterworths monographs in chemical engineering*, 1st ed., London: Butterworths, 1983.
  43. Decloux, M., Baxerres, J.L., and Gibert, H., "Etude Hydrodynamique d'une couche a circulation

- multiétagée, *Entropie*, Vol. 124, 1985, pp. 61-69.
44. Lamboursain, L., St-Onge, F., and Jolicoeur, M., "A Lab-built Respirometer for Plant and Animal Cell Culture," *Biotechnology Progress*, Vol. 18, 2002, pp. 1377-1386.
45. Aboka, F.O., Yang, H., de Jonge, L.P., Kerste, R. van Winden, W.A., van Gulik, W.M., Hoogendijk, R., Oudshoorn, A., and Heijnen, J.J., "Characterization of an Experimental Miniature Bioreactor for Cellular Perturbation Studies," *Biotechnology and Bioengineering*, Vol. 95, No. 6, 2006, pp. 1032-1042.

## About the Authors



**Marisha Ben-Tchavtchavadze** graduated from the University of Ottawa with a double major in biochemistry and chemical engineering in 2002. She then started her career in research at the Biotechnology Research Institute in Montreal. In 2004, she began her Chemical Engineering Master's degree at École Polytechnique de Montréal in Mario

Jolicoeur's research group. She is one of the founding members of the ISPE École Polytechnique Student Chapter and winner of the Central Canada Chapter local poster competition. She is now looking forward to starting her engineering career in September of 2007. She can be reached by e-mail at: [marisha.ben-t@polymtl.ca](mailto:marisha.ben-t@polymtl.ca).



**Michel Perrier** obtained his B.Eng. and M.Sc.A degrees from École Polytechnique and his PhD from McGill University, all in chemical engineering. He began his career in 1986 with Shell Canada as a process control engineer where he worked on design and implementation of control systems. From 1990 to 1993, he worked for Pulp and Paper

Research Institute of Canada as a research engineer on monitoring of control loop performance and on the development of adaptive control techniques. Since 1993, he has been a professor of chemical engineering at École Polytechnique and adjunct professor at McGill University since 1998. He has been a visiting professor at the Centre for Integrated

Dynamics and Control in the Department of Electrical Engineering at the University of Newcastle in Australia and at the Centre for Systems Engineering and Applied Mechanics at the Université Catholique de Louvain in Belgium. His research interests are in the field of dynamics, control, and optimization of biotechnological processes. He is currently Vice-Chair of the International Federation for Automatic Control (IFAC) Technical Committee on Biosystems and Bioprocesses. He can be reached by e-mail at: [michel.perrier@polymtl.ca](mailto:michel.perrier@polymtl.ca).



**Mario Jolicoeur** obtained his B.Eng. and M.Sc.A degrees from the École Polytechnique de Montréal all in chemical engineering. His PhD was obtained from École Polytechnique and Université Paul Sabatier in Toulouse in plant biotechnology. He began his career in 1990, between his Masters' and his PhD studies, with BioExpert Inc. as a biochemical

engineer where he worked on design of bioreactors. From 1990 to 1994, he worked for the Biotechnology Research Institute of the Canada Research Council as cell culture engineer. In 1994, he began his PhD studies. He has been a professor of chemical engineering at École Polytechnique since 1998. He was a visiting professor at the Biotechnology Process Engineering Center in the department of Chemical Engineering at the Massachusetts Institute of Technology (MIT) in 1999 and at the Laboratory of Chemical and Biological Engineering in the department of Chemical Engineering at the Ecole Polytechnique Fédérale de Lausanne (EPFL) in 2004-2005. His research interests are in the field of optimization of biotechnological processes for plant and animal cells using metabolic engineering. He currently holds a Canadian Research Chair on the Development of Metabolic Engineering Tools. He can be reached by e-mail at: [mario.jolicoeur@polymtl.ca](mailto:mario.jolicoeur@polymtl.ca).

École Polytechnique de Montréal, Chemical Engineering, 2900, boul. Édouard-Montpetit, Pavillon JAB, Mario Jolicoeur, Bio-P<sup>2</sup> Research Unit, Montreal, Quebec H3T 1J4, Canada. 