

# Automated microbioreactor systems for pharmaceutical bioprocessing: profiling of seeding and induction conditions in high-throughput fermentations

**Introduction:** Automated microbioreactor systems are designed for intensive bioprocess characterization. They facilitate reduction of development timelines without loss of valuable information. The RoboLector automated microbioreactor system was used for joint investigation of induction profiling and inoculation from seed cultures of different ages, which is only rarely recognized in literature for optimization. **Results:** The microbioreactor system allows reliable detection of growth phases and accurate inoculation procedures in combination with a true walk-away performance. Inocula taken from seed cultures resting in stationary growth phase for up to 10 h had no influence on induction profiling experiments, where late induction is preferred for maximum space-time-yield of recombinant enzyme production. **Conclusion:** The presented method allows for conduction of precise inoculation procedures and thus, for detailed studies on influential bioprocess parameters. The findings indicate that standardization in methods is more promising than standardization of cultivation conditions to scout for new productive bioprocesses.

## Background

In the biopharmaceutical industry, biotechnological processes for therapeutic agents, such as antibodies or hormones, are pressured to be ready as soon as possible. Reasons for this are faster production of material for clinical trials and subsequent earlier market entry and thus, prolonged patent protection for innovative protein drugs. In a market estimated US\$44 billion back in 2004 [1], this is a great stimulation. However, due to necessarily strong regulations with the production of therapeutic agents for human use (e.g., testing and approval procedures for development and supply of vaccines [2]), a fundamental knowledge of cell factories, bioprocessing behavior and their interaction is required, which is targeted by PAT [3] and QbD [4] initiatives.

There are several factors to optimize in biotechnological production of therapeutic agents, including interactions of factors. Steps along the line of production which were improved individually may have an overall negative effect in combination. For example, high cell density cultivations (HCDC) can

cause problems in following purification steps due to overload of cells and/or product concentration, when membrane separations or preparative chromatography are designed to work with low viscosity fluids. Additionally, a host strain optimized for HCDC may be suboptimal in a medium cell density cultivation (MCDC), which is preferred in purification stages in this context.

## The role of microbioreactor systems in bioprocess development: an overview

The nature of **microbioreactor systems** (MBR) is to enable experimentation in a high-throughput manner. That makes them the tool of choice when it comes to fast generation of intense knowledge in **bioprocess development**. Such high-throughput is clearly needed by naming some optimization targets in biopharmaceutical process development: gene dosage [5] and promotor strength [6] of the expression cassette, engineering of secretion signals [7], host variations [8], medium composition [9], feeding solutions [10] and control strategies during cultivation [11].

Johannes Hemmerich<sup>1</sup>  
& Frank Kensy\*<sup>1</sup>

<sup>1</sup>m2p-labs GmbH, Arnold-Sommerfeld-Ring 2, 52499 Baesweiler, Germany

\*Author for correspondence:

kensy@m2p-labs.com

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## Key Terms

**Microbioreactor systems:** Laboratory machines for well-controlled and parallel conduction of cultivation experiments in microliter-scale, can be highly integrated with other hardware devices and software solutions.

**Bioprocess development:** Definition of a biotechnological production process. The upstream processing part includes engineering of host strain and evaluation of cultivation conditions like composition of medium or feeding trajectories. The downstream processing part includes recovery, purification and formulation/finishing the target product.

From bench over pilot to production scale the classical stirred tank reactor (STR) will remain for long time the gold-standard of bioprocess optimization, as for this kind of reactor extended knowledge and experience exists. Furthermore, STRs are integrated main parts of existing qualified production lines in most of the cell-based biotechnological processes. From this point of view, MBR have to mimic STR performance to be considered relevant for bioprocess development.

Perhaps the most direct scale-down approach is the miniaturization of the stirred tank itself. Puskeiler *et al.* introduced such a system with up to 48 parallel units with an operating volume of several milliliters [12]. The single units can be equipped with baffled or non-baffled reaction tanks and/or different types of stirrers. The integration of a liquid-handling robot and a microplate reader allows intermittent at-line monitoring of biomass (via optical density) and other assay-accessible substances, whereas pH and dissolved oxygen (DO) are measured online with optical sensor spots (optodes) [13]. As stirrer speed of each unit is controlled independently, aeration and mixing can be adjusted individually. In addition, the liquid-handling robot facilitates manipulation of the individual cultures based on collected data, for example, with titration agents.

Another two bioreactor concepts, the shaken vessel and the bubble column, are used in combination for a MBR with up to 24 parallel units [14,15]. Here, aeration and agitation is controlled by different shaking speeds and additional submerged gassing. Again, online monitoring of pH and DO is realized with optodes. Based on these online signals, pH of the culture units can be adjusted by controlling the amount of NH<sub>3</sub> or CO<sub>2</sub> in the inlet gas stream when operating in 'bubbling mode'. Again, working volumes of several milliliters are employed. Additionally, each mini-column is equipped with a heat conductor which enables temperature control, at least within a distinct range for two neighboring columns. In contrast to the aforementioned system, there is no description found for robotic integration yet. Therefore, for bolus feeding and sam-

pling, the cultivation cassette has to be removed from the incubator and placed in laminar flow hood to prevent contaminations.

In this study, we employ the BioLector technology [16], which uses microplates as an array of parallel operated cultivation units. The transparent bottom of the microplate (specifically: the 48-well FlowerPlate microplate [17]) gives optical access for online detection of biomass concentration (via scattered light) and fluorescent molecules, as well as pH and DO (both using optodes). It should be noted that the measuring principle for biomass estimation would interfere with other light-scattering components in the fermentation broth like bubbles or agitated stirrers. Additionally, the MBR was integrated into a liquid-handling robot, which enables manipulation of the individual cultivation units based on gathered data and at-line determination of assay-accessible substances, comparable to the milliliter stirred tank microbioreactor system described above. Next to the different agitation and aeration used compared with the aforementioned two MBR, the here used system operates with lower working volumes (typically 800–1500 µl compared with approximately 5–10 ml). The combination of a BioLector MBR system with a liquid handling robot was first introduced by Huber *et al.* [18], who named this concept 'RoboLector'. The capabilities of a RoboLector beyond the basic BioLector technology are determined by the capabilities and degree of integration of further technical equipment of the liquid handling unit, which is not predefined to a specific manufacturer.

For all of the three described MBR, several application examples are found in literature [12–15,18–20]. Also, scalability of the systems to conventional STR was demonstrated [12,20,21], which is crucial for a meaningful application and acceptance of MBR in bioprocess development.

#### Treatment of seed cultures as optimization parameter in bioprocess development

Seed cultures, which serve for generation of initial cell mass in a production process, are rarely recognized as a parameter to be considered for optimization, although varying results are found in literature concerning the treatment of seed cultures. For example, Rohe *et al.* observed different productivities in cultivations, which were inoculated from different seed cultures [6]. By contrast, fermentation runs started from the same source showed equal productivities. Therefore, it was concluded biological variations in seed cultures affected recombinant protein production in main cultures, although the same clone was applied. However, this issue was not further investigated. In another study, Buso *et al.* found there was no need to conduct

pre-culturing steps [22]. Direct inoculation of main cultures from a transformation mix did not affect amount or quality of target protein. By contrast, for *Streptomyces calvuligerus*, status of seed cultures was demonstrated to affect subsequent main cultures, as shown by Neves *et al.* [23]. By using inocula from a late-exponential growth phase they were able to increase batch-to-batch productivity. However, monitoring physiological status of seed culture is mandatory in that case.

To face the problem of differentially growing seed cultures, a method of growth synchronization was proposed by Huber *et al.* [19] and Sirkus *et al.* [24]. Seed cultures were conducted using glucose slow-release systems, which forces the cultures to linear growth sooner or later. When all cultures entered this phase, time point of synchronized growth is reached and main culture are inoculated.

In another work by Huber *et al.* the method of “biomass-specific” replication was introduced [18]. Here, the inoculum volume depends on the biomass concentration of the seed culture. By doing so, the same initial biomass concentration is archived in subsequent main cultures. Advantageous to this method is uniform growth in main culture, as shown by the authors. However, when viability of the cells is affected by growth phase (e.g., in exponential or stationary growth phase), the biomass-specific replication fails, as it does not consider these growth phases for inoculation.

In conclusion, it is recognized that treatment of seed cultures may affect productivity of a bioprocess. One approach targets the seed cultures directly, for example, by the method of growth synchronization. The other way is to even out differentially growing seed cultures by modifying the treatment of main cultures, for example, by the methods of biomass specific replication or biomass specific replication.

### Motivation & outline of this study

Especially, the combinatorial examination of seed culturing and typical high-throughput experimentation is missing in literature. Although there are several studies, including harmonization of seed cultures, surprisingly there is not report found whether this is necessary or not. For complex investigations with several factors to be examined, leaving out one factor only reduces workload and increases throughput.

The study was conducted with the RoboLector MBR system [18,20] to monitor differentially growing seed cultures and evaluation of their impact on subsequent typical high-throughput cultivation experiments. First, reliable detection of stationary growth phase of seed cultures was verified including assessment of the ability of the system to perform inoculation procedures in a reproducible and reliable (i.e., without supervision

by an experimenter) way. Secondly, the **induction profiling** for optimization of productivity in an *Escherichia coli*-based bioprocess was expanded by the parameter of seed culture’s growth status to evaluate if that parameter affects results. Inoculum for the main cultures was taken from seed cultures after up to 10 h after those entered stationary phase. This mimics often performed overnight cultivation of seed cultures, which lack in monitoring of growth. A graphical experimental outline is given in **Figure 1**. The new method gives access to defined inoculation conditions and thus, can be used for setup and evaluation of routine cell culturing protocols.

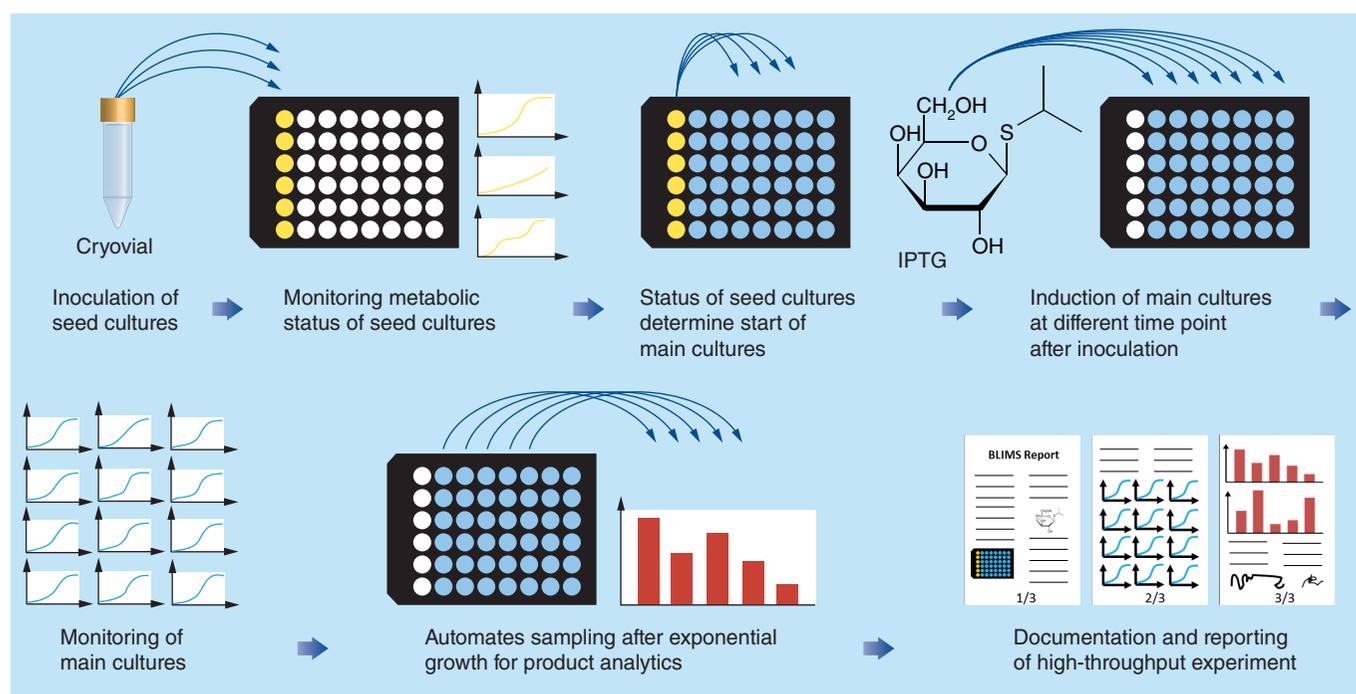
### Material & methods

The used RoboLector system was a combination of the BioLector MBR system (G-BL-100, m2p-labs, Baesweiler, Germany) [16] and a Multiprobe II Ex liquid handling robot (PerkinElmer, MA, USA). For cultivations, 48-well FlowerPlates with optodes for pH and DO measurement were used (MTP-48-BOH, m2p-labs), sealed with a sterile, gas permeable membrane with a preslitted silicone layer acting as a septum for robotic pipetting actions (F-GPRS48-10, m2p-labs). The BioLector device monitored online scattered light (proportional to biomass concentration), pH and DO. Measurements were taken every 8 min for all 48 wells. Cultivation conditions were: shaking frequency 1100 rpm, shaking diameter 3 mm, filling volume 1000  $\mu$ l, temperature 37°C and relative humidity over 85%.

Microorganism was *E. coli*, expressing a recombinant enzyme intracellularly upon induction with Isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG). Chosen concentration was 1 mM final. Sampling was programmed to execute just after exponential growth of main cultures, and samples were transferred into 96-well microplates placed on a custom-made cooling station. Cooling was controlled at 0°C. Cell lysis for analysis of recombinant enzyme activity was performed enzymatically by addition of 1 volume of lysis buffer (125 mM NaH<sub>2</sub>PO<sub>4</sub>/K<sub>2</sub>HPO<sub>4</sub> pH 8.0; 750 mM NaCl; 0.25% v/v TritonX-100; 12.5 mM EDTA; 1.5 g/l lysozyme; 20 min at 37°C with shaking), followed by benzonase treatment to reduce viscosity caused by released nucleic acids (0.5 volumes of 50 mM MgCl<sub>2</sub> \* H<sub>2</sub>O; 12.5 U/ml benzonase; 20 min at 37°C with shaking). Soluble cell fraction was obtained by centrifugation (10 min; 3000 rpm; 4°C).

#### Key Term

**Induction profiling:** Systematic approach for identification of factors for optimal recombinant gene expression, mainly to maximize productivity of target protein.



**Figure 1. Experimental outline for induction profiling cultivation experiments.** Inoculation of main cultures is made from differentially growing seed cultures.

Medium composition is described elsewhere [21], with additional buffering capacity of 100 mM 3-(N-morpholino)propanesulfonic acid (pH 7.4) and appropriate antibiotics to maintain selection pressure. In main cultures 20 g/l glucose served as carbon source, for seed cultures glycerol amount was adjusted to 15 g/l due to different used volumes of cryostock, in which glycerol is used as cryoprotectant.

Activity measurement of recombinant enzyme was performed with a p-Nitrophenyl-substrate. Liberated p-Nitrophenol was quantified by kinetic absorption measurement at a wavelength of 420 nm in 96-well standard microplates using a multiplate reader (Spectraflour Plus, Tecan, Crailsheim, Germany). Assay conditions were as follows: 37°C, 10 µl of diluted cell lysate, 200 µl of assay buffer (pH 8.0) containing p-Nitrophenyl-substrate. Measurements were compared with a dilution series of target enzyme from commercial source with known activity.

## Results & discussion

### Normalization of cultivations by monitoring metabolic status of seed cultures

For the first set of high-throughput cultivation experiments, three seed cultures were employed with different initial cell densities. Other fermentation parameters (especially medium and amount of carbon source) were kept equal. As a result, seed cultures entered stationary growth phase at different time points, but with the same final biomass concentration, according to the

well-known model of microbial growth with a limiting substrate [25].

With end of growth, transfer of seed broth into seven replicates of main cultures was programmed. To detect end of growth in the seed cultures, monitoring of DO is used, more precisely the measurement of a drop below 50% air saturation (a.s.), followed by a rise above 80% a.s. was defined as clear indication of entering stationary growth phase. This method is superior over monitoring biomass concentration, as it allows for detection of respiration activity of the culture, which is a direct indicator for metabolic activity. Following main cultures are started in equal fermentation conditions, including same amount of inoculum with same metabolic activity of cell broth. Resulting main cultures are only distinguished by time of beginning. Growth kinetics of all seven replicates of the three sets of main cultures do not show deviations, as Figure 2 depicts. To be precise, all cultures started immediately to grow after inoculation, reflected by falling DO signals and increasing scattered light signal. Growth lasted quite exactly for 6 h in all cultivations, with same final biomass concentration. Liquid transfer from seed to main cultures was programmed to be performed without interruption of shaking to avoid artifacts caused by settling cells. The coefficient of variation (CV) of final biomass concentration among all 21 cultivations (seven replicates in three cultivation sets) is 2.9%, while CV-values for the individual sets are 2.4, 3.2 and 2.0% (Figure 2). With superimposed fermenta-

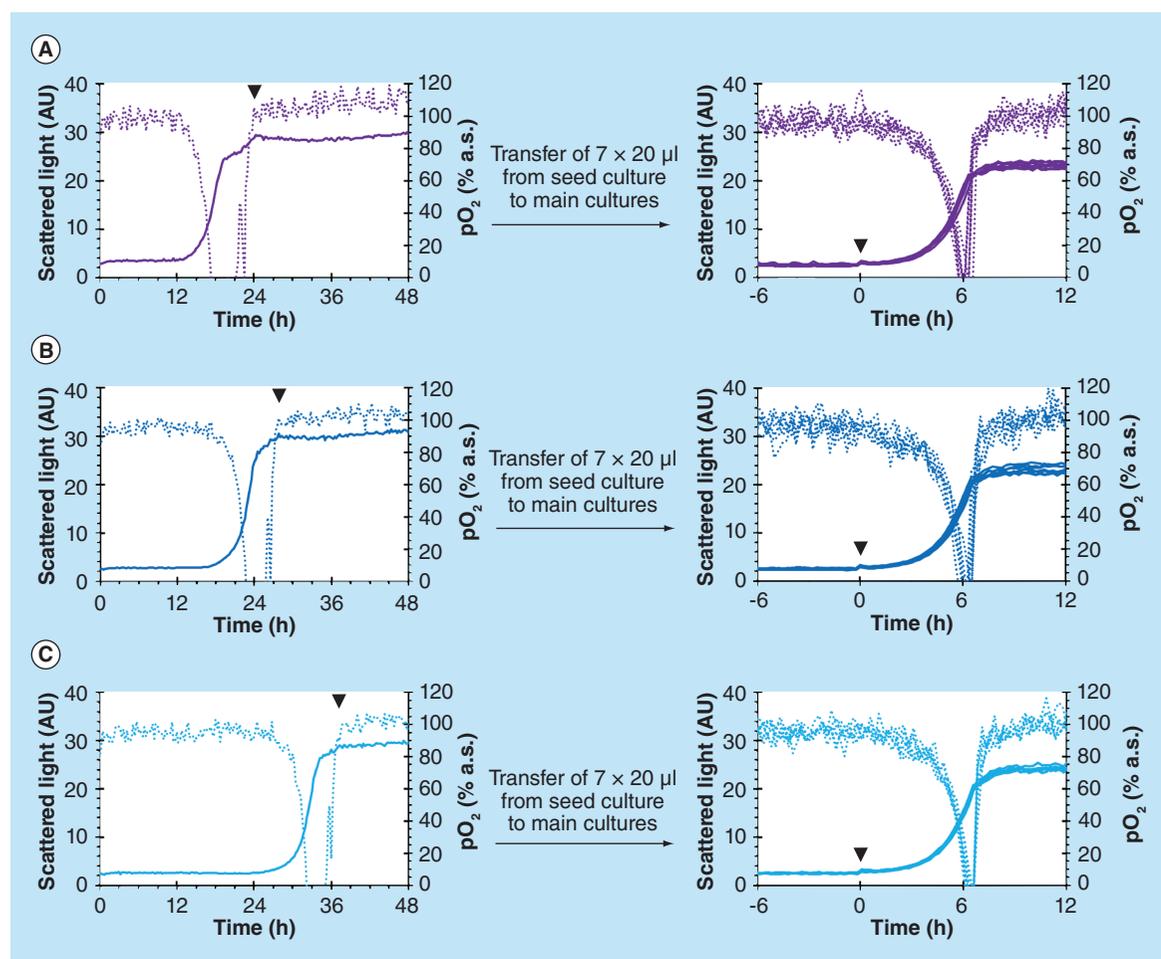
tion kinetics among each seven replicates and the three cultivation sets, pipetting accuracy of small volumes is shown to be excellent. This is especially important in microscale fermentation, where small volume handling is daily routine. End of exponential growth phase could be detected safely and deviations from biological replicates can be neglected (Figure 2), which is required in high-throughput investigations.

Workload is done by the RoboLector MBR system automatically, so transfer of cells from seed to main cultures is executed even during the night or weekends. That means, workflow progression in sequential cultivations is only determined by the dynamics of the individual cultures. With programmable sterilization procedures for the robotic pipetting tips (e.g., tip incubation with ethanolic or alkaline solutions [20]), it is possible to grow several clones or cell types at the same time. A possible application would be benchmark-

ing expression hosts for a given protein of interest, in particular with automated sampling and subsequent analytics at-line to the high-throughput cultivation.

#### Growth status of seed culture & its influence on induction profiling experiments

With determined excellent standard deviation of biological replicates, confirmed safety in detection of growth phases and according automated handling actions, the full potential of the RoboLector MBR system was applied: influence of seed culture on induction profiling (a typical high-throughput cultivation experiment [18,20]) was investigated. Four sets of main cultures were prepared, while each set of main cultures ought to be inoculated from individual seed cultures at different stages in stationary growth phase, which is transfer of cells that are resting in stationary growth phase for up to 10 h. This simulates often performed



**Figure 2. Growth kinetics regarding biomass concentration (solid lines) and dissolved oxygen (dotted lines) of three seed cultures (left graphs) and their subsequent main cultures (right graphs, seven replicates each).**

Seed cultures were started with different volumes from cryovial (A: 50  $\mu$ l; B: 20  $\mu$ l; C: 5  $\mu$ l) and thus, reached end of growth at different time points (indicated with black triangles, A: 24.0 h; B: 27.8 h; C: 37.2 h). Time point of inoculation of the three sets of main cultures was set to zero for direct visual growth comparison and is also indicated with black triangles.

overnight growth of seed cultures, where online monitoring of fermentation parameters is missed and the possible influence on main cultures is not in scope. As a result, the inoculation of main cultures is performed with an unknown viability. In addition, it is not known if and when seed culture entered stationary phase during overnight incubation.

For the RoboLector method, inoculation of main cultures was programmed to execute either just with entering stationary phase of seed culture or several hours after growth saturation, which is 3, 6 or 10 h. The following main cultures are used for induction profiling. IPTG addition was programmed to execute at 0.5, 1, 2, 3, 4 or 6 h after inoculation for the four sets of main cultures. Although no biomass degradation in seed cultures could be detected by online monitoring of biomass concentration (Figure 3), there is no indication for 'fitness' or viability of cells. When using overnight grown seed cultures, a defined amount of cells is often used for inoculation. This amount of cells is determined by measuring optical density because it is a quick method, but gives no access to viability of the culture. This holds also true for scattered light measurement of the MBR system. On the opposite, the method of colony forming units (CFU) detection on agar plates needs days of incubation.

During exponential growth activity of cultures is considered to be maximal. Here, this assumption is supported when growth patterns for seed and main cultures plotted in Figure 2 are compared. The seed culture started with 50 µl from a cryovial exhibited a delay of 12 h before cell growth is detectable. By contrast, subsequent main cultures begun with 20 µl of seed broth start growing after inoculation immediately, which is due to maximum activity of the inoculum. Therefore, growth phase of seed cultures could affect main cultures, which brings another parameter to consider in a high-throughput cultivation experiment. Moreover, indecisive results could arise. Hence, it is necessary to evaluate impact of seed culture's status beside other parameters.

The four sets of induction profiling cultivations were sampled just after end of growth individually, which again is detected as described above with a fall and rise in DO signal. Time point of induction was of great influence on amount of recombinant enzyme. While enzymatic activity did not exceed 300 U/ml for inductions during the first 4 h, it was in the range of 900–1200 U/ml when IPTG was added after 6 h, which means a three- to four-fold increase. Also, cultures were growing slowly after IPTG addition, which is reflected in prolonged cultivation times needed to reach maximal biomass concentration. When induced 0.5 h after inoculation, process time doubles from

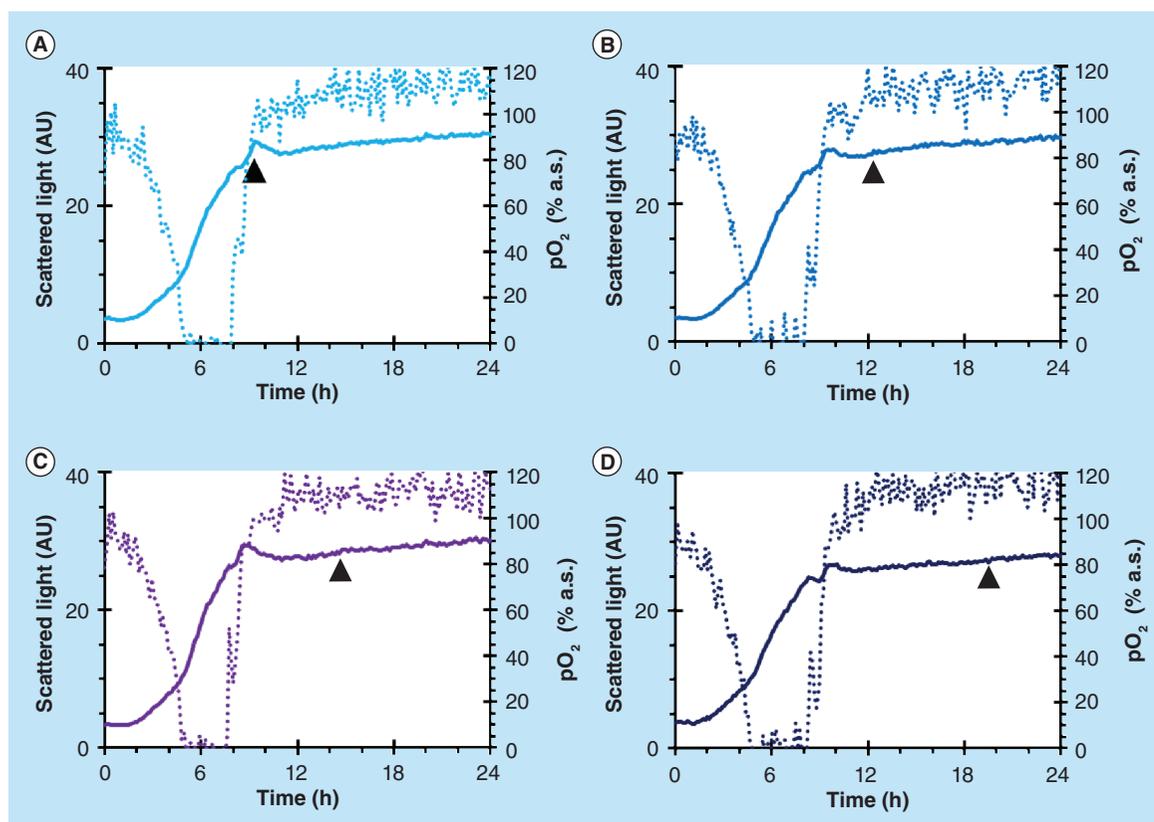
approximately 12 to 24 h compared with latest induction at 6 h. Remarkably, final biomass concentration did not vary significantly (data not shown). Both early induction and prolonged process time affect space-time-yield negatively. Induction at 6 h led to a productivity of approximately 80 U/ml/h, while other conditions did not exceed approximately 20 U/ml/h, which is fourfold less. By contrast, status of seed cultures did not change these proportions, as summarized results show in Figure 4.

The conclusion is, that seed culture as investigation factor can be omitted, which allocates a degree of freedom. The only point to consider is that seed culture has to reach growth saturation, which is easily archived routinely with simple overnight cultures. Time point for induction of main cultures is much more important to tweak productivity of recombinant enzyme synthesis. The presented data indicated in this specific case late induction is preferred. By contrast, in other studies early induction was beneficial with another target protein [18], although *E. coli* was used as host as well. Furthermore, for the production of another enzyme in *C. glutamicum* optimal time point of induction was determined between early and mid-exponential growth phase [20]. It should be noted that in the two mentioned studies, estimation of productivity was not performed from samples drawn directly after the exponential growth, but from samples after the completion of the whole experiment. This may have a non-negligible effect on evaluation of the experimental data and would be worth for consideration. In conclusion, there seems no general applicable workflow for bioprocess optimization, but a standardization of a method for seeking an optimized bioprocess should be the target. In addition, it is likely that not all factors of influence and their interaction can be considered, even with high-throughput platform technologies.

## Conclusion & outlook

In pharmaceutical bioprocessing, cell-based methods account for the vast majority of produced agents. Development in the upstream part of production is mainly dominated by modification, introduction or deletion of genetic elements of production strains and the search for optimal cultivation process parameters with these strains. In this field, microbioreactor systems (MBR) are emerging, as they provide high-throughput experimentation and demonstrated scalability to stirred tank reactors (STR), which are still predominant from bench to production scale.

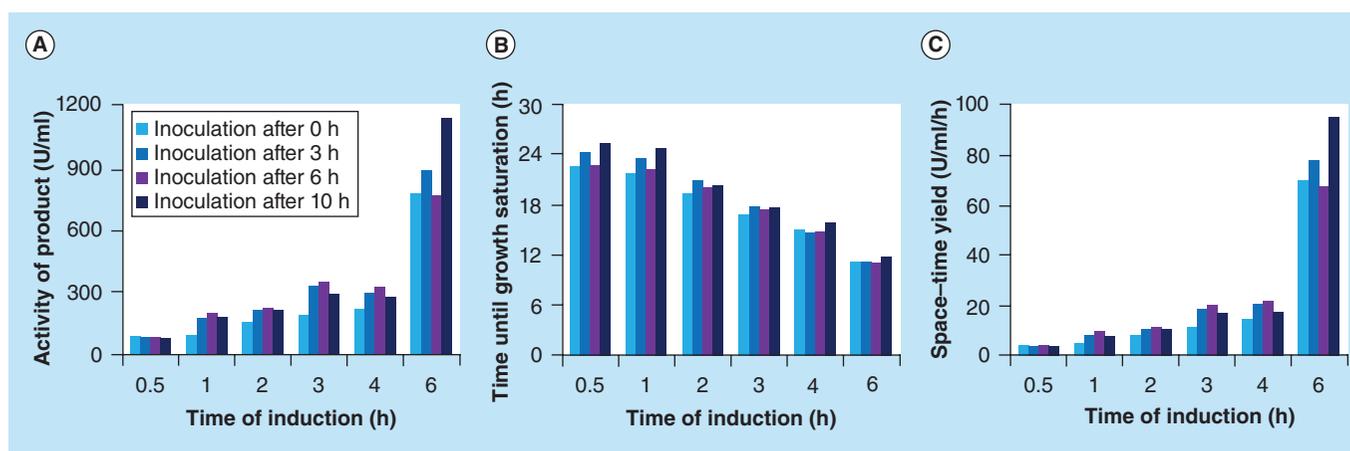
The use of an automated MBR system in upstream bioprocess development is presented. With detection of growth phase and automated liquid handling of small volumes determined to be accurate and reproduc-



**Figure 3. Online fermentation kinetics of seed cultures.** Solid lines: biomass concentration; dotted lines: DO signal; black triangles: time of transfer from seed cultures into corresponding main cultures after 0 h (A), 3 h (B), 6 h (C) or 10 h (D) after seed cultures entered stationary phase.

ible, the status of seed cultures for induction profiling experiments was assessed. Productivity was affected by time point of induction, where late in induction resulted in highest recombinant activity and lowest process time. There was no significant influence of the seed cultures status on output of induction profiling, regardless if the inoculum just reached saturation of growth or 10 h before.

The presented method demonstrated how online data acquisition in automated MBR systems can be extremely beneficial for accelerating bioprocess development, because they allow for fast identification of parameters, whether these are worth optimizing or not. The highly varying results found in literature arising from the question ‘How to treat seed cultures in bioprocessing?’ indicate one conclusion: standardization



**Figure 4. Evaluation of productivity for induction profiling experiments with inoculation from different stages in seed cultures.** (A) Activity of recombinant product; (B) time needed for saturation of growth; and (C) calculated space-time-yield.

in optimization methods would be a more promising target rather than standardization of cultivation techniques, especially with a growing number of influential parameters.

### Future perspective

In general, MBR systems are suitable for applications in several stages during bioprocess development. First, parameters of influence are identified to decide whether these need attention or not in further development. Then parameters which turned out to affect productivity are examined in optimization runs (including their interactions). Afterwards, along transferring the biological process into pilot and production scale, MBR can serve as scale-down model to re-evaluate the process behavior, allowing continuous supervision of process consistency. Current ongoing research,

development, evaluation and application of MBR systems pave the way for these to replace laborious and bulky bioreactors in bioprocess development. With a growing number of laboratory machines designed for high-throughput applications, seamless integration and automated data exchange with MBR becomes an important role.

### Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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## Executive summary

### Introduction & background

- Experimentation in high quality and quantity is needed for intense understanding in bioprocess development, here microbioreactor systems are the emerging tools-of-choice.
- The evaluation of seed cultures as a parameter for investigation in bioprocess optimization is only rarely recognized, although highly varying results are found in literature.
- An automated microbioreactor system (RoboLector) is validated and used for combinatorial induction and seed culture profiling for maximizing expression of a recombinant enzyme in *Escherichia coli*.

### Results & discussion

- The applied microbioreactor system provides excellent and reliable performance in detection of growth phase and execution of inoculation procedures, demonstrated by superimposed fermentation kinetics and a CV of 2.9% (n = 21) for final biomass concentration.
- Late induction was both beneficial for high volumetric enzymatic activity and low process time.
- Inoculum had no influence, regardless whether taken from seed cultures which just entered stationary growth phase or up to 10 h before.
- The optimization parameter 'seed culture' was evaluated thoroughly ('effect check') and confirmed not to need further attention.

### Conclusion & outlook

- Results of the study and corresponding literature indicate that standardization of optimization methods for bioprocess development is a more promising approach compared with standardization of cultivation methods.

## References

- Papers of special note have been highlighted as: • of interest
- 1 Pavlou AK, Reichert JM. Recombinant protein therapeutics – success rates, market trends and values to 2010. *Nat. Biotechnol.* 22, 1513–1519 (2004).
  - 2 Marshall V, Baylor NW. Food and drug administration regulation and evaluation of vaccines. *Pediatrics* 127, S23–S30 (2011).
  - 3 US FDA. Guidance for Industry PAT – a framework for innovative pharmaceutical development, manufacturing, and quality assurance. [www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070305.pdf](http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070305.pdf)
  - 4 Yu LX. Pharmaceutical quality by design: product and process development, understanding, and control. *Pharmaceut. Res.* 25, 781–791 (2008).
  - 5 Kim HY, Sohn JH, Kim CH *et al.* Rapid selection of multiple gene integrant for the production of recombinant hirudin in *Hansenula polymorpha*. *Biotechnol. Bioprocess. Eng.* 5, 1–6 (2000).
  - 6 Hartner FS, Ruth C, Langenegger D *et al.* Promoter library designed for fine-tuned gene expression in *Pichia pastoris*. *Nucleic Acids Res.* 36, e76 (2008).
  - 7 Degering C, Eggert T, Puls M *et al.* Optimization of protease secretion in *Bacillus subtilis* and *Bacillus licheniformis* by screening of homologous and heterologous signal peptides. *Appl. Environ. Microbiol.* 76, 6370–6376 (2010).
  - 8 Lee SY, Lee KM, Chan HN, Steinbüchel A. Comparison of recombinant *Escherichia coli* strains for synthesis and accumulation of poly-(3-hydroxybutyric acid) and morphological changes. *Biotechnol. Bioeng.* 44, 1337–1347 (1994).

- 9 Kennedy MJ, Prapulla SG, Thakur MS. Designing fermentation media. A comparison of neural networks to factorial design. *Biotechnol. Tech.* 6, 293–298 (1992).
- 10 Ramchuran SO, Holst O, Karlsson EN. Effect of postinduction nutrient feed composition and use of lactose as inducer during production of thermostable xylanase in *Escherichia coli* glucose-limited fed-batch cultivations. *J. Biosci. Bioeng.* 99, 477–484 (2005).
- 11 Chen R, John J, Rode B *et al.* Comparison of polysialic acid production in *Escherichia coli* K1 during batch cultivation and fed-batch cultivation applying two different control strategies. *J. Biotechnol.* 154, 222–229 (2011).
- 12 Puskeiler R, Kaufmann K, Weuster-Botz D. Development, parallelization, and automation of a gas-inducing milliliter-scale bioreactor for high-throughput bioprocess design (HTBD). *Biotechnol. Bioeng.* 89, 512–523 (2005).
- 13 Hortsch R, Weuster-Botz D. Growth and recombinant protein expression with *Escherichia coli* in different batch cultivation media. *Appl. Microbiol. Biotechnol.* 90, 69–76 (2011).
- 14 Chen A, Chitta R, Chang D, Amanullah A. Twenty-four well plate miniature bioreactor system as a scale-down model for cell culture process development. *Biotechnol. Bioeng.* 102, 148–160 (2009).
- 15 Isett K, George H, Herber W, Amanullah A. Twenty-four-well plate miniature bioreactor high-throughput system. Assessment for microbial cultivations. *Biotechnol. Bioeng.* 98, 1017–1028 (2007).
- 16 Kensy F, Zang E, Faulhammer C, Tan R, Büchs J. Validation of a high-throughput fermentation system based on online monitoring of biomass and fluorescence in continuously shaken microtiter plates. *Microb. Cell. Fact.* 8, 31 (2009).
- 17 Funke M, Diederichs S, Kensy F, Müller C, Büchs J. The baffled microtiter plate. Increased oxygen transfer and improved online monitoring in small scale fermentations. *Biotechnol. Bioeng.* 103, 1118–1128 (2009).
- 18 Huber R, Ritter D, Hering T *et al.* A novel platform for automated high-throughput cultivations in microtiter plates with high information content. *Microb. Cell. Fact.* 8, 42 (2009).
- **First presentation of the automated microbioreactor concept used in this study.**
- 19 Huber R, Scheidle M, Dittrich B, Klee D, Büchs J. Equalizing growth in high-throughput small scale cultivations via precultures operated in fed-batch mode. *Biotechnol. Bioeng.* 103, 1095–1102 (2009).
- 20 Rohe P, Venkanna D, Kleine B, Freudl R, Oldiges M. An automated workflow for enhancing microbial bioprocess optimization on a novel microbioreactor platform. *Microb. Cell. Fact.* 11, 144 (2012).
- **Presents a more advanced version of the RoboLector concept.**
- 21 Kensy F, Engelbrecht C, Büchs J. Scale-up from microtiter plate to laboratory fermenter. Evaluation by online monitoring techniques of growth and protein expression in *Escherichia coli* and *Hansenula polymorpha* fermentations. *Microb. Cell. Fact.* 8, 68 (2009).
- 22 Busso D, Stierlé M, Thierry J, Moras D. A comparison of inoculation methods to simplify recombinant protein expression screening in *Escherichia coli*. *Biotechnology* 44, 101–106 (2008).
- 23 Neves AA, Vieira LM, Menezes JC. Effects of preculture variability on clavulanic acid fermentation. *Biotechnol. Bioeng.* 72, 628–633 (2001).
- 24 Šiurkus J, Panula-Perälä J, Horn U, Kraft M, Rimšeliene R, Neubauer P. Novel approach of high cell density recombinant bioprocess development. Optimisation and scale-up from microlitre to pilot scales while maintaining the fed-batch cultivation mode of *E. coli* cultures. *Microb. Cell. Fact.* 9, 35 (2010).
- 25 Monod J. The growth of bacterial cultures. *Annu. Rev. Microbiol.* 3, 371–394 (1949).

