Implementation of a Fully Automated Microbial Cultivation Platform for Strain and Process Screening

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Advances in molecular biotechnology have resulted in the generation of numerous potential production strains. Because every strain can be screened under various process conditions, the number of potential cultivations is multiplied. Exploiting this potential without increasing the associated timelines requires a cultivation platform that offers increased throughput and flexibility to perform various bioprocess screening protocols. Currently, there is no commercially available fully automated cultivation platform that can operate multiple microbial fed-batch processes, including at-line sampling, deep freezer off-line sample storage, and complete data handling. To enable scalable high-throughput early-stage microbial bioprocess development, a commercially available microbio reactor system and a laboratory robot are combined to develop a fully automated cultivation platform. By making numerous modifications, as well as supplementation with custom-built hardware and software, fully automated milliliter-scale microbial fed-batch cultivation, sample handling, and data storage are realized. The initial results of cultivations with two different expression systems and three different process conditions are compared using 5 L scale benchmark cultivations, which provide identical rankings of expression systems and process conditions. Thus, fully automated high-throughput cultivation, including automated centralized data storage to significantly accelerate the identification of the optimal expression systems and process conditions, offers the potential for automated early-stage bioprocess development.

1. Introduction

Advances in genetic engineering and biotechnology promote the continued development of genetic elements to improve microbial expression systems. These advancements create rising numbers of possible host candidates, while, at the same time, automation and high-throughput approaches accelerate strain generation rates. The ability to combine different genetic elements further multiplies the number of assayable strains. The development of a single medical compound or biocatalyst can necessitate the screening of hundreds of candidates under scalable process conditions. In addition, because every strain can be screened under various process conditions, the number of cultivation runs is further multiplied. Taken together, these factors confront the field of microbial process development with unprecedented high-throughput issues.

To address this need, a multitude of microbio reactor (MBR) systems have emerged to accelerate bioprocess development. The small size of MBR systems offers great potential to increase the throughput of scalable cultivations by parallelization. At the same time, the small volumes and a large number of parallel cultivations set high demands on liquid handling (LiHa) precision, analytical sensitivity, and automation degree, since manual operation is unfeasible. Additionally, sophisticated interfaces are imperative to handle the vast amounts of generated data and extract crucial process information using appropriate visualization tools. Consequently, every system available to date brings with it a number of limitations that have to be considered when selecting hardware for a desired process. As a result, systems suitable for cell line cultivation may be unsuitable for microbial cultivation and vice versa. Fast metabolism and growth rates, which are typical for microbial bioprocesses, require shorter supply and response intervals from the cultivation system. In contrast, the daily withdrawal of samples is considered to be a high sampling rate in cell culture systems, which translates to one sample per hour for microbial processes. The fast kinetics of microbial cultivation have the advantage of high production rates; although this aspect makes their requirements more demanding in terms of process control systems (PCSs) and sampling reaction times to capture the

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process phase data of interest. Consequently, the automation of microbial bioprocesses is more challenging and, yet, is imperative to handle the increasing bioreactor counts using miniaturization and parallelization. To tap into the benefits of microbial cultivation, a miniaturized bioreactor system with a preferably high volumetric oxygen transfer coefficient ($k_{l}a$) is needed to approximate laboratory-scale steel tank reactors. MBR volumes should provide sufficient sample volume and quick access for repeated and fast-automated sampling, measurement and sample storage to enable overnight processes without human manipulation. Simultaneously, the system volume should be as small as possible to allow for high-throughput parallelization and easy handling. Finally, the combination of automated high-throughput with a shorter cultivation process duration results in even higher possible cultivation counts per week; therefore, disposable material should be used within reasonable limits.

To keep the system volume as small as possible and still provide sufficient volume for sampling, the 8–15 mL scale was chosen. In this scale segment, the two stirred-tank systems ambr15 (Tap Biosystems, Royston, UK) and bioREACTOR (2mag AG, Munich, Germany) are available. Originating from cell line cultivation, the ambr15 system offers a high degree of automation off the shelf. Featuring plug closed sampling ports and only one automated pipetting tip, sampling and liquid addition rates represent a limitation for fast microbial cultivation kinetics. A modified ambr15f version with two micropump feed lines per reactor vessel solved the latter issue for up to two liquids and was recently reported for the evaluation of recombinant *Escherichia coli* (*E. coli*) fed-batch cultivation. However, the cultivations were conducted under power per unit volume adapted conditions, resulting in a $k_{l}a$ of 0.04–0.05 s$^{-1}$.[9] Feautring only one plate position for chilled sample or media storage limits sample numbers. To cover the extended demand of automated sample processing and storage, customization in the form of the addition of a second robot for sample handling would be necessary to adapt the ambr15f for the fully automated microbial cultivation process.

Initially designed for microbial cultivation, the gas-inducing stirrers of a bioREACTOR offer a $k_{l}a$ of >0.4 s$^{-1}$ and sterile headspace aeration that features open sampling ports for quick access. The combination of autoclavable gas distributors and stirrers with disposable reactor vessels provides the practical benefits of disposables while keeping the material costs low.[6,7] Lacking any robotic augmentation or liquid pumps, custom integration into a laboratory robot system is inevitable for the bioREACTOR as well. However, the successful integration in Tecan and Hamilton systems or combinations of both for automated cultivation with integrated sample preparation has already been reported.[8–10] In contrast to laboratory-scale reactors, the addition of liquid by robotic pipetting allows for only intermittent feeding, which was accepted as scalable fed-batch performances to the laboratory scale were achieved previously.[11,12] Further distribution gradients observed in production-scale reactors are reported to also cause fluctuating process conditions perceived by individual cells.[13,14] These fluctuations even offer the potential to improve scalability by modifying them to suit a specific target laboratory- or large-scale reactor.[15] This can be achieved by increasing or reducing feed pulse step time, also with the aid of substrate release systems when necessary to overcome robotic minimum step times.[16–18]

The objective of this study was the creation of a fully automated microbial cultivation platform for microbial process development that allows “hands-off” operation without manual intervention, including the complete process control and sample handling from the calibration of the disposable reactor vessel sensors to the last sampling at the end of the cultivation process. Consequently, all data processing during the course of the cultivation and at-line measurements should be performed automatically, while at the same time allowing the addition of data from off-line measurements. Finally, all of the data should be stored in a centralized database together with the data from other scales to provide convenient access for data analysis software (SW). We report the verification of the data generated by the fully automated cultivation platform by comparing the cultivation of two *E. coli* strains under varying process conditions to the results generated in 5 L steel tank reactors.

To develop this fully automated high-throughput cultivation platform, numerous modifications of commercially available hardware components were necessary. In addition, the platform was completed using custom-built hardware components and custom-programmed superordinate process control software (PCS) that operates with an integrated data management system.

2. Experimental Section

2.1. MBR and Laboratory Automation Hardware

Presterilized and packed disposable bioreactors with immobilized pH and dissolved oxygen (DO) sensors (Mini-Bioreactors HTBD LG1-PIS3-Hg; PreSens GmbH, Regensburg, Germany) for use in up to four bioreactor blocks (bioREACTOR8; 2mag AG) equipped with fluorescence readers (MCR-LG1-v2; PreSens GmbH) were used for microbial cultivation. Each block was equipped with eight bioreactors for up to 32 cultivations in parallel. The bioreactor blocks were placed on a working table of the HEPA filter equipped with a LiHa system (Freedom EVO; Tecan, Crailsheim, Germany). The LiHa systems operation SW (EVOware; Tecan) was used to directly execute the scripts or in a remote-control mode executing worklists. Two refrigerated circulating baths (VersaCool; Thermo Fisher Scientific GmbH, Schwerte, Germany) were used, one for temperature control and another for head-space cooling of the bioreactor blocks. For at-line optical density measurements, a microplate spectrophotometer (SPECTRAMax PLUS384; Molecular Devices Corporation, San Jose, USA) with remote-controlled operating SW (Softmax Pro; Molecular Devices Corporation) was used. For deep-freeze storage of off-line samples, a deep freezer storage unit equipped with a robotic manipulator (RoMa, STR44-DF; Liconic Instruments, Montabaur, Germany) was integrated into the LiHa systems worktable.

2.2. Organisms

Experiments were performed using *E. coli* BL21(DE3) cells (Novagen, Merck KGaA, Darmstadt, Germany). The gene encoding for FabZ together with the native leader sequence of the OmpA protein, which was placed under the control of the T7 promoter and with a T7 terminator, was integrated into the attTN7
Each block was equipped with bioreactors and was aseptically filled with a sterile medium in a laminar flow hood. Subsequently, the autoclaved gas distributor with the gas-inducing impellers was assembled. Previously sealed by an autoclave tape, the blocks were transferred to the previously disinfected working table of the LiHa system. All aseptically prepared media were also placed on the working table in covered vessels. The PCS was initiated, and the respective process parameters were entered for each reactor. During parameterization, all reactors were set to 25 °C, 1900 rpm, and 62.5 mL h⁻¹ sterile air headspace gassing. The headspace cooling of the bioreactors blocks was set to 4 °C to minimize evaporation. The previously autoclaved vial rack, which had been stored at −80 °C, was aseptically filled with opened and frozen cryo vials, and the covered vial rack was then placed on a cooled tray. Immediately before starting the fully automated bioprocess, all seals and covers were removed.

Beginning with the preparation phase, the temperature control was set to 37 °C for 60 min to allow the fluorometric sensors to equilibrate in the gassed and agitated media. In the consecutive calibration phase, all reactors were set to 2800 rpm and were gassed with 250 mL h⁻¹ nitrogen for 20 min to calibrate the lower 0% limit of the DO sensors. Next, all reactors were gassed with 250 mL h⁻¹ air for 20 min to calibrate the upper 100% limit of the DO sensors, after which agitation and gassing were reset to 1900 rpm and 62.5 mL h⁻¹ air. At the end of the calibration phase, the pH sensors were recalibrated (one-point) to the actual medium pH, which was checked by a reference electrode and previously entered during the parameterization. In the following equilibration phase, the pH control was set to a pH of 6.8 and activated to adjust the media pH in case that the actual media pH differs from the batch start set point.

To compensate for evaporation, 70 µL h⁻¹ of purified water was added by the LiHa arms system liquid. Similar to the benchmark cultivations, the DO level was regulated to ≥35% in a control cascade, increasing the stirrer speed from 1900 rpm to 2800 rpm followed by additional oxygen supplementation (up to 50% v/v). As in the benchmark cultivations, the pH was controlled at 6.8 using 25% ammonia and 3 M phosphoric acid. In cases where different pH set points were used for product expression, the pH was adjusted to the respective setpoints prior to induction, using ramps with a duration of 2 h. Thirty-five milliliters of preculture at an optical density at 550 nm (OD₅₅₀) of 2 were supplemented with a sterile syringe for inoculation.

The synthetic media used for all the cultivations was prepared as described by Strieder et al. The medium was supplemented with 1 mL L⁻¹ antifoam agent (PPG 2000; Dow Chemical Co., Midland, USA) and was autoclaved for sterilization in place (SIP) prior to the start of cultivation.

Feeding was initiated when a culture that was grown to 5.7 g L⁻¹ cell dry mass (CDM) in 2.5 L of batch medium entered the stationary phase, as indicated by a DO peak. Starting with a 12 h-long exponential feed phase, the carbon-source-limited growth rate was adjusted to 0.18 h⁻¹. Subsequently, a constant feed rate of 50 mL h⁻¹ with 600 g L⁻¹ glucose solution was set for 15 h. The CDM yield coefficient on the glucose was 0.35 g g⁻¹, and the 2.0 L feed medium provided glucose and components sufficient to yield a final CDM of 97 g L⁻¹.

Beginning with the constant feed phase, a 2-h-long pH ramp to the respective induction phase set points was initiated. At the end of the ramp, the reactors were induced with 25 mL isopropyl β-D-1-thiogalactopyranoside (IPTG; 75 µM). The first of the manually drawn samples was withdrawn before induction (T₀) and then at 4 h (T₁), 7 h (T₂), 10 h (T₃), and 13 h (T₄, end of cultivation) after the IPTG pulse. Manual OD₅₅₀ measurements (Genesys 10S UV-Vis; Thermo Fisher Scientific GmbH, Schwerte, Germany) and 300 µL culture suspension samples were taken, and the latter were stored in reaction tubes at −20 °C for the off-line product titer analysis (Figure S1A, Supporting Information).

2.4. Medium and Fully Automated Cultivation Conditions at a 10 mL Scale

Each block was equipped with bioreactors and was aseptically filled with a sterile medium in a laminar flow hood. Subsequently, the autoclaved gas distributor with the gas-inducing impellers was assembled. Previously sealed by an autoclave tape, the blocks were transferred to the previously disinfected working table of the LiHa system. All aseptically prepared media were also placed on the working table in covered vessels. The PCS was initiated, and the respective process parameters were entered for each reactor. During parameterization, all reactors were set to 25 °C, 1900 rpm, and 62.5 mL h⁻¹ sterile air headspace gassing. The headspace cooling of the bioreactors blocks was set to 4 °C to minimize evaporation. The previously autoclaved vial rack, which had been stored at −80 °C, was aseptically filled with opened and frozen cryo vials, and the covered vial rack was then placed on a cooled tray. Immediately before starting the fully automated bioprocess, all seals and covers were removed.

Beginning with the preparation phase, the temperature control was set to 37 °C for 60 min to allow the fluorometric sensors to equilibrate in the gassed and agitated media. In the consecutive calibration phase, all reactors were set to 2800 rpm and were gassed with 250 mL h⁻¹ nitrogen for 20 min to calibrate the lower 0% limit of the DO sensors. Next, all reactors were gassed with 250 mL h⁻¹ air for 20 min to calibrate the upper 100% limit of the DO sensors, after which agitation and gassing were reset to 1900 rpm and 62.5 mL h⁻¹ air. At the end of the calibration phase, the pH sensors were recalibrated (one-point) to the actual medium pH, which was checked by a reference electrode and previously entered during the parameterization. In the following equilibration phase, the pH control was set to a pH of 6.8 and activated to adjust the media pH in case that the actual media pH differs from the batch start set point.

To compensate for evaporation, 70 µL h⁻¹ of purified water was added by the LiHa arms system liquid. Similar to the benchmark cultivations, the DO level was regulated to ≥35% in a control cascade, increasing the stirrer speed from 1900 rpm to 2800 rpm followed by additional oxygen supplementation (up to 50% v/v). As in the benchmark cultivations, the pH was controlled at 6.8 using 25% ammonia and 3 M phosphoric acid. In cases where different pH set points were used for product expression, the pH was adjusted to the respective setpoints in 2-h-long ramps prior to induction. For each cultivation, 160 µL of cryo stock culture with an OD₅₅₀ of 1–1.25 was automatically supplemented by the LiHa arm for inoculation.

As in the benchmark cultivations, feeding was automatically initiated when the culture that was grown to a CDM of 5.7 g L⁻¹ in 8 mL of batch medium entered the stationary phase as indicated by a pH peak. Starting with a shortened 4-h-long exponential feed phase, the carbon-source-limited growth rate was adjusted to 0.18 h⁻¹. Subsequently, a constant feed rate of 78 µL h⁻¹ with a 600 g L⁻¹ glucose solution was set for 15 h. The feed medium (1.4 mL) provided glucose and components sufficient to yield a final CDM of 36 g L⁻¹.

Similar to the 5 L scale start of the constant feed phase, a 2-h-long pH ramp to the respective induction phase set points was initiated. At the end of the ramp, the reactors were automatically induced with 76 µL IPTG (100 µM), and samples were withdrawn as for the 5 L scale time points (Figure S1B, Supporting Information).

The addition of the feed medium, inducer, and titration agent was provided by the LiHa arm according to the parameterization of the PCS. The microplate spectrophotometer was used for...
automated at-line optical density measurements to monitor biomass. Three hundred microliter samples of culture suspension in deep well plates were transferred to a deep freezer storage unit (set to −20 °C) by the RoMa arm for off-line product titer analysis.

2.5. Off-Line Sample Preparation and Content Quantification

For the quantitative analysis of the FabZ soluble analyte, the samples were prepared using a Tecan pipetting robot in the 96-well format. Cell lysis was necessary for content quantification, which was performed by incubation with 1/10 v/v Lysonase (Merck) in FastBreak cell lysis reagent (Promega) (30 min at room temperature with shaking at 450 rpm). Samples were diluted in the Gyros analysis buffer (RexxipA) for quantification. Content quantification was performed using a Gyrolab xPlore by an automated immunoassay with two Fab-specific antibodies for capture (biotinylated for immobilization within the Gyros CD-microstructure) and detection (Alexa647 fluorescence labeled for quantification). The Gyros protocol (200-3W-002-A) was performed according to the manufacturer’s instructions. The standard curve was analyzed with the Gyros Evaluator SW using a five-parameter fit. Quantification was performed in the linear range of the standard curve (15 ng mL⁻¹ to 1000 ng mL⁻¹). The immunoassay showed high intra-assay (CV < 10%) and interassay precision (CV < 10%) with a spike recovery of between 90% and 110%.

3. Results and Discussion

3.1. Automated Cultivation Platform

To build the envisioned fully automated cultivation platform, we combined commercially available automated devices and applied minor modifications to some components. We completed the setup with additional in-house developed hardware and custom-built superordinate PCS (Figure 1A). To achieve individual temperature and agitation control of each reactor block, an in-house developed docking station equipped with Peltier elements was attached to the reactor block control units. To streamline the contamination-free insertion of the reactor blocks, guide rails with snap-locks allowed for quick and simultaneous connection of multiple fluid and data ports at once. To supply each reactor block with individual air, oxygen, and nitrogen supplies, a gas supply station was developed that allowed for the controlled gassing of separate reactor blocks for automated DO sensor calibration and DO control. To eliminate manual interference during cultivation, a custom-built PCS was developed to enable parameterization for the complete process. From sensor calibration and inoculation to batch, feed, and induction phase through the end of cultivation, the whole process was individually configured by trigger–action–cascades, including sampling plans for at-line OD measurements or deep-frozen sample storage for later off-line analytics. Through remote control of the laboratory robot and microplate spectrophotometer SW, the PCS enabled automated liquid addition and sampling. PCS control of the deep freezer storage unit and its RoMa arm also allowed automated sample deep freezing. To store sufficient liquid volumes needed during the bioprocess, such as feed media, disinfection, and titration agents, autoclavable 170 mL, 500 mL, and 1000 mL vessels were used. In-house manufactured autoclavable inoculum vial racks with removable thermal storage provided uniform thawing of cryo stocks.

3.2. Sterile System Setup Routines and System Fluid Channel Disinfection with Liquid Monitoring

To enable antibiotic-free bioprocesses, automated cleaning and disinfection procedures for system setup of the laboratory robot

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Figure 1. A) Hardware and SW composition of the automated cultivation platform with in-house developments (indicated in yellow). B) Data handling and storage concept of LIMS, custom-built PCS (yellow), and database with the integration of off-line analytics and access of database-based analysis SW.
with system liquid monitoring were developed, as well as custom-
built autoclavable washing stations and waste storage vessels.

A presterilized disposable 50 L bag was aseptically connected to
a presterilized disposable 0.2 µm liquid filter and an autoclaved
valve fitting in a laminar flow cabinet. The 50 L bag was first
connected to the purified water reservoir by the filter and was
filled (Figure 2). Utilizing the valve fitting, the bag was aseptically
connected to the system liquid tubing of the laboratory robot by
flushing and hold steps with a 0.4% v/v peracetic acid (PAA)
(38–40%; Merck) solution and purified water.

To provide sterile starting conditions for the laboratory robot
and its LiHa system liquid tubing, an automated cleaning and
disinfection routine, including prompts directing the initial
manual setup steps, was used prior to every cultivation. Two 2 L
flasks with cleaning solution (Setup & daily system clean; LVL
Technologies, Crailsheim, Germany) and a 0.4% v/v PAA
solution were prepared in a safety cabinet and connected to the
laboratory robots system liquid tubing. The empty working
table, robotic arms, and LiHa tips were manually cleaned with
disinfection wipes (Incidin OxyWipe S; Ecolab, Reinach,
Switzerland). Microplates for a inline and off-line sampling
were inserted into the plate holder and deep freezing storage.

An autoclaved washing station, two autoclaved waste contain-
ers, and a water monitoring filter (Biosart 100 Monitor;
Sartorius Stedim) were placed on the working table. Immedi-
ately before starting the cleaning and disinfection routine,
the washing station and waste container sealing were removed.

Commencing a series of automated washing steps and hold
times with the cleaning agent and PAA solution, the system
liquid channels, and LiHa were cleaned and disinfected, while
simultaneous UV radiation provided surface disinfection of the
work table. At the end of the cleaning and disinfection script,
11 mL of system liquid per fixed tip was filled into the water
monitoring filter, which was subsequently removed together
with the waste vessels containing dispensed system liquid
and cleaning agent. The monitoring filter was placed on an agar
plate (irradiated 3P tryptic soy agar with neutralizers;
BioMérieux, Marcy-l’Étoile, France) and was cultured overnight
at 37 °C to assess the disinfection of the system liquid.

3.3. LiHa Procedures to Prevent Cross Contamination and
Unwanted Sample Dilution

Since every fixed tip handles media supply and sampling for
multiple bioreactors, cleaning and disinfection procedures for the
LiHa arm had to be developed to prevent cross contamination
during cultivation while preventing the dilution of the pipetted
samples with system liquid. After sampling for OD measurement
and inoculation (Figure 3A), the fixed tips of the LiHAs
were cleaned in the washing station with system liquid. The cleaned
tips were then disinfected in an aqueous solution of 35%
2-propanol, 25% w/w 1-propanol (Incidin Liquid; Ecolab), and
70% v/v ethanol (Figure 3B). To clean and disinfect the section of
the fixed tips immersed in the bioreactor during the media supply
and sampling, the in-house manufactured autoclavable washing
station and vessels feature deeper cavities. Since tips immerse into
only the reactor headspace for media supply, without immersion
into the culture suspension, the mixing step in propanol was
skipped during subsequent cleaning and disinfection. Increasing
the biomass and culture suspension viscosity during cultivation
increases the risk of system liquid contamination via the carryover
of living cells adhering to the inner tip surface. This is especially
ture when increasing sample volume, since the cell suspension is
aspirated deeper into the tip and tubing, while at the same time,
this surface is less accessible and exposed to the mixing steps in
propanol and ethanol for disinfection. Therefore, a 70% v/v
ethanol gap was aspirated to prevent contamination of the system
liquid during the sampling of 300 μL culture suspension for off-
line content quantification (Figure 3C). The ethanol gap was
disposed at the subsequent cleaning and disinfection step. Because
gas bubbles within the aerated culture suspension can
cause errors in OD measurements, these had to be disposed of
beforehand. Additionally, the system liquid can cause artificial
sample dilution when handling small sample volumes and
dilution series as reported by Ouyang et al. Therefore, the
sampling script used for taking OD measurements was modified
to include an excess volume and a mixing step to dispose of the
gas bubbles (Figure 3D). The fixed tips were disinfected as
described previously.

To monitor sterile operation and to prevent cross contamination,
three reactor blocks were operated identically but only odd rows of
the first and even rows of the third bar were inoculated. Since every
fixed tip of the liquid handler provides evaporation compensation,
titration agent addition and sampling for one inoculated and two
sterile reactors, insufficient cleaning and disinfection would result
in cross contamination of the two sterile reactors. As indicated by
the sensor signals and OD measurements, metabolic activity and
increasing biomass were observed only in the inoculated reactors
(Figure S2, Supporting Information).

3.4. pH Control Concept

Because of the intermittent liquid addition by the LiHa arm,
back coupling and feedback effects had to be considered to

![Figure 2. Aseptically assembled valve installation (V1 + V2). 50 L bag, and filter (F) connected by quick connectors (--) to the purified water reservoir, position 1 (P1) of the automated six-way valve (V3) (I-25052; Valco Instruments Inc., Houston, USA), and 2 L of PAA solution for aseptically connecting to the laboratory robot system liquid tubing. Red lines indicate the disinfection loop with valve V2 open after installation of the new bag. Black lines indicate the purified water supply loop of the laboratory robot for flushing and LiHa with valve V2 closed and valve V1 opened. Additionally, PAA and cleaning solution (clean) were connected to positions P2 and P3 of the six-way valve for automated cleaning and disinfection routines of the laboratory robot, indicated by blue lines.](image-url)
realize pH control for the pulsed addition of the titration agent. In addition, the pipetting of lower volumes decreases the accuracy of the LiHa and also increases the LiHa arm travel time and washing steps. To meet these requirements, the function below was determined empirically to allow the calculation of titration agent volume based on the actual pH difference from the setpoint at the time of addition. By default, the pH difference was checked every 180 s, and a threshold for a minimum liquid addition of 5 µL was used to limit the number of titration steps and increase pipetting accuracy.

\[
\text{Volume}_{\text{base}} = ((\Delta \text{pH} \times 56.25)^2 + \text{volume}_{\text{threshold}}) \times c
\]

\[
\text{Volume}_{\text{acid}} = ((\Delta \text{pH} \times 56.25)^2 + \text{volume}_{\text{threshold}}) \times 0.25 \times c
\]

To avoid feedback effects, the acid volume addition was reduced by a factor of 0.25, and a 300 µL threshold for the maximum liquid addition was used, which also prevented dispensing commands exceeding the 1000 µL volume of the LiHa syringes. In case of different titration agent concentrations, the correction factor \( c \) can be modified, as well as the pH control timer interval.

3.5. Batch End Detection for Automated Feed Start

Because the eight bioreactors of one block share agitation speed and gassing, the detection of batch glucose depletion by the characteristic DO peak is not reliable. Since the DO control always adjusts to the reactor with the lowest DO level, the DO levels in the other reactors increase. Since the metabolization of batch glucose causes acidification, and the pH is individually controlled per reactor, monitoring the pH signal in between titration steps was performed to indirectly detect depletion of the carbon source in the batch medium. The control algorithm assessed three true/false criteria. First, the bioprocess had to be in the batch phase; second, at least one base volume addition had occurred; and third, the slope of the pH signals had turned positive for the last 18 measurements at 5 s intervals. To prevent the false detection of a positive slope after the addition of a base, 0.33 times the last base addition interval plus a 20 s offset was skipped before the pH signal slope monitoring began. To detect the carbon source depletion that occurred in this nonmonitored timespan, a timer of 2.5 times the last base addition interval was started after each base addition to detect carbon source depletion.

Utilizing the algorithm, the characteristic rise in the pH caused by the metabolization of acetate formed during the batch phase was sufficient to correctly detect the end of the batch phase (Figure 4).

Due to the scheduling of the laboratory robot, reactors were measured simultaneously where applicable despite peaking in a short consecutive order. Thirty-two reactors spiking in a short time period resulted in OD measurements and the start of the feed supply to also represent a peak workload for the laboratory robot. This result can be seen by the OD measurement of reactor B, which also happens to end acidification simultaneously with a base addition, causing delayed detection and further delayed OD sampling, as the robot handles the other reactor blocks first (Figure 4). In contrast to its delayed detection and OD measurement, the batch end time point is calculated back accordingly, as seen by the added feed volumes. Despite the high workload of the robot, only 12–18 min from batch end detection, OD sampling, and measurement were needed until the supplementation of the titration agent and feed medium was resumed. While the titration agent and feed
medium are added simultaneously to all the reactors within one block, the correct addition of different volumes reflects the order in which the bioreactors pH spikes occurred, as seen in the staggering of the feed curves (Figure 4).

3.6. Data Storage Concept

The capability of the system to generate vast amounts of data in a short time raised the need for automated systematic data management. In addition, the need to parameterize up to 32 bioreactors at once and up to 96 bioreactors per week increases the demands for a user-friendly SW solution. Lastly, it was crucial to structure and store the generated process data with the corresponding metadata in a way that lays the foundation for business intelligence applications and data integrity.

To handle all these tasks, a system to manage all process metadata, data processing, data storage, and data retrieval for all incoming data was developed (Figure 1B). A laboratory information management system (LIMS; LabWare Inc., Wilmington, USA) was used to manage the metadata, including strain and instrument information. Additionally, the LIMS was used to plan cultivation project hierarchies and generate the corresponding process and sample identification documents (IDs) for all cultivations. Once generated, these IDs were transferred to the automated cultivation platform.

Up to 32 process IDs and the respective sample IDs were loaded into the PCS, and all cultivation runs were parameterized individually or grouped by metadata utilized from the LIMS. Fully parameterized processes were saved in recipes and loaded to quickly parameterize replicates or were modified to create similar processes with one or more varying parameters.

After the start of the process, all online data were directly stored in the database, as well as a local copy for backup. At-line generated data from photometric assays were processed in the same manner, while the sampling time point was logged for the results timestamp, despite an approximately 5 min time delay of the assays readout. Bioreactor vessel information and the sample timestamps for later off-line analytics were stored locally during the process. At the end of the process, these data were automatically transferred to the LIMS, and all the metadata were transferred to the database and merged with the already stored online and at-line data for convenient retrieval by analysis SW. After the analysis of the off-line samples, the result for each sample ID was transferred to the LIMS and merged with the bioreactor vessel information and time stamp. All completed off-line data were automatically transferred to the database for joint analysis.

Using this network of LIMS, PCS and the database together with automated data processing routines, all on-line, at-line, off-line and metadata can be stored and linked by unique identifiers that provide the basis for data mining applications and sustainable data management.

3.7. Verification of the Validity of the Results Generated by the Automated Cultivation Platform

To verify that the data generated by the automated cultivation platform match the data generated in the benchmark 5 L reactors, a set of cultivations was performed at both scales. The

Figure 4. Feed volume, OD, and pH during the depletion of batch glucose. The depletion of glucose was indicated by the end of acidification from the measured pH at the end of the batch phase, which was determined for all reactors (A–F), and an automated OD measurement was initialized followed by starting the intermittent feed addition. Since the end of the acidification of reactor B occurred simultaneously with a base addition, its detection was delayed, and the OD measurement did not occur until the sampling of reactor C–F in the other reactor blocks was performed. As indicated by the order of the added feed volume, the calculated batch end time point correctly places reactor B somewhere between reactors A and C.
control strain with the genome integrated FabZ product and the HF strain with the additional plasmid-based HF were cultivated with three different pH levels during induction.

Figure 5 displays a selection of parameters for three different cultivations of the control strain at the three pH levels for both scales. While the manual sampling at the 5 L scale for the pre-induction of the OD and soluble products was taken at the end of the workday 10 h before induction, the automated sampling at the 10 mL scale was performed briefly before induction. In the 10 mL-scale reactors, oxygen transfer was not increased by headspace overpressure, and the reduced exponential feed phase resulted in OD values half as high as observed at the 5 L scale. Higher soluble product titers were measured in cultivations with higher pH in both scales, while pH correlated variation in OD was limited to the 10 mL scale. The more pronounced DO fluctuations in small scale cultivations are caused by the intermittent glucose feed. The minimum pipetting volume in the 10 mL reactors led to an increased pH fluctuation range. Despite the described differences between the scales, the ranking with respect to titer, were the same. To eliminate a possible impact of the different OD levels at both scales, the specific soluble product titer was calculated for the end of cultivation samples for both scales and both strains (Figure 5C). Again, the ranking correlated with pH during the production phase and was the same for both scales. The specific product titer was generally higher in the small-scale cultivations. With respect to the expression system used, the strain with the HF showed significantly higher titers in all direct comparisons.

**Figure 5.** OD, product titer, feed, DO, pH, and temperature of A) benchmark 5 L and fully automated B) 10 mL cultivation of the control strain pH screening at pH 6.35 (red), 6.8 (black), and 7.25 (blue) during induction phase after a temperature ramp. C) The specific soluble product concentration at the end of cultivation for both strains and at both scales at pH 6.35 (red), 6.8 (gray), and 7.25 (blue) (left to right n = 1, 3, 1, 3, 1, 3, 12, 4, 4, 6, 4).
4. Conclusion

In this study, we present a fully automated microbial cultivation platform that is capable of performing up to 32 fed-batch cultivations simultaneously, including at-line and off-line sample handling, as well as processing of all incoming data for centralized database storage.

We observed that currently commercially available automated hardware and SW systems do not meet the desired needs. While there are single devices and systems that individually fulfill single aspects of the specific requirements, they were not designed to contribute in a fully automated platform. Therefore, the realization of a fully automated cultivation platform represented a challenge and required significant effort in hardware and SW adaptation as well as custom development.

The initial performance of the automated microbial cultivation platform produced results was comparable to benchmark 5 L cultivations with respect to the performance of different expression systems, as well as different process conditions. Therefore, the platform is a suitable tool for strain and process condition screening to support early-stage process development. With the capacity of up to 96 cultivations per week, the automated cultivation platform can significantly accelerate the generation of process data. Furthermore, the automated data handling and database storage are appropriate procedures to efficiently identify crucial process information by data analysis and mining tools, as well as ensuring data integrity for operation in an industrial environment.

The attained high-throughput cultivations and database connection further show a high potential to conduct cultivation projects that utilize randomized designs to automatically partition DoE plans for fully automated processing and reporting.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

automation, data management, high-throughput, microbioreactor, milliliter bioreactor