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Microbial technologies for biotherapeutics production: Key tools for advanced biopharmaceutical process development and control

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Current trends in the biopharmaceutical market such as the diversification of therapies as well as the increasing time-to-market pressure will trigger the rethinking of bioprocess development and production approaches. Thereby, the importance of development time and manufacturing costs will increase, especially for microbial production.

In the present review, we investigate three technological approaches which, to our opinion, will play a key role in the future of biopharmaceutical production. The first cornerstone of process development is the generation and effective utilization of platform knowledge. Building processes on well understood microbial and technological platforms allows to accelerate early-stage bioprocess development and to better condense this knowledge into multi-purpose technologies and applicable mathematical models. Second, the application of verified scale down systems and in silico models for process design and characterization will reduce the required number of large scale batches before dossier submission. Third, the broader availability of mathematical process models and the improvement of process analytical technologies will increase the applicability and acceptance of advanced control and process automation in the manufacturing scale. This will reduce process failure rates and subsequently cost of goods. Along these three aspects we give an overview of recently developed key tools and their potential integration into bioprocess development strategies.

Introduction

Parallel to the emergence of novel biopharmaceuticals, the last decades led to an outstanding expansion of knowledge in the field of biopharmaceutical production [1–3]. In the next years, the integration of this knowledge in biopharmaceutical development and production will be a key factor to address increasing challenges, such as:

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-Enhanced competition and time pressure
The rapid increase in the number of biopharmaceutical drug candidates led to a diversification of the company landscape and an acceleration of competition. For example, the emergence of biosimilars forced major biopharmaceutical players to rethink business and development strategies [4] and enhanced the importance of manufacturing costs [5]. Additionally, the pharmaceutical industry will be continuously challenged to deliver fast solutions to global health threats, as demonstrated by the recent COVID-19 pandemic.

-Decreasing biopharmaceutical R&D productivity
The return of investment on new biologicals is continuously decreasing due to an increase in drug development costs as well as a decrease on average sales per new product [6]. These trends will force pharmaceutical companies towards increased cost awareness in each and every part of the product life cycle, including development [7] and manufacturing costs [8].

-Decreasing patient group and production batch sizes due to increased drug specificity
Advances in the understanding of disease pathology and the availability of precise diagnostic tools enable the development and use of targeted therapies. For example, genetic read and write tools will facilitate the use of nucleic-acid-based information for individualized therapies [9–11]. This trend will require a complete rethinking of various aspects along the supply chain of such therapies (e.g. [12]). Generally, we anticipate that the decrease in patient group sizes will ultimately increase the importance of process development and manufacturing costs within the total product development budget [13].

Overall, these factors will increase the importance of cost and time required for the development and manufacturing of novel and improved biopharmaceuticals. In view of the increasing variety of biotherapeutics where microbial production technologies are applied [14–16], decreasing manufacturing costs and shortening development times will be key factors to increase the competitiveness of microbial manufacturing. To effectively tackle the addressed challenges, the technological advances of the last decade have to be adopted on the shop floor. Due to the underlying biological complexity [17], the high number of required unit operations [18] and the strict requirements towards quality and product safety, biopharmaceutical production has very tight requirements [19,20]. Any advancements and their implementation is less straightforward as in other productive sectors [21].

From a Contract Manufacturing Organizations (CMO) perspective, three approaches need to be in focus to facilitate the adoption of recent technological advancements:

- Build on well understood biological and process platforms to enable fast process development for multiple products and the effective utilization and storage of available scientific knowledge through mathematical descriptions.
- Facilitated process design and characterization supported by scale-down models and in silico simulations, thereby anticipation of process robustness early in the process life cycle.
- Reduced number of failed batches and manual interaction through site- and scale-independent control strategies & automation, based on the gained platform knowledge that is condensed in mathematical models and well-established in-process analytics.

Within this review, we give an insight in recent technological advances in microbial production processes along the three above mentioned topics. By discussing relevant examples, the review aims to point out how process development can be efficiently based on well-understood biological and technological platforms (Section 'Biological and technological platforms'), how this knowledge can be used for a faster development of robust and optimal processes (Section 'Scale down models and digital process design') and how advanced monitoring, control and process automation (Section 'Advanced monitoring, control and automation') will allow increased efficiency in the manufacturing of biotherapeutics.

Biological and technological platforms

The increasing amount of biotechnological process knowledge is captured in the growing body of scientific literature, patent applications as well as company databases in the pharmaceutical industry. The effective usage of biological and technological platform knowledge for the development of processes and products will help to reduce both cost and development time by avoiding continuously recurring developments of production hosts, molecular and analytical methods as well as unit operations.

Once these platforms are established, they need to be maintained to guarantee their validity and applicability across different applications. To do so, a systematic knowledge management is needed [22], which was reviewed recently in the context of the Quality by Design principles [23]. The review points out, that the establishment of mathematical models to store and maintain knowledge in an applicable format is not fully exploited and still strongly restricted to the academic sector. Nevertheless, biological and process modelling can be a key to systematically leverage technologies across different applications and pharmaceutical products along the full product and process lifecycle [24,25]. To do so, the established and maintained mathematical models need to be integrated process development activities as well as the shop floor, including direct information transfer from the physical plant and human interactions. This can then be regarded as a digital twin [26].

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Table 1. Process knowledge platform for microbial USP parameters.

Product class	Host and expression loci	Temperature	Feed profile	Applied inducer concentration	Reference
Nano-bodies, fABs	*E. coli*/secretion into periplasm	25–32 °C	Batch-like or static q_s; $q_{s,Glu}$ = 0.15 g/g/h	Arabinose (0.07–0.26%, w/v)lactose $q_{s,lac,max}$ = 0.33 g/g/h; IPTG, 1.25 mM	[34,35]
	P. pastoris/extracellular	25–30 °C	Static feeding q_s = 0.03–0.06 g/g/h, dynamic increase of q_s up to $q_{s,max}$ increases q_p	Methanol 0.5–1% (v/v)	[42,44–47]
Host toxic protein	*E. coli*/IB (inactive)	30–31.5 °C	Static q_s, q_s = 0.25–0.45 g/g/h Dynamic: shift from high to low q_s	IPTG, 0.5 mM	[48–51]
	E. coli/IB (active)	15–25 °C	Batch like growth at μ_{max}	Lactose auto-induction, IPTG 0.4 mM	[52–54]
pDNA	*E. coli*/intracellular	42–45 °C	Static feeding with q_s = 0.26 g/g/h, trends show lower μ enhances q_p	no induction but temperature shift to enhance PCN	[9,41,55]

Biological platforms

A basic prerequisite for the establishment of a successful production process is the preparation and the selection of an appropriate production clone. Process robustness and efficiency can be already targeted at this level, for example by (i) decreasing product-related impurities (e.g. codon optimization could avoid mismatches during translation) and (ii) by easing process-related downstream approaches (e.g. product secretion for *Escherichia coli* [27]), or by (iii) reducing the metabolic variability of the host cells (e.g. knock-out of unnecessary metabolic pathways). A number of strain engineering and clone selection tools have been summarized in excellent reviews [15,28]. Here we give a short overview of biological platforms and their preferred process conditions for relevant microbial products, which are also summarized in Table 1.

For the production of nucleic acids, the pharmaceutical industry uses predominantly *E. coli* as a production host [9], represented also in the rising number of filed patents for pDNA production using *E. coli* over the last decade [29]. Aside from nucleic acid production, *E. coli* is also used for the production of non-glycosylated proteins, where besides different hormones [30] especially antibody fragments (fABs) are of rising interest [14,31]. Strain and protein engineering techniques allowed to efficiently transfer fABs into the periplasm, making use of its oxidizing environment and thus allowing disulfide-bond formation, which is needed for correct folding of the fABs [32,33]. For these processes a temperature decrease throughout induction, were found to yield in higher fAB titers [34,35].

Even though extracellular or periplasmic product location is desirable to ease further purification steps, overall process yield might be favorable when applying intracellular protein expression in *E. coli* hosts, despite time- and cost intensive downstream procedures [36]. As the host cell cannot cope with the high amounts of recombinant protein produced, the formation of inclusion bodies (IBs) – misfolded protein aggregates in *E. coli* – is frequently observed [37,38]. Although refolding into the active state of the protein is usually required, inclusion body formation also comes along with benefits, such as less proteolytic degradation, exceptionally high product titers and the possibility of expressing host-cell toxic proteins [36,39]. Cultivation temperature hereby influences enzymatic activity of the aggregated proteins, where a decrease of cultivation temperature (below the 30 °C mark) resulted in higher activity [37,40]. Whereas relatively low cultivation temperatures reportedly boost recombinant protein production, it was shown that pDNA manufacturing can be increased by applying a temperature shift in the range of 42–45 °C. Plasmid copy numbers (PCN) of 400–500 per cell for genetically optimized strains were reported [9,41].

In addition, *Pichia pastoris* is also a promising unicellular host, when it comes to recombinant protein production, as products can be secreted into the supernatant [31,42]. Dynamic feeding approaches developed for *P. pastoris* have shown to increase cell specific productivity [43] and extracellular protein concentrations of up to 4 g/L [44].

Upstream process (USP) technologies

Microbial fermentation in red biotechnology can be carried out in batch, fed-batch or continuous mode. Currently fed-batch processes in stirred tank reactors are mostly used for microbial production as they reach higher cell densities under controlled growth condition [56], compared to batch, and offer better process stability, compared to continuous processes [57]. Even though continuous manufacturing is currently trending [58], challenges remain in the realization of continuous cultivation processes [58]. Recombinant host organisms still lack in long term stability and are therefore often not suited for continuous cultivations. There are recent works tackling this issue, both on a genetic [59,60] or process-technological level [39]. A separation of growth and production in cascaded reactor setups was also shown as a promising option to enable stable continuous production [61].

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These upstream process modes are well-understood and diverse models have been established to describe growth, product formation and physical properties such us thermodynamics, fluid dynamics, gas transfer and others during the cultivation process [17,62]. Transforming these mathematical descriptions of the current upstream process platforms into digital twins can lead to a more efficient process development and scale-up, which will be further discussed in section 3.

Midstream process (MSP) technologies

The product purification steps of manufacturing technologies using microbial hosts are regarded much more expensive compared to the upstream (product expression) steps [63].

High-pressure homogenization, causing cell lysis via cavitation is commonly employed in the industry for the release of intracellularly accumulated proteins [64,65]. Inclusion bodies have a highly dense structure and hence can be fairly easily separated from other host cell proteins via centrifugation [36,66,67]. Making use of the state of the art centrifugation and separation techniques, IB purities of 80% prior to solubilization have been reported [64].

Still, the refolding yield is a major obstacle when it comes to the feasibility of an IB-process. Highly concentrated chaotropic reagents (e.g. urea or guanidine-HCl) have to be used in order to solubilize aggregated protein. However, the application of less concentrated solubilization reagents at high pH and other 'mild solubilization' techniques have shown to enhance refolding yields [68,69]. Still, refolding yields are highly dependent on the target protein and no general applicable solubilization and refolding procedure has been established. Techniques enhancing refolding yields have been introduced as stated below [70,71]:

i. on column refolding, to separate interfering substances
ii. the addition of 'chaperone-like' particles to enhance solubility
iii. usage of proper refolding aiding chemicals (e.g. L-arginine as aggregation suppressor)
iv. dilution techniques to prevent protein aggregation

Refolding by dilution is mathematically described and still the most common application in industry [72]. However, target product concentrations have been reported to be as low as 0.01–0.1 g/L, in order to enable feasible refolding [73]. Low target product concentrations in the refolding step result in large processing volumes at manufacturing scale, necessitating the use of large tanks and requiring proper buffer handling. Therefore, either model-based approaches in refolding [74] or continuous refolding could become a future perspective, reducing the scale and buffer consumption of the process step [75].

Usually, soluble proteins are expressed into the periplasm (*E. coli*) or extracellularly (*P. pastoris*), hence no cell disruption is necessary [35]. As the expression hosts remain intact during product harvest, the complexity of the process-related impurity matrix for the subsequent DSP process steps is lower when compared to full cell disruption. Moreover, the expressed product can be easily separated from the production cells with filtration techniques such as depth- or tangential flow filtration [76]. Still, disintegration of stressed cells at the end-of-filtration and the subsequent release of process-related impurities have to be carefully monitored.

For the purification of plasmid DNA, cell disruption is commonly carried out with alkaline lysis by high pH levels (e.g. pH 12). Thereby, host cell DNA is denaturing irreversibly, whereas plasmid DNA is able to renature [77]. A major obstacle in pDNA purification is that high DNA concentrations implement elevated viscosities, being especially critical in large scale [48]. Thus, determination of DNA concentration and subsequent dilution is mandatory. Neutralization is commonly employed with chilled ammonium or potassium-acetate buffers implementing precipitation of cell debris and RNA [49]. Precipitation yield throughout neutralization can be boosted efficiently via addition of sodium-dodecylsulphate (SDS) throughout alkaline cell lysis as SDS is able to denature and linearize impurity proteins [78].

Downstream process (DSP) technologies

Depending on the product locus and the determined purity of target product, there is a variety of downstream procedures applicable to meet the demands set by regulatory authorities.

For any process employing gram-negative bacteria as a host, anion exchange chromatography (AEXC) is commonly used to purify biopharmaceuticals from lipopolysaccharides [50]. Even though AEXC is a common chromatography method to purify products derived from *E. coli*, irreversible adsorption of pDNA in the outer layer of stationary phase is an obstacle in pDNA purification using AEXC [79,80]. Stationary phases composed of monoliths promote excellent mass transfer and further exhibit a high binding capacity for large molecules, which is highly beneficial for the purification of pDNA [29].

Cation exchange principles have also been reported to decrease endotoxin levels sufficiently, so depending on the pI of the target protein, any ion chromatography method can be chosen for endotoxin removal [81]. Mhatre et al. reported that fABs can be efficiently purified using cation exchange chromatography in combination with an increasing pH gradient [82]. On the other hand, affinity chromatography principles have been described to yield in sufficient purification of fABs [83]. Independent of the target product, size-exclusion methods or filtration steps are additionally available procedures to either conduct (i) buffer changes or to achieve (ii) final target product purification [50]. Moreover,

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model-based approaches, making use of online-derived signals, would potentially boost chromatographic purification efficiency even more [84].

Process analytical technologies (PAT)

Besides cost and time-consuming offline measurements, the current industrial standard measurements of microbial up and downstream processes are on-line measurement of physical and physicochemical parameters (e.g. temperature, pH, headspace pressure, dissolved oxygen, liquid and gas mass flows) [85–88]. Additionally, measurements such as infrared, fluorescence, dielectric spectroscopic techniques can be used to monitor nutrient, metabolite or biomass concentrations in the fermentation broth [89–91]. For example, near-infrared and fluorescent spectroscopy were used for the monitoring of different analytes in bacterial and yeast fermentations [92]. Fluorescent spectroscopy was successfully implemented for the real-time quantification of the expressed protein concentration [93–96].

The MSP and DSP steps are critical in terms of product quality impact [70]. Thus, PAT tools need to be provided for these unit operations [97]. As an example, protein refolding steps are often run as black boxes lacking any monitoring for proper process understanding and control. At-line, reverse phase HPLC analysis was already applied to monitor the refolding process [98]. However, the time delay of the analysis and the complex analytical hardware requirement are a major drawback of this approach [38]. In another study, the dissolved oxygen content in the refolding tank could be used to predict the protein quality by the DO signal, even at large scales [99]. Fourier-transform infrared spectroscopy (FT-IR) can predict the secondary structures of proteins therefore it was used to determine the endpoint of refolding marked by the start of product aggregate formation [100–102].

As demonstrated in this chapter, a wide body of biological and processing knowledge as well as a robust process analytical toolset is available for microbial process development. These are partly condensed in mathematical models to further assist MSP and DSP steps [74,103]. However, a consistent roadmap or examples about the integration of this generic knowledge into an easily accessible and applicable platform database is not available yet. The condensation of process knowledge into mathematical equations is applied currently at the next stage of bioprocess development, process design and characterization – as discussed in the next chapter.

Scale down models and digital process design

No matter what production hosts are used, which upstream strategy is followed and which downstream unit operations are included, the development of the full process includes lab-scale experiments for process design and a subsequent technology transfer and scale up. These steps are still highly relying on human experience and expertise, as well as on know-how regarding the mentioned biological and technological platforms. To reduce this dependency on human experience and expertise and to reveal new possibilities that reach beyond human capabilities, digital methods for process design and characterization in combination with highly automatized and parallelized lab equipment are of central interest.

Scale-down models

For fast and cost-efficient process development the aim is to conduct the planned experiments in qualified scale-down systems to mimic large-scale conditions and thus ease scale up [104–106].

Physical scale-down systems have the potential to reveal adaptation mechanisms [107]. For example, which cells that are being used are able to adjust to rapidly fluctuating environmental conditions. Furthermore, scale-down systems can identify possible root causes for maintaining similar process performance at different scales. Those systems can analyse inhomogeneous zones in multi-compartment systems or analyse integral effects of inhomogeneities in single-compartment systems [108,109].

Although some experimental studies exist that aim to mimic large-scale conditions [110–113], the integration of such effects in process modelling and digital scale-down models is of great interest but still a very challenging task [114]. Recent studies showed such possibilities by combining CFD simulations with kinetic models [115], which can be potentially validated by the usage of spatially resolved measurements using either multiple measurement points or submerged sensor balls [116]. In addition to that, single cell analytics (i.e. online flow cytometry) have been established to monitor subpopulation formation [110,111]. This revealed that having different subpopulations negatively influences process robustness of USP steps [117,118]. Based on these single cell analytics, novel feedback controls can be possibly established to counteract to the formation of subpopulations [119].

Physical scale down models, which today are fully automated and miniaturized bioreactor systems [107,120,121], need to be representative to the manufacturing scale [19] and therefore qualified in the same way [122]. To achieve this, good industry standards need to be applied to keep scale-independent factors constant. Additionally, data needs to be provided to prove that the performance of the scales is comparable or to show that consistent scale effects can be identified and quantified (Fig. 1A). This can be done by equivalence testing with a two-one-sided *t*-test (TOST) [123] (as it is exemplarily displayed in Fig. 1B) or by comparing estimated model parameters [107], therefore knowledge gained from experiments in these scale-down models can be transferred to manufacturing scale.

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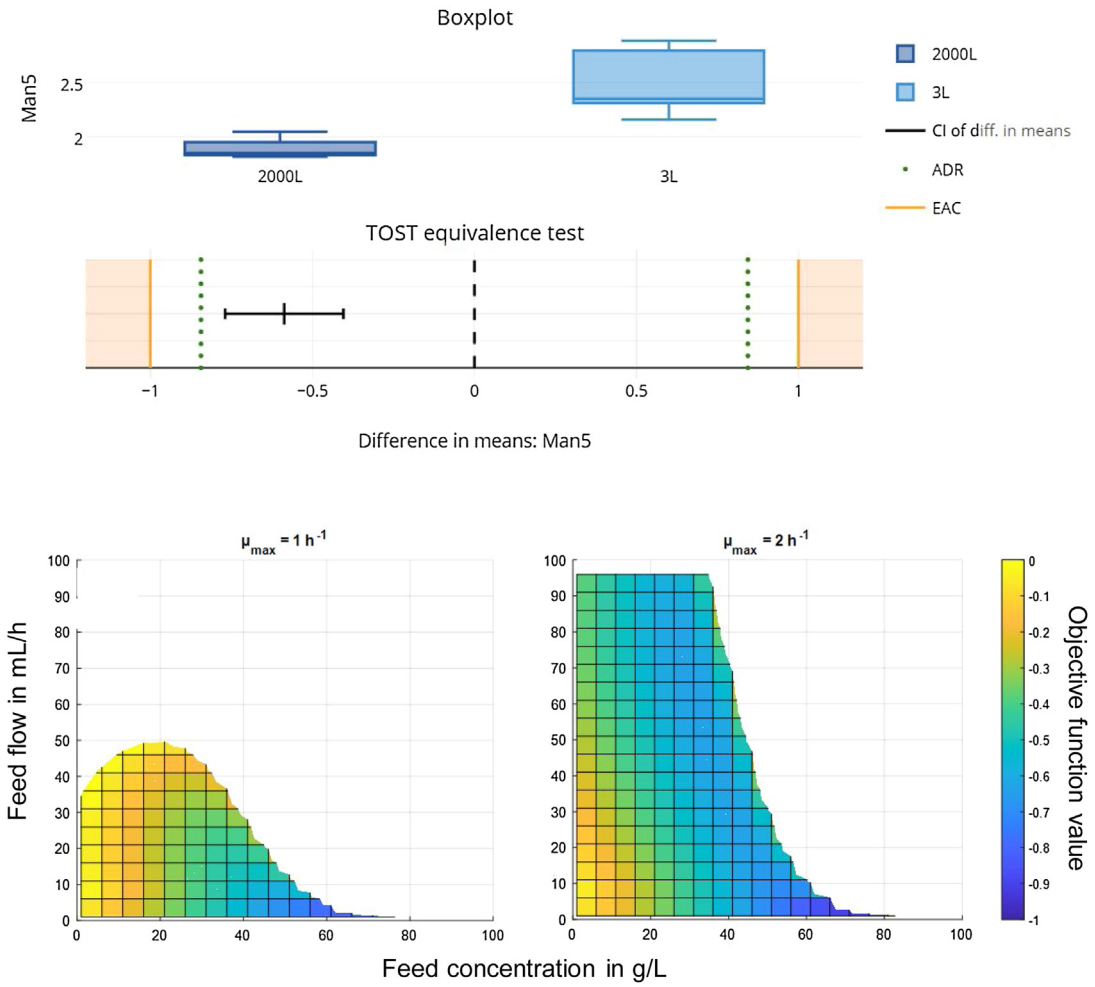

Fig. 1. (A) Results of a TOST test shows if the 95% confidence interval of difference in means (black error bar) is within the pre-set equivalence acceptance criteria (EAC, orange lines). If that is the case, as in this example, the equivalence test is passed. (B) Simulated design space of continuous lactic acid bacteria cultivations [124] under consideration of different maximum growth rates. Based on such simulations, appropriate characterization experiments can be planned. Minimal values in blue indicate regions with highest lactose to biomass conversion. Values above 0 are not displayed as they indicate non-feasible regions with potential washout or extensive lactose accumulation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Digital process design

Quality by design (QbD) [19,20,22,125] has been discussed since many years for ensuring product quality by deeper process understanding. However, its full potential is still not fully exploited [126]. Failing to efficiently plan and execute activities on the stage of process characterization leads to increased time-to-market and lack of process understanding and robustness. To de-risk process development and to characterize and transfer developed processes with reasonable effort, model-based simulation and digital design have high relevance.

A crucial step in process development and process design is the determination of the process design space in order to meet the targeted quality [127]. Today the determination of the process design space follows a series of experimentally intense steps. Although design of experiments (DoE) enables to determine the design space within a reduced set of experiments [128], a DoE provides very limited information if the selected levels and factors were not appropriate. This unnecessarily prolongs process development and hinders the identifications of the real optimum and its consideration in subsequent regulatory filings.

An in silico investigation of the system behaviour based on model simulations can complement the classical DoE approach. The simulation model can be based on physical and natural laws governing the process, or on pre-existent

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knowledge or just on hypothesized relations [129,130]. A hypothetical design space based on different influencing factors and deflection levels can be established [131] before execution of the DoE. Within Fig. 1B, an example of a precalculated design space is shown for a continuous lactic acid cultivation (taken form [124]). There, the feasible combinations for the dilution rate and feed concentration are shown for two different maximum growth rates. Specific DoE experiments can be planned, based on the in silico study and besides classical data-driven DoE evaluation, the hypothesized model can be validated. Overall, this procedure increases the chance to hit the real optimum [132]. Recently this approach was successfully applied to a mammalian cell culture process to plan different, feasible feed addition scenarios [133]. In addition to that, based on a penicillin production case study, Bano et al. showed how to reassess the operational space during running processes [134].

The combination of digital design with directed characterization experiments will ultimately lead to a reduction of required experiments prior to dossier submission. Nevertheless, with the application of continuous and scale-out approaches, the criticality of scale-up effects might decrease as manufacturing processes will be developed 'at scale' and the scaling to industrial capacity will be done by time and rather as a scale-out than a scale-up philosophy. In combination with digital process design and the usage of platform knowledge, development at scale becomes possible as only a reduced number of development batches are required [135].

Monte Carlo simulations to anticipate process robustness

In contrast to multivariate regression models, a model based on physical laws, chemical and biochemical reaction kinetics is easily scalable and trustworthy predictive [136], thus can be extrapolated and transferred, to a certain extent. Before moving into pilot scale, potential process modes (batch, fed-batch, continuous) can be benchmarked [25,124], potential scale effects can be investigated [115,137], as well as process bottlenecks can be revealed [138]. Then, the optimal process can be designed according to the model [132,139,140]. Although upstream processes are in focus of these developments, examples exist also for downstream [141]. In recent publications, the value of these predictive models to assess process sensitivities or robustness against uncertainties could be shown for various biochemical processes [18].

A core concept of these investigations is Monte Carlo simulations. Similarly to process validation where process reproducibility in terms of product quality is assessed, a predictive model allows to propagate uncertainties [142] and assess potential effects of possible changes, such as raw material quality [143], model parameter uncertainties [144,145] as well as process deviations. It further allows the definition of the necessary control strategies and the normal operating regions [18] along the entire process. In turn, it reduces the number of experiments needed for a comprehensive process validation and for the defined and proven production capability [18]. Model simulations could be used to evaluate the influence of parameter uncertainties on the process states of an *E. coli* fed-batch [144] and cultivations of Streptomyces sp. [146]. The current review mainly focusses on the characterization of single unit operations. Robust process outcome and the demonstrated manufacturing capability can however only be reached by a consistent process chain. A seamless interplay between the unit operations needs to be intensified. For this purpose, integrated process models, as known from other industries, need to be applied to integrated bioprocesses. With this procedure, also economical aspects can be included as shown by Biwer et al. [147] for the very competitive penicillin production process.

Advanced monitoring, control and automation

Due to the complexity of biopharmaceutical products and their manufacturing processes, advanced process monitoring and control methods are mandatory to consistently deliver the desired product quality [85,86]. Besides the control of the physical and physicochemical parameters, such as pH, temperature and dissolved oxygen, current control still strongly relies on quality by testing and on manual control, based on single measurements [148]. To elevate process control to an advanced level, current process monitoring and control strategies have to be transformed from using individual sensors into a systematic approach to monitor and subsequently control key parameters that cover the entire manufacturing process. This can only be done if a digital twin runs parallel to the respective process step or the entire process chain. These digital twins can consist of soft-sensors [149], real-time mass balances [150], state observers [151] or model based [152] or model predictive controllers [153]. Thus, data from appropriate process sensors have to be fetched into the underlying model. This makes the model capable of doing online prediction of process variances and allows an immediate control, ensuring increased process stability and surveillance, which is crucial to achieve consistent product quality.

Digital twins for process monitoring

After data collection using Process Analytical Technologies (see Section 'Process analytical technologies (PAT)'), the measured raw information needs to be converted into the desired monitoring outputs to achieve full process surveillance. Hereby, the mentioned PAT measurements, which are also schematically displayed in Fig. 2A, provide real-time information of the ongoing process, whereas the deployed model contains prior platform knowledge, which are mainly technical and biological relationships and boundaries of the system [154,155] in form of a mathematical process description. For the integration of the measurements, it is not important whether measurements are performed in-line, on-line, at-line

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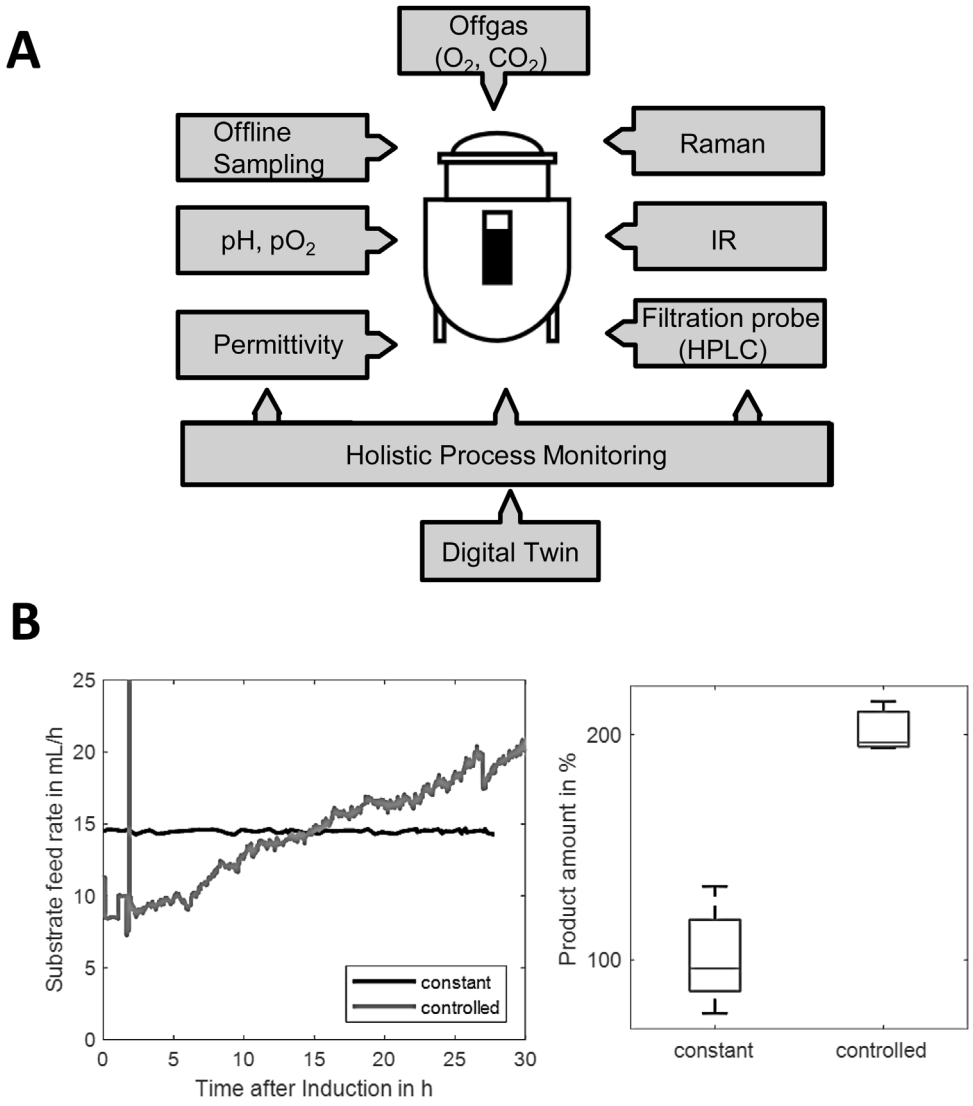

Fig. 2. (A) Possible PAT scenarios as digital twin inputs for bioprocess monitoring. (B) Possible effect of advanced process control demonstrated with an antibody fragment producing *E. coli* fed-batch process, compared to a constant feed rate during induction. With the same amount of applied feed, the product amount can be doubled, and the biological reproducibility increased.

or off-line, but it is important that they are able to detect deviation in time with the required accuracy [156].

To establish a digital twin for monitoring, different steps need to be considered [157]. Based on observability analysis [158] it can be analysed, which measurement combinations can be selected according to their information content [159]. Through subsequent simulation studies, required measurement intervals, measurement accuracies as well as effect of outliers on the expected monitoring output can be investigated [160].

Simple examples of successful, digital twin-based monitoring are based on mass balancing [161–163]. By considering the law of the conservation of mass, missing conversion rates can be determined. Under usage of off-gas balancing, Ahele et al. [163] showed to efficiently reduce batch-to-batch variations. In contrast to that, data-driven approaches based on latent variable techniques (principle component analysis) show similar possibilities towards golden batch monitoring [164].

By elemental balancing, substrate inflow, off-gas composition, biomass growth and concentrations [162,165], metabolic shifts [166] and conversion yields [162] can be directly calculated in carbon source limited fed-batch processes. Although, relying on first principle mass balances, these approaches do not include product information and other cell internal reaction mechanisms.

Models with underlying reaction kinetics, enable the description of cell internal behaviour [17]. The limiting factor of

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these models are often their over-parameterization, therefore such models need to be simplified towards the scope of application [167,168]. In addition, suitable filtering algorithms, such as the extended and unscented Kalman filter as well as particle filters [169,170] that are able to cope with non-linearities, have to be considered. Similarly to the above-mentioned elemental balances, simple growth models were able to correctly predict growth behaviour including standard online analytics such as off-gas composition [171,172].

In more recent publications, the monitoring scenarios were extended by near-infrared and Raman spectroscopic measurements [92,157]. The main target was to include product information, which was measured by NIR for ethanol [92] and by Raman for penicillin [157]. In combination with the underlying process model that describes the growth and production kinetics of the investigated yeast and *P. chrysogenum* species, accurate real-time monitoring of all relevant variables could be achieved. Besides taking the spectral information directly as a measurement input, Destro et al. showed a possibility to combine data-driven and knowledge-driven (mechanistic) models within a state estimator [173]. This extension led to better fault detection capabilities in continuous and fed-batch simulations.

Other approaches successfully dealt with the incorporation of delayed offline measurements for real-time monitoring [174–176]. Hereby, results from offline samples could be successfully used for different state estimators [169] or for the reparameterization of real-time models [177]. Other works included online and time-delayed offline analytics for accurate monitoring. To achieve this, minor and major model update steps had to be defined [175,176]. Based on this configuration, accurate surveillance of product formation in penicillin production processes was achieved with daily product measurements and a delay of 6 h [174,178]. For *E. coli* fed-batches a similar scenario enabled accurate and real-time determination of current transfer rates, which led to optimal supply of oxygen [179].

Advanced and predictive process control

After developing a digital twin for monitoring, feedback control can be based on the monitoring outputs and preventive control actions can be directly based on the digital twin [180]. Any deviations can lead to significant product loss, or, in worst case, to a failure of the whole batch. Therefore, beside of designing a process to deliver the aimed product quality according to Quality by Design, rigorous control and efficient event prediction tools are needed to continuously deliver the targeted quality [148,181].

Biochemical processes are very sensitive against changes in process parameters, so little overshoots in temperature or pH or even nutrient depletion can have an irreversible effect on the production organisms [181]. This sensitiveness requires to act in a predictive manner as corrective measures based on standard feedback controllers (e.g. step control or PID) can be too late in time or by wrong tuning or changing system dynamics, they can fail to rigorously converge to the aimed set-point [182]. Besides the tight control of environmental factors, cell- or product-specific variables, such as growth [165] and production rates [183] as well as by-product formation [182,184] or impurities are critical control variables. As microbial upstream processes are mostly operated in fed-batch rather than batch or continuous mode, the substrate feed rate is often the manipulated variable [185]. The potential effect of this manipulation can be followed on Fig. 2B, where the controlled, varying feed rate resulted in twice of the product amount compared to applying the constant feed rate, although the feed amount was the same in both cases.

In addition to the use of statistical control charts or latent variable control as valuable tools for golden batch control as shown for repetitive *P. pastoris* fed-batches [164], there is a trend towards model-based predictive control [148]. This advanced control methodology can optimize the manipulated variables towards the predefined goal, formulated as an objective function [153]. Today, examples for so called Model Predictive Control (MPC) [186] can be found in biotechnological applications. Dewasme et al. showed an MPC implementation for hybridoma cells [184]. The feed addition was controlled close to overflow to ensure optimal growth, which in a simulation study, including model and process uncertainties showed to significantly reduce variations in productivity. Yoo et al. [187] showed how an MPC can be used to optimize the lipid content in microalgae cultures. del Rio-Chanona et al. [177] implemented a self-optimizing model for optimal hydrogen production with cyanobacteria. Ulonska et al. [188] compared the performance of an MPC to model-based control in an *E. coli* fed-batch with the aim to control both glucose and lactose uptake, where the latter was used as inducer. The authors in [182] also compared different controllers in a penicillin production process and showed in real experiments that the MPC-driven process led to higher product concentrations and yields. The implemented PID and feed-forward controllers were only able to track the setpoints for substrate uptake as well as nitrogen and product precursor concentrations, whereas the MPC could foresee and prevent potential formation of side products. Within the field of downstream processes, model predictive control could be adopted for chromatography [189], where compared to manual operation higher purities and yields could be achieved.

Besides acting on single unit operations, integrated models can be used to supervise the whole process chain and can predict and foresee important adaptations and optimal conditions of subsequent steps to reach the target quality in the most efficient way [18].

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Conclusion

In this contribution, we discussed three cornerstones which will play a key role in the necessary adaptation of bioprocess development and manufacturing to current trends in the pharmaceutical industry. To our opinion, the targeted and tailored application of these tools will allow the development of robust manufacturing processes for biotherapeutics production and will subsequently contribute to enhanced development efficiency that will show an improvement in the competitiveness of microbial manufacturing technologies.

First, an overview on the available platform knowledge for microbial process development was provided. Examples for the capture and storage of generic biological and process knowledge in the form of mathematical models were shown. We postulate that proper knowledge management and data science tools should be used to condense the growing body of scientific knowledge into mathematical models to make generic platform knowledge easily accessible and to enable the utilization of true platform technologies for accelerated process development.

Second, enhanced process knowledge and facilitated process development can be achieved through the digitalization of process design and targeted process characterization. These tasks strongly rely on verified scale-down systems as well as in silico modelling approaches. In silico process models enable the effective utilization of platform knowledge and reduce the need of physical experiments that are required for the process understanding that can lead to process validation and dossier submission.

Third, the ultimate goal of bioprocess development is the definition of a process control strategy for the robust delivery of the product in the targeted quality and appropriate quantity. This goal requires the development of advanced and predictive control strategies. To utilize the full potential of digital twins, control strategies should involve automated decision and feedback control along the entire manufacturing process chain.

Our vision is that platform knowledge will be captured increasingly in mathematical process models, which will be maintained and continuously improved through feeding back the generated process knowledge throughout the entire lifecycle. This knowledge cycle should be supported by accessible and interconnected databases and the application of standardized knowledge management and data science tools. Easily accessible platform knowledge will be utilized in digital process development approaches and will ultimately lead to the acceleration of bioprocess development.

Chapter	Current challenge in bioprocess development	Proposed tool	Prerequisites for implementation	Additional potentials in application
Biological and Technological Platform Knowledge	Extraction of process knowledge from bioprocess datasets	Data preprocessing, multivariate analysis, model-based process evaluation	Data awareness, easily accessible data, software tools	Facilitated global data sharing and knowledge exchange, clearer representation of results
	Storage and management of gained knowledge	Mathematical models	Definition of model establishment and maintenance workflows	A change of paper and human based knowledge to digitalized, transferable and accessible knowledge, stored within process models
Scale Down Models and Digital Process Design	Numerous large scale batches until dossier submission due to limited applicability of small-scale process data	Validation of scale-down systems and characterization of potential scale-up effects	Scale-down systems and scale-up models need to be accepted by the regulatory authorities	Process transfer along identified and verified site-independent platform scales, scale-out scenarios enabling development and transfer at scale
	High number of batches required to identify process design space	Digital process design to identify operational points and parameter ranges for experimental designs	Predictive simulation model	Alignment of different modelling approaches (hybrid modelling) dealing with model and process uncertainty, further usage in process validation steps
Advanced Monitoring, Control and Automation	Process knowledge not available for real time interpretation of process data and optimal decision making	Soft sensors and state estimators converting raw data into reliable real-time information	High level of digitalization to fetch real-time data with platform knowledge	Decreased number of human control points and manual in process analytics, real-time batch release based on online evaluated data
	High demand of manual work due to the lack of automation and feedback control	Model based and predictive process control	Increased trust in process models and control tools, availability of industrial use cases, and roadmap for regulatory dossier filing	Application of model-based approaches from early development stage onwards and through the entire process lifecycle

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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