Using computational fluid dynamics simulation improves the design and subsequent characterization of a plug-flow type scale-down reactor for microbial cultivation processes

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Abstract

The scale-up of bioprocesses is still one of the major obstacles in biotechnological industry. Scale-down bioreactors were identified as valuable tools to investigate the heterogeneities observed in large-scale tanks in laboratory-scale. Additionally, computational fluid dynamics (CFD) simulations can be used to gain information about fluid flow in tanks used for production. Here we present the rational design and comprehensive characterization of a scale-down setup, in which a flexible and modular plug-flow reactor is connected to a stirred tank bioreactor. With the help of CFD the mixing time difference between differently scaled bioreactors were evaluated and used as scale-down criterium. Additionally, it was used to characterize the setup at conditions were experiments could technically not be performed. This was the first time a scale-down setup was tested on high cell density Escherichia coli cultivations to produce industrial relevant antigen-binding fragments (Fab). Reduced biomass and product yields were observed during the scale-down cultivations. Additionally, the intracellular Fab fraction was increased by using the setup. The results show that including CFD in the design and characterization of a scale-down reactor can help to keep a connection to production scale and also gain intensive knowledge about the setup, which enhances usability.

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Abbreviations:

Antigen binding fragment Fab

Asymmetry 10 Asymmetry at 10% peak height

Bo Bodenstein number

CDM Cell dry mass

CFD Computational fluid dynamics

CIP Cleaning in place

DO Dissolved oxygen

E. coli Escherichia coli

ELISA Enzyme linked immunosorbent assay

EMG exponential modified gaussian function

ϵ turbulent energy dissipation rate [m^2/s^3]

k kinetic energy unit

μ Growth rate [h^{-1}]

MP Measurement point

PFR Plug-flow reactor

Po Power number [-]

Re Reynolds number [-]

rpm revolutions per minute

RT Residence time

sL Standard liter

SM Static mixer

SSM Semi-synthetic medium

STR Stirred tank reactor

T Temperature

TNFα Tumor necrosis factor α

$Y_{X/S}$ Biomass to substrate yield

Abstract

The scale-up of bioprocesses is still one of the major obstacles in biotechnological industry.

Scale-down bioreactors were identified as valuable tools to investigate the heterogeneities observed in large-scale tanks in laboratory-scale. Additionally, computational fluid dynamics (CFD) simulations can be used to gain information about fluid flow in tanks used for production.

Here we present the rational design and comprehensive characterization of a scale-down setup, in which a flexible and modular plug-flow reactor is connected to a stirred tank bioreactor. With the help of CFD the mixing time difference between differently scaled bioreactors were evaluated and used as scale-down criterium. Additionally, it was used to characterize the setup at conditions were experiments could technically not be performed. This was the first time a scale-down setup was tested on high cell density *Escherichia coli* cultivations to produce industrial relevant antigen-binding fragments (Fab). Reduced biomass and product yields were observed during the scale-down cultivations. Additionally, the intracellular Fab fraction was increased by using the setup.

The results show that including CFD in the design and characterization of a scale-down reactor can help to keep a connection to production scale and also gain intensive knowledge about the setup, which enhances usability.

Introduction

Scale-up is still one of the major challenges in biotechnological production.[1] The main reason is, that when using the classical scale-up criteria considering, e.g. constant volumetric power input or impeller tip speed during the increase of bioreactor volume, it is not possible to keep at the same time also the mixing time constant.[2, 3] As a consequence, gradients of substrate, dissolved oxygen and other parameters develop in large scale tanks.[4, 5] Accordingly, the living organisms used in fermentation processes respond to these gradients, which can have several drawbacks on the bioprocess including a reduced biomass yield or increased side product formation.[4, 6, 7]

An approach to tackle this problem is the development of different scale-down setups, which simulate the heterogeneous conditions observed in large-scale using laboratory-scale systems.[8-10] Commonly applied techniques are pulse feeding of substances, like substrate into a stirred tank reactor (STR), [11, 12] or the use of two-compartment reactors. For the latter, different setups exist, where either two STRs are connected,[13, 14] or a STR is combined with a plug-flow reactor (PFR).[15-18] A comprehensive review about the use of several different scale-down setups can be found in Neubauer, et al.[19] In literature there are also reports, where more than two compartments can be used for such experiments.[20]

The flow in these devices is usually characterized by tracer pulse experiments with water, but these do not take into account the change of fermentation broth viscosity during cultivation.[16, 21] The achievement of direct linkage between the production-scale and the scale-down setup remains difficult as well, as experimental data about industrial production equipment is commonly rare.[1, 22] Computational fluid dynamics (CFD) modelling is considered to be a suitable tool to close this gap.[22, 23] Several studies were performed, where mixing inside large-scale tanks was described by using CFD[23-25]

Our aim was to develop a flexible and modular plug-flow reactor for scale-down purposes, which could be easily connected to a laboratory-scale bioreactor used for process development and optimization. The development was done in coordination with a production scale (4 m^3) bioreactor. The main scale-down criterion was based on the mixing time difference between a laboratory- and the production-scale bioreactor. The difference was used as mean residence time (RT) the cells spend inside the PFR. CFD simulation was not only used to calculate the mixing time in the two different scaled bioreactors, it was also used for detailed characterization of the PFR. RT distributions inside the main part of the plug-flow compartment were calculated taking different flow rates, fluid viscosities and the presence of static mixers (SM) into account. To the authors knowledge this is the first study where CFD was used to characterize and optimize the second compartment of a scale-down setup. Tracer-pulse experiments were done to compare and validate the CFD simulations.

To test the setup for an industrially relevant process, high cell density *Escherichia coli* (*E. coli*) cultivations producing antigen-binding fragments (Fab) were performed. Fabs have high potential for biopharmaceutical industry due to their less complex structure and the potential to be produced in cost efficient microbial cultivations compared to full length antibodies.[26, 27]Nevertheless, Fab production in *E. coli* is still challenging.[28, 29] This provides an optimal starting point to investigate scale effects on process efficiency by means of using the designed scale-down reactor.

Material and Methods

Design of the scale-down reactor

The STR (Bioengineering, Switzerland) used in this study had a maximal working volume of 20 L and was equipped with two six-blade Rushton type impellers. It was a stainless-steel tank with a height to diameter ratio of 2.8 and standard 25 mm Ingold ports for connection of sensors, as well as other equipment. For online monitoring and control a pH sensor (Easyferm Plus PHI ARC 120, Hamilton Bonaduz AG, Switzerland) and a dissolved oxygen (DO) sensor (Visiferm DO ARC 120, Hamilton Bonaduz AG, Switzerland) were used.

The authors are aware that plug-flow indicates an axial dispersion of 0,[30] which is hardly achievable in reality. Nevertheless, it is common practice in relevant literature[16, 20, 31, 32] to use the term PFR as synonym for tubular reactor. Therefore, the authors apply this practice in this work as well. The whole scale down setup can be seen in Figure 1 and Figure S 1, the following labeling is according to these figures. The main part of the PFR consisted of four insulated straight stainless-steel DN25 DIN tubes (7) (Bilfinger Industrietechnik Salzburg GmbH, Austria) connected via three stainless steel bows DN25 DIN (8) (Bilfinger Industrietechnik Salzburg GmbH, Austria). At the beginning and the end of this main part a sampling device (5 + 10) and a sensor device (6 + 9) (both SIBA Sonderanlagen GmbH, Austria) were located. In the sampling device up to three sensors could be mounted. The connection of the PFR to the STR was done with connectors aligned to two-way valves (2 + 13), which could be mounted in standard 25 mm Ingold ports. The transfer pipes were DN20 Pharmaline PTFE tubes (4) (Tecno Plast Industrietechnik GmbH, Germany) to withstand the sterilization and cleaning in place (CIP) procedure. For adding feed solution inside the PFR a T-shaped adapter could be mounted (Figure 1 B). For recirculation of the fermentation broth a peristaltic pump (3) (Masterflex I/P, with Masterflex HP pump head, Cole-Parmer, USA) was used. To compensate for variation of the flow rate due to abrasion of the pump hose (Masterflex Norprene Food, Cole-Parmer, USA) and change of fluid properties during the bioprocess, the flow inside the PFR was controlled via a sterilizable magnetic-inductive flowmeter (12) (Promag H300, Endress+Hauser, Austria). To enhance radial mixing inside the PFR, stainless-steel SM (Figure 1 C) (Stamixco AG, Switzerland) could be inserted inside the straight tube compartments. Their impact was studied by performing the experiments with and without the SM. Sterilization was done for 40 minutes with hot steam at 121 °C and 1.2 barg. For CIP the PFR was rinsed with sodium hydroxide, phosphoric acid and deionized water. Pressure was monitored via an inline pressure sensor (11) (Labom Mess- und Regeltechnik GmbH, Germany) and temperature (T) was measured with a resistance thermometer (Labom Mess- und Regeltechnik GmbH, Germany). At the two measurement points (MP) 1 and 2 pH (Easyferm Plus PHI ARC 120, Hamilton Bonaduz AG, Switzerland) and DO sensors (Visiferm DO ARC 120, Hamilton Bonaduz AG, Switzerland) were mounted. The volume of PFR was equal to 20.8% of the maximal working volume of the STR.

CFD characterization of STR and PFR

To characterize the flow field and intensity of the mixing in the STR and the PFR we performed CFD simulations of both by using the program Ansys Fluent v. 2021. The sketches of STR and PFR together with their geometries are presented in Figure 1 B and Table S 1. To reflect the measurement of the mixing time where no sparging was used, the CFD simulations were realized using single phase with water as the working fluid, having viscosity and density equal to 0.72 mPa.s and 994 kg/m^3, respectively. The flow in the STR was simulated using the realizable k -ϵ model [33]combined with the standard wall function to describe the flow in the boundary layer near the solid walls. Impeller rotation was modelled through sliding mesh approach. The simulations were performed for stirring speed ranging from 800 to 1500 revolutions per

minute (rpm) to cover typical cultivation conditions. To describe the flow by taking the complex internal structure of the impeller and all used probes into account, a mesh consisting of 4.7 million hexahedral elements was used. Mesh independence study confirmed no impact of further mesh refinement on the flow pattern. Since only small vortex formation was experimentally observed at the gas-liquid interface, the top interface was simulated via symmetry boundary conditions. The mixing time was simulated using time dependent evolution of tracer inside the STR.

Due to the complexity of the flow in the peristaltic pump, used to drive the fluid through the scale-down setup, only the main body of the PFR ranging from (5) to (10) in Figure 1 B was used for CFD modelling of the flow field and the tracer mixing time. Two geometries of PFR were considered. For the first one four SM were placed in the straight parts of the PFR (Figure 1 B), while in the second geometry the SM were omitted. The flow in the PFR was modelled via SST k -ωturbulence model.[33] To resolve the near-wall region the mesh was build such that more elements were located in this region resulting in total of 3.2 million polyhedral elements. Similar to the STR also in this case only single phase was considered for CFD simulations. This choice was supported by the positioning of the connector between STR and PFR at the bottom of the STR (Figure S 1). Due to this measure, only liquid was entering the PFR during cultivation process. In contrast to the STR, several variations of the fluid properties were considered in the simulations. In particular, we performed two simulations using the flow rates of 1.37 L/min and 4.11 L/min, which were compared with the experimentally measured time evolution of a tracer. After CFD model validation, we performed further simulations considering a faster flow rate, which was actually used during cultivation experiments. Additionally, two scenarios of fluid viscosity were considered as well. Firstly, 1 mPa.s representing the viscosity of the media at fermentation start. In the second case, we considered properties of fermentation broth, which was characterized at the end of the fermentation process. It was found that it changed its rheological properties from Newtonian to non-Newtonian with shear thinning behavior. To take this into consideration we adopted in our CFD simulation the viscosity dependency on shear rate as measured by Rheometer (viscosity [mPa.s] = 2.6+(6e6/(1+70000*shear rate [1/s]))). Under these conditions the viscosity was varying from 2.6 mPa.s up to 10 mPa.s in the PFR. Tracer viscosity in both cases was equal to 18 mPa.s, and thus reflecting high glucose concentration in the feed.

Tracer-pulse experiments

To validate the CFD simulation results of the mixing inside the 20 L STR, pulse experiments with a salt tracer were performed.[34] 20 mL of 4 M NH_4SO_4 solution were pulsed into the reactor filled with 20 L deionized water and tempered to 37 °C. A conductivity sensor (inLab 710 together with SevenExcellence conductivity meter, Mettler Toledo, Switzerland) was used to track the pulse response and the mixing time was determined when 95% homogeneity was reached.[22, 35, 36] The experiments were performed in three independent replicates for 800 rpm and 1200 rpm stirrer speed. Data acquisition was done with LabX direct pH3 software (Mettler Toledo, Switzerland).

In the case of PFR, RT experiments were performed using the setup with and without SM. A pulse injection of 20 mL 4 M NH_4SO_4 solution was done via the T-shaped feed addition point positioned directly after the first connector (Figure 1 B and Figure S 1). A conductivity sensor (inLab 710 together with SevenExcellence conductivity meter, Mettler Toledo, Switzerland) was used to monitor the resulting tracer peaks at the two measurement points MP1 and MP2 (Figure 1 B). The flow rate inside the plug-flow compartment was adjusted either to 1.37 L/min or 4.11 L/min. Due to short tracer RT and slow response of the conductivity probe, measurements at higher flow rates were not possible. Each experiment was performed in four replicates. Each data set was normalized between 0 and 1 prior to further evaluation. Evaluation was done with the program Peak fit (Systat Software Inc., USA), by fitting the exponential modified gaussian (EMG) function to the experimental data and obtaining the mean RT and the variance by calculation of the first and the second moment of the function. Additionally, to compare the shape of the resulting peaks, the peak asymmetry at 10% peak height (Asymmetry 10) was obtained as well. The obtained mean RTs were compared to the theoretical ones and the one calculated via CFD. The characterization of the flow behavior inside the PFR was done via a dimensionless number calculated according to Levenspiel,[30] taking the axial dispersion into

account. This number is considered as altered form of the Bodenstein number (Bo) by several different authors in this field[16, 21, 32] and is defined as:

$$Bo = \frac{2 * \tau^2}{{\sigma_\tau}^2}$$

Equation 1

where τ is the mean residence time and σ_τ is the variance of the residence time distribution. If the value of Bo is bigger than 10, the flow is considered to be plug-flow like.[32]

Strain and culture conditions

E. coli BL21(DE3) (New England Biolabs GmbH, USA) was used for this study. The integration of the Fab FTN2, which targets Tumor necrosis factor α (TNFα), into the production host's genome is described in detail in a previous publication.[37]

For cultivation the 20 L stainless-steel fully automated bioreactor already described above was used. As preculture, cells from glycerol cell banks were grown in 200 mL semi-synthetic media (SSM) in 2 L baffled flasks, at 37 °C and 180 rpm shaking frequency. The media composition was the same as described by Fink et al.[29] Approximately 280 mg of cell dry mass (CDM) were used to inoculate the bioreactor. During batch phase, the reactor contained 10 L media with following components calculated per g CDM: 94.1 mg/g KH_2PO4, 31.8 mg/g 85% H_3PO_4, 150 mg/g yeast extract, 41.2 mg/g Na_3-Citrate*2 H_2O, 46.0 mg/g $MgCl_2$*6 H_2O, 20.2 mg/g $CaCl_2$*2 H_2O, 45.3 mg/g NH_4SO_4, 50 µL/g trace element solution with the same composition as used by Marisch et al.[38]. Glucose*H_2O was added to achieve 120 g CDM by assuming a yield coefficient ($Y_{X/s}$) of 0.303 g/g. Additionally, 10 mL PPG 2000 were added to the batch medium as anti-foam agent. The feed solution consisted of the same composition as the batch medium, except for omitting yeast extract, NH_4SO_4 and PPG 2000. The amount of NH_4SO_4 required for the feed medium was additionally added to the batch medium. In the first feed phase the cells were grown with an exponential growth rate (µ) of 0.17 h^{-1} for 2.21 generations. This was followed by a second exponential growth phase with µ of 0.05 h^{-1}. With this feed profile 1506 g CDM should be achieved at fermentation end. Pulse induction was done 19 h after feed start with 1 µmol IPTG per g CDM calculated for the planned biomass at fermentation end. During batch phase temperature was set to 37 °C and was shifted to 30 °C at feed start. The DO was set to a minimum of 40% and was controlled by stirrer speed (800 rpm – 1200 rpm) and manual variation of aeration rate (5 standard liter (sL)/min – 25 sL/min), as well as variation of the headspace pressure (0.5 barg – 1.2 barg). During the whole process, the pH was maintained at 7.0±0.2 with the addition of 25% ammonia. The pH probes were calibrated with pH 4 and pH 7 buffer solution (Hamilton Bonaduz AG, Switzerland). For the DO sensors, a 2-point calibration was performed in Batch media at 37 °C, 0.25 barg, 5 sL/min aeration (100%) and after sparging with nitrogen (0%). For scale-down experiments performed with the combination of STR and PFR, the PFR was connected at feed start. For the cultivations without the PFR (reference), the feed solution was added directly in the STR. For the scale-down cultivations the feed was injected in the beginning of the PFR via the feed addition point (Figure 1 B). To investigate the effect of the SM on the cultivation, fermentations with and without the mixers were performed as well. To compensate for the SM-volume and to achieve the same mean RT, the flow rate was adapted accordingly. Each cultivation was performed in duplicates.

Off-line fermentation analysis

During reference cultivations and scale-down cultivations several samples were taken at the same time points for comparison. The description of gravimetrical CDM analysis and OD 600 measurements[38] as well as the sampling for product analysis[29] were already described in literature.

The cell lysis protocol using lysozyme and the Fab quantification by a sandwich enzyme-linked immunosorbent assay (ELISA) was done as described by *Fink et.al* [37]. Due to discontinuation of Anti-human IgG mouse antibody [2A11] (Abcam, Cambridge, UK), it was replaced for this study by Mouse Anti-human

IgG Fab secondary antibody [SA1-19255] (Thermo Fisher Scientific Inc., USA), which was diluted 1:800. For quantification of the intracellular Fab fraction the lysed cell pellet was analyzed, for the extracellular fraction the thawed supernatant was used. The sum of both fractions corresponds to the total amount of Fab produced.

Results

Mixing time in the stirred tank bioreactor (STR)

As the mixing time was our scale-down criterion, it was important to determine the mixing time in the 20 L STR. Therefore, a CFD model was created to calculate the mixing times at various stirring speeds. The operating conditions for the CFD simulation are summarized in Table 1.

According to the calculated impeller Reynolds number (*Re)* , which cover the range from 145967 to 273689, the flows in the STR were turbulent under all studied conditions. Despite the complex STR internal parts, the flow generated by the two Rushton impellers is dominated by strong radial pumping from the impeller blades towards the vessel periphery, followed by the formation of two circulation zones above and below each impeller (Figure S 2).[39] The radial pumping zone is characterized by the highest values of the turbulent kinetic energy (k) and turbulent energy dissipation rate (ϵ) (Figure S 2 C and D). Summary of the vessel averaged ¡ϵ ¿ together with the obtained Power number (Po)[40, 41] calculated from the torque acting on the impeller surface is presented in Table 1.

To get the mixing time, simulations of tracer mixing inside the considered system were performed. The example of the time evolution of normalized tracer mass fractions at four probe positions (Figure 1 A) is presented in Figure S 3. Due to the closer distance of the top probes to the point of tracer addition and formation of mixing zones by the action of Rushton impellers,[41] tracer mass fraction was the highest at the top part of the vessel, while the tracer mass fraction at the bottom of STR was gradually rising. Mixing time was determined when the normalized tracer concentration reached 95% vessel homogeneity at the last probe. The mixing times plotted as a function of ¡ϵ ¿ obtained for all simulated conditions in STR, follow a power-law scaling with the slope close to that measured experimentally by Nienow et al (Figure S 4).[41, 42] This scaling was used to determine the mixing time in the 20 L vessel at the minimum energy input used for the 4 m^3 bioreactor.

The obtained results were validated by tracer-pulse experiments. Even though experimental values are slightly lower than the calculated ones, they closely follow the same trend with decreasing the mixing time by increasing ¡ϵ ¿.[41, 42] Therefore, the difference between mixing time in laboratory scale STR and the large-scale STR at lowest energy input during operation was used as basis for defining the RT of the *E. coli* cells in the PFR.

Design of the scale-down reactor

The PFR (Figure 1 and Figure S 1) was designed to contain approximately 20 % (finally achieved 20.8 %) of the maximal working volume of the 20 L working volume STR, as this is considered to be a suitable value also in other studies.[16, 21, 31] To keep the connection to the large scale systems, we adjusted the RT of the cells inside the PFR to be equal to the mixing time difference between the 20 L and a 4 m^3 STR at minimum energy input during operation, which was in our case equal to 37 s. By taking the PFR volume and the mixing time difference into account, it resulted in a flow rate of 6.66 L/min. To handle this flow-rate the PFR was built from stainless-steel pipes in combination with rigid PTFE transfer tubes. This measure had also the advantage that sterilization and cleaning procedures could be implemented easily. To keep the setup still flexible and modular the connections between the different parts (tubes, bows, etc.) were done with standard Tri-Clamp connections. This enabled us to move the sensor, sampling and feed ports to various positions along the PFR. Additionally, the variation of the STR to PFR volume ratio could be easily adjusted by removing or adding pipe segments to the setup. The high flow rate made it necessary to acquire a powerful pump. For sterility purposes we used a peristaltic pump, with which we could vary the mean RT of the cells inside the PFR between 15 s and 8.3 minutes. This enabled us to achieve the planned 37 s RT, but also

added further flexibility to our setup. Stainless-steel SM could be inserted inside the straight stainless-steel pipes, which will enhance the radial mixing inside PFR and reduce axial dispersion, thus promote plug-flow behavior inside the reactor. The actual influence of the SM on the fluid flow behavior inside the tubular reactor, as well as the influence on*E. coli* high cell density processes is evaluated in this study. Gradients of substrate, pH and DO were built up during cultivation by adding feed solution at the entrance of the PFR. Temperature gradients were avoided by insulation of the pipes.

Characterization of the scale-down reactor

To verify, if the flow inside the tubular reactor can be considered as plug-flow and to analyze the RT distribution inside the plug-flow compartment, tracer-pulse experiments at two different flow rates were performed. Due to technical limitation of our conductivity probe, the flow rates were chosen to result in mean RT equal to 1 and 3 minutes, corresponding to laminar and nearly fully turbulent conditions (Table 2). The measurements were performed at MP1 and MP2 (Figure 1 B) using the PFR with and without the SM. In Table 2 the theoretical RT is compared with the mean RT of the resulting peaks. The variance and the Asymmetry 10 were shown and gave information about the peak width and shape. As Bo was in every case bigger than 10, the presented results confirmed the plug-flow-like behavior inside the tubular reactor.[32] The experimentally determined mean RT at 4.11 L/min fit very well to the theoretical value, additionally the peak variance got smaller at higher flow rate as expected. In contrast, at the low flow rate non-ideal reactor behavior existed as the peak was passing the second measurement point MP2 some seconds earlier as expected. The data also showed that the SM reduced the axial dispersion and thus kept the RT distribution narrow. This can be seen when comparing the variance, the experimental error, the Asymmetry 10 and the Bo number. Without SM a much more pronounced peak tailing was observed, especially at low Re number.

Since the slow response of the conductivity probe prevented the execution of tracer pulse experiments at the flow rate used in scale-down cultivations, a CFD simulation of the PFR was performed instead. By validation of the CFD simulation for the above discussed flow rates, the model could be used to extrapolate for the higher flow rate. As the experimental and the modelling setup were different (we did not consider to model the transfer tubes and the peristaltic pump), the comparison was done by evaluation of the time when the tracer passed between MP1 and MP2 (Table 2 B). Furthermore, the tracer signal obtained at position MP2 was used to evaluate the variance of the distribution for both the model and the experiments.

The mean RT between MP1 and MP2 fit very well between the theoretical, experimental and modelled values at the higher flow rate. For the lower flow rate the tracer passed earlier than the theoretical and CFD modelled values would suggest. However, as the experimental error for these conditions was quite large, the relative deviation was considered to be acceptable. Please note, that the absolute values for variance cannot be compared directly as the tracer in the experimental setup had to pass a longer distance, but the trend was the same. Variance decreased significantly by using higher flow rate and SM, as it was determined experimentally. Therefore, these results confirm that the CFD model could be used to predict the flow behavior inside the PFR especially for higher flow rates.

Additionally, an example of the contour plot of velocity magnitude, turbulent kinetic energy and ϵ obtained for above mentioned flow rates is presented in Figure S 5, Figure S 6 and Figure S 7. The impact of the SM on the radial mixing inside PFR was clearly seen, particularly in the horizontal parts of the tubular reactor. This mixing enhancement is further documented in Figure S 8 A and B, where the time evolution of the tracer mass fraction at MP1 and MP2 is presented. While tracer distribution at the tubular reactor inlet was rather narrow, the axial dispersion increased along the flow direction resulting in broader tracer distribution at the MP2. Furthermore, this distribution became even broader with tailing for PFR without SM.

CFD for flow extrapolation

Once validated, the model was used for an extrapolation of the flow rate with 6.66 L/min, but also to study the effect of varying fluid viscosity. Two limiting cases of fluid viscosities, covering constant viscosity of media with a value of 1 mPa.s and shear rate dependent viscosity at fermentation end, were used. Comparison

of the tracer mass fraction as a function of time is shown in Figure 2. It can be seen, that while for low viscosity of the fluid there was only small difference in the tracer mass fraction profile at MP2, at higher fluid viscosity there was a big improvement of the mixing when the SM was used. This is indicated by the more symmetric and narrower peak that occurs when using SM.

Scale-down and reference cultivations

To test the performance of the scale-down setup under production conditions, high cell density *E. coli* cultivations to produce Fab were performed with (scale-down) and without (reference) the tubular reactor connected to the STR. Additionally, the influence of an altered RT distribution on the bioprocess was evaluated in experiments with and without SM.

The use of the scale-down setup with SM in comparison to the standard laboratory scale cultivation using only the STR (reference) lead to a biomass yield ($Y_{x/s}$) reduction of 11% (Figure 3 A). Only 65 g/L CDM were reached instead of 73 g/L. The omittance of the SM led to a further decrease of 2%. Nevertheless, as this change was in the range of the experimental error, it could be concluded that a broader RT distribution did not influence the biomass yield to a high extent

To investigate the influence of the scale-effects on the recombinant Fab production, we used the intracellular and extracellular Fab yield. The total amount of Fab was the sum of both fractions. When using the scale-down setup, the total specific Fab yield was reduced by 20% (Figure 3 B). In fact, accounting also for the biomass reduction a volumetric total Fab yield reduction of 28% compared to the reference cultivation without the PFR was observed. The broader RT distribution induced by the removal of the SM decreased the yield further, but again just marginally. Interestingly, the extracellular Fab fraction was larger during reference cultivation compared to the scale-down setup (Figure 3 D). For the reference, 26% of the specific total Fab was found in the extracellular fraction, whereas for the scale-down cultivations it was only around 1%.

Interesting insights in the heterogeneities induced by the PFR were gained by the online data of pH and DO at different positions along the scale-down setup (Figure 3 D and E). 9 h after feed start, the feed profile was changed from an exponential growth rate of $\mu = 0.17$ h^{-1} to $\mu = 0.05$ h^{-1}. This switch was reflected by a sharp increase in both pH and DO online data. At the end of the first growth phase, an acidification of 0.3 pH units were seen after 33 s (MP2) in the PFR. In the second growth phase, the pH continuously decreased as the fermentation proceeded and reached a value of 6.9 at fermentation end.

At the end of the first feed phase, the DO was consumed in the PFR already after 5 s mean RT (MP1). By switching to a lower growth rate, the oxygen consumption in the PFR was lowered as well. The oxygen concentration was decreasing until the end of the cultivation. At MP2, the dissolved oxygen concentration in the PFR was 0% along the whole cultivation course.

Discussion

In this work, we designed and constructed a flexible and modular plug-flow type scale-down reactor that can be easily adapted to various conditions and research questions. The relative long oxygen limited zone (Figure 3 F) mimics the feeding zone in large industrial scale reactors, where oxygen depletion can occur, especially at high cell densities.[2, 5, 19] The size of this zone can also vary with the feed rate, as more substrate leads to higher metabolic activity, higher oxygen consumption and therefore bigger oxygen depleted zones. This mechanism can be mimicked by our scale-down setup as well. Additionally, a pH gradient along the PFR was reported (Figure 3 E). The observed acidification could be explained by the production of organic acids and CO_2.

In contrast to other publications, we also investigated the influence of scale-effects on an industrially relevant Fab production process. In these experiments we could demonstrate a reduction in $Y_{X/s}$ of 11%. This is in accordance with Bylund et al.[4] who reported a biomass reduction of 15%- 20% by scaling up a 30 g/L *E.coli* process from 15 L to 8 m^3. We could show that the total Fab production was reduced by the scale down setup and that the distribution between intra- and extracellular Fab was altered. The reduction of recombinant

protein production caused by scale-effects was already reported in literature.[13] Nevertheless, the shift to higher intracellular product when using a scale-down setup is a notable finding. It could be explained by the higher productivity during the reference cultivations and the resulting higher metabolic load for the cells, that lead to the release of product from the periplasm by cell lysis. However, it could also be attributed to an increase in cell robustness originating from varying conditions in the scale-down setup. Existence of this effect was reported previously by several authors performing flow cytometry with different staining methods during*E.coli* cultures.[17, 43, 44] Brognaux et al[44] linked this behavior to a higher membrane permeability during well-mixed fed-batch cultivations. With respect to product purification, the increase of cell robustness could lead to beneficial effects, as almost the whole amount of product is then located in one compartment. The absence of cell lysis would further facilitate subsequent downstream operations.

The CFD model of the STR is in closed agreement with Rutherford et al.[39] , who experimentally measured *Po* for two Rushton impellers with parallel flow configuration, supporting the turbulent conditions. Additionally, the determined mixing time difference of 37 s between the different scales is in a reasonable range as mixing times (t_{95}) of approximately 50 s for a 12 m^3 and 150 s for a 30 m^3 scale STR with Rushton impellers at comparable specific power input were reported.[45]

To characterize the scale-down setup even further we did not only use standard tracer-pulse experiments, but also CFD simulation to model a flow rate, where experiments could not be performed due to technical limitations. Furthermore, the influence of SMs were investigated, as well as changes in fluid viscosity were addressed, which was not considered in previous work. The omittance of the SM lead to asymmetric peaks, suggesting strong segregation of the flow. This is documented by the contour plot of the tracer mass fraction presented in Figure S 9 and Figure S 10. While nearly no difference in tracer profile was observed for low viscosities there was clear segregation of the flow in PFR without static mixer, under high viscosity conditions. This not optimal behavior at low *Re* numbers could also lead to dead zone development, which would be an explanation for the earlier tracer passing observed during the experiments with the lower flow rate. These results do not only provide valuable information on the setup at real cultivation conditions, they can also be used to further optimize the design of the scale-down reactor. In particular, Figure S 10 indicates possible improvement of the bow geometry to reduce the segregation for highly viscose media. Particularly, a more radial shape of the bows or additional mixers in this section would avoid this not optimal behavior of tracer by-passing especially at high viscosities and low Re numbers.

With the extensive knowledge gained about the setup, we were able to investigate how a broader RT distribution would influence the bioprocess, by removing the SM for cultivation. As reported by Limberg et al.,[31] who compared a STR-STR with a STR-PFR scale down setup, no significant impact of the RT distribution on biomass and product formation could be shown. In our experiments a possible explanation for this behavior could be that the viscosity of the fermentation broth is increasing over time and that the broader RT distribution will develop only at a later stage of the cultivation, which might be too short to make a difference. Another more general explanation could be that we and Limberg et al.[31] followed a bulk approach, as we analyzed samples from the whole fermentation broth. This means that on average all the cells were the same time inside the PFR and no effect on population average dependent parameters just as biomass yield can be seen. Nevertheless, the heterogeneity on microbial level could still be altered.[46] More experiments need to be done to answer this question.

More knowledge about the conditions in industrial large-scale bioreactors is needed as well, to adapt the scale-down setup even further. This is pointed out in a recent review from Nadal-Rey et al.[47] as well, where also the importance of computational methods in this field is mentioned. We have shown that computational methods are not only necessary to gain information about the large scale itself, but also about the tools that should be used to mimic these conditions. This knowledge can enable engineers and scientists to optimize scale-down tools further. In the future even more collaborations between industry and academia will be necessary to solve the problems that occur during scale-up. This close connection will make it possible to mimic the inhomogeneous conditions in industrial scale and speed up bioprocess development, especially scale-up. The broad flexibility offered by the introduced scale-down setup can make it to a powerful tool for

further studies in this direction.

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Conflict of interest statement

The authors declare no commercial or financial conflict of interest.

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Tables

Table 1: Summary of operating conditions of the STR including also the mixing times determined by CFD simulation and tracer-pulse experiments. The deviations indicate the experimental uncertainty.

N [rpm]	Re [-]	V_{tip} [m/s]	¡ϵ¿ [m^2/s^3]	Po [-]	t_{CFD} [s]	t_{exp} [s]
800	145967	3.7	6.7	10.2	8.2	6 ± 1
1200	218951	5.6	22.8	10.2	5.7	4 ± 1
1500	273689	7.0	44.4	10.2	4.6	-

Table 2: Experimental results (A) and CFD validation (B) of the PFR characterization. The results show the average of 4 replicates, the deviations are indicated by ± standard deviation of the 4 independent replicates.

A	**Theoretical RT [s]**	**Mean RT [s]**	**Variance [s^2]**	**Asymmetry 10**	**Bo [-]**	**Re [-]**
1.37 L/min MP1	29	31 ± 1	60 ± 11	2.3 ± 0.1	33 ± 4	1286
4.11 L/min MP1	10	11 ± 0	3.6 ± 0.3	2.3 ± 0.1	71 ± 9	3858
1.37 L/min MP2 + SM	150	141 ± 5	137 ± 30	1.9 ± 0.2	305 ± 65	1286
4.11 L/min MP2 + SM	50	53 ± 0	12.4 ± 1.7	2.1 ± 0.2	457 ± 57	3858
1.37 L/min MP2	160	137 ± 17	732 ± 157	3.9 ± 0.3	52 ± 3	1286
4.11 L/min MP2	53	57 ± 1	51 ± 11	2.4 ± 0.4	131 ±-22	3858
B	**Mean RT: MP2 – MP1 [s] Theoretical**	**Mean RT: MP2 – MP1 [s] Exp.**	**Mean RT: MP2 – MP1 [s] CFD**	**Variance MP2 [s^2] Exp.**	**Variance MP2 [s^2] CFD**	**Variance MP2 [s^2] CFD**
1.37 L/min + SM	121	110 ± 6	125	137 ± 30	109.4	109.4
4.11 L/min + SM	40	42 ± 0	41	12.4 ± 1.7	7.7	7.7
1.37 L/min	131	106 ± 18	127	732 ± 157	275.1	275.1
4.11 L/min	43	46 ± 1	44	50.7 ± 11.3	19.09	19.09

Figure legends

Figure 1: Illustration of the scale-down reactor setup. The inside of the STR with the probe positions for CFD mixing time simulation (A) and the whole scale-down setup with corresponding labelling (B) are shown. The values of the dimensioning can be found in Table S 1.

Figure 2: Comparison of the tracer mass fraction as a function of time at position MP2 in the PFR. For constant fluid viscosity of 1 mPa.s (A) and shear rate dependent viscosity at fermentation end (B).

Figure 3: Overview of the cultivation results. The biomass concentration (A), the total produced Fab (B), the intracellular Fab fraction (C) and the extracellular Fab fraction (D) are shown. Data points represent the average of biological duplicates. The error bars show the deviations to the average of the individual experiments. The online data for the pH (E) and DO values (F) are given as well. In F the DO values for MP2 were at 0% during the whole cultivation.