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High-performance recombinant protein production with *Escherichia coli* in continuously operated cascades of stirred-tank reactors

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Abstract The microbial expression of intracellular, recombinant proteins in continuous bioprocesses suffers from low product concentrations. Hence, a process for the intracellular production of photoactivatable mCherry with *Escherichia coli* in a continuously operated cascade of two stirred-tank reactors was established to separate biomass formation (first reactor) and protein expression (second reactor) spatially. Cascades of miniaturized stirred-tank reactors were implemented, which enable the 24-fold parallel characterization of cascade processes and the direct scale-up of results to the liter scale. With PAmCherry concentrations of 1.15 g L^{-1} cascades of stirred-tank reactors improved the process performance significantly compared to production processes in chemostats. In addition, an optimized fed-batch process was outperformed regarding space–time yield (149 mg L^{-1} h^{-1}). This study implicates continuous cascade processes to be a promising alternative to fed-batch processes for microbial protein production and demonstrates that miniaturized stirred-tank reactors can reduce the timeline and costs for cascade process characterization.

Keywords Cascades of stirred-tank reactors · Continuous culture · *Escherichia coli* · High-throughput bioprocess design · Protein expression

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Introduction

Continuous bioprocessing is commonly considered as a promising alternative to batch and fed-batch bioprocessing. Major advantages are the often higher space–time yield (STY) and consequently reduced plant dimensions, the possibility of continuously removing unstable products from the reaction medium, and the increased operational flexibility [4]. Although continuous processes are frequently employed for low-value products such as single cell protein or ethanol [11, 21], continuous production processes for high value products such as recombinant proteins are not state of the art for industrial applications [4]. Besides the well-known challenges of continuous bioprocesses like the long-term operability and assurance of sterility, the cost-intensive and time-consuming development and optimization of efficient processes restrict the broad applicability of continuous bioprocesses in industry [4, 9]. In addition, the continuous exchange of medium will reduce the achievable product concentrations and consequently the economics of the downstream processing, if the biocatalyst respective products are not retained in the bioreactor by, e.g., filtration [1, 2].

In particular, the continuous production of intracellular recombinant proteins is challenging, because a separation of the retention times of the product and the biocatalyst is impossible. Furthermore, recombinant protein expression will implicate a considerable metabolic burden to the cells, if strong promotors—e.g. the Isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible T7 RNA polymerase system for *Escherichia coli* (*E. coli*)—are employed [18–20]. Consequently, the growth of cells is inhibited and genetic instabilities of cells occur due to the strong selection pressure [4, 13]. These issues are circumvented by temporally

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separating the growth phase and the production phase using inducible expression systems in commonly applied batch or fed-batch processes [5, 15, 20]. In general, batch and fed-batch processes are started by the inoculation of the stirred-tank reactor with a defined cell concentration. To achieve stable reaction conditions, pH, temperature, and dissolved oxygen concentration are frequently maintained constant throughout the processes. The provided batch substrate is metabolized for cell growth. Subsequently, fed-batch mode can be initiated to increase the cell concentration. Recombinant protein expression can be induced at a defined process time or at defined cell concentrations to avoid a negative influence of the protein expression on the initial cell growth [e.g. 5, 15, 20].

Regarding continuous bioprocessing, growth and protein expression can be decoupled by separating the two process phases spatially, applying a cascade of two sequentially operated continuous bioreactors. In particular, the first reactor is used for the growth of cells, whereas the protein expression is induced in the second reactor [7, 10, 13]. However, the use of cascades of bioreactors for recombinant protein expression is restricted to academic studies so far. To bring this technology to industrial application, robust processes gaining high product concentrations and space–time yields have to be implemented. As the development and optimization of continuous processes is time-consuming and cost-intensive [4, 9], there is a high demand for parallel and miniaturized bioreactors offering a high degree of freedom regarding the mode of operation to overcome these limitations [22].

Hence, a well-characterized, miniaturized bioreactor system for the continuous operation of up to 48 parallel continuously operated stirred-tank reactors with a reaction volume of 10 mL [17], will be modified for the operation of a cascade of two continuous stirred-tank reactors. In this study, a process for the production of the recombinant protein photoactivatable mCherry (PAmCherry) with *E. coli* BL21 (DE3) under the control of the T7 RNA polymerase system in a cascade of two continuously operated stirred-tank reactors will be characterized on a milliliter scale. The first reactor of the cascade will be applied for the formation of biomass. The produced cells will be transferred to the second reactor, where the recombinant protein expression will be induced. Selected processes will be transferred to the liter scale to verify the scalability of milliliter scale results. Subsequently, the production process will be improved at liter scale.

Materials and methods

Bacterial strain

An *E. coli* BL21(DE3) strain harboring a pET28a(+) plasmid with the gene for PAmCherry (Clontech, Saint-Germain-en-Laye, France) carrying an additional His_6-tag was used for all bioprocesses. The strain is henceforth abbreviated as *E. coli* PAmCherry.

Media, seed culture, and inoculation

Seed cultures were grown either in defined medium containing 2 g L^{-1} glucose or in low-salt LB medium (5 g L^{-1} yeast extract, 10 g L^{-1} peptone, 5 g L^{-1} NaCl).

All bioprocesses on a milliliter and liter scale were carried out in defined medium according to Riesenberg et al. [14], which was modified for the applied strain by Schmideder et al. [17]. Glucose served as batch substrate at a concentration of 25 g L^{-1}. Ammonia [12.5% (v/v)] was used to adjust the pH to 7.0. The defined medium was also used as feeding medium with 25, 50 or 500 g L^{-1} glucose. IPTG was added to the feeding solutions in the desired concentration for the induction of protein expression. All reagents were of analytical grade from various suppliers.

Seed culture preparation and inoculation were performed as described previously [17].

Bioreactors, process monitoring, and control

For bioprocesses on a milliliter scale, two continuously operated stirred-tank reactors on a milliliter scale implemented by Schmideder et al. [17] were connected and operated as a continuous cascade of two stirred-tank reactors (see Fig. 1). Sterile single-use stirred-tank reactors with baffles (provided by 2mag AG, Munich, Germany) were operated with gas-inducing stirrers [8, 12] in a parallel bioreactor system (bioREACTOR 48, 2mag AG, Munich, Germany) at 3000 rpm. The temperature was maintained to 30 °C. Dissolved oxygen was monitored and the pH was maintained to 7.0 with ammonia [12.5% (v/v)] making use of a liquid handler (Genesis, Tecan GmbH, Crailsheim, Germany) and the software fedbatch XP (DASGIP–an Eppendorf company, Jülich, Germany) [16]. A continuous flow of sterile air was delivered to the headspace of each reactor at a rate of 0.1 L min^{-1}. Losses in liquid volume by evaporation were prevented by saturating the inlet air with water at room temperature and setting the headspace cooling of the reactors to 20 °C [16, 17]. Multi-channel peristaltic pumps (MP8, DASGIP—an Eppendorf company, Jülich, Germany) were used for the feeding and efflux of medium. A topping with apertures for the pipet tip of the liquid handler and three bent micro-pipes was manufactured of the autoclavable and chemically stable material polyetheretherketone and was integrated into the exhaustion unit of the parallel bioreactors. Micro-pipes for feeding extended below the liquid surface of the reaction medium, whereas the micro-pipes for the removal of medium were fixed at a defined height to maintain the reaction volume

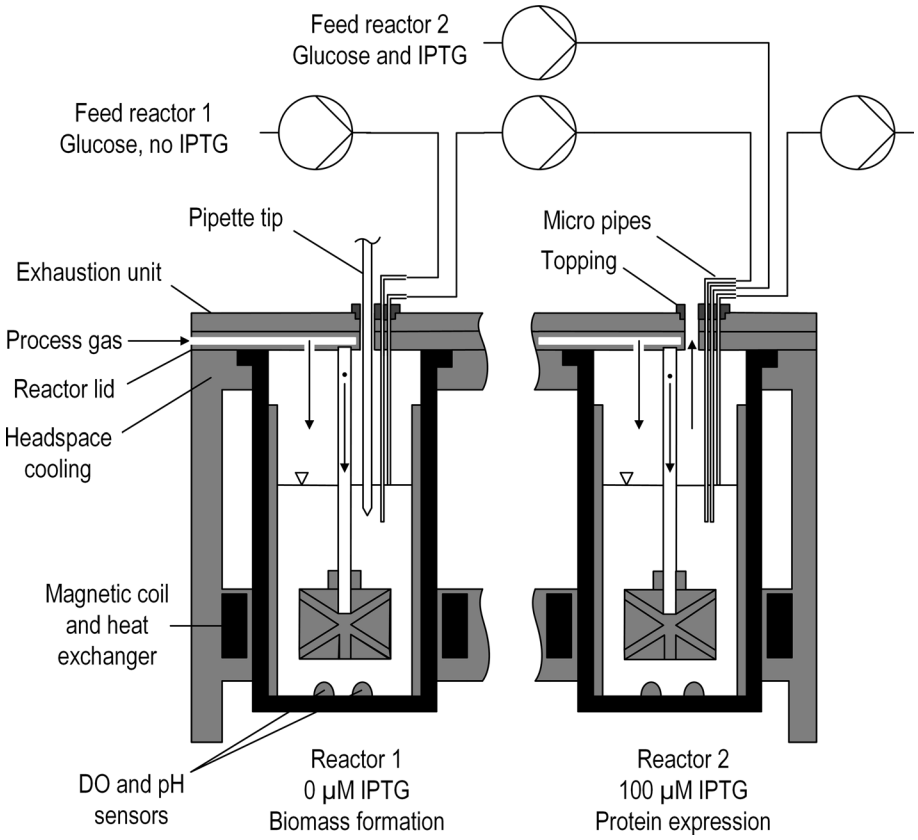

Fig. 1 Schematic setup of a cascade of two continuously operated stirred-tank reactors on a milliliter scale. Shown is one cascade of 24, which can be operated in parallel. The first reactor was used for biomass formation (no IPTG) at a preset dilution rate. The culture broth was transferred to the second reactor, where the expression of the recombinant protein was induced by the addition of 100 µM IPTG at the beginning of the continuous operation. Defined medium with glucose and variable concentrations of IPTG was fed to the second reactor to realize the supply of a carbon source and to keep a constant inducer concentration of 100 µM throughout the continuous operation

to 10 mL [17]. The maximal oxygen transfer rate of parallel stirred-tank bioreactors with a liquid volume of 10 mL and an impeller speed of 3000 rpm was estimated to be 270 mmol O_2 L^{-1} h^{-1} based on the oxygen transfer coefficient $k_L a$ of 0.35 s^{-1} (unpublished data) and an oxygen saturation concentration of 7.0 mg L^{-1} at 30 °C.

Bioprocesses on a liter scale were performed in parallel stirred-tank reactors with a nominal volume of 1.5 L (DASGIP®Bioblock, DASGIP–an Eppendorf company, Jülich, Germany). Each reactor was equipped with two six-blade Rushton turbines. Ammonia [12.5% (v/v)] was used to maintain the pH to 7.0 and the dissolved oxygen concentration was maintained above 20% air saturation by increasing the stirrer speed up to 1200 rpm, the aeration up to 8.0 vvm, and the partial pressure of oxygen in the process air up to 0.3 bar. Multi-channel peristaltic pumps (MP8, DASGIP—an Eppendorf company, Jülich, Germany) were used for the feeding and efflux of medium. In each case, two continuously operated stirred-tank reactors were connected to realize a two-stage cascade.

Cultivation protocols

Cultivation protocols for the identification of suitable process conditions and the evaluation of the scalability of bioprocesses were identical at milliliter and liter scale. The reaction volume of both reactors of each cascade was set to 10 mL and 0.5 L, respectively. After a batch phase (25 g L^{-1} glucose), the continuous mode was started with 25 g L^{-1} glucose in the feeding medium. The first reactor for biomass formation was operated at preset dilution rates of 0.2 and 0.3 h^{-1}, respectively. The efflux of the first reactor was transferred to the second reactor of the two-stage cascades to ensure the supply of the second reactor with exponentially growing cells. The expression of the recombinant protein was induced by the single addition of 100 µM IPTG to the second reactor at the beginning of the continuous process phase. Defined medium with a glucose concentration of 25 g L^{-1} was fed to the second reactor at a dilution rate of 0.1 h^{-1}. In addition, IPTG was added to the feeding medium of the second reactors to final concentrations of 300 and 400 µM, respectively, to maintain a constant inducer concentration of 100 µM in the second reactor throughout the continuous operation. Hence, dilution rates of 0.3 and 0.4 h^{-1} were achieved in the second reactor, which corresponds to dilution rates of 0.15 and 0.2 h^{-1} of the two-stage cascades. A schematic sketch of the process configuration at milliliter scale is depicted in Fig. 1.

An improved process for the production of PAmCherry in a two-stage cascade of stirred-tank reactors was performed on a liter scale. The reaction volume was set to 0.5 L for the first reactor and 1.0 L for the second

reactor, respectively. After a batch phase (25 g L^{-1} glucose), an exponential feeding of glucose was initiated with 3.5 g L^{-1} h^{-1} to maintain the preset growth rate to $\mu_{set} = 0.15$ h^{-1}. After 5.3 h, the maximum feeding rate of 7.4 g L^{-1} h^{-1} of glucose was achieved. Hence, a total of 50 g L^{-1} of glucose was supplied to each reactor for the initial biomass formation. Subsequently, the continuous operation with 50 g L^{-1} glucose in the feeding medium was started. The dilution rate of the first reactor was set to 0.2 h^{-1}. Induction of the protein expression was performed by adding 100 µM IPTG to the second reactor at the beginning of the continuous operation. Furthermore, defined medium containing 50 g L^{-1} glucose and 200 µM IPTG was fed to the second reactor at a preset dilution rate of 0.1 h^{-1}. Consequently, the dilution rate of the second reactor and the two-stage cascade were 0.2 and 0.13 h^{-1}, respectively.

Analytics

The measurements of the optical density at 600 nm (OD_{600}), the cell dry weight (CDW),the PAmCherry concentration as well as the enzymatic determination of the acetate, and the glucose concentration at milliliter and liter-scale were performed as described previously [15–17].

Results

Parallel bioprocess characterization on a milliliter scale and transfer to liter scale

The production of the recombinant protein PAmCherry with *E. coli* was characterized in parallel, continuously operated cascades of two stirred-tank reactors on a milliliter scale. A batch phase (25 g L^{-1} glucose) was conducted in both reactors of the cascades for initial biomass formation, before the continuous operation (25 g L^{-1} glucose in feeding media) was started. The first reactor of the cascade was operated without induction of recombinant protein expression at a dilution rate of $D = 0.3$ h^{-1}. The exponentially growing cells were continuously transferred to the second reactor. After addition of 100 µM IPTG to the second reactor to induce recombinant protein expression at the beginning of the continuous operation, medium containing 400 µM IPTG was fed to the second reactor at a dilution rate of $D = 0.1$ h^{-1} to maintain the IPTG concentration to 100 µM. Hence, the dilution rate of the cascade was maintained to 0.2 h^{-1}.The process was carried out in duplicate to characterize the parallel reproducibility of steady state studies on a milliliter scale.

A constant CDW of 11.0–12.5 g L^{-1} was observed after the start of the continuous operation in the first reactor of the cascade with a dilution rate of $D = 0.2$ h^{-1} (see Fig. 2), whereas a slight increase of the CDW up to 13.3 g L^{-1} until one hydraulic residence time could be detected in the second reactor. Subsequently, the CDW decreased to a constant steady state concentration of 11.3 g L^{-1}. As expected, only basal expression of PAmCherry (<10 mg L^{-1}) was detected in the first reactor without addition of inducer. In contrast, the PAmCherry concentration increased to a maximum of 298 mg L^{-1} after 1.8 residence times in the second reactor, before a steady state concentration of 261 ± 9 mg L^{-1} was achieved, which corresponds to a STY of 52 mg L^{-1} h^{-1}. The growth of cells was clearly limited by glucose throughout the whole continuous operation as glucose concentrations in the medium of <50 mg L^{-1} were below the substrate affinity constant of 90 mg L^{-1} of glucose for the employed *E. coli* strain [17]. Furthermore, concentrations of the inhibitory by-product acetate were below 0.1 g L^{-1}, whereby a negative influence of acetate on the growth and product formation could be excluded [6]. The parallel reproducibility of continuously operated cascades of two stirred-tank reactors on a milliliter scale could be demonstrated, as the dynamics of biomass and PAmCherry production in the two parallel cascades were equivalent within the estimation errors. Furthermore, high precision regarding the preset dilution rates and reaction volumes (deviation <3%) was demonstrated.

Further bioprocesses were performed to evaluate the influence of the dilution rate on the protein production. Thereby, the dilution rate of the first reactor of the cascade was reduced to 0.2 h^{-1} ($D_{cascade} = 0.15$ h^{-1}) and the IPTG concentration in the feeding medium of the second reactor was reduced to 300 µM to obtain a constant inducer concentration of 100 µM. After approximately three residence times, constant biomass concentrations of 10.8–11.6 g L^{-1} and protein concentrations <12 mg L^{-1} were detected in the first reactor of the cascade (see Fig. 3). In the second reactor, a constant biomass concentration of 10.8 ± 0.4 g L^{-1} was measured after 3.5 residence times. A PAmCherry concentration of 502 ± 36 mg L^{-1} was reached at steady state. In summary, the process performance of the production of PAmCherry with *E. coli* could be improved regarding product concentration (192%), STY (144%), product selectivity (Y_{PS}, 192%), and the product yield coefficient (Y_{PX}, 204%) by decreasing the dilution rate of the cascade from 0.2 to 0.15 $h^{-1}$and consequently increasing the residence time of cells in the second reactor for protein expression of the cascade from 2.5 to 3.3 h (see Table 1).

The direct scalability of cascade processes from milliliter to liter scale with the maximum oxygen transfer rate at the milliliter scale as scale-up criterion could be demonstrated, as the dynamics of biomass production as well as recombinant protein expression was identical within the

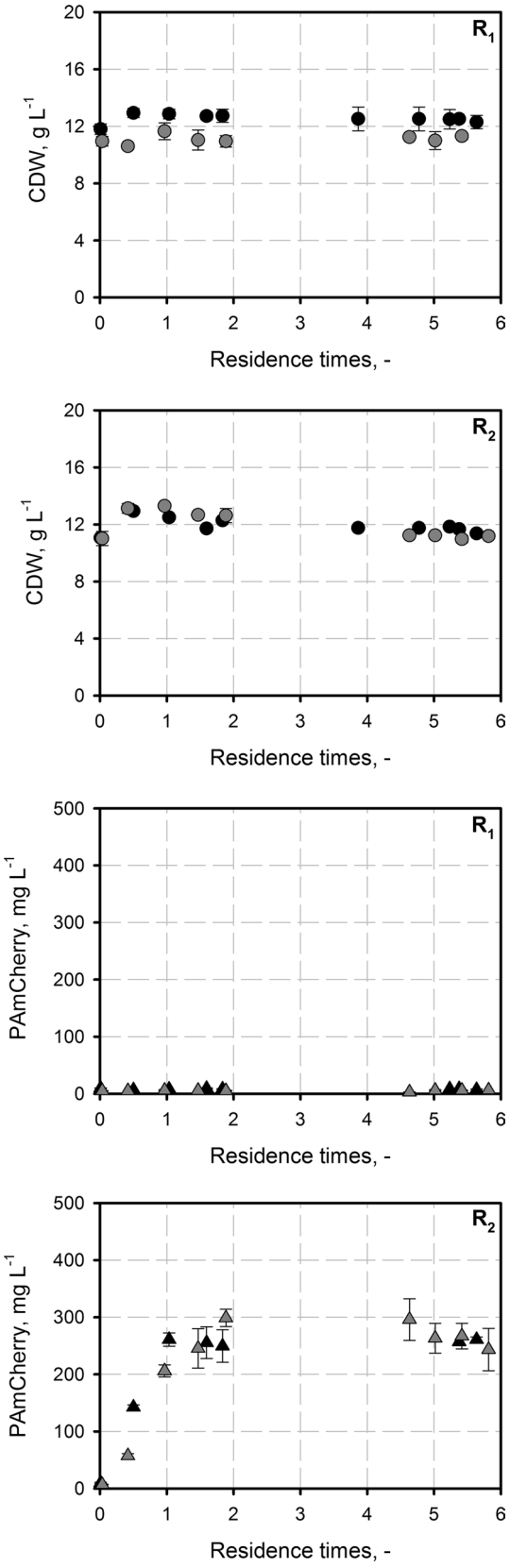

Fig. 2 Parallel bioprocesses for the production of PAmCherry with *E. coli* in a cascade ($D_{cascade} = 0.2\ h^{-1}$, 25 g L^{-1} glucose in feeding medium, pH 7.0, $T = 30$ °C) of two continuously operated stirred-tank reactors on a milliliter scale (number of parallel cascades $n = 2$; reactor 1 (R_1): $V_1 = 10$ mL, $D_1 = 0.3\ h^{-1}$, 0 µM IPTG; reactor 2 (R_2): $V_2 = 10$ mL, $D_2 = 0.4\ h^{-1}$, 100 µM IPTG): CDW (*filled black circle*, *filled grey circle*) and PAmCherry concentrations (*filled black triangle*, *filled grey triangle*) with standard deviations as function of residence times

estimation error for the cascade processes with a dilution rate of $D = 0.15\ h^{-1}$ (see Fig. 3).

Improvement of cascade processes

In general, the main goals of bioprocess optimization are the maximization of product concentrations, space–time yields, and product selectivity. In addition, high product yield coefficients can improve the economics of the following product separation. The implemented cascade processes for the production of PAmCherry with *E. coli* showed reasonable performances in comparison to the production of the recombinant protein in CSTRs (see Table 1, [15]). However, a further improvement of the continuous bioprocess was carried out at liter scale to outperform an optimized fed-batch process described previously (see Table 1, [15]). On the one hand, higher product concentrations and space–time yields should be achieved by increasing the CDW during continuous operation. At this juncture, the glucose concentration was doubled to 50 g L^{-1} in the feeding medium, which was not applicable at milliliter scale due to extensive foam formation at high cell densities during continuous operation. For biomass formation prior to continuous operation, the initial batch phase (25 g L^{-1} glucose) was followed by an exponential feeding phase with a preset growth rate of $\mu_{set} = 0.15\ h^{-1}$ until a total concentration of 50 g L^{-1} glucose were added to the reactor. On the other hand, the product selectivity and product yield coefficient should be improved by further increasing the residence time of expressing cells in the second reactor. For this purpose, the first reactor of the reactor cascade was operated at a volume of 0.5 L and a dilution rate of $D_1 = 0.2\ h^{-1}$. The volume of the second reactor was increased to 1.0 L and its dilution rate was set to $D_2 = 0.2\ h^{-1}$, which corresponds to a dilution rate of $D_{cascade} = 0.13\ h^{-1}$ for the cascade of two stirred-tank reactors.

A constant CDW of 21.6–23.7 g L^{-1} was observed after the start of the continuous operation in both reactors of the cascade (see Fig. 4). As expected, only basal expression of PAmCherry (<40 mg L^{-1}) was detected in the first reactor without addition of inducer. The PAmCherry concentration

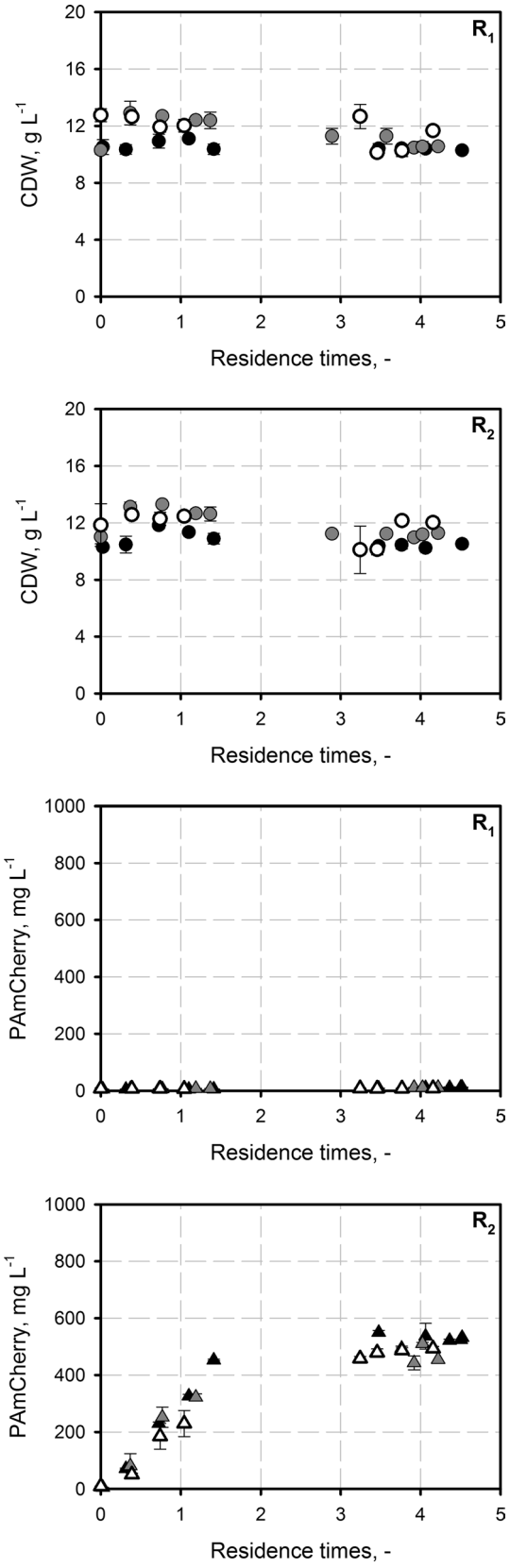

Fig. 3 Parallel bioprocesses for the production of PAmCherry with *E. coli* in a cascade ($D_{cascade} = 0.15\ h^{-1}$, 25 g L^{-1} glucose in feeding medium, pH 7.0, $T = 30$ °C) of two continuously operated stirred-tank reactors on a milliliter scale ($n = 2$; reactor 1 (R_1): $V_1 = 10$ mL, $D_1 = 0.2\ h^{-1}$, 0 µM IPTG; reactor 2 (R_2): $V_2 = 10$ mL, $D_2 = 0.3\ h^{-1}$, 100 µM IPTG) and on a liter scale ($V_1 = 500$ mL, $D_1 = 0.2\ h^{-1}$, 0 µM IPTG; $V_2 = 500$ mL, $D_2 = 0.3\ h^{-1}$, 100 µM IPTG): CDW at milliliter (*filled black circle*, *filled grey circle*) and liter scale (*empty circle*) as well as PAmCherry concentration at milliliter (*filled black triangle*, *filled grey triangle*) and liter scale (*empty triangle*) with standard deviations as function of residence times

in the second reactor increased until approximately two residence times, before a steady state concentration of 1146 ± 10 mg L^{-1} was achieved, which corresponds to a STY of 149 ± 1 mg L^{-1} h^{-1}. Hence, the product concentration (228%) and the STY (199%) could be increased significantly by doubling the biomass concentration. In contrast, the higher residence times of expressing cells in the second reactor (5 vs. 3.3 h) only led to a slight increase of the product selectivity and product yield coefficient (see Table 1).

In summary, the improved continuous production of PAmCherry with *E. coli* in a cascade of two stirred-tank reactors outperformed an optimized fed-batch process described previously [15] regarding space–time yield. As expected, product concentration, product selectivity, and product yield coefficient of the fed-batch process could not be achieved with continuous processes (see Table 1). However, a further increase of the biomass concentration could lead to an improvement of the production of PAmCherry in cascades of stirred-tank reactors.

Discussion

Parallel bioreactors were successfully modified to facilitate the characterization of the microbial production of recombinant proteins in continuous cascades of stirred-tank reactors on a milliliter scale. A high precision regarding preset dilution rates and reaction volumes was achieved at milliliter scale. Exemplarily, the intracellular production of PAmCherry with *E. coli* was characterized in cascades of stirred-tank reactors on a milliliter scale. Previous studies on recombinant protein expression with *E. coli* in cascades of two stirred-tank reactors described high dilution rates of 0.40 and 0.63 h^{-1} in the second reactor to be advantageous for protein expression, because of reduced plasmid loss [7, 10, 13]. In contrast, the process performance for the production of PAmCherry with *E. coli* was improved by a reduction of the dilution rate from 0.40 to 0.3 h^{-1} in

Table 1 Comparison of bioprocesses for the production of PAmCherry with *E. coli* in continuous stirred-tank reactors (CSTR), in continuously operated cascades of two stirred-tank reactors operated at different dilution rates (D), and an optimized fed-batch processes regarding cell dry weight (CDW), PAmCherry concentration ($c_{PAmCherry}$), space–time yield (STY), product selectivity (Y_{PS}), and product yield coefficient (Y_{PX})

Parameter	CSTR* ($D = 0.15\ h^{-1}$)	Cascade ($D = 0.20\ h^{-1}$)	Cascade ($D = 0.15\ h^{-1}$)	Cascade ($D = 0.13\ h^{-1}$)	Fed-batch*
Glucose in feeding medium (g L^{-1})	25	25	25	50	–
CDW (g L^{-1})	11.3 ± 0.4*	11.3 ± 0.2	10.8 ± 0.4	22.2 ± 0.1	53.1 ± 0.9*
$c_{PAmCherry}$ (mg L^{-1})	33.2 ± 0.4*	261 ± 9	502 ± 36	1146 ± 10	8400 ± 300*
STY (mg L^{-1} h^{-1})	5.0 ± 0.1*	52 ± 2	75 ± 5	149 ± 1	120 ± 4*
Y_{PS} (mg g^{-1})	1.32 ± 0.02*	10.4 ± 0.4	20.0 ± 1.4	22.9 ± 0.2	75 ± 2*
Y_{PX} (mg g^{-1})	3.3 ± 0.1*	23 ± 1	47 ± 3	52 ± 1	159 ± 5*

The parameters for continuous processes were calculated at steady state. The data for bioprocesses in CSTRs and fed-batch processes were taken from [15] (*)

the second reactor and consequently a reduction of the dilution rate of the whole cascade from 0.20 to 0.15 h^{-1}. As expected, the increased residence time of producing cells in the second reactor resulted in increased product concentrations and product yield coefficients. In addition, the STY and product selectivity were improved by factors of 1.5 and 2.0, respectively. In this study, the reduction of the dilution rate in the second reactor was realized by simultaneously reducing the dilution rate in the first reactor for biomass formation. Hortacsu and Ryu described an increased specific plasmid content of non-producing cells at low dilution rates in the first reactor of the cascade [7]. Hence, the increased product formation for the cascade with a dilution rate of 0.15 h^{-1} might be caused by a higher specific plasmid content of cells from the first reactor of the cascade.

In summary, a specific adjustment of process conditions for the production of recombinant proteins in cascades of stirred-tank reactors has to be performed for every target protein. Hortacsu and Ryu proposed to first characterize the optimal dilution rate with regard to the highest specific production rate in chemostats. This dilution rate shall be employed for the second reactor of the cascade. The dilution rate of the first reactor of the cascade can be optimized in terms of balancing plasmid stability (high dilution rates) and specific plasmid content (low dilution rates) [7]. By varying the reaction volume of both reactors of a cascade, the optimal dilution rate can be preset for both, biomass formation in the first reactor and protein expression in the second reactor. The suitability of the implemented cascades of stirred-tank reactors on a milliliter scale for the parallel characterization and implementation of recombinant protein production processes was shown, as the processes could directly be transferred to stirred-tank reactors on a liter scale using the maximal oxygen transfer rate as scale-up criterion. Furthermore, the miniaturized bioreactors were previously shown to be suitable for the characterization of protein expression in continuous cultures [15]. Hence, the bioreactors can be applied for the parallel characterization and optimization of microbial production processes of recombinant proteins in continuous cascades of stirred-tank reactors in a time- and cost-reducing manner. The degree of freedom regarding process control could further be increased by enabling the adjustment of different reactions volumes of the reactors of cascades on a milliliter scale, which is restricted to a value of 10 mL with the current setting [17].

Although the cascade processes outperformed the production of PAmCherry with *E. coli* in CSTRs [15] significantly, a further improvement of the bioprocesses was achieved at liter scale, where high cell densities could be realized without foaming and different reaction volumes could be preset for the two reactors of a cascade. On the one hand, the CDW at steady state was increased to 22.2 ± 0.1 g L^{-1} by implementing an exponential feeding phase prior to continuous operation and setting the glucose concentration of the feeding medium to 50 g L^{-1} during continuous operation. On the other hand, the dilution rate of the second reactor was decreased to 0.2 h^{-1}. Due to the increased CDW, the PAmCherry concentration and the STY could be increased to 1146 ± 10 mg L^{-1} and 149 ± 1 mg L^{-1} h^{-1}, respectively. In contrast, the product selectivity and the product yield coefficient could only be slightly improved by decreasing the dilution rate in the second reactor. As the dilution rate of the first reactor ($D_1 = 0.2\ h^{-1}$) was equal compared to the best performing process at milliliter scale, the gene dosage and consequently the specific production rate of cells entering the second reactor might also be equal for both processes [7].

As expected, the improved cascade process could not outperform a previously described fed-batch processes for the production of PAmCherry with *E. coli* regarding product concentration, product selectivity, and product yield coefficient, because of the constant washout of producing cells during continuous operation [15]. Although the achieved product yield coefficient of 52 ± 1 mg g^{-1} is in the range of high-performance fed-batch processes

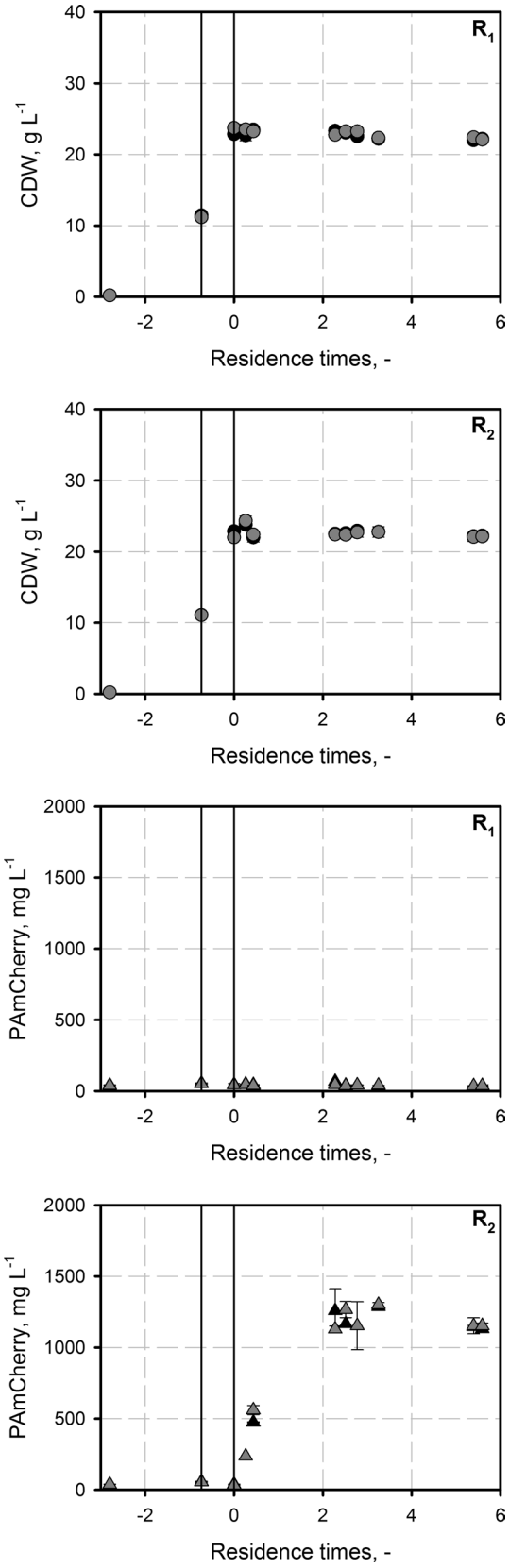

Fig. 4 Parallel bioprocesses for the production of PAmCherry with *E. coli* in a cascade ($D_{cascade} = 0.13\ h^{-1}$, 50 g L^{-1} glucose in feeding medium, pH 7.0, $T = 30$ °C) of two continuously operated stirred-tank reactors on a liter scale (reactor 1 (R_1): $V_1 = 500$ mL, $D_1 = 0.2\ h^{-1}$, 0 µM IPTG; reactor 2 (R_2): $V_2 = 1000$ mL, $D_2 = 0.2\ h^{-1}$, 100 µM IPTG): CDW (*filled black circle*, *filled grey circle*) and PAmCherry concentration (*filled black triangle*, *filled grey triangle*) with standard deviations as function of residence times. Process time was set to zero at the beginning of the continuous phase. The *vertical lines* indicate the end of batch phase (−0.8 residence times) and fed-batch phase (0 residence times)

for the production of intracellular proteins with *E. coli* ($Y_{PX} = 50 - 190$ mg g^{-1} [3]), a further improvement of the specific protein content would reduce the costs for downstream processing [4]. In general, higher concentrations of intracellular products can be achieved by applying a cascade of more than two reactors or implementing methods for the partial retention of cells to increase the residence time of producing cells in the reactor cascade [1]. In contrast, the STY could be improved by a factor of 1.24 in cascades of two stirred-tank reactors compared to fed-batch bioprocessing [15], which reflects the main advantage of continuous processes [4].The performance of the cascade process could further be optimized by increasing the CDW and consequently the concentration of intracellularly produced PAmCherry. In this study, the CDW was limited to approximately 22 g L^{-1}, because of the maximum oxygen transfer rate of the reactors at liter scale, which allowed for maximal glucose feeding rates of 10 g L^{-1} h^{-1} without oxygen limitation, which correspond to glucose concentrations of 50 g L^{-1} in the feeding medium (for $D = 0.2\ h^{-1}$). The CDW and the product concentration could further be increased by operating the bioreactor under elevated pressure and increasing the partial pressure of oxygen in the process gas. Consequently, glucose feeding rates of 20 g L^{-1} h^{-1}, which correspond to glucose concentrations of 100 g L^{-1} in the feeding medium (for $D = 0.2\ h^{-1}$), would be practicable, resulting in a further doubling of the CDW.

In conclusion, this study illustrates that the spatial separation of biomass formation and protein expression in a cascade of two stirred-tank reactors facilitates the efficient, continuous production of intracellular proteins with *E. coli*. The drawback of time-consuming and cost-intensive process development can be overcome, as miniaturized stirred-tank reactors were shown to be suitable for the characterization of continuous cascade processes for the production of recombinant proteins with bacteria. The low reaction volume (8–15 mL) and the operation of a maximum of 24 two-stage reactor cascades in parallel

will reduce the timeline and the costs for cascade process characterization and implementation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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