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Investigation of the physiological response to oxygen limited process conditions of *Pichia pastoris* Mut$^+$ strain using a two-compartment scale-down system

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Inhomogeneities in production-scale bioreactors influence microbial growth and product quality due to insufficient mixing and mass transfer. For this reason, lots of efforts are being made to investigate the effects of gradients that impose stress in large-scale reactors in laboratory scale. We have implemented a scale-down model which allows separating a homogeneous part, a stirred tank reactor (STR), and a plug flow reactor (PFR) which mimics the inhomogeneous regimes of the large-scale fermenters. This scale-down model shows solutions to trigger oxygen limited conditions in the PFR part of the scale-down setup for physiological analysis. The goal of the study was to investigate the scale-up relevant physiological responses of *Pichia pastoris* strain to oxygen limited process conditions in the above mentioned two-compartment bioreactor setup. Experimental results with non-induced cultures show that the specific growth rate significantly decreased with increasing the exposure time to oxygen limitation. In parallel more by-products were produced. Examining physiological scalable key parameters, multivariate data analyses solely using on-line data revealed that different exposures to the oxygen limitation significantly affected the culture performance. This work with the small scale-downs setup reflects new approaches for a valuable process development tool for accelerating strain characterization or for verifying CFD simulations of large-scale bioreactors. As a novel methodological achievement, the combination of the two-compartment scale-down system with the proposed multivariate techniques of solely using on-line data is a valuable tool for recognition of stress effects on the culture performance for physiological bioprocess scale-up issues.

[**Key words:** Scale-down; Two-compartment reactor; *Pichia pastoris*; Stress response; Oxygen limitation]

The quality of recombinant pharmaceutical products is strictly regulated by directives and standards of international and governmental bodies. Predictable yields for cells and product as well as consistent product quality call for robust industrial-scale bioprocesses. The desired product quantity and quality are strongly affected by the heterogeneities in the concentration profiles which occur in production-scale bioreactors due to insufficient mixing (1). Regarding aerobic bioprocesses, the limits of mass transfer capacity and fluid dynamics are the most crucial that affect industrial-scale performance, resulting in concentration gradients for oxygen (2,3) and for the limiting substrate (4). For example, in industrial-scale fed-batch cultivations, the concentration of the limiting substrate fluctuates due to the imperfection of mixing. This is largely affecting cell physiology as the feed solution is usually applied in concentrated form to minimize dilution effects (5,6).

Therefore, the bioprocess performance differ between small-scale (laboratory scale) and the large-scale. While the hydraulic scale-up is mastered since decades, studying physiological effects focussing on scale-up relevant information still remained somewhat unexploited (7,8). In order to investigate the impact of stress effects of large-scale reactors, scale-down model systems should be designed (9,10). For example, scale-down models can focus on mimicking the desired inhomogeneities, such as pH gradients (11), mixing time (12) or the limiting substrate gradient (13). On one hand, the gradients in large-scale reactors are modelled and investigated by carrying out oscillatory experiments in laboratory scale. For that, a single compartment well-mixed and aerated fermenter is used which is considered as an ideal small-size bioreactor. Regarding the two most critical compounds in aerobic bioprocesses, the effects of oscillating dissolved oxygen tension (14) and the oscillatory feed profile of the limiting substrate (15) on physiology and productivity were reported. On the other hand, the inhomogeneities can be mimicked in laboratory scale with two-compartment systems, where one part represents the homogeneous part, e.g., a continuous stirred tank reactor (STR) and the other part – another STR or a plug flow reactor (PFR) mimics the inhomogeneities in the large-scale reactors (7,16,17). The latter configuration allows perturbation of a part of the reaction volume and therefore follows a scale-down approach of partly not well-mixed zones in large-scale.

The effects of gradients of limiting substrate, oxygen as well as pH on cell physiology and productivity were recently investigated in the case of *Escherichia coli* (5–8,15), *Bacillus subtilis* (11,18) and

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Saccharomyces cerevisiae (13,16). To our knowledge, such studies have not been reported on *Pichia pastoris* yet. This strain has also become a prosperous host organism due to its beneficial properties, e.g., easy cultivation to high cell densities, several available tools for molecular manipulations, high level of production of recombinant proteins, moreover, production of complex proteins with eukaryotic post-translational modifications (19–21).

In the era of the emerging recombinant techniques, the question of faster screening and characterizing different recombinant strains are also getting more highlighted in laboratory scale bioreactors. Regarding the acceleration of bioprocess scale-up to large-scales from physiological point of view, the speed of process development also requires reliable and quick tools and methodologies. These techniques should not be necessarily targeted full physiological quantitation but only scale-up relevant physiological studies.

We have implemented a small-size two-compartment setup based on the relevant two-compartment scale-down setups and according to the available modelling and simulation tools and results (10,18). The goal of this study was to investigate the scale-up relevant physiological responses of a *P. pastoris* strain to oxygen limited process conditions with this small two-compartment scale-down setup. Repetitive batches were carried out with two different setup configurations and different residence times in the PFR. As a new approach for strain characterization, as demonstrated, the repetitive batches in a scale-down system offer faster alternatives for acquiring bioprocess scale-up relevant physiological information. Hysteresis effect can be however hypothesized among the repetitive experiment items, the dependency and experiment correlation was checked and discussed. First, the perturbation impact on the maximum specific growth rate, rates and yields were analyzed. Subsequently, multivariate data analysis was performed with the collected data. The dependence of the repetitive batch experiments were also pre-checked with correlation test and found to be sufficiently low for multivariate analysis. These mentioned simple statistical and multivariate approaches can provide a base for scale-up methodologies from the physiological point of view.

MATERIALS AND METHODS

Strain and media The experiments were carried out with *P. pastoris* Mut^+ SMD 1168 h (22). Preculture: Yeast nitrogen base medium (YNBM), per litre: potassium phosphate buffer (pH 6.0), 0.1 M; YNB w/o amino acids and ammonia sulphate (Difco TM), 3.4 g; $(NH_4)_2SO_4$, 10 g; biotin, 400 mg; glucose, 20 g. Batch medium: Basal salt medium (BSM), per litre: 85% phosphoric acid, 26.7 mL; $CaSO_4 \cdot 2H_2O$, 1.17 g; K_2SO_4, 18.2 g; $MgSO_4 \cdot 7H_2O$, 14.9 g; KOH, 4.13 g; glycerol as C-source, 10 g, Antifoam Struktol J650, 0.2 mL; PTM1, 4.35 mL; NH_4OH as N-source. Trace element solution (PTM1), per litre: $CuSO_4 \cdot 5H_2O$, 6.0 g; NaI 0.08 g; $MnSO_4 \cdot H_2O$, 3.0 g; $Na_2MoO_4 \cdot 2H_2O$, 0.2 g; H_3BO_3, 0.02 g; $CoCl_2$, 0.5 g; $ZnCl_2$, 20.0 g; $FeSO_4 \cdot 7H_2O$, 65.0 g; biotin, 0.2 g; H_2SO_4, 5 mL (23). As for the elemental composition of the biomass, the elemental analysis and the ash determination procedures were accomplished with previously washed and then lyophilized biomass samples according to the standard procedures (24).

Two-compartment scale-down setup The following small-size two-compartment scale-down system was implemented and characterized (Fig. 1A). The repetitive batch experiments were carried out in a fully instrumented 3.6 L autoclavable laboratory Labfors bioreactor (Infors, Switzerland) with 1 L batch working volume which represents the homogeneous part of the setup (STR). The on-line data monitoring and process control were executed with a Process Information Management System (Lucullus, Biospectra AG, Switzerland). The pH and temperature were controlled by PID controllers and the pH value in the reactor was maintained at the physiological optimum value of 5 ± 0.1 with the addition of NH_4OH with a certain known concentration. The reactor volume and the base consumption were continuously monitored with the utilization of laboratory scales with 0.1 g resolution (Sartorius, Germany and Mettler Toledo, Switzerland). The cultivation parameters in the bioreactor were 30°C and 1200 rpm of agitation speed and 1.5 vvm air inlet flow. In order to trigger different conditions from the homogeneous part of the setup, a plug flow reactor (PFR) with a defined working volume ratio was connected to the bioreactor. The broth was continuously circulated in silicone tubings (Roth, Germany and Tygon, Saint-Gobain, France) with the average inner diameter of 0.64 cm, outer diameter of 1 cm and 3.3 m length at a defined residence times between the two parts of the scale-down setup with a peristaltic pump (Ismatec, Switzerland). There are no special materials required and no extra tubings needed for connecting the stirred tank reactor with the plug flow part of the setup, as the PFR itself consists of silicone tubing. To observe the conditions of the culture broth in the PFR before returning to the STR, the PFR was equipped with a sampling port and a stainless steel Flow cell (Hamilton Bonaduz, Switzerland) with the connection of the combined pH as well as temperature sensor and pO_2 sensor (Hamilton Bonaduz, Switzerland). The hydrodynamic characterization of the PFR is described in a latter section. As for the previously described two-compartment scale-down setups, working volumes of 2.5–15 L were reported with focussing on mimicking only restricted number of compound gradients affecting production (7,16,25,26). Although this setup with 1 L working volume operates in the same working volume range as reported, its great variability features wide variety of perturbations like pH, substrate, temperature as well as oxygen related stresses at the same time.

Setup design: different PFR configurations The study focused on mimicking regimes with low dissolved oxygen concentrations which are lower than the critical dissolved oxygen concentration limit for observing only purely oxidative metabolism, executed with the small-size two-compartment scale-down setup. The critical dissolved oxygen concentration was reported between 30% and 40%, depending on the cultivation conditions and goals (27,28). The gradients in the dissolved oxygen concentration can be reasoned mainly by the insufficient mixing and secondarily by the unsatisfactory aeration. Therefore the low dissolved oxygen concentration was set in the PFR via the following two different configurations (Fig. 1B and C): Configuration A: Circulation of the broth with bubbles as dispersed system between the STR and the PFR with different residence times – with the aim of modelling the occurrence of low dissolved oxygen concentration regimes due to insufficient mixing in large-scale bioreactors. In this case, however, oxygen is still present, the mass transfer between the gas and liquid phase can only be carried out mainly via diffusion due to the lack of satisfactory mixing. Configuration B: Circulation of the broth as only liquid without bubbles in the PFR with different residence times – with the aim of modelling regimes with both insufficient aeration and mixing in production-scale reactors. This scale-down setup configuration models those regimes in the large-scale bioreactors where the oxygen is not provided at all.

The setup has been characterized before implementation, with respect to peristaltic pump calibrations for the residence times and bubble elimination. Furthermore, investigations of the response times of the sensors in the flow cell, hydrodynamic as well as sedimentation calculations were also executed. The circulation flow in the PFR has been proved to be laminar (from 130 to 1010, $Re < 2.4 \cdot 10^4$) with any of the flow rates of chosen residence times (from $7.6 \cdot 10^{-3}$ L/min to 0.2 L/min). The Re number calculation was executed with the usage of tubing diameter of the PFR, broth density as well as viscosity, and the linear velocity in the PFR.

Though the flow in the PFR is in the laminar flow regime, in order to have nearly ideal plug flow in the recycle loop, Kenics static mixers were built in the recycling loop in order to enhance turbulence of the liquid flow. This prevents any axial dispersion and at the same time, provides nearly ideal plug flow. With implementing these static mixers, there is negligible residence time distribution in PFR. Therefore, the average residence time was taken into account as the residence time of PFR for the discussion of the experimental results.

As the broth circulates in the PFR as a liquid–solid system, comparing the linear velocity in the loop for any residence times (10^{-2}–10^{-1} m/s range) to the occurrence of sedimentation (approximately 10^{-4} m/s range), the total amount of sedimented biomass in the PFR can be considered as negligible. The dry-weight measurements in the PFR also showed that less than 1% of the total produced biomass is sedimenting in the PFR zone during one batch experiment. Hence, it can be concluded that the broth which enters to the PFR zone represented the well-mixed broth from the STR part of the setup.

Experimental design: repetitive batches With the goal of investigating the impact of low dissolved oxygen concentration regimes on the overall performance, repetitive batch experiments were carried out with different setup configurations and different residence times in the PFR. In order to avoid the impact of the inoculum variations, the first experiment of the repetitive batch workflow was repeated in both setup configurations (Fig. 1B and C). As the working volume was always 1 L, 100 mL from the previous batch remained as inoculum in the bioreactor, 900 mL of fresh medium was added under sterile conditions.

Off-line analytics Off-line sampling was done regularly from the bioreactor and from the PFR. The following components were measured: biomass dry weight, substrate concentration (glycerol) and metabolite concentrations. Acetate was found to be the only significant metabolite. Other metabolites (e.g., ethanol) were only found in trace amounts which could not be quantitatively determined with our analytical methods (below 1 mg/L). All procedures were performed in duplicates.

To quantify the dry weight, cellular mass was measured gravimetrically in the reactor and in the PFR. Five and two mL of culture broth (from the bioreactor and from the PFR, respectively) were added to pre-weighted and dried glass tubes, followed by the immediate centrifugation at 5000 rpm for 10 min at 4°C (Sigma Centrifuge, Germany). The supernatant was then removed from the top and used for HPLC and enzymatic analysis of the substrate and the metabolite. The pellets were re-suspended twice in 5 mL of ice-cold distilled water using a vortex to wash them,