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Quasi-continuous fermentation in a reverse-flow diafiltration bioreactor

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ABSTRACT

Heterogeneities occur in various bioreactor designs including cell retention devices. Whereas in external devices changing environmental conditions cannot be prevented, cells are retained in their optimal environment in internal devices. Conventional reverse-flow diafiltration utilizes an internal membrane device, but pulsed feeding causes spatial heterogeneities. In this study, the influence of conventional reverse-flow diafiltration on the yeast *Hansenula polymorpha* is investigated. Alternating 180 s of feeding with 360 s of non-feeding at a dilution rate of 0.2 h^{-1} results in an oscillating DOT signal with an amplitude of 60%. Thereby, induced short-term oxygen limitations result in the formation of ethanol and a reduced product concentration of 25%. This effect is enforced at increased dilution rate. To overcome this cyclic problem, sequential operation of three membranes is introduced. Thus, quasi-continuous feeding is achieved reducing the oscillation of the DOT signal to an amplitude of 20% and 40% for a dilution rate of 0.2 h^{-1} and 0.5 h^{-1}, respectively. Fermentation conditions characterized by complete absence of oxygen limitation and without formation of overflow metabolites could be obtained for dilution rates from 0.1 h^{-1} to 0.5 h^{-1}. Thus, sequential operation of three membranes minimizes oscillations in the DOT signal providing a nearly homogenous culture over time.

1. Introduction

Process intensification by applying continuous fermentations with cell retention has been frequently investigated in the last years [1–8]. Although, industrial production in biotechnology is mostly performed as batch or fed-batch process, it was shown that continuous processes are economically promising if cell retention devices are applied [9]. Furthermore, continuous fermentations may provide several advantages such as constant product quality, smaller equipment size, higher space–time-yields, lower downtime and faster adaption of cells [8,10,11]. However, also in continuous fermentations heterogeneities of environmental conditions can occur. Particularly, externally arranged cell retention devices are prone to spatial heterogeneities as microorganisms are exposed to changing environmental conditions during passage through the device [12]. In contrast, cells are retained in their optimal environment in internally arranged cell retention devices. Previous works [6,7,13] introduced a membrane bioreactor concept applying reverse-flow diafiltration (RFD)[1] for internal or *in situ* product recovery. Harvesting product solution and feeding fresh medium is alternatingly accomplished over the same submerged hollow-fiber membrane. Thus, cell retention is gently enabled and simultaneously a fouling layer on the membrane is minimized. Hence, long-term stability is ensured and was proven for 2 month in a previous work [7]. However, alternation of harvesting and feeding results in a pulsed feeding of substrate. Therefore, concentrations fluctuate over time causing heterogeneous fermentation conditions.

Heterogeneities in bioreactors were examined extensively in literature [14–19]. The main focus of these studies was on large-scale bioreactors, where spatial heterogeneities of e.g. nutrients or the pH value occur due to poor mixing conditions at large scale. Hence, regions with high glucose concentrations and glucose-limited

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[1] Reverse-flow diafiltration: RFD.

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regions exist simultaneously within the same bioreactor. Studies investigating heterogeneities of nutrients by applying glucose pulse experiments or bioreactors with poor mixed regions were conducted with *Saccharomyces cerevisiae* [20–23], *Escherichia coli* [24–27] and other bacteria [28–32] and yeast. In these studies, it could be shown that overflow metabolites occur due to locally high substrate concentrations. Furthermore, short-term oxygen limitations of the microorganisms could be detected, causing a reduction of biomass and product concentration or the formation of overflow metabolites [14,20,23,25,26,28,30,31,33]. However, also positive effects of short-term oxygen limitation could be noticed as glucose is in excess and, thus, the number of dead cells decreased [14,26]. Furthermore, feed pulses were demonstrated to lead to a higher product yield on consumed substrate and higher activity per protein [34] and improve protein release by 60% [35].

The current study aims at improving the conventional reverse-flow diafiltration by realizing a quasi-continuous reverse-flow diafiltration. Temporally constant concentrations are achieved in the bioreactor by the sequential operation of three membranes. In the first part of this study, the pulsed feeding of RFD and its impact on the yeast *Hansenula polymorpha* is investigated. In particular, fermentation conditions which result in repeated short-term oxygen limitations due to pulsed feeding were examined and compared to oxygen unlimited conditions. In the second part, a process scheme for sequential operation of three membranes is introduced. The sequential operation is experimentally investigated for several dilution and stirring rates.

2. Materials and methods

2.1. Membrane

Hollow fiber membranes made from polyethersulfone with a nominal pore size of 0.2 μm on the inside were used (Pentair, Enschede, Netherlands) [7]. The pore size of the outside surface was of approximately 1–2 μm and the overall porosity about 40%. The asymmetric structure of the membrane is less prone to fouling for outside-in filtration. The membrane had an inner and outer diameter of 1.5 mm and 2.35 mm, respectively. Pressurized air at 1.5 bar was used to reveal leakages. Preservatives were removed prior to each experiment by flushing the membrane with deionized water sufficiently.

2.2. Culture and medium

In this study the yeast *H. polymorpha* pCoM11sc3625, an engineered strain kindly provided by PharmedArtis GmbH (Aachen, Germany), was used. A single-chain antibody variable fragment is secreted by this yeast [36].

Experiments with *H. polymorpha* were conducted in Syn6 medium [37]: 15.3 g L^{-1} $(NH_4)_2SO_4$, 2.0 g L^{-1} KH_2PO_4, 6.6 g L^{-1} KCl, 6.0 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.7 g L^{-1} NaCl; 27.3 g L^{-1} 2-morpholinoethanesulfonic acid (MES) and 20 g L^{-1} glucose autoclaved separately. Furthermore, 1 L of medium contained 10 mL autoclaved calcium chloride stock solution (100 g L^{-1} $CaCl_2 \cdot 2H_2O$), 10 mL sterile filtered microelements solution (6.65 g L^{-1} Titriplex III, 6.65 g L^{-1} $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$, 0.55 g L^{-1} $CuSO_4 \cdot 5H_2O$, 2 g L^{-1} $ZnSO_4 \cdot 7H_2O$, 2.65 g L^{-1} $MnSO_4 \cdot H_2O$), 10 mL sterile filtered vitamin stock solution (0.04 g L^{-1} biotin dissolved in 5 mL 2-propanol and 5 mL deionized water mixed with 13.35 g L^{-1} thiamine dissolved in 90 mL deionized water) and 10 mL sterile filtered trace elements solution (0.065 g L^{-1} $NiSO_4 \cdot 6H_2O$, 0.065 g L^{-1} $CoCl_2 \cdot 6H_2O$, 0.065 g L^{-1} H_3BO_3, 0.065 g L^{-1} KI, 0.065 g L^{-1} $Na_2MoO_4 \cdot 2H_2O$). All chemicals used were of analytical grade.

2.3. Culture conditions

Pre-cultures were grown in Syn6 medium buffered with 27.3 g L^{-1} MES. Cultures were incubated in 250 mL Erlenmeyer flasks filled with 10 mL at 37 °C. The flasks were shaken with a Kuhner shaker (LS-X, Birsfelden, Switzerland) at 350 rpm and a shaking diameter of 50 mm. Pre-cultures were harvested while exponentially growing [38].

Continuous cultures were conducted in a 3 L fermenter (Applikon Biotechnology, Schiedam, Netherlands) filled with 1.5 L Syn6 medium without buffer at 37 °C. The aeration rate was set to 1 vvm. The pH was adjusted to 5.5 with 5 M NaOH. The set point of the dissolved oxygen tension (DOT) was set to 30% and maintained by varying the stirring rate if not stated otherwise. To prevent foaming, Plurofac LF1200 (BASF, Ludwigshafen, Germany) was used if necessary. The dilution rate D was set to 0.2 h^{-1} if not stated otherwise. To achieve stable fermentation conditions, each dilution rate was kept constant for five retention times.

2.4. Analytics

Glucose and ethanol concentrations were measured offline using HPLC (Dionex, Sunnyvale, USA) equipped with an Organic Acid-Resin HPLC separation column 250 × 8 mm (CSChromatographie, Langerwehe, Germany) and a Shodex RI-71 detector (Techlab, Erkerode, Germany). Dry cell weight (DCW) was determined four fold [39].

Antibody concentrations were measured offline through size exclusion as the antibody is the only protein within the expected size range. Size exclusion was conducted by means of Äkta FPLC provided with a HiPrep 26/10 Desalting column (both GE Healthcare Life Science, Buckinghamshire, UK). A 0.1 M MES buffer was used as running buffer. Antibody concentration was calculated on the basis of UV peaks measured by Monitor UPC 900 (also GE Healthcare). Prior calibration is required [7].

DOT was measured online with a dissolved oxygen sensor based on the working principle of a Clark electrode (AppliSens, Applikon Biotechnology, Schiedam, Netherlands). Prior to each experiment the sensor was calibrated.

Exhaust gas was analyzed online regarding its oxygen and carbon dioxide concentration by an exhaust gas analyzer (Emerson XStream; Emerson, Weßling, Germany). Out of these values the oxygen transfer rate (OTR) and the respiratory quotient (RQ) were calculated according to Regestein et al. [40]. The OTR is commonly used to characterize aerobic cultures, as it describes oxygen consumption through metabolic activities, such as microbial growth [41]. The RQ contains information about the metabolism. RQ values close to 1.0 indicate oxidative growth on glucose, values above 1.0 imply the formation of overflow or other fermentative metabolites such as ethanol [42].

2.5. Membrane bioreactor

A schematic diagram of the membrane bioreactor set up used in this study is shown in Fig. 1. Cells were cultured in the bioreactor, outside the membrane. The membrane module was constructed as described by Carstensen et al. [6,13], but modified to allow operation of three membranes in parallel (pumps work simultaneously) or individually (pumps work in a time shifted sequence) [7]. Each membrane had a length of 1 m and was coiled around baffles and fixed by attached hooks. One end of each membrane was individually attached to a feed tube, the other one to a permeate tube (Fig. 1, continuous lines). Each tube was connected to one peristaltic pump to ensure independent operation of each membrane. All materials integrated into the membrane bioreactor were tested for their biocompatibility before application [39].