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Research Article

pH fluctuations imperil the robustness of *C. glutamicum* to short term oxygen limitation

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ABSTRACT

The presence of complex gradients for, e.g., nutrients, oxygen or pH in industrial scale fed batch processes are a major challenge for process performance. To consider such impact of scale-up during laboratory scale process development, scale-down bioreactor simulation, i.e. mimicking inhomogeneous conditions, became the method of choice. However, most scale-down studies simulate combined inhomogeneities of more than one parameter, so that the impact of the individual parameters remains unclear. The presented scale down study addresses this challenge by separating the influence of glucose, pH and oxygen fluctuations in terms of their specific impact in a well-established two compartment scale down device. This was carried out for an 1,5-diaminopentane production process using the industrial production host *Corynebacterium glutamicum*. Strikingly, oxygen depletion alone showed no effect on the process performance while changes of only one pH unit in acidic as well as alkaline direction reduced the biomass and product formation. Even more pronounced phenotypes up to −13% of μ and −39% of $Y_{X/S}$ were observed, when an oscillatory acidic pH shift was combined with dissolved oxygen fluctuations. These losses are accompanied by a missing regulation of fermentative pathways. In conclusion, large-scale *C. glutamicum* processes seem to be most sensitive to pH variation.

1. Introduction

Besides pharmaceutical ingredients, the production of bulk chemicals for fuels and polymers possesses the largest market potential in industrial biotechnology (Erickson et al., 2012; Festel et al., 2012). In order to meet the continuously growing demand the development of more efficient bioprocesses and production strains has to go hand in hand with an expansion of existing production capacities (Takors, 2012). But increase in scale is usually accompanied by a decreasing mixing quality due to physically limited power input and aeration rate. Combined with punctual addition of nutrients and high metabolic turnover rates of microbial organisms, gradients may form along the enlarged reactor profile. Reported manifests are a reduction of product and biomass yield as well as growth and product formation rate or even quality (Hewitt and Nienow, 2007; Junker, 2004; Lara et al., 2006a; Takors, 2012).

To remedy this problem, the nature of inhomogeneities as well as their impact on the respective production organism has to be further investigated. A straightforward approach is the use of detailed computational fluid dynamic (CFD) models focusing on mixing characteristics and gas-liquid mass transfer (Noorman, 2011; Sarkar et al., 2016; Vrábel et al., 2000). However, as these predicted inhomogeneities neglect or highly simplify the complex reciprocal influence between production organism and culture environment, laboratory studies are still an integral part for scale-up prediction. On the other hand CFD can give valuable information for the proper layout of scale-down reactors.

In order to investigate the disturbed conditions at laboratory scale a broad spectrum of scale-down setups was established during the last three decades. The most simplified ones focus on periodic fluctuations of substrate availability by pulse wise feed addition (Buse et al., 1992; Lara et al., 2009; Neubauer and Junne, 2010). Currently, more developed setups simulate complex oscillations of cells through environmental gradients by merging several inhomogeneous parameters. Of particular interest during former studies was to mimic substrate enriched areas around the feeding zone and the associated drop of dissolved oxygen due to increased metabolic activity. Therefore, a

Abbreviations: c, concentration; CDW, cell dry weight; DAP, 1,5-diaminopentane; DHAP, dihydroxyacetone phosphate; FBP, fructose 1,6-bisphosphate; GAP, glyceraldehyde 3-phosphate; LC, liquid chromatography; L-LAC, L-lactate; MAL, malate; MS, mass spectrometry; PFR, Plug flow reactor; PYR, pyruvate; q_{DAP}, biomass specific DAP formation rate; STR, stirred tank reactor; SUC, succinate; TCA, tricarboxylic acid cycle; V, volume; $Y_{DAP/S}$, substrate specific DAP yield; $Y_{X/S}$, substrate specific biomass yield; $Y_{DAP/X}$, biomass specific DAP yield; $Y_{lac/X}$, biomass specific L-lactate yield; $Y_{suc/X}$, biomass specific succinate yield

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concentrated feed stream was introduced into an additional reactor compartment which was connected to an ideal stirred tank reactor (STR). Oscillations between the compartments within a defined residence time of the cell suspension mimick the circulation through different zones in industrial scale bioreactors (Lara et al., 2006b; Neubauer and Junne, 2010; Takors, 2012). Depending on the used organism the introduced gradients had differing influence on the process performance. For *Escherichia coli* (Delvigne et al., 2005; Hewitt et al., 2000; Lejeune et al., 2010; Moon et al., 2009; Soini et al., 2008) and *Saccharomyces cerevisiae* (George et al., 1993; Käß et al., 2014a) partial oxygen limitation and substrate limitation lead to an increased side product formation consequently resulting in losses of biomass and product formation. *Corynebacterium glutamicum* also tends to form high amounts of side-products under comparable conditions, but biomass formation was not altered when glucose was limited (Käß et al., 2014a; Käß et al., 2014b; Lemoine et al., 2015; Limberg et al., 2017).

Only few of the published studies focus on inhomogeneity of parameters besides dissolved oxygen and substrate concentration. However, dissolved carbon dioxide (dCO_2) and pH perturbations were identified as critical scale-up factors, since in large-scale bioreactors dCO_2 gradients along the vertical profile are caused by the changing hydrostatic pressure. These concentration fluctuations are reported to affect various metabolic reaction balances involving CO_2 as substrate or product (Baez et al., 2011; Blombach and Seibold, 2010). pH-inhomogeneities could be traced back to chemical and biological origins. Chemical factors are the punctual addition of acidic or alkaline compounds for adjusting pH. Furthermore, the addition is usually controlled by view pH probes in spatial distance to the addition zone, e.g. located near an impeller and the fluid surface. Combined with the comparatively long mixing time of the industrial scale this consequently results in the formation of differing pH zones (Amanullah et al., 2001; Lara et al., 2006a; Takors, 2012). Moreover, biological factors such as the production of pH altering compounds by the organism itself due to substrate excess and limiting oxygen levels imperil the pH homogeneity during bioprocesses (Inui et al., 2004; Käß et al., 2014b). Combining chemical and biological factors, pH alterations in the range of pH = 4 in the bulk liquid up to pH = 9 near the alkali addition were predicted (Lara et al., 2006a; Reuss et al., 1994). During cell culture processes such fluctuating environmental compartments, were experimentally determined to be less pronounced (Brunner et al., 2017; Sarkar et al., 2016). Confronting *B. subtillis* (Amanullah et al., 2001) or *E. coli* (Erickson et al., 2012; Festel et al., 2012; Park and Lee, 2010) with combinations of pH and oxygen gradients resulted in drastically lowered growth rates, substrate uptake and final biomass yields.

Considering the gathered knowledge from more than 3 dozens published scale-down studies, the investigation of inhomogeneous cultivation conditions on microbial processes seems to be a well developed field of research. However, it is astonishing that a ranking of the impact of critical gradients remains unclear for most production organisms. The now presented study ties up with this drawback by not only investigating the effect of merged inhomogeneities on *Corynebacterium glutamicum,* but also separating the influence of pH and oxygen inhomogeneities under fed batch conditions. This was realized by the use of a well characterized parallel STR–STR scale-down device (Limberg et al., 2016), enabling a higher cultivation throughput and the individual control of cultivation conditions in both compartment.

A 1,5-diaminopentane producing *C. glutamicum* strain was chosen, as the product is one of the most promising building blocks for bio-based polymer production (Kind et al., 2014). Furthermore *C. glutamicum* is a widely spread production organism in industry (Eggeling and Bott, 2015; Takors et al., 2007; Wendisch et al., 2016) and highly relevant for polymer production, as it was proven for polylactic acid (Mayr et al., 1994) and succinate (Litsanov et al., 2012) production.

2. Materials and methods

2.1. Strain and media composition

The 1,5-diamonipentane (DAP) producing strain *C. glutamicum* DM1945*Δact-ldcC*^{opt} is a derivative of the L-lysine producing *C. glutamicum* DM1945 and was constructed as described previously (Lemoine et al., 2015; Limberg et al., 2017). All cultivations were carried out in minimal medium CGXII (Keilhauer et al., 1993) without MOPS buffer, containing an initial amount of 5 g L^{-1} glucose and 20 g L^{-1} $(NH_4)_2SO_4$ (Sigma, Missouri, U.S.A) for the initial batch phase. During the subsequent fed-batch phase, basic CGXII medium containing 300 g L^{-1} glucose and 40 g L^{-1} $(NH_4)_2SO_4$ was fed. Protocatechuic acid (PCA) was not added to the feed medium to ensure glucose as sole C-source (Unthan et al., 2014). In order to prevent foam formation 0.4‰ (v/v) antifoam AF204 (Sigma, Missouri, U.S.A.) was added before inoculation. The pH was maintained in both compartments by controlled addition of 6 M HCl and 6 M NaOH.

Cryo culture stocks for inoculation, were derived from exponentially growing and fully controlled bioreactor cultivation using CGXII medium. Harvested and washed cells were dissolved in CGXII medium containing 20% glycerole but no glucose and subsequently conserved at − 80 °C.

2.2. Description of the scale-down setup, culture conditions and cultivation procedure

All cultivations were carried out in a parallel cultivation platform (DASGIP, Jülich, Germany) and directly inoculated from cryo culture stock to a calculated OD_{600} of 0.05 (Fig. 1 B). The uniformity of the initial batch phase served as indicator for experimental consistency of cultivation process. When the batch glucose was consumed (17.75 h after inoculation) a glucose limiting exponential feeding profile for $\mu = 0.2\ h^{-1}$ (Eq. (1)) was activated. The profile was started using an initial pump rate Q of 3×10^{-3} L h^{-1}. During all cultivations the temperature was maintained at a level of 30 °C.

$$F = Q \times e^{\mu \cdot t} \quad (1)$$

Reference cultivations (REF) were carried out as biological triplicates ($n_B = 3$) in single stirred tank reactors (STR) incorporating a working volume of 1 L. Furthermore, the pH was maintained to 7 and pO_2 at a level of 30% by controlling the agitation rate. Cultivation conditions and results of REF were previously described by Limberg et al., 2017. Scale-down cultivations were carried out in a well characterized STR–STR scale down device consisting of two connected stirred tank reactors with a combined working volume of 1 L (Limberg et al., 2016; Limberg et al., 2017) (Fig. 1A). The distribution of culture volume between the compartments was set to 0.78 L in the STR1 and 0.22 L in the smaller STR2 including the connecting tubes. Average residence time in the STR2 compartment was set to 3 min. Minimum biological replicates for scale-down cultivations was two ($n_B = 2$). The additional stirred tank reactor (STR2) was connected to the main stirred tank reactor (STR1) 16.25 h after inoculation. As this process switch is congruent with the starting point for scale-down process comparison, this time point was consequently defined as t = 0.

During scale-down processes the STR1 compartment was continuously well aerated (pO_2 = 30%) and pH controlled to 7. A spectrum of differing scale-down conditions was realized by combining different values for pH and O_2 availability in the STR2 (Fig. 1A). During scale-down cultivations (SD) the feed stream was introduced into the STR2. In order to avoid unwanted O_2-transfer in the STR2 (SD1, 2, 4 & 6), the head space was gently flushed with 0.5 vvm of N_2. In case of well aerated conditions in the STR2 (SD3, 5, 7), pO_2 was maintained to a minimum of 30% saturation by controlling the aeration rate via a micro sparger. Variation of the pH level in the STR2 (SD2 − SD7) was