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Control of specific carbon dioxide production in a fed-batch culture producing recombinant protein using a soft sensor

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ABSTRACT

The feeding of a fed-batch cultivation producing recombinant protein was controlled by a soft sensor set-up. It was assumed that the control approach could be based on the cell's production of carbon dioxide and that this parameter indicates the metabolic state occurring at induced protein expression. The soft sensor used the on-line signals from a carbon dioxide analyser and a near-infrared (NIR) probe for biomass to estimate the specific production rate (q_{CO_2}).

Control experiments were carried out with various q_{CO_2} set-points where we observe that the feeding of nutrients to the culture could easily be controlled and resulted in a decreased variability compared to uncontrolled cultivations.

We therefore suggest that this control approach could serve as an alternative to other commonly applied methods such as controlling the cell's overflow metabolism of acetate or the cell's specific growth rate. However, further studies of the internal metabolic fluxes of CO_2 during protein expression would be recommended for a more precise characterization of the relationship between q_{CO_2} and protein expression in order to fully interpret the control behaviour.

1. Introduction

Low batch-to-batch variance is highly desirable in large-scale production of proteins (Jenzsch et al., 2006; Gnoth et al., 2007). Several attempts have been made to achieve this by controlling the feeding of nutrients in fed-batch cultivations from on-line data. A common approach has been to control overflow metabolism of acetate in *Escherichia coli* cultures (Xu et al., 1999; Rocha and Ferreira, 2002; Gerigk et al., 2002), of ethanol in yeast (Axelsson et al., 1988) and of lactate in mammalian cell cultures (Ozturk et al., 1997) by adjusting the feeding rate. Another approach has been to control the specific growth rate of the cultivation from biomass sensors (Gerigk et al., 2002; Fan and Yuan, 2005), calorimetric measurements (Biener et al., 2010; Paulsson et al., 2014) and effluent gas analysis (Levisauskas et al., 1996; Aehle et al., 2011). A third approach has been estimation of the culture's growth state from responses in dissolved oxygen to feed transients measured by a DO-electrode (Konstantinov et al., 1990; Åkesson et al., 1999, 2001). Other examples of growth control are exploitation of multi-sensor based trajectories (Bachinger et al., 2000; Cimander and Mandenius, 2002; Cimander et al., 2003; Sagmeister et al., 2013), mass balance equations (Cornelissen et al., 2003) and estimations from mixed acid products (Xu et al., 1999; Gustavsson and Mandenius, 2013).

In this work, we try another approach where we assume that the cell's specific production of CO_2 (q_{CO_2}) is a useful indicator of the cellular state during expression of a recombinant protein. We also assume that a control of the q_{CO_2} variable could be a new way to decrease the variability of product titre and biomass growth. The q_{CO_2} parameter is estimated by using a soft sensor (Chéruy, 1997; Dochain, 2003; Luttmann et al, 2012) where the total production of CO_2 from the culture is measured by a gas analyser at the bioreactor effluent and the biomass concentration in the reactor by an in situ near infrared (NIR) probe (Scarf et al., 2006). Measurement of CO_2 production, expressed as CO_2 evolution or production rate (*OUR*, CPR), in microbial and cellular cultures is an established technique in bioreactor engineering (Jenzsch et al., 2007; Aehle et al., 2011). Use of NIR probes for biomass monitoring in bioreactors has been reported for a variety of organisms up to cell concentrations of 80 g L^{-1} (Finn et al., 2006).

Also the soft sensors have previously been applied for biomass estimation. For example, Sundström and Enfors (2008) monitored the pH balance of an *E. coli* culture via the added titration volume and from that estimated the specific growth rate. Others have applied soft sensors or soft sensor-like methods with spectrometric and electrochemical methods (Clementschitsch et al., 2005;

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Kiviharju et al., 2008; Ödman et al., 2009; Warth et al., 2010; Sonnleitner, 2013).

In this article, we report the observation that the q_{CO_2} signal derived from the soft sensor could be used to control the feeding of substrate to a fed-batch cultivation with *E. coli* expressing the recombinant green fluorescence protein (GFP). We also observe that this control approach reduces the variability in biomass and GFP production. From these observations, we suggest that the q_{CO_2} provides another possibility for controlling fed-batch cultivations as an alternative to other previously applied control methods. However, a more detailed understanding of the cell's metabolic behaviour in relation to intracellular fluxes of CO_2 during the expression phase of a recombinant protein would be desirable to fully explain the results.

2. Materials and methods

2.1. Cell culture and media

The *E. coli* strain HMS 174(DE3) (Novagen, Madison, WI, USA) transformed with plasmid pET30a (Novagen) containing GFP-mut3.1 (Clontech, USA), under control of the *T7/lac* promoter and a 25 bp *lac* operator sequence was used. The transformed strain was obtained from the Department of Biotechnology, University of Natural Resources and Life Science, Vienna.

A semi-synthetic medium (Nemecek et al., 2008) was used for the pre-culture while a modified medium was used in the fed-batch cultivation. All reagents and chemicals were purchased from Merck, if not otherwise stated. Media components were added in relation to the working volume of the bioreactor. The pre-culture medium was composed of 3.0 g L^{-1} KH_2PO_4, 4.5 g L^{-1} $K_2HPO_4{\cdot}3H_2O$, 2.5 g L^{-1} $C_6H_5Na_3O_7{\cdot}2H_2O$, 1.0 g L^{-1} $MgSO_4{\cdot}7H_2O$, 4.5 g L^{-1} $(NH_4)_2SO_4$, 3.7 g L^{-1} NH_4Cl, 1.5 g L^{-1} yeast extract, 6.0 g L^{-1} glucose and 0.5 mL L^{-1} of a trace element solution. The batch medium was composed of 5.0 g L^{-1} glucose, 6.67 g L^{-1} K_2HPO_4, 0.25 g L^{-1} KH_2PO_4, 1.2 g L^{-1} NaCl, 1.1 g L^{-1} K_2SO_4, 0.5 g L^{-1} yeast extract, 10 g L^{-1} $(NH_4)_2SO_4$, 0.15 g L^{-1} $MgSO_4{\cdot}7H_2O$, 0.013 g L^{-1} $CaCl_2{\cdot}2H_2O$ and 0.125 mL L^{-1} of the trace element solution, and the feed medium was composed of 100 g L^{-1} glucose, 27.5 g L^{-1} $(NH_4)_2SO_4$, 1.5 g L^{-1} $MgSO_4{\cdot}7H_2O$, 0.026 g L^{-1} $CaCl_2{\cdot}2H_2O$ and 2.50 mL L^{-1} of the trace element solution. The trace element solution contained 40.0 g L^{-1} $FeSO_4{\cdot}7H_2O$, 10.0 g L^{-1} $MnSO_4{\cdot}H_2O$, 10.0 g L^{-1} $AlCl_3{\cdot}6H_2O$, 4.0 g L^{-1} $CoCl_2$, 2.0 g L^{-1} $ZnSO_4{\cdot}7H_2O$, 2.0 g L^{-1} $Na_2MoO_2{\cdot}2H_2O$, 1.0 g L^{-1} $CuCl_2{\cdot}2H_2O$ and 0.50 g L^{-1} H_3BO_3 dissolved in 5 N HCl.

2.2. Cell bank preparation

The transformant was cultured in a rotary incubator at 200 rpm overnight at 37 °C. The culture was grown in a shake-flask containing 200 mL of the semi-synthetic medium supplemented with 50 μg L^{-1} kanamycin and reached a cell concentration of 2 g dry weight L^{-1}. The optical density at 600 nm (OD_{600}) after 19 h was approximately 4 absorbance units. Vials of aliquots with 1.2 mL of the cell suspension and 0.6 mL of glycerol (60%) were prepared and stored at −70 °C.

2.3. Fed-batch cultivation

All cultivations were carried out in a 10 L in situ sterilized bioreactor (Model LMS 2002, Belach Bioteknik AB, Solna, Sweden) equipped with standard instrumentation. The control software (BioPhantom, version 2000; Belach Bioteknik AB) was implemented with the soft sensor algorithms described below. A pre-culture was prepared from one cell bank vial in a shake flask with 200 mL medium and grown overnight to an OD value of 8–12. This culture was used to inoculate the bioreactor at an initial batch medium volume of 4 L. During the subsequent fed-batch 2 L of feeding medium was added. The pH was controlled at 7.0 ± 0.1 by addition of 1 M sulphuric acid or 20% ammonia. The aeration rate was 1 vvm and the temperature was 37 °C. Dissolved oxygen (DO) was controlled to 30% by adjusting the stirrer speed (300–1200 rpm). Foaming was controlled by addition of a 50% anti-foam solution (Glanapon, Busetti & Co GmbH, Vienna, Austria).

Exponential feeding of the fed-batch was started when the DO level increased due to depletion of the batch medium. This was a well-defined starting point which was easy to reproduce. The feeding rate of the feed pump (P4 U1-MXV, Alitea, Sweden) was adjusted so the culture maintained a constant specific growth rate of 0.3 h^{-1}. The rate was calculated from the weight of the medium flask as recorded on-line by a scale (XL-3100, Denver Instrument). A tuned PID-controller adjusted the feed pump speed according to a set growth rate.

GFP expression was induced by addition of 0.03 g L^{-1} isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma) when the OD_{600} had reached a value of 20 absorbance units. This corresponded to approximately one culture generation and occurred 3–4 h after starting of feeding.

2.4. On-line sensors

2.4.1. Standard bioreactor devices

The reactor was equipped with standard DO and pH electrodes as well as probes for temperature, headspace pressure, and volume level. Signals were acquired every second.

2.5. On-line CO_2 analyser

The outlet gas from the reactor was measured by a gas analyser (CP460-O_2/CO_2; Belach Bioteknik AB, Sweden) which analysed carbon dioxide levels.

2.6. On-line near-infrared monitoring

Biomass was measured at 900–1100 nm by a sterilizable in situ near-infrared probe (Cell Growth Monitor Model 650; Wedgewood Technology, CA). The probe used a pathlength of 5 mm. The NIR signal was filtered to reduce noise and the sensitivity was adjusted by non-linear compensation. The probe was inserted close to the stirrer turbine of the bioreactor to ensure that the optical window of the probe was efficiently flushed with culture media. Other signal disturbances due to changes in flow pattern caused by impeller rate adjustments were compensated for by a filter function implemented in the instrument (Warth et al., 2010).

2.7. Off-line analyses

Samples for measurement of optical density at OD_{600} (using Ultraspec 1000, Pharmacia Biotech, UK) and the biomass dry weight were taken from the bioreactor intermittently through a steamed pipe. Diluted un-centrifuged GFP samples were measured in a fluorimeter (Fluostar Galaxy, BMG Labtechnologies GmbH, Offenburg, Germany) at excitation/emission wavelengths of 470/515 nm in 1-mL samples. As reference was used uninduced culture media. The fluorescence units of the fluorimeter were calibrated versus a GFP standard solution (recombinant GFP; Millipore Inc., USA).