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Development and *in silico* analysis of a new nitrogen-limited feeding strategy for fed-batch cultures of *Pichia pastoris* based on a simple pH-control system

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ABSTRACT

A novel fed-batch strategy based on a pH control system was devised in order to address some of the disadvantages associated with available control strategies for *Pichia pastoris* cultures. Changing settings of the controller, the t_{ON}/t_{OFF} time ratio, adjusted the speed in which the ammonium hydroxide was added for pH control and the sole nitrogen source. Setting the t_{ON}/t_{OFF} ratio to 0.5/0.5, 0.5/10 and 0.5/15 resulted in a decrease in the growth rate as the t_{OFF} value increased. Therefore, the design allows running the culture at lower growth rates in a controlled way. Two high cell density cultures with t_{ON}/t_{OFF} ratio of 0.5/10 and 0.5/20 were also fed ammonia with the same controller. Setting t_{ON}/t_{OFF} at 0.5/10 and 0.5/20 led to an increase in final biomass and protein as the t_{OFF} decreased. A metabolic model assessed the effect of nitrogen-limitation in high cell density cultures on the yeast metabolism. Model results show that fluxes of intracellular reactions such as glycolysis, the tricarboxylic cycle and the pentose-phosphate pathway are higher at t_{ON}/t_{OFF} = 0.5/10. Additionally, higher sensitivity of growth and protein production to oxygen at t_{ON}/t_{OFF} = 0.5/10 proves that oxygen limitation led to higher protein production in this case.

1. Introduction

The methylotrophic yeast *Pichia pastoris* is recognized as an effective host for the production of many heterologous proteins mainly due to its high expression levels, its versatile promoters and its respiratory growth on various substrates [1]. *P. pastoris* has been used for production of various recombinant proteins such as recombinant human erythropoietin (rHuEPO) [2], recombinant human growth hormone (rhGH) [3], recombinant human superoxide dismutase (rhSOD), recombinant human serum albumin (rHSA) [4], and recombinant human monoclonal antibody 3H6 Fab fragment (FAB) [5].

The most common feeding strategies for fed-batch cultures and production of recombinant proteins using *P. pastoris* include maintaining constant dissolved oxygen (DO) values (DO-stat) [6], constant specific growth rate feeding (μ-stat) [7], constant methanol concentration feeding [8], and employing an oxygen-limited fed-batch (OLFB) [9] and a temperature limited fed-batch (TLFB) [10]. In DO-stat cultures, DO control becomes insensitive when there is accumulation of the carbon source. Moreover, high-density cultures of *P. pastoris* exhibit oscillatory behavior in DO-stat feeding, which leads to irreversible loss of culture productivity and thus lower yields. μ-stat feeding, commonly carried out in open loop settings, is difficult in terms of robustness and process stability. Open loop systems may be unable to respond to perturbations of the culture, which may lead to deviation from the initial set-point and in the case of *P. pastoris* cultures to over-accumulation of methanol. In the case of constant methanol concentration feeding, due to the nonlinearity and complexity of the process dynamics, which are subject to inherent and externally imposed variability, the optimal settings of the PID controller are determined by trial and error tuning or by other empirical methods. OLFB cultures were not successful in the production of all types of heterologous proteins from *P. pastoris* fed-batch cultures. In TLFB cultures, low cultivation temperatures make scale-up difficult, due to restricted heat exchange capacity [1].

Fed-batch cultures in which the specific growth rate (μ) is maintained at a value that allows maximizing the specific biomass productivity without leading to an overflow of metabolic formation

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are an economically interesting strategy to ensure overall productivity and maximized product yield. Consequently, over the past decade different strategies have been suggested for controlling the specific growth rate of yeast cells and maximizing protein production [11,12]. Control strategies to regulate μ are usually based on the carbon source limited substrate feeding. However, the controller proposed within the scope of this study is based on nitrogen limited feeding.

The implications of limitations of the nitrogen source on the metabolism of yeast cells have been intensively studied [13–15]. For example, limited nitrogen levels are indeed known to lead to glycogen accumulation [16,17] and to lipid accumulation in oleaginous microorganisms [18], as well as to a higher protein turnover [14]. Gene expression is also differently regulated in nitrogen limited situations, where an increase in the expression of ribosomal and glycolytic genes and a decrease in the expression of genes involved in nitrogen source degradation have been observed [19]. Recently, a process has been suggested to enhance rhamnolipid production, in which control of the specific growth rate at low values with concomitant nitrogen limitation would be required to improve the product yield through changes in metabolic fluxes [20].

In order to further analyze the characteristics of these control strategies from a system perspective, it is required to model the yeast metabolism [21]. Some studies employ genome-scale metabolic models for this purpose [4,5,22]. In this research, the genome-scale metabolic model of iLC915 [5] is used to study the effect of nitrogen limitation on *P. pastoris* metabolism and was consequently able to simulate fermentation profiles.

The present study was conducted to develop a new nitrogen-limited fed-batch strategy for high cell density cultures of *P. pastoris* that produce recombinant human serum albumin (rHSA) based on a simple pH control system. This strategy is proposed in order to omit the disadvantages associated with the available control strategies such as over-feeding, low bioreactor temperature and absence of a feed-back from the bioreactor [1]. Time constant settings of this pH control system were changed to lower the specific growth rate in a controlled manner while assessing the impact of the control strategy on maximal cell density and recombinant protein production. Additionally, *in silico* experiments were carried out using a genome-scale metabolic model in order to investigate cell metabolism and sensitivity of microbial growth and protein production to oxygen under limited nitrogen conditions.

2. Materials and methods

2.1. Microorganism

P. pastoris GS115/His$^+$Mut^s producing extracellular recombinant human serum albumin (rHSA) under the control of *AOX1* promoter (Invitrogen Corporation, CA, USA) was used in this study.

2.2. Cultivation methods

2.2.1. Inoculum preparation

The inoculums were prepared from a 1 ml frozen cell stock vial that was cultured in 100 ml of a medium contained per liter: 0.460 g K_2HPO_4, 0.236 g KH_2PO_4, 2.68 g yeast nitrogen base, 2 ml glycerol and 0.2 ml biotin (0.002 g/50 ml) [7]. The inoculated liquid media was then incubated at 30 °C on a rotary shaker at 250 rpm for 24 h.

2.2.2. Fermenter cultivation

A 2–l working volume fermenter (Bioengineering AG, Switzerland) with a pH-controller (M7832N, MOSTEC AG, Switzerland) was used. The fermentation medium consisted per liter: 40 g glycerol, 13 g KH_2PO_4, 2.7 g K_2HPO_4, 0.1 g NaCl, 2 g $MgSO_4{\cdot}7H_2O$, 2 g K_2SO_4, 0.2 g $CaCl_2$ and 2 ml of a trace element solution: 6 g $CuSO_4{\cdot}5H_2O$, 0.09 g KI, 3 g $MnSO_4{\cdot}H_2O$, 0.02 g H_3BO_3, 0.24 g $MoNa_2O_4{\cdot}2H_2O$, 0.5 g $CoCl_2$, 10 g $ZnCl_2$, 20 g $FeSO_4{\cdot}7H_2O$, 0.2 g biotin, and 5 ml H_2SO_4 [7]. The carbon source feed media were glycerol feed (100 ml glycerol and 0.5 ml trace elements solution), methanol feed (100 ml methanol and 0.5 ml trace elements solution) and sorbitol feed (100 ml sorbitol solution, 800 gl^{-1}, and 0.5 ml trace elements solution). The pH of the medium was controlled at 5.0 using concentrated ammonium hydroxide solution and concentrated phosphoric acid with the normality of 1. The temperature was maintained at 28 °C, the dissolved oxygen (DO) concentration above 20% air saturation, agitation rate 750 rpm and the aeration rate at 1 lmin^{-1}.

2.3. Analytical methods

2.3.1. Biomass determination by dry cell weight (DCW)

Two milliliter of culture was centrifuged (10,000 × *g*, 1 min), and the pellets were washed and centrifuged twice with distilled water and then dried at 100 °C to constant weight.

2.3.2. Glycerol and sorbitol quantification

Glycerol and sorbitol in the culture media were determined by HPLC analysis (Agilent Technologies, Palo Alto, USA) using a refractive index detector. An ion exchange chromatography column (Supelcogel C-610H, Supelco, Bellefonte, USA) with a guard column (Superlguard C610H, Supelco, Bellefonte, USA) was used at 30 °C. The eluent was 15 mM sulphuric acid solution in ultrapure water at a constant flow rate of 0.5 mlmin^{-1}.

2.3.3. Methanol quantification

An enzymatic method was used for the determination of methanol in the culture media [23]. The reagent solution contained 1.2 gl^{-1} of 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (98%), 1.5 Uml^{-1} Horseradish peroxidase and 0.3 μll^{-1} H_2O_2 (30%) in PBS solution (pH 7.4). The AOX solution contained 1 Uml^{-1} alcohol oxidase in PBS solution (pH 7.4). The reaction was initiated by mixing 1 ml reagent solution, with 5 μl samples and 30 μl AOX solutions. After 3 min, the absorbance was read at 405 nm and methanol concentration was estimated using a linear standard equation. Dilutions were made to samples to bring the methanol concentration between 0.05–0.50 gl^{-1}, the working range of this method.

2.3.4. Ammonia quantification

An enzymatic ammonia assay kit from Sigma and accompanied protocol was used to determine the concentration of ammonia in samples.

2.3.5. Protein quantification

Total protein concentration was determined by the Bradford assay using the Sigma-Aldrich Bradford Reagent. For SDS-PAGE analysis, samples were mixed 1:1 with Bio-Rad's Laemmli sample buffer and used in precast gels (Precise Tris-HEPES Protein Gels) from Thermo-Scientific. Brilliant Blue G-Colloidal concentrate from Sigma was used for staining.

3. Theory/calculation

3.1. pH controller system

The feeding strategy proposed in this study consists of supplying the required nitrogen source for growth through the addition of ammonium hydroxide which is regulated by a pH control system of the bioreactor. In yeast cultures with limited nitrogen levels, ammonium consumption and proton release occurs