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Feeding strategies for the enhanced production of recombinant human serum albumin in the fed-batch cultivation of *Hansenula polymorpha*

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ABSTRACT

An intermittent feeding strategy in fed-batch cultivation has been widely applied to control the accumulation of overflow toxic metabolites and oxygen limitation. However, successive depletions of carbon source by carbon-limited feeding might induce considerable stress to host cells, such as nutrient starvation, which may evoke the enhanced degradation of secreted recombinant proteins by proteolysis. To prevent this degradation, we evaluated novel nutrient feeding strategies for the production of MOX promoter-driven recombinant human serum albumin in the fed-batch cultivation of *Hansenula polymorpha*. Compared to conventional feeding strategies such as exponential or intermittent feeding strategy, feeding strategies developed in the present study significantly reduced the degradation of recombinant products and yielded high levels of intact recombinant human serum albumin in fed-batch cultivation.

1. Introduction

Fed-batch cultivations have generally been preferred over batch or continuous cultivations because optimal cell growth rates and nutrient fluxes can be achieved by controlling the nutrient-feeding rate to reduce the metabolic load [1–3]. Well-controlled fed-batch cultivation can accomplish high cell density over 100 g l^{-1} dry cell weight and product yield up to 13.5 g l^{-1} of recombinant phytase by repeated fed-batch cultivation [4]. Several nutrient feeding strategies have been developed to augment cell growth and product yield leading to the implementation of various control algorithms to precisely control and to reduce deviations or errors of output responses [1,5–7].

Exponential feeding and intermittent feeding strategies have been adopted to control fed-batch cultivations [1,2]. The growth-associated introduction of nutrients in an exponential feeding strategy can reduce metabolic shocks from dumped feeding of nutrient in the early stage of cultivation, in which the metabolic activity is not fully developed. It however, is very difficult to predict and maintain desired specific growth rate over the whole cultivation period which is a major variable in exponential feeding strategy since inappropriate supply of nutrient may result in excess or deficiency. Therefore, intermittent feeding is the most popular strategy in fed-batch cultivation. Primary control parameters used for feedback control of intermittent feeding strategies are pH, dissolved oxygen (DO), partial pressure of carbon dioxide (pCO_2), respiratory quotient (RQ), and nutrient concentration [8–10]. Because of its responsiveness, versatility, and real-time availability, oxidation response-based feedback control, which is based on the oscillatory behavior of DO level caused by the substrate depletion, is widely used [11,12]. The production of overflow metabolites from carbon source metabolism, which limit cell growth and protein expression, can be controlled with a nutrient-limited feeding strategy by periodic exhaustion of a key substrate, thus allowing continued growth and maximum expression of recombinant products.

Hansenula polymorpha has recently emerged as an attractive host organism for the production of heterologous proteins due to its favorable cultivation properties and strong promoters for industrial applications [13,14]. Moreover, the lack of hyper-immunogenic terminal mannose epitope in glycan structure produced by *H. polymorpha* shows significant promise for the pharmaceutical production of recombinant glycoproteins [15]. *H. polymorpha* is also a model organism for basic research on peroxisomal biogenesis and function, as well as on nitrate assimilation pathways not present in *Pichia pastoris* [16]. For this methylotrophic yeast, foreign genes have been typically expressed under the control of methanol oxidase (MOX) promoter relevant to

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the methanol metabolism. *H. polymorpha* can use methanol as a sole carbon and energy source, but cannot manage to high concentration of methanol, due to the detrimental effect of oxidized toxic metabolites, formaldehyde and hydrogen peroxide, generated from methanol oxidation [17,18].

To relieve the shock of oxidized products on the cell for both high production of recombinant proteins and high-cell density cultivation, an accurate control of methanol supply is necessary. Besides the relief of the shock from methanol oxidative products, maintenance of the gene expression under the control of the MOX promoter is also crucial in controlling the methanol supply. Fed-batch cultivation, in which a nutrient is a growth-limiting substrate and is fed below the critical level of nutrient uptake rate, has been employed successively to control the substrate supply. Among feeding strategies in fed-batch cultivation, intermittent feeding strategy allows control over oxygen limitation as well as the accumulation of overflow toxic metabolites. However, a successive depletion of carbon source might induce a chronic starvation repeatedly and thus a considerable stress to the host cell, potentially causing cell lysis and enhancing the degradation of secreted proteins by proteases released into the culture medium.

In the present study, we developed a novel feeding strategy for fed-batch cultivation, Algorithm-based Feeding mode (AF mode), to prevent the excess methanol exposure, and evaluate this strategy using the MOX promoter-driven recombinant human serum albumin (rHSA) production in *H. polymorpha* as model system. Furthermore, to reduce the degradation of rHSA, we also developed a tuning system of critical feed concentration, Critical feed rate-based Continuous feeding mode (CCF mode). These novel feeding strategies were able to produce fully intact rHSA at high yields in fed-batch cultivations.

2. Materials and methods

2.1. Strains

Yeast strains used were *H. polymorpha* DL-1L (*leu2*), a derivative of DL-1 (ATCC 26012) for a wild type strain and the *H. polymorpha* G0T7 strain harboring one copy of a MOX promoter-rHSA expression cassette, derived from *H. polymorpha* DL-1L, for a rHSA producer [13].

2.2. Media and fermentation conditions

Cultivations were performed in a 5-l fermentor (Bioflo III, New Brunswick Scientific, USA). The main culture with 2-l YPG (4% Yeast extract, 2% Bacto-peptone, 2% glycerol) medium was inoculated with 100 ml of pre-culture grown to middle-exponential phase in the same medium. During cultivation, the culture pH was adjusted to 6.0 with 2N HCl and 30% (v/v) of ammonia solutions. The temperature was controlled at 37 °C and the agitation speed was maintained at 800 rpm. Air was supplied at a flow rate of 1.5 vvm. The dissolved oxygen probe (Ingold, USA) was calibrated using 100% as air saturation at 37 °C, and 0% as equilibration with pure N_2.

After exhaustion of initial glycerol in the medium at the end of the batch phase, four feeding modes were applied for fed-batch cultivation. Two of them are novel feeding modes developed in this study, AF mode and CCF mode, and others are conventional modes [13,19,20]. In the AF and CCF modes, the fed-batch phase was performed under methanol-limited conditions. These modes are described in detail at the next section. The computer program for AF mode was constructed with AFS-Biocommand Software (New Brunswick Scientific, USA). The exponential feeding mode and intermittent feeding mode were performed as follows. Methanol was supplied below 0.1 h^{-1} of specific growth rate in exponential feeding mode. In intermittent feeding mode, 8 g l^{-1} of methanol was supplied by pulsation when methanol in the culture medium was exhausted and the dissolved oxygen tension began to increase. Other variables for exponential feeding were obtained from the batch cultivation of *H. polymorpha*. Dissolved oxygen was maintained with air and pure oxygen above 20% in the exponential and intermittent feeding modes. All cultivations were conducted repeatedly three times.

2.3. Feeding strategies

2.3.1. AF mode performance

The AF mode is comprised of three individual control modules (Fig. 1). The first module regulates exponential feeding (Fig. 1A), the second module controls the upper limit of methanol feeding rate to reduce the shock of dumped feeding to the cells in the late exponential phases (Fig. 1B), and the third module directs the DO-feedback fed-batch process (Fig. 1C). These three modules interact to control the feed rate of actual output. The output is transmitted to a feed pump built into a NBS fermentor. Fig. 2 illustrates the typical output responses of AF mode. The actual output of this algorithm showed the united mode of both exponential feeding, as a predetermined control, and intermittent feeding, as a feedback control. The feed rate was determined by exponential calculations in the first module, and the third module regulates the methanol supply based on increases in the DO level. And the second module participates in the final decision of dosing an amount of methanol. This module is useful for limiting the overfeeding of methanol during the late exponential growth phases. This module can distribute methanol into several doses and then increases the frequency of dosing based on the metabolic capacity of the cells. The initial feed rate (F_i) was calculated based on the data obtained in batch cultivations. For fed-batch cultivation, the feed rate was then calculated according to the equation, $F = (\mu XV e^{\mu t})/(YS)$, where μ is the desired specific growth rate (h^{-1}) on methanol, X the cell density (g l^{-1}), V the culture volume (l), t the time elapsed from the start of feeding of methanol (h), Y the yield of biomass on methanol, and S is the methanol concentration (g l^{-1}) in the feed solution [2,21]. The upper limit of the feed rate, F_U, was set to maintain the level

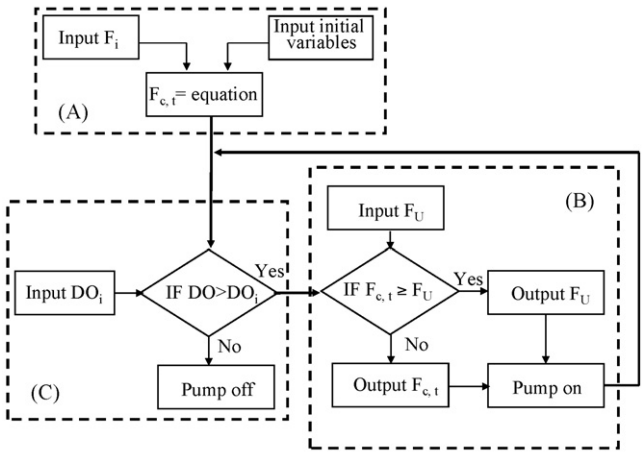

Fig. 1. Schematic diagram of algorithm-based feeding mode (AF mode) in fed-batch cultivation. (A) The module of exponential feeding; (B) the module to control the upper limit of feed rate; (C) the module of DO-feedback control; F_i, initial feed rate; F_C, current feed rate; F_U, upper limit of feed rate; DO, dissolved oxygen; DO_i, set point of dissolved oxygen.

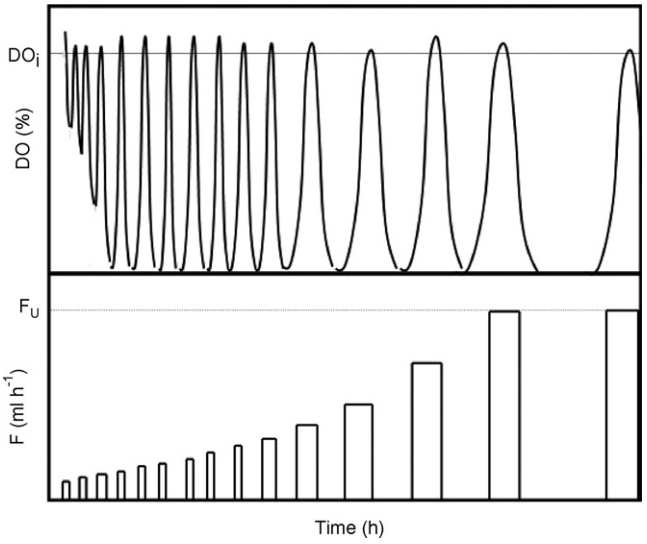

Fig. 2. Output responses of the AF. F_U, upper limit of feed rate; DO_i, set point of dissolved oxygen.