Periodic Methanol Induction Control for Enhanced Production of Porcine Interferon by Pichia pastoris Postprint

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Date: 2019-01-17T00:00:00+00:00

Abstract

The production of porcine interferon (pIFN-) using methanol-utilizing Pichia pastoris is typically carried out under high cell density conditions with methanol concentration controlled at a constant value of 5-10 g/L during the induction phase, during which the dissolved oxygen concentration (DO) naturally drops to a level close to zero. If the highly aerobic Pichia pastoris remains in a high methanol/low DO induction environment for an extended period, its metabolic activity deteriorates; intracellular methanol accumulates severely; and the production efficiency of pIFN- expression becomes low. To address this issue, a methanol cyclic induction control strategy was proposed to enhance pIFN- production. Methanol was first controlled at a high concentration for 7 h; then the methanol feeding rate was reduced to control DO at approximately 20% for about 4 h, with a total of 6 cycles repeated. Using the aforementioned cyclic control strategy, the metabolic activity of Pichia pastoris could be maintained at a high level for an extended period; intracellular methanol remained at an extremely low level (0.003 g/g-DCW), alleviating the methanol toxicity effect; and pIFN- activity reached a maximum level of 3.90×10^7 IU/mL, which was 1.86 times that achieved under constant methanol concentration control.

Full Text

Enhancing pIFN- Production by *Pichia pastoris* via Periodic Methanol Induction Control

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Abstract

In the production of porcine interferon- (pIFN-) by methylotrophic *Pichia pastoris*, induction is typically performed at high cell density with methanol concentration controlled at 5–10 g/L, causing dissolved oxygen concentration (DO) to naturally decline to near-zero levels. However, this highly aerobic yeast system suffers from deteriorating metabolic activity, severe intracellular methanol accumulation, and low target protein expression efficiency when subjected to prolonged high methanol/low DO conditions. To address these issues, we propose a novel periodic methanol induction control strategy. The method alternates between two distinct phases: a 7-hour period of high methanol concentration (5–10 g/L) with low DO (~0%), followed by a 4-hour period where methanol feeding rate is reduced to maintain DO at approximately 20% (creating a low methanol/high DO environment). This cycle was repeated six times. Implementation of this periodic control strategy successfully maintained high metabolic activity in recombinant *P. pastoris* while keeping intracellular methanol at extremely low levels (0.003 g/g-DCW), thereby alleviating methanol toxicity. Consequently, pIFN- activity reached a maximum of 3.90×10 IU/mL, representing an 86% improvement over conventional constant methanol concentration control.

Keywords: *Pichia pastoris*, methanol, periodic control, DO, pIFN-

Introduction

Constant-value control of state variables (such as concentrations, specific growth rates, or substrate consumption rates) represents the most common approach for fermentation process optimization. However, periodic control—whether self-generated or forced—can offer distinct advantages over constant-value control in certain specialized applications. Periodic control involves switching state variables between different environmental conditions at a defined frequency and repeating this pattern for multiple cycles.

In the field of fermentation process control, several studies have demonstrated the effectiveness of periodic strategies. Cheng et al. [1] employed periodic glucose feeding to enhance plasmid stability and -galactosidase production in recombinant *Saccharomyces cerevisiae*. By exploiting the differential response of plasmid-bearing and plasmid-free cells to environmental changes, they alternated between glucose excess (1 g/L) and starvation states at ~6-hour intervals for 4–5 cycles. This approach maintained low plasmid loss rates (12%), increased total cell concentration by 32.8%, and improved -galactosidase yield by 80.6% compared to conventional batch fermentation. Ye et al. [2] utilized periodic switching between aerobic and anaerobic conditions to enhance vitamin B production by *Propionibacterium freudenreichii*. While anaerobic conditions promoted rapid cell growth, prolonged cultivation led to propionic acid accumulation that inhibited growth. Although aerobic conditions could oxidize propionic acid, continuous aeration reduced growth and product synthesis. Periodic

environmental switching increased cell concentration by 189%, reduced propionic acid levels from 5.08 g/L to 2.78 g/L, and doubled vitamin B production compared to pure anaerobic fermentation. Shen et al. [3] observed that high-concentration ethanol continuous fermentation exhibited periodic oscillations when dilution rate was switched from 0.027 h ^{1} to 0.04 h ^{1}, whereas direct initiation at 0.04 h ^{1} achieved steady state. The oscillatory process improved average residual glucose by 14.8%, ethanol concentration by 12.6%, and productivity by 12.3% compared to the steady-state process. Ma et al. [4] achieved 57% higher human pro-urokinase expression in *P. pastoris* by alternating stimulation with dual carbon sources (methanol/glycerol).

Methylotrophic *Pichia pastoris* is widely used for heterologous protein expression [5–7], with induction typically conducted at high cell density. In MutS-type *P. pastoris* expressing pIFN- , methanol concentration is usually maintained at 5–10 g/L. However, this highly aerobic system experiences near-zero DO saturation when cells are continuously exposed to constant high methanol concentrations. This condition not only reduces metabolic activity but also impairs the first oxidation step of intracellular methanol metabolism (methanol + O $\rightarrow$ formaldehyde) due to oxygen limitation, leading to severe toxic methanol accumulation and low induction efficiency. To overcome these limitations, we propose a novel periodic methanol induction control strategy that alternates methanol concentration and DO between "high/low" and "low/high" levels at different cycle durations. This approach aims to maintain recombinant *P. pastoris* metabolic activity, alleviate intracellular methanol accumulation, enhance methanol induction efficiency, and ultimately increase pIFN- concentration and antiviral activity. By comparing this periodic control strategy with constant methanol concentration control (8–10 g/L) and constant DO control (~20%), we validate its effectiveness and provide valuable insights for efficient heterologous protein expression in *P. pastoris*.

Materials and Methods

1.1 Experimental Strain and Culture Media

Strain: Recombinant *Pichia pastoris* KM71H (IFN -pPICZ A) was constructed by the Key Laboratory of Animal Husbandry and Veterinary Medicine, Shanghai Academy of Agricultural Sciences.

Media compositions: - Plate activation medium (g/L): glucose 20, peptone 20, yeast extract 10, agar 20, natural pH. - Seed medium (g/L): glucose 20, peptone 20, yeast extract 10, natural pH. - 5-L fermenter initial medium (g/L): glycerol 20 mL/L, MgSO 1, K SO 1, (NH) SO 5, CaSO 0.1, H PO 2% (V/V), PTM1 trace salts 10 mL/L, pH 6.0. - Glycerol feeding medium (g/L): glycerol 500 mL/L, MgSO 0.03, (NH) SO 0.5, KH PO 0.5, PTM1 10 mL/L, pH 6.0. - Induction feeding medium: pure methanol + 10 mL/L PTM1, pH 5.5.

1.2.1 Seed Culture

A rice-grain-sized inoculum was transferred from YPD plates to a 500-mL shake flask containing 50 mL seed medium and cultivated at 30 °C, 220 rpm for 24 h to prepare the seed culture for fermenter inoculation.

1.2.2 High-Cell-Density Fed-Batch Cultivation in 5-L Fermenter

Fed-batch cultivation was performed in a 5-L fermenter (BIOTECH-5BG, Shanghai Baoxing Bio-Engineering Equipment Co.) equipped with pH and DO electrodes (Mettler Toledo, Switzerland). The initial working volume was 2.3 L with 13% (V/V) inoculum. Aeration was maintained at 3 vvm (air), with initial agitation at 300 rpm. Agitation speed was manually increased to keep DO above 10%. After depletion of initial glycerol, an improved DO-Stat strategy [8] was implemented for glycerol feeding. Pure oxygen was supplied when maximum agitation (700 rpm) could not maintain DO above 10%. pH was maintained at 6.0 with 5% (V/V) ammonia solution, and temperature was controlled at 30 °C. Upon reaching high cell density (~125 g-DCW/L, OD 500) after approximately 25 h, glycerol feeding was stopped, followed by 1–2 h of "starvation cultivation" to completely deplete residual glycerol.

1.2.3 Methanol Induction for pIFN- Production

At the end of starvation cultivation, DO rose sharply. Pure oxygen was then switched to air, temperature was reduced to 20 °C, and pH was adjusted to 5.5. Temperature was controlled using a low-temperature circulating bath (MP-10C, Shanghai Yiheng Technology). A small amount of methanol was added; after DO began to decrease and oxygen uptake rate (OUR) increased (indicating cellular adaptation), induction feeding was 正式启动. Three induction strategies were implemented:

1. **Constant methanol concentration control (Strategy I):** Methanol concentration was maintained at 5–10 g/L throughout induction using an online methanol electrode detection system (FC-2002, East China University of Science and Technology) with ON-OFF control.
2. **Constant DO control (Strategy II):** Methanol was fed at an extremely low rate using an industrial computer (EVOC 810A, Advantech) with AD-DA interface card (PCL-812PG, Advantech) to drive an adjustable peristaltic pump (BT00-50M, Baoding Langer). The control law was F = F* + Kc × (DO -DO), F 0, where F is methanol feed rate, F* is the "baseline" rate (0.7 mL/min), Kc = 0.05, and DO = 20%. This maintained DO near its setpoint throughout induction.
3. **Periodic methanol induction control (Strategy III):** The fermentation environment was alternated between "high methanol/low DO" and "high DO/low methanol" states. Methanol concentration and DO were controlled at approximately 5–10 g/L/~0% and 0 g/L/~20%, respectively,

with defined cycle durations repeated for 5–6 cycles, achieving total induction times of 70–80 h.

1.2.4 Analytical Methods

Cell dry weight (g-DCW/L) was determined from OD measurements (UNICO 7200 spectrophotometer) using the linear relationship: g-DCW/L = 0.25 × OD . Extracellular methanol concentration was measured by gas chromatography (GC112A, Shanghai Precision Scientific Instrument Co.) with FID detector and Alpha-Col AC20 capillary column (SGE, Australia) according to reference [9]. Intracellular methanol concentration was determined after cell disruption and centrifugation using the method of Suye et al. [10], followed by GC analysis as above. Specific methanol consumption rate was calculated as described in reference [11] by measuring weight loss of the induction medium bottle using an electronic balance (JA1102, Shanghai Haikang Electronic Instrument Factory) interfaced with the industrial computer/AD-DA card. pIFN- was qualitatively analyzed by SDS-PAGE (Bio-Rad Mini-PROTEAN) according to reference [12] and quantified using a G:Box gel documentation system with GeneTools software (SynGene, UK). Antiviral activity of pIFN- was determined as described in reference [13]. Off-gas O and CO partial pressures were measured online using a tail gas analyzer (LKM2000A, LOKAS, Korea), enabling calculation of oxygen uptake rate (OUR) and carbon dioxide evolution rate (CER) using standard formulas [14]. Alcohol oxidase (AOX) activity was measured using the method of Suye et al. [10], with one unit defined as the amount producing 1 mol H O per minute from methanol. Formaldehyde dehydrogenase (FLD) and formate dehydrogenase (FDH) activities were determined according to reference [15]. All measurements (except online parameters) were performed in triplicate, with mean values reported.

Results and Discussion

2.1 pIFN- Expression Performance Under Constant Methanol Control (Strategy I)

When cell concentration reached ~125 g-DCW/L, methanol concentration was controlled at 5–10 g/L (Run #1), causing DO to drop to 0% within 3–5 h after induction initiation. pIFN- concentration increased gradually but began declining after 60 h of induction. Final cell concentration, pIFN- concentration, and antiviral activity reached 150 g-DCW/L, 0.79 g/L, and 2.10×10 IU/mL, respectively (Fig. 1 [Figure 1: see original paper]). SDS-PAGE analysis (Fig. 2 [Figure 2: see original paper]) showed that the intensity of the target pIFN- band (16 kDa) increased with induction time (Run #1), but the enhancement was modest.

Previous studies [16] demonstrated that induction at low temperature (20 °C) enhances alcohol oxidase (AOX) activity, increases target protein yield, reduces extracellular protease secretion, and improves overall fermentation performance.

However, this condition also dramatically increases oxygen demand, resulting in extremely low DO levels throughout the induction period.

Methanol metabolism in *P. pastoris* involves two pathways: formaldehyde dissimilation for energy generation and target protein synthesis [12,17]. Exogenous methanol enters the cytoplasm and is oxidized to formaldehyde by AOX in peroxisomes. A portion of formaldehyde enters the dissimilation pathway (Pathway A), where FLD converts it to formate, which is then oxidized to CO by FDH, releasing NADH to support assimilation. The remaining formaldehyde enters the protein synthesis pathway (Pathway B) for heterologous protein production. However, several issues arise: (1) High AOX activity at low temperature intensifies oxygen consumption, and extremely low DO levels impede efficient methanol→formaldehyde oxidation; (2) The extracellular/intracellular methanol concentration gradient drives continuous methanol influx when external concentration is high (8–10 g/L), while inefficient oxidation causes severe intracellular accumulation and "methanol toxicity," reducing pIFN- expression. Intracellular methanol concentration reached 0.01387 g/g-DCW under these conditions (Fig. 3b [Figure 3: see original paper]); (3) Supplementing pure O could maintain high DO and efficient methanol→formaldehyde oxidation, and since FLD and FDH activities are substantially higher than AOX activity (Figs. 3–4 [Figure 4: see original paper]), toxic intermediates (methanol, formaldehyde, formate) could be fully oxidized, alleviating accumulation and improving pIFN- expression [12]; (4) However, continuous pure O supplementation is costly and poses safety risks.

Periodic methanol induction control offers a solution: (1) Reducing methanol concentration to ~0 g/L allows rapid DO recovery and oxygen influx, restoring cellular metabolic activity; (2) Extracellular methanol deficiency stops further diffusion into cells while accumulated intracellular methanol continues to be utilized for energy generation and protein synthesis, relieving "methanol toxicity" and maintaining effective induction; (3) Once intracellular methanol is depleted, methanol concentration can be rapidly restored to high levels to repeat the cycle.

2.2 pIFN- Expression Performance Under Constant DO Control (Strategy II)

Using the extremely low methanol feeding rate described above (F = F* + Kc × (DO -DO), F 0), DO was maintained at ~20% (Strategy II, Run #2). This kept DO between 10–35% while methanol concentration remained at 0.0–1.0 g/L throughout induction. After 76 h, final cell concentration and pIFN- concentration were only 120.3 g-DCW/L and 0.34 g/L, respectively (Fig. 1). The pIFN- band intensity showed minimal increase (Fig. 2, Run #2), indicating poor expression. Although high DO should theoretically support efficient methanol→formaldehyde oxidation, the low extracellular methanol concentration created insufficient transmembrane gradient, limiting methanol influx and resulting in suboptimal intracellular methanol levels. Consequently, pIFN- expression performance was far inferior to Strategy I.

2.3 pIFN- Expression Performance Under Periodic Methanol Induction Control (Strategy III)

As described, periodic methanol induction control alternates the fermentation environment between "high methanol/low DO" and "high DO/low methanol" states (Strategy III, Run #3).

2.3.1 Determination of Cycle Durations for Different States Unlike chemical reactions, biological systems respond slowly to operational changes. In this system, rapidly adding pure methanol to increase concentration is relatively fast, whereas decreasing methanol concentration through consumption is time-consuming. The 20 °C induction temperature was selected partly because high AOX activity accelerates methanol consumption.

Since all methanol-related reactions occur intracellularly, we propose a simple equation for intracellular methanol accumulation (Eq. 1a). Here, C OH, C OH, ΔC OH, r OH, and T represent extracellular methanol concentration (g/g-DCW), average intracellular methanol concentration (g/g-DCW), intracellular methanol concentration increment during a specific induction period (g/g-DCW), specific methanol consumption rate (h ^{1}), and induction time interval (h), respectively. K is the diffusion coefficient from extracellular to intracellular space (h ^{1}). Since extracellular methanol concentration far exceeds intracellular concentration, Eq. 1a simplifies to Eq. 1b (neglecting C OH). Using experimental data from Run #1 (constant methanol control) (Figs. 3b and 5a [Figure 5: see original paper]), we determined K . With ΔC OH = 0.005 g/g-DCW (0.0025→0.0075, Fig. 3b), C OH = 0.064 g/g-DCW (~8 g/L methanol for 125 g-DCW/L cells), r OH = 0.025 h ^{1}, and T = 25 h (10–35 h, Run #1), K was calculated as 0.028 h ^{1}.

For cycle duration determination, we assumed: (1) Under high methanol (8–10 g/L), intracellular methanol increment per cycle (T) should not exceed 0.001 g/g-DCW (ΔC OH = 0.001), with C OH = 0.064 g/g-DCW and r OH = 0.025 h ^{1}. From Eq. 1b, T should be 5.2 h. (2) Under low methanol (0–1 g/L), intracellular methanol decrement per cycle (T) should be ~0.001 g/g-DCW (ΔC OH = -0.001), with C OH = 0.032 g/g-DCW (average concentration) and r OH = 0.010 h ^{1} (Fig. 5a), assuming no further methanol diffusion. From Eq. 1b, T was calculated as 3.1 h. The high/low methanol cycle length ratio was 1.68. Based on these theoretical calculations and practical considerations, final cycle durations were set at T = 7 h and T = 4 h (ratio = 1.75).

2.3.2 Enhanced Methanol Utilization Rate and Cellular Metabolic Activity Literature reports [18] indicate that specific methanol consumption rate reflects intracellular methanol metabolism, with higher rates generally correlating with higher target protein production. Fig. 5a compares specific methanol consumption rates across three runs. The periodic control strategy (Run #3) achieved the highest average rate (0.0245 h ^{1}), followed by constant methanol control (0.0220 h ^{1}), while constant DO control showed the lowest rate (0.0105

h [1]), less than half of the other two runs. Figs. 5b and 5c compare OUR and CER levels for Run #1 (constant methanol) and Run #3 (periodic control) (Run #2 data omitted due to low respiratory activity). Run #1 OUR and CER reached stable values after 15 h induction, then gradually declined. Run #3 OUR and CER exhibited periodic fluctuations corresponding to environmental switching but maintained overall higher levels than Run #1. Both runs showed similar respiratory quotients (RQ) stabilized at 0.4–0.5 (Fig. 5d), indicating simultaneous enhancement of both dissimilation and assimilation pathways. CER originates from the formaldehyde dissimilation pathway (Pathway A) releasing NADH, while OUR supports methanol→formaldehyde oxidation and oxidative phosphorylation, converting NADH to ATP for target protein synthesis and cell growth [12]. Notably, during the final 20 h of induction, Run #3 OUR remained higher than Run #1, while RQ dropped below 0.4, suggesting improved NADH utilization efficiency and energy metabolism—another key factor enabling sustained pIFN- concentration increase in the late induction phase.

2.3.3 Improved pIFN- Expression Performance via Periodic Methanol Induction Control Following the established strategy, methanol concentration was ON-OFF controlled at high levels (5–10 g/L) for 7 h, then DO was maintained at ~20% by reducing methanol feed rate for 4 h. This cycle was repeated six times for a total induction period of 79 h. Using this strategy, pIFN- protein concentration increased steadily, reaching final values of 190 g-DCW/L cell concentration, 2.01 g/L pIFN- concentration, and 3.90×10 IU/mL antiviral activity—significantly higher than the other two runs (Fig. 1). The antiviral activity achieved with periodic control was 1.86-fold higher than that of constant methanol control (Run #1). SDS-PAGE analysis showed progressively intensifying pIFN- bands (16 kDa) (Fig. 2, Run #3), confirming the substantial concentration increase.

Previous studies [19] indicate that methanol utilization-slow (MutS) *P. pastoris* prefers higher methanol concentrations for induction. The pIFN- production strain used in this study is a MutS type, for which higher methanol concentrations are beneficial. Insufficient methanol concentration yields weak induction, while excessive concentrations cause "methanol toxicity," with the optimal range being 5–10 g/L [16]. Run #1 maintained methanol within this optimal range but suffered from declining metabolic activity and intracellular methanol accumulation, resulting in limited pIFN- improvement even at 20 °C [16]. The proposed periodic strategy maintains methanol at optimal concentrations for extended periods (7 h) to strengthen induction intensity, while brief intervals (4 h) at high DO/low methanol restore metabolic activity and relieve intracellular methanol accumulation. During these low-methanol phases, cells continue utilizing residual intracellular methanol for effective induction. Thus, periodic methanol induction control achieves three simultaneous benefits: metabolic activity recovery, intracellular methanol reduction, and sustained effective induction, demonstrating superior performance to constant methanol control at optimal concentrations and highlighting its novelty and practicality.

Conclusion

This study proposes a novel periodic methanol induction control strategy to improve *P. pastoris* expression of pIFN- . At 20 °C, methanol concentration and DO were periodically switched between high/low and low/high levels: 7 h of ON-OFF methanol control at 5–10 g/L followed by 4 h of DO control at ~20% through reduced methanol feeding, repeated for six cycles. This periodic strategy: (1) enhanced and sustained recombinant *P. pastoris* metabolic activity and specific methanol consumption rate at high levels; (2) reduced intracellular methanol to extremely low concentrations (0.003 g/g-DCW), eliminating "methanol toxicity" and enabling complete utilization of accumulated methanol; (3) achieved maximum pIFN- activity of 3.90×10 IU/mL, representing an 86% improvement over the suboptimal constant methanol concentration control strategy.

Acknowledgments

This research was supported by the Jiangsu Modern Industrial Fermentation Collaborative Innovation Center and the National First-Class Discipline Program of Light Industry Technology and Engineering (#2018-17).

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Note: Figure translations are in progress. See original paper for figures.