Enhanced human lysozyme production by *Pichia pastoris* via periodic dissolved oxygen concentration control

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Abstract

In human lysozyme (hLYZ) production by *P. pastoris*, severe reactive oxygen species (ROS) accumulation occurred during the cell growth phase when using DO-stat control mode, leading to a limited expression of hLYZ. In this study, a novel periodic dissolved oxygen concentration (DO) control strategy was proposed to solve these problems. This strategy intermittently/periodically switched the cultivation environment from "high DO-low glycerol concentration" (DO-stat control mode) to "low DO-high glycerol concentration" for 4 cycles. With the strategy: (1) the highest cell concentration (˜120 g-DCW L^{-1}) with healthy and functional skeleton was achieved at the end of cell growth phase by controlling ROS levels below 48.35 Fluorescence intensity g-DCW^{-1}; (2) cells metabolic activities in the successive induction phase were largely enhanced; (3) hLYZ activity reached the highest level of 2.18×105 IU mL^{-1}, which was about 2-fold than that obtained with the DO-stat control mode, when the same induction strategy was adopted.

KEYWORDS:

Pichia pastoris ; reactive oxygen species; glycerol feeding culture; periodic control; human lysozyme

1 INTRODUCTION

Heterologous protein production by methanotrophic yeast *P. pastoris* is basically divided into two discrete and successive phases: (1) a cell growth phase to achieve high cell-density cultivation using glycerol as the sole carbon source; (2) a successive methanol induction phase to initiate the targeted recombinant protein production by feeding methanol.[1-3] In general, achievement of high cell-density cultivation at the end of cell growth phase (beginning of the methanol induction phase) is acknowledged as the precondition for efficient heterologous protein production in the successive methanol induction phase. Therefore, different online and offline glycerol feeding control strategies (DO-stat feeding, constant feeding or exponential feeding etc.) were carried out to achieve a higher cell concentration at the end of cell growth phase.[4,5]Among these control strategies, the online DO-stat glycerol feeding strategy, namely the environment of "high DO-low glycerol concentration" (DO 20-80%, glycerol concentration 0-1 g L^{-1}), have been considered as the most simple and effective method to achieve high cell-density cultivation within short time.[5] However, it was well known that severe reactive oxygen species (ROS) accumulation occurred when cells were exposed to the oxygen-enriched (high DO) environments for long time.[6,7] In this case, the structural and functional cell components were obviously damaged, leading to the loss of cell growth ability or even cell death. As a result, in human lysozyme (hLYZ) production by the aerobic *P. pastoris* , severe ROS accumulation must be managed when using the traditional DO-stat glycerol feeding strategy.

In adaptive laboratory evolution, the increased toxicity due to environmental stresses could be effectively ameliorated by implementing a recovery treatment after environmental stimulation.[8]In this study, a similar control strategy, namely periodic DO control strategy, was developed to repress the severe ROS accumulation under the oxygen-enriched environments by alternatively switching the cultivation environment from "high

DO-low glycerol concentration" (DO-stat control mode) to "low DO-high glycerol concentration" for 4 cycles., aiming to achieve a higher cell concentration at the end of cell growth phase and further enhance hLYZ production in the successive methanol induction phase.

2 EXPERIMENTAL SECTION

2.1 Strains and media

The methanol utilization slow type (Mut^S) *P. pastoris* strain (KM71) was used as a host strain for expressing hLYZ.[9] The compositions for seed medium, batch medium, and glycerol/methanol feeding medium were same as our previous report.[10]

2.2 Cell cultivation and hLYZ induction methods

The fed-batch cultivation for hLYZ production by Mut^S*P. pastoris* was implemented in a 5 L fermentor (BLBIO-5GJ-3-H, Bailun Bio Co., China). The working volumes were about 3.0 L. Inoculation and aeration rate were at 20% (v v^{-1}) and 4 vvm. Temperature and pH were maintained at and 6.0. In the glycerol batch phase, DO was maintained above 10% by manually increasing agitation rate. When glycerol was exhausted and DO suddenly rose up, the glycerol fed-batch phase was initiated. With the help of glycerol feeding control strategies A, B or C, to be described below, the glycerol feeding rate was automatically/manually regulated by the programmable peristaltic pump (BT00-100 M, Langer Co., China) and the duration of this phase were about 23 h. The methanol induction phase was initiated by feeding methanol after glycerol was used out. During the methanol induction phase, methanol concentration was controlled around 5-10 g L^{-1} using the same procedures described in our previous reports.[1,10] Air was used for aeration throughout the entire methanol induction phase, the aeration rate, temperature and pH remained unchanged.

Strategy A (high DO-low glycerol concentration): with the help of DO-stat control mode, DO was controlled at 20-80% and glycerol concentration naturally stayed at 0-1 g L^{-1}. When the base-line of DO could not be control over 20%, pure oxygen was used to aerate.

Strategy B (low DO-high glycerol concentration): glycerol concentration was controlled at 2-5 g L^{-1} by manually regulating glycerol feeding rate based off-line measured data and DO naturally stayed at almost zero level under the ordinary air-aeration conditions.

Strategy C (periodic DO control): with the aid of strategy A and B, the strategy periodically switched the cultivation environment from "high DO-low glycerol concentration" (DO 20-80%, glycerol concentration 0-1 g L^{-1}; T_1[?]5 h) to "low DO-high glycerol concentration" (DO ˜0%, glycerol concentration 2-5 g L^{-1}; T_2[?]1 h) for 4 cycles.

2.3 Analytical methods

Concentrations of cell, glycerol, methanol, reactive oxygen species (ROS), and oxygen uptake rate (OUR), specific glycerol/methanol rate were determined by the reported method.[10-12] Cell viability was measured by fluorescein isothiocyanate (FITC) labeling technique.[13] The activities of alcohol oxidase (AOX), formaldehyde dehydrogenase (FLD), formate dehydrogenase (FDH), hLYZ and SDS-PAGE analysis were measured or analyzed using the same procedures described in our previous reports.[9,10]

3 RESULTS AND DISCUSSION

3.1 Achievement of high cell concentration with functional cellular skeletons during glycerol feeding phase by the periodic DO control strategy

The environment of "high DO-low glycerol concentration" based on DO-stat control mode (strategy A) was firstly implemented to achieve high cell-density culture during the cell growth phase. As shown in Fig1A, when using this strategy, DO was controlled at 20-80% and glycerol concentration naturally stayed at 0-1 g L^{-1}during the glycerol feeding phase. However, severe reactive oxygen species (ROS) accumulation occurred when cells were exposed to the oxygen-enriched (high DO) environment for long time, leading to the loss of cell growth or even cell death (Fig.1D). More concretely, ROS concentration rapidly rised to the highest

level of 152.95 Fluorescence intensity g-DCW^{-1} at 18 h when implementing the environment of "high DO-low glycerol concentration". After that, cells growth almost stopped. Meanwhile, about 17.09% of the cells cannot tolerate such a ROS concentration at the end of glycerol feeding phase when using this control strategy (Fig.1G). The environment of "low DO-high glycerol concentration" (strategy B) was also implemented by controlling glycerol concentration at 2-5 g L^{-1} and DO naturally reduced to almost zero level during most of the period in the glycerol feeding phase (Fig.1B). When using this strategy, maximum ROS concentrations declined to a very low level of 28.25 Fluorescence intensity g-DCW^{-1} (Fig.1E), which could be due to decrease in cell metabolic activity at oxygen limitation (low DO) environments. However, with this strategy, final cell concentration finished at a very low level of 46.25 g-DCW L^{-1} and cell mortality was at the highest level of 26.08% (Fig.1E and 1H), which were far beyond the expectation. In this case, the shortage of oxygen (low DO) could not stratify the lowest requirement for cell growth and maintenance metabolism, a lower ROS level cannot achieve high cell-density cultivation.

Aiming to compromise the positive effects of above glycerol feeding strategies, a novel periodic DO control strategy (strategy C) was implemented via periodically switching the cultivation environment from "high DO-low glycerol concentration" (DO 20-80%, glycerol concentration 0-1 g L^{-1}; T_1[?]5 h) to "low DO-high glycerol concentration" (DO ˜0%, glycerol concentration 2-5 g L^{-1}; T_2[?]1 h) for 4 cycles (Fig.1C). As shown in Fig.1F, the highest cell concentration of 118.96 g-DCW L^{-1} was achieved at the end of glycerol feeding phase with this periodic control strategy. Meanwhile, maximum ROS concentration declined to a lower level of 48.35 Fluorescence intensity g-DCW^{-1} and cell mortality was controlled below 10% throughout the entire glycerol feeding phase (Fig.1F and 1I).

Figure 1

3.2 Reducing maintenance coefficient during the glycerol feeding phase by the periodic DO control strategy

According to substrate (glycerol) mass balance, maintenance coefficient using different glycerol feeding strategies could be determined by the following equation:

(1)

Here, ν , μ , $Y_{X/S}$ and $m$represented specific glycerol consumption rate, specific cell growth rate, cell yield on glycerol and maintenance coefficient. As shown in Fig.2, to maintain cellular homeostasis under the environments of high ROS levels (152.95 Fluorescence intensity g-DCW^{-1}), the distribution of glycerol towards to cell maintenance reached the highest level of 0.0328 g g-DCW^{-1}h^{-1} when implementing the "high DO-low glycerol concentration" control strategy (strategy A). In contrast, with the aid of the periodic DO control strategy (strategy C), ROS levels were controlled below 48.35 Fluorescence intensity g-DCW^{-1}during the glycerol feeding phase. Accordingly, maintenance coefficient with this strategy was also kept at a lower level of 0.0198 g g-DCW^{-1} h^{-1}. It should be noted that the data using the "low DO-high glycerol concentration" control strategy (strategy B) cannot be fitted with Eq. 1 and not shown in Fig.2 because cell concentration decreased obviously during the glycerol feeding phase (Fig. 1E).

Figure 2

3.3 Enhancing hLYZ production by the periodic DO control strategy

After cell concentration reached a relatively high density at the end of cell growth phase, the successive methanol induction phase was initiated by feeding methanol to induce heterogeneous protein synthesis/production. As shown in Fig.3, cell concentration was kept at an almost constant level throughout the entire methanol induction phase when using the same methanol induction control strategy of controlling methanol concentration at 5-10 g L^{-1}. As a result, achievement of high cell-density cultivation at the end of cell growth phase (beginning of the methanol induction phase) was an important factor for efficient heterologous protein production in the successive methanol induction phase. As shown in Fig.3, when using the periodic DO control strategy, final hLYZ activity reached the highest level of 2.18×10^5 IU mL^{-1} with a higher cell concentration (˜120 g-DCW L^{-1}) at the end of cells growth phase (Fig.3). This activity was about 2-fold of that obtained with the "high DO-low glycerol concentration"/DO-stat control strategy when using

the same methanol induction strategy. In the case of using the "low DO-high glycerol concentration" control strategy, final hLYZ activity finished at very low levels of 4.71×10^3 IU mL^{-1} and the duration of methanol induction phase was only at about 20 h due to the extremely low oxygen uptake rate (OUR) and the stoppage of utilizing methanol (Fig.3). In addition, SDS-PAGE analysis using different glycerol feeding strategies was also carried out to confirmed above results. As shown in Fig.3C, the hLYZ protein band obtained with the periodic DO control strategy (strategy C) were much intensive than those of the other two batches (strategy A and B).

Figure 3

3.4 Metabolic interpretation of enhancing hLYZ production in the successive methanol induction phase by the periodic DO control strategy

3.4.1 Reducing the adaptation time to methanol induction environments

When the second methanol induction phase was initiated by feeding methanol, Mut^S *P. pastoris* cells has to take a long-term time to adapt the environment of methanol induction by synthesizing alcohol oxidase (AOX) to drive methanol metabolism. In general, the stoppage of this adaptation phase could be easily and indirectly reflected by the drop of DO and the increased OUR (online data). As shown in Fig.4, when using the periodic DO control strategy (strategy C), the drop of DO and the increased OUR occurred at about 3 h during the methanol induction phase, which was only one half of that obtained with the "high DO-low glycerol concentration" control strategy (strategy A) when using same methanol induction strategy. As the batch using "low DO-high glycerol concentration" control strategy (strategy B), the drop of DO and the increased OUR were not observed throughout the entire methanol induction phase (data not shown), leading to the failure of hLYZ production.

3.4.2 Enhancing P. pastoris metabolic activities in the successive methanol induction phase

The major chemical reactions in heterogeneous protein by *P. pastoris* and relevant key enzymes involved have been widely reported previously.[1,10] Functions of the key enzymes involved in methanol metabolism were as follows: AOX catalyzes the first chemical reaction in methanol metabolism (CH_3OH+O_2-$HCHO+H_2O_2$); FLD and FDH contribute to NADH and ATP regeneration ability in the formaldehyde dissimilation pathway and the role of this path was to supply a source of energy for heterogeneous proteins production. As shown in Fig.4II, when using the "high DO-low glycerol concentration" control strategy, specific activities of AOX, FLD and FDH were significantly repressed, leading to a limited methanol/oxygen consumption and hLYZ expression. As for the batch using the periodic DO control strategy, the specific activities of AOX, FLD and FDH were obviously enhanced. Accordingly, the average specific methanol consumption rate and OUR increased for about 40% with the periodic control strategy, which eventually led to an efficient hLYZ production.

Figure 4

In a summary, a novel periodic DO control strategy for efficient hLYZ production was proposed. With this novel control strategy: a higher cell concentration (˜120 g-DCW/L) with healthy and functional skeleton was achieved at the end of cell growth phase by controlling ROS concentration below 48.35 Fluorescence intensity $g\text{-}DCW^{-1}$; (2) *P. pastoris* metabolic activities in the successive methanol induction phase were largely enhanced, leading to an efficient hLYZ production when the same methanol induction strategy was applied.

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Figure Captions

Figure 1 Curves of fermentation data during the glycerol feeding phase when using different glycerol feeding strategies.

A, D & G: "high DO-low glycerol concentration" control strategy; B, E & H: "low DO-high glycerol concentration" control strategy; C, F & I: periodic DO control strategy. - : DO; : glycerol concentration; *: cell concentration; $^{:}ROSconcentration$; : $cellmortality$.

Figure 2 Glycerol metabolism patterns when using different glycerol feeding strategies.

: "high DO-low glycerol concentration" control strategy; $^{:}$"$lowDO - highglycerolconcentration''controlstrategy; * : periodicDOcontrolstrategy$.

Figure 3 Variations of cell concentration, hLYZ activities and SDS-PAGE analysis results of hLYZ production during the methanol induction phase when using different glycerol feeding strategies.

/strategy A: "high DO-low glycerol concentration" control strategy; $/strategyB$: "$lowDO - highglycerolconcentration''controlstrategy; */strategyC : periodicDOcontrolstrategy.$

Figure 4 Variations of major fermentation/induction data of hLYZ production during the methanol induction phase when using different glycerol feeding strategies.

(I) Variations of DO and OUR. A: "high DO-low glycerol concentration" control strategy; B: periodic DO control strategy. - : DO; . . . : OUR.

(II) Variations of specific activities of AOX, FLD, FDH and specific methanol consumption rate. : "high DO-low glycerol concentration" control strategy; *: periodic DO control strategy.

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