Controlling Specific Growth Rate for Recombinant Protein Production by *Pichia pastoris* Under Oxidation Stress in Fed-batch Fermentation

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Abstract

Methanol can be used by *Pichia pastoris* as the carbon source and inducer to produce recombinant proteins in high-cell-density fermentations. However, methanol oxidation at high specific growth rates can lead to the reactive oxygen species (ROS) accumulation, resulting in cell damage. Here, we study the relationship between methanol feeding and ROS accumulation by controlling specific growth rate during the induction phase. A higher specific growth rate increased the level of ROS accumulation caused by methanol oxidation. While the cell growth rate was proportional to specific growth rate, maximum total protein production and highest enzyme activity were achieved at a specific growth rate of 0.05 1/h as compared to that of 0.065 1/h. Moreover, oxidative damage induced by over-accumulation of ROS in *P. pastoris* during the methanol induction phase caused cell death and reduced protein expression ability. ROS scavenging system analysis revealed that the higher specific growth rate, especially 0.065 1/h, resulted in increased intracellular catalase activity and decreased glutathione content significantly. Finally, Spearman’s correlation analysis further revealed that the reduced glutathione might be beneficial for maintaining cell viability and increasing protein production under oxidative stress caused by ROS toxic accumulation. Our findings suggest an integrated strategy to control the feeding of the essential substrate based on analyzing its response to oxidative stress caused by ROS toxic accumulation, as well as develop a strategy to optimize fed-batch fermentation.

Keywords High cell density fermentation · Reactive oxygen species · Oxidative damage · Cell viability · Glutathione
Introduction

Methylotrophic yeast *Pichia pastoris* is one of the most extensively used expression systems for heterologous protein production, such as human growth hormone and lipase [1–3]. Methanol serves both as a carbon source for yeast growth in high-cell-density fermentation of *P. pastoris* and an inducer to produce heterologous proteins [4, 5]. Methanol feeding has become one of the optimization targets for large-scale production of protein in high cell density fermentation. However, the accumulation of intracellular toxic by-products from methanol oxidation may cause oxidative damage to cells [6, 7].

The shift from growth solely on glycerol to growth and heterologous protein production on methanol has a drastic impact on yeast. High methanol levels lead to the accumulation of intracellular toxic oxidative by-products such as formaldehyde and peroxide to cause cell death, whereas low methanol levels reduce protein productivity by triggering proteolytic degradation of the recombinant protein [8, 9]. To control methanol within an adequate range in fed-batch fermentation, methanol feeding is the only parameter to optimize. Increasing the methanol consumption blindly may cause the accumulation of ROS and reduce methanol utilization. Therefore, methanol concentration must be carefully controlled at an adequate range to achieve the best synergy between methanol consumption and cell growth [10–12]. For this purpose, the prerequisite is to understand how methanol concentration affects cell growth and protein production [13, 14]. The stress response of cells to environmental stress is a basic regulation mode of microbial life activities. Methanol provides a carbon skeleton for cell growth and target protein synthesis. Methanol entering in *P. pastoris* cell will be oxidized to formaldehyde in the peroxisome molecule and produces H$_2$O$_2$. H$_2$O$_2$ is known as ROS. The remaining formaldehyde is assimilated to maintain recombinant cell metabolism and thus the targeted protein synthesis by a cyclic pathway that starts with condensation of formaldehyde. Therefore, methanol feeding strategies regulate cell growth and heterologous protein levels by affecting cell metabolism. The methanol feeding strategy optimized by the parameters will be limited by the methanol-induced intracellular protein/cell synthesis pathway. Most cell growth for product formation are either directly or indirectly related to specific growth rate, which depends on the level of methanol consumption during *P. pastoris* fermentation [15]. Therefore, exploration of future optimal induction modes from cell growth and methanol consumption is the guarantee for large-scale production of heterologous proteins.

Our study focused on the effects of methanol consumption under different specific growth rates on cell growth and intracellular ROS scavenging systems of *P. pastoris* in high cell density fermentation. We identified the correlation of fermentation conditions with ROS-associated parameters. Then, we investigated the expression profile of genes involved in the ROS scavenging system at the mRNA level. Our findings suggest an integrated strategy to control the feeding of the essential substrate based on analyzing its response to oxidative stress caused by ROS toxic accumulation.

Materials and Methods

Strain and Chemicals

*P. pastoris* X-33 (Invitrogen, Carlsbad, CA) strain expressing lipase (named MAS1) gene from marine *Streptomyces* sp. strain W007 was used in our study [16, 17]. Yeast extract
peptone dextrose (YPD) medium for seed culture contained (g/L) yeast extract 10.00, peptone 20.00, and glucose 20.00. The basal salt medium (BSM) for batch fermentation contained (g/L) glycerol 40.00, 85% $\text{H}_2\text{PO}_4$ 26.70 mL, $\text{CaSO}_4$ 0.93, $\text{K}_2\text{SO}_4$ 18.20, MgSO$_4$$\cdot$7$\text{H}_2\text{O}$ 14.90, KOH 4.13, the *Pichia* trace metal (PTM1) salt solution 4.35 mL, and pH 5.0. PTM1 salt solution contained (g/L) CuSO$_4$$\cdot$5$\text{H}_2\text{O}$ 6.00, NaI 0.08, MnSO$_4$$\cdot$4$\text{H}_2\text{O}$ 3.00, ZnSO$_4$$\cdot$7$\text{H}_2\text{O}$ 20.00, FeSO$_4$$\cdot$7$\text{H}_2\text{O}$ 65.00, CoCl$_2$$\cdot$6$\text{H}_2\text{O}$ 0.50, boric acid 0.02, and H$_2$SO$_4$ 5.00 mL. All other chemicals of analytical grade were supplied from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China) unless otherwise indicated.

**Fed-batch Fermentation of Lipase MAS1**

The first inoculum culture was prepared from one colony of *P. pastoris* suspended in 50 mL sterilized YPD broth containing 100 μg/mL zeocin (Invitrogen, Carlsbad, CA). The culture was incubated at 30 °C in a 500-mL baffled shake-flask on rotary shaker at 250 rpm. When the OD$_{600}$ value reached 6.0, a second inoculum culture was prepared by transferring the first inoculum 10% (v/v) into a 2-L baffled shake-flask containing 400 mL sterilized YPD medium. The second inoculum culture was then incubated under the same condition as the first inoculum culture for 12 h.

The BSM (3 L) was inoculated with 10% (v/v) second inoculum into a 5-L bioreactor (BIOTECH-5BG, Baoxing Co., Shanghai, China). The initial OD$_{600}$ of the culture in bioreactor was about 0.5. A four-phase fermentation protocol (glycerol batch, glycerol fed-batch, starvation period, and methanol feeding based on growth kinetic) was used. The bioreactor was operated at 30 °C and pH around 6.0 via adding 28% (v/v) ammonia throughout the high cell density fermentation. Dissolved O$_2$ cascade was constantly maintained above 30% air saturation. The glycerol or methanol feeding to the bioreactor was controlled by a pump. Once glycerol was depleted from culture broth, indicated by a sharp increase in DO, the glycerol fed-batch phase was started at a constant flow rate of 18 mL/(L•h) with 50% (v/v) glycerol containing 12 mL/L PTM1 solution until the biomass reached about 180 g/L (wet cell weight). After 1 h starvation period following the above high cell density fermentation, the culture was supplied with 100% (v/v) methanol containing 12 mL/L PTM1 solution to induce lipase expression corresponding to different designed specific growth rates (0.015, 0.035, 0.05, and 0.065 1/h). We ran the fed-batch fermentation employing a methanol sensor (MC-168, PTI, USA) to control methanol feeding to maintain the methanol level at 2–4 g/L. A proportional, integral, and derivative control mode was applied for the methanol control system. Samples were taken at regular intervals every 24 h.

**Biomass, Protein Concentration, Lipase Activity, and SDS-PAGE Analysis**

Wet cell weight (WCW) of the cell suspension was determined by centrifugation of 10 mL cell broth in a pre-weighed centrifuge tube at 8000×g at 4 °C for 10 min, and the fermentation supernatant was collected and used for subsequent experiments. Total protein concentration in the supernatant was determined by the Bradford assay [18]. After lipase activity was measured using the alkali titration method [19], the obtained fermentation supernatant was analyzed by SDS-PAGE. Olive oil (Macklin, Shanghai, China) was emulsified with 4% (w/v) polyvinyl alcohol at the ratio of 1:3 (v/v). Each reaction contained 4 mL of emulsified olive oil, 5 mL of phosphate buffer at 50 mM and pH 6.0, and 1 mL of enzyme solution, and was carried out at 65 °C. The reaction was terminated by adding 15 mL of 95% (v/v) ethanol. The released fatty acids were neutralized by 0.05 M NaOH.
One unit of lipase activity is defined as the amount of enzyme releasing 1 μmol of fatty acid per minute.

**Cell Viability and Cell Death**

The cell viability was measured using the methylene blue dye exclusion technique [20]. The fresh fermentation broth was diluted and mixed with 0.1% (w/v) methylene blue in equal volume. After standing at room temperature for 10 min, the cells were observed under a microscope (MLS1, Mshot, China) through hemocytometer. To determine the effect of specific growth rate on cell death, cells were collected at the end of fermentation from each run, and washed with PBS buffer (100 mM, pH 6) and suspended in the same buffer (10⁶ cells). Cell death was determined by propidium iodide staining kit (Sangon, Shanghai, China) with a fluorescence microscope (LSM800, ZEISS, Jena, Germany).

**Cellular ROS Detection**

Cells were collected at the end of fermentation from each run. The H₂O₂ content was calculated by measuring the absorbance at 390 nm. The cells were suspended in 3% trichloroacetic acid solution (2.5 mL), and centrifuged at 12,000×g and 4 °C for 10 min. Then, 1 mL of the supernatant was mixed with equal volume of PBS buffer (pH 7), followed by adding 2 mL 1 mol/L potassium iodide. The cells were resuspended in PBS buffer (50 mM, pH 7.8) containing 1 mmol/L EDTA and 1% (v/v) PVP, sonicated and then centrifuged at 12,000×g and 4 °C for 10 min. The supernatant was used for O₂•−, superoxide dismutase (SOD), and catalase (CAT) analysis, and protein concentration in the supernatant was determined by the Bradford assay. The effect of constant methanol feeding rate on O₂•− content was measured by determining nitrite production [21].

**Determination of Lipid Peroxidation**

Cells were collected at the end of fermentation from each run, washed with PBS buffer, and suspended in the same buffer. Malondialdehyde (MDA) was quantified by measuring thiobarbituric acid reactive substances as described previously [22]. The cell suspension treated with snailase and digestion buffer (Sangon Biotech, Shanghai, China) were centrifuged at 10,000×g for 15 min at 4 °C. One volume of the supernatant was mixed with two volumes of TBA reactive solution (0.25 M chlorhydric acid, 15% (v/v) trichloroacetic acid, and 0.375% (w/v) thiobarbituric acid). Subsequently, the samples were incubated for 20 min at 100 °C in a dry bath, and then the mixture was cooled on ice and centrifuged at 12,000×g for 30 s at 4 °C. Supernatant absorbance was measured at 532 nm.

**SOD, CAT, and GSH Assays**

Cell samples are processed according to the “Cellular ROS Detection” section. SOD activity, CAT activity, and reduced glutathione (GSH) content were determined by their cellular analysis kits, respectively (Nanjing Jiancheng BioENG, Co., Nanjing, China).
RNA Isolation and Quantitative Real-Time PCR (RT-qPCR)

Cells were collected at the time point corresponding to the maximum enzyme activities. Total yeast RNA was extracted using RNAiso Plus (Takara, Dalian, China) according to the manufacturer’s instructions. The cDNA was synthesized using PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Dalian, China). RT-qPCR was conducted in CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA), and SYBR Green PCR Master Mix kit (Takara, Dalian, China) was used for the real-time PCR analysis. The primers used for RT-qPCR are listed in Table 1. The relative expressions of target genes were calculated by the $2^{-\Delta\Delta C_{t}}$ method [23].

Statistical Analysis

All analytical determinations were carried out in triplicate. The results were reported as the mean ± SD of these measurements. Significant differences in the means were accomplished by using an analysis of variance procedure ($p < 0.05$). The differences were assessed by ANOVA and denoted as follows: * $p < 0.05$ versus specific growth rate $0.035 \text{ h}^{-1}$.

Results

Fed-batch Fermentation of Lipase by Controlling the Specific Growth Rate

The time course of total cell growth and the methanol level was controlled by the sensor. Exponential growth was observed within 144 h of methanol feeding time. Based on the obtained maximum growth rate ($\mu_{\text{MeOH,m}}$) and maximum methanol specific consumption rate ($v_{\text{MeOH,m}}$) in the induction phase, we conducted fed-batch fermentations with different desired growth rates, $\mu_{\text{MeOH,d}} \leq \mu_{\text{MeOH,m}}$, by feeding methanol at a feed rate, $F_{\text{MeOH}}$, estimated as in [24–27]:

$$F_{\text{MeOH}} = v_{\text{MeOH}}X_{0}V_{0}e^{v_{\text{MeOH}}dt} = \mu_{\text{MeOH,d}}v_{\text{MeOH,m}}X_{0}V_{0}e^{v_{\text{MeOH}}dt}/\mu_{\text{MeOH,m}}$$  \hspace{1cm} (1)

where $X_{0}$ and $V_{0}$ are the cell density and broth volume at the beginning of the methanol fed-batch phase, and $t$ is the feed time of methanol. An actual $\mu_{\text{MeOH}}$ and $v_{\text{MeOH}}$ were obtained from each run, and the linear dependence of $\mu_{\text{MeOH}}$ on $v_{\text{MeOH}}$ is shown in Fig. 1. The methanol $v_{\text{MeOH}}$ was exponentially fed into the bioreactor to support $\mu_{\text{MeOH}}$ using Herbert-Pirt linear relationship:

$$v_{\text{MeOH}} = 0.7281 \mu_{\text{MeOH}} + 0.0981$$  \hspace{1cm} (2)

| Gene   | Forward primer (5’-3’)       | Reverse primer (5’-3’)                |
|--------|-----------------------------|--------------------------------------|
| Actin  | GTCCAGCATAAACACGCAG        | CAGTGGGAAAAACCCACGAA                  |
| cSOD   | CGAAACATCTCAGAAAG          | ACCCTTGGCAACACTCTCA                   |
| mSOD   | AACACAGGAGGTGGAGAGGC       | CAAAGGGACCAACTACC                     |
| CAT    | GCTACTAACCCTGAGGACGC       | TTGAAGTTACGACACCCAG                   |
| GPX1   | CCCATAGATAAGAAAAGCG        | CCAAATGTTACGAGGAA                     |
| GLR1   | AACTCGCACCACGGTAT          | TCTCAATCGCAGGACT                     |
According to Eqs. (1) and (2), in the fed-batch fermentation process, the average biomass yield and maintenance coefficient of *P. pastoris* were 1.37 (g WCW/g MeOH) and 0.0981 (g MeOH/g WCW/h), respectively. Equation (2) was substituted into Eq. 1, to give Eq. 3:

\[ F_{\text{MeOH}} = (0.7281 \mu_{\text{MeOH}} + 0.0981)X_0 V_0 e^{\mu_{\text{MeOH}} t} \]  

(3)

**Effect of Specific Growth Rate on Fermentation Performance**

Figure 2 shows the time courses of WCW, lipase activity, and total protein concentration throughout the production phase with different specific growth rates. The specific growth rate had a direct impact on the cell growth rate and biomass. Higher specific growth rate led to greater cell growth. At the end of methanol fed-batch phase, the attainable cell densities for specific growth rates 0.015, 0.035, 0.05, and 0.065 1/h were 392, 436, 448, and 479 g/L WCW, respectively. During the first 24 h after induction, protein production and lipase activity were similar for the four rates. However, the maximum total protein concentration value and lipase activity reached were 2.8 mg/mL and 1166.5 U/mL for the specific growth rate of 0.05 1/h. Lipase MAS1 was found to have one N-glycosylation site by N-glycosylation site analysis, but SDS–PAGE showed that there was one form of recombinant protein with molecular masses about 29 kDa. The recombinant protein accounted for approximately 90% of the total protein in the culture supernatant which was estimated by the software quantity one (Bio-Rad). *P. pastoris* secreted few native proteins which facilitate downstream processing (Fig. 2D). As shown in Table 2, when the specific growth rate increased in the range of 0.015 to 0.05 1/h, the average specific production rate increased significantly. When the specific growth rate was 0.05 1/h, the average specific production rate reached 0.052 mg/g WCW/h.

**Effect of Specific Growth Rate on Cell Viability and Cell Death**

As mentioned above, specific growth rate had a direct impact on the cell growth and methanol consumption level. Methanol serves as a carbon source for yeast growth, and high methanol toxicity maintained low cell viability, which could help to cell death.
Figure 3A shows the effect of specific growth rate on cell viability in a 5-L bioreactor. At all four rates, the cell activity decreased with the extension of induction time. Among the rates of 0.015, 0.035, and 0.05 1/h, the specific growth rate showed an apparent flow rate-dependent positive effect on P. pastoris cell viability. The cell viability decreased significantly after 72 h of induction for rates of 0.015, 0.035, and 0.05 1/h. For 0.065 1/h, the transition of rapid decline in cell viability was advanced after 48 h of induction. Compared to other specific growth rates, the cell viability decreased more slowly at 0.05 1/h, and it had the relatively highest cell viability at the end of methanol fed-batch phase. Figure 3B shows

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Table 2 Effect of specific growth rate on MAS1 production

| Specific growth rate (1/h) | Harvest time after induction (h) | Cell density (g WCW/L) | Volumetric MAS1 production (g/L) | Specific MAS1 production (mg/g WCW) | Average specific production rate (mg/g WCW/h) |
|---------------------------|---------------------------------|------------------------|----------------------------------|-----------------------------------|--------------------------------------------|
| 0.015                     | 144                             | 384 ± 6                | 1.8 ± 0.1                        | 4.69                              | 0.033                                      |
| 0.035                     | 120                             | 424 ± 10               | 2.4 ± 0.1                        | 5.64                              | 0.047                                      |
| 0.05                      | 120                             | 444 ± 16               | 2.8 ± 0.1                        | 6.26                              | 0.052                                      |
| 0.065                     | 96                              | 465 ± 16               | 2.2 ± 0.1                        | 4.75                              | 0.049                                      |

Data are mean ± standard deviation of results from independent duplicate fermentations.
that each group of cells had different amounts of red fluorescence after staining with propidium iodide. Normal cells could not be stained, and the dead cells showed strong red fluorescence. The number of cells showing propidium iodide-positive (red fluorescence) was higher at 0.065 1/h, and the cells mainly showed strong fluorescence intensity. Moreover, the propidium iodide-positive cells in other feeding rates showed lower red fluorescence compared with the specific growth rate of 0.065 1/h. Among the four specific growth rates, the rate of 0.05 1/h had the lowest number of cell deaths at the end of methanol fed-batch phase. From the above results, the specific growth rate of 0.05 1/h appears to be most preferable because it maintained the highest cell viability and the lowest level of cell death.

**Effect of Specific Growth Rate on Lipid Peroxidation and ROS Accumulation**

Accumulation of ROS in cells caused by methanol feeding to maintain higher specific growth rate can form oxidative damage. As shown in Fig. 4, four specific growth rates did not cause a significant increase in $\text{O}_2^{-}$ content in *P. pastoris*. *P. pastoris* had higher cellular
levels with 0.065 1/h in the induction phase compared with that in other methanol feeding induction conditions. Moreover, higher specific growth rates resulted in increased cellular H$_2$O$_2$ levels. Notably, for specific growth rate of 0.065 1/h, the cellular H$_2$O$_2$ levels were significantly higher than other induction groups. The cellular H$_2$O$_2$ levels reached were 2.2 nmol/min/mg-WCW for specific growth rate of 0.065 1/h. However, the cellular H$_2$O$_2$ level was 1.0 nmol/min/mg-WCW at specific growth rate of 0.05 1/h. Compared to 0.035 1/h, the rate of 0.05 1/h obtained a slight increase in cellular H$_2$O$_2$ level. MDA is a by-product of lipid peroxidation and reflects the degree of oxidative damage caused by ROS. The MDA levels were determined in order to evaluate oxidative damage in the cell of recombinant P. pastoris exposed to four specific growth rates (Fig. 4). The MDA levels were similar for the specific growth rates of 0.015, 0.035, and 0.05 1/h at the end of fermentation. By contrast, 0.065 1/h at 96 h showed a significant higher MDA level with respect to the other induction groups.

**Effect of Specific Growth Rate on Cellular ROS Scavenging System**

Yeast cells have a range of enzymatic and non-enzymatic systems that respond to ROS. An appropriate specific growth rate may stabilize the ROS scavenging system. We assayed activities of SOD and CAT, and the content of reduced GSH. Except for the increase of CAT activity, the results showed that higher specific growth rates caused nearly no change of SOD activity, and an obvious decrease in concentration of GSH with the specific growth rate of 0.065 1/h (Table 3). Moreover, the CAT activity with a specific growth rate of 0.05 1/h showed no comparable difference with that of 0.035 1/h, whereas the CAT activity with the specific growth rate of 0.065 1/h increased obviously.

**Correlation of Fermentation Performance with ROS-Associated Parameters**

Figure 5 gives the heatmap constructed by Spearman’s correlation analysis, to explore the correlations of fermentation performance and with ROS-associated parameters. The relevant parameters causing the accumulation of ROS in P. pastoris cells were related to the specific growth rate. Generally, high cell biomass and higher cell viability were beneficial for increasing total protein content and lipase activity. However, cell viability was negatively correlated...
with the cell biomass. Higher cell viability is beneficial for increasing protein content and lipase activity. Also, those interventions were further regulated by GSH levels.

**RT-qPCR Analysis of Intracellular Gene Expression**

Figure 6 shows that the ROS scavenging system-related gene was amplified from the cDNA, and all samples were compared with the specific growth rate of 0.035 1/h. Figure 6 shows that higher specific growth rates caused a downregulation of glutathione peroxidase (GPX1) and glutathione reductase (GLR1) at the mRNA levels. CAT expression at the mRNA level was higher for rates of 0.05 and 0.065 1/h compared with that of 0.035 1/h. Specific growth rate of 0.065 1/h had a significantly higher expression of CAT at the mRNA level compared with that specific growth rate of 0.035 1/h ($p < 0.05$). Moreover, cSOD, GPX1, and GLR1 mRNA level was higher for rate of 0.05 1/h in the methanol induced phase than that of 0.065 1/h.

**Discussion**

*P. pastoris* is extensively used to produce various heterologous proteins. Amounts of biopharmaceutical drugs and industrial enzymes have been successfully produced by fed-batch high-cell-density fermentation of this cell factory [28–31]. Although high-density

| Specific growth rate (1/h) | SOD (U/mg-protein) | CAT (U/mg-protein) | GSH (mg/g-WCD) |
|----------------------------|--------------------|--------------------|----------------|
| 0.015                      | 1.8 ± 0.2          | 1.1 ± 0.1          | 1.5 ± 0.2      |
| 0.035                      | 1.7 ± 0.1          | 1.6 ± 0.2          | 3.2 ± 0.3      |
| 0.05                       | 1.9 ± 0.2          | 1.8 ± 0.1          | 3.0 ± 0.3      |
| 0.065                      | 2.0 ± 0.1          | 2.7 ± 0.3          | 0.8 ± 0.2      |

Data are mean ± standard deviation of results from independent duplicate fermentations

Fig. 5 A Spearman’s correlation heatmap was used to represent significant statistical correlation values ($r$) between fermentation performance and ROS-associated parameters with different methanol feeding rate. The intensity of the color represents the degree of association between fermentation performance and ROS-associated parameters [numerical values: WCD, wet cell density; PC, total protein concentration; LA, lipase activity]
fermentation of *P. pastoris* is considered a promising development, it also has its limitations. It is not reliable to judge methanol concentration by dissolved O\textsubscript{2} alone in the bioreactor, because the accumulation of intracellular toxic by-products from methanol oxidation may cause cytotoxic to cells. Therefore, it is necessary to seek more perfect methods to promote protein production based on reducing the accumulation of toxic by-products of methanol oxidation.

The specific growth rate had a direct impact on the cell growth rate and cell biomass. The specific growth rate of 0.015 1/h had the lowest cell biomass. Compared to 0.035 and 0.05 1/h, the specific growth rate of 0.015 1/h obtained a lower total protein concentration and enzyme activity. These results indicated that lack of carbon/energy sources could not meet the sufficient requirements for the cell metabolism, and could also cause declines in cellular metabolic activity. As a result, while cell viability at the specific growth rates of 0.015, 0.035, and 0.05 1/h was equivalent at 72 h of induction, the cell viability at 0.015 1/h decreased more rapidly in the next 48 h than at 0.035 and 0.05 1/h. By contrast, the specific growth rate of 0.065 1/h resulted in a rapid increase in total protein concentration and enzyme activity during the first 96-h induction time. However, the 0.035 and 0.05 1/h, especially specific growth rate of 0.05 1/h appears to be most preferable because it produced the highest total protein concentration and highest enzyme activity in the induction phase. Current research recognized that excessive methanol would damage the AOX1 transcriptional efficiency and cellular metabolic activity, particularly when cells are exposed to high methanol concentration for a long time, as excessive methanol and dissolved oxygen may lead to the accumulation of formaldehyde, the first intermediate of methanol metabolism, to certain toxic levels [32]. Our study showed that a higher specific growth rate (0.065 1/h) resulted in a rapid decrease in cell activity. One reason might be that higher methanol toxicity maintained low cell viability, which could help to cell lysis. The conclusions and results could be found in related reports [33]. However, we further investigated that the accumulation of ROS from methanol oxidation may cause oxidative damage to cells. Different from the accumulation of formaldehyde to certain toxic levels, in this study, the decline in cell activity caused by oxidative damage was also considered one of the crucial factors for the metabolic toxicity of methanol.
Accumulation of ROS in cells caused by methanol feeding can form oxidative damage. MDA is a by-product of lipid peroxidation and reflects the degree of oxidative damage caused by ROS. For the specific growth rate of 0.065 1/h, the cellular ROS accumulation and MDA levels were significantly higher than other induction conditions, indicating that the higher specific growth rates led to a significant increase in lipid peroxidation in \textit{P. pastoris}. Generally, the cellular ROS scavenging system attenuates the oxidative damage by scavenging ROS, but excessive H$_2$O$_2$, which is not scavenging in time, can damage the lipids on the cell membrane [34]. Since membrane phospholipids are rich in unsaturated fatty acids and the hydrophobic membrane has a high solubility for oxygen atoms, so it is most vulnerable to free radicals [35]. Therefore, we found that oxidative damage caused by the accumulation of ROS at specific growth rate of 0.065 1/h could lead to cell apoptosis at an early stage and reduced protein expression ability.

Therefore, controlling intracellular ROS accumulation is beneficial for improving protein production in \textit{P. pastoris} during the methanol induction phase. Evidence from our study demonstrated that the specific growth rate of 0.05 1/h can stabilize the ROS scavenging system. Notably, the ROS accumulation level that affects total protein concentration and enzyme activity was closely related to cell viability. Those interventions were further regulated by GSH levels. Other protein factors are also effective defense strategies to ameliorate the toxic effects of ROS, such as GPX1 and CAT. While GPX and CAT utilize H$_2$O$_2$ as substrate, GSH protects cells from oxidative stress and endogenous toxic metabolites through HCHO metabolism and detoxification of ROS [36]. Actually, GPX1 also acts as a prophylactic antioxidant enzyme, with GSH to attenuate ROS accumulation, and is converted from the oxidized (GSSG) to reduced form (GSH) by GLR1 [37]. In this study, instead of increase, the concentration of GSH decreased along with ROS accumulation, suggesting higher specific growth rates induced damage to the cell ROS scavenging system. GLR1 and GPX1 control the cycle of transition between GSH and GSSG; the significantly high expression levels of their encoding gene indicate the high efficiency of transition between GSH and GSSG, thus explaining the reason for the high GSH content at specific growth rates of 0.015 to 0.05 1/h. It was reported that \textit{p53} reactivation and induction of massive apoptosis (PRIMA-1Met) induce myeloma cell death by impairing GSH/ROS balance in human myeloma cell lines [38]. Similarly, titanium dioxide nanoparticles might cause a PRIMA-1Met-like effect in \textit{P. pastoris}, independent of titanium dioxide nanoparticles [39]. However, in this study, methanol acts as a carbon source and inducer for \textit{P. pastoris}. We supposed that metabolic by-product (H$_2$O$_2$) form methanol oxidation may cause the accumulation of ROS and induce oxidative damage to cells via impairing ROS scavenging system, especially GSH system, thereby reducing protein expression ability.

\textbf{Conclusions}

Methanol feeding as a crucial operating parameter increases heterologous protein productivity by \textit{P. pastoris}, but the accumulation of intracellular toxic by-products from methanol oxidation may cause oxidative damage to cells. Our study demonstrates that controlling the specific growth rate of 0.05 1/h promoted protein production by selectively balancing ROS scavenging system and reducing oxidative damage to cells. Importantly, specific growth rate within suitable range can maintain a high content of GSH. Our findings help understand methanol-induced intracellular bioreaction network in high-cell-density fermentation. This strategy may serve as reference for similar fermentation processes.
Author Contribution Rongkang Hu and Yonghua Wang designed the study. Rongkang Hu did experimental work. Rongkang Hu, Ruiguo Cui, and Yonghua Wang collected and analyzed the data. Rongkang Hu wrote the manuscript with support from Qingqing Xu, Dongming Lan, Ruiguo Cui, and Yonghua Wang. All the authors read and approved the manuscript for publication.

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Data Availability The data produced and/or analyzed in the current study are available from the corresponding author on reasonable request.

Code Availability Not applicable.

Declarations

Ethics Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Conflict of Interest The authors declare no competing interests.

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