Control of the Ratio of Inducer to Cell Concentration to Enhance the Phytase Production in Recombinant *Pichiapastoris*

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Abstract Phytase production by *Pichiapastoris* was used as a case to study the mechanism and strategy for optimization of heterologous protein production. It was found that the ratio of inducer-methanol to cell concentration had a significant influence on phytase production. In this case, we found that the optimum initial cell concentration and methanol concentration were 85 g/L and 10 g/L, respectively. During induction period, an easy-to-control methanol feeding method was proposed according to the optimal ratio of methanol to cell concentration at a range of 0.063 -0.132 g/g, and phytase activity and productivity reached 53984 U/mL and 529.25 U/(mL·h), respectively. The method for optimization of phytase production through controlling the optimal ratio maybe provide an alternative idea to enhance other heterologous protein production with *P. pastoris*.

Keywords: *Pichiapastoris*, feeding strategy, methanol concentration, cell concentration, heterologous proteins

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1. Introduction

In biological system, hydrolysis of phytic acid (the principle storage form of phosphorus in legumes, cereals, oil seeds and nuts) to myo-inositol and inorganic phosphate is an important reaction for energy metabolism, metabolic regulation and signal transduction pathways [1]. The reaction was primarily catalyzed by phytase [2]. So far, phytase has been widely used in feed industry, and also applied to food and beverage industries and so on [3]. Along with the fast development of genetic engineering, genes encoding phytase have been cloned and expressed from many microorganisms' strains [4]. Due to the advantages of producing large quantities of heterologous proteins and capability for large-scale production, *P. pastoris* is widely being used for research and production of various foreign proteins [5,6,7]. Follow the manual instructions; two-step fermentation is the fundamental method to express heterologous protein in *P. pastoris* [8]. A cell high-density growth with glycerin or glucose as carbon source is just the first stage, and the second is generally called induction phase, in which methanol is added for biosynthesis of the recombinant protein under the control of a tightly regulated alcohol oxidase (AOX1) promoter. When the carbon source is switched from glucose to methanol, the response of recombinant cultures to induction of heterologous proteins involves significant changes in cellular physiology, growth rate and metabolism [9,10,11,12].

It has been proved that cell concentration at the beginning of the second phase plays a significant role in recombinant protein production in many literatures [13,14]. Moreover different glucose feeding strategies have also been explored at the first phase of high-density cultivation. Thus, in order to reach high productivity of heterologous proteins, the cell concentration must be further increased [13,15,16,17]. For methanol induction phase, it has been one of the hottest topics in biochemical engineering to explore the method for methanol feeding so as to realize the high-efficient expression of heterologous protein in *P. pastoris* [16,18,19,20]. However, though various bioprocess strategies possibly related to metabolism have been studied for the methanol feeding, based on kinetics parameters such as oxygen consumption and methanol consumption, low expression efficiency of target protein should occur, because *P. pastoris* cells compete to each other for methanol which is the only carbon source for cell growth and protein expression. Therefore, it is conceivable that methanol would be primarily used for heterologous protein expression not for cell growth under the non-limited methanol concentration, if cell concentration at the beginning of methanol induction phase reached very high levels [21,22]. Meanwhile, whatever the cell concentration is, the methanol concentration could be adjusted timely to ensure that the recombinant protein could be effectively induced, if the methanol was added through the ratio of methanol to cell concentration.

In this study, with the purpose of further increasing phytase production by *P. pastoris*, the impact of the initial

cell concentration and the ratio of methanol to cell concentration during the methanol induction phase were carefully investigated. Our results showed that adopt the methanol feeding method based upon the optimal ratio of methanol to cell concentration could be usefully expressed phytase. Furthermore, we hold that the strategy maybe applied to other heterologous protein fermentation processes.

2. Materials and Methods

2.1. Strain

P. pastoris H311 (Mut⁺) used in this study was developed and provided by the Yunnan Normal University research group.

2.2. Media

YPD medium, containing yeast extract 10 g/L, peptone 20 g/L and glucose 20 g/L, was used for seed culture. The basal salts medium for fermentation contained (g/L): glucose 50, KH₂PO₄ 40, MgSO₄ • 7H₂O 14.9, CaSO₄ • 2H₂O 0.39, (NH₄)₂ • SO₄ 30 and 4.3 mL/L of PTM1 trace metal solution. PTM1 solution contained (g/L): CuSO₄•5H₂O 6, KI 0.09, MnSO₄•H₂O 3, H₃BO₃ 0.02, MoNa₂O₄•2H₂O 0.2, CoCl₂ 0.5, ZnCl₂ 20, FeSO₄•7H₂O 65, biotin 0.2 and H₂SO₄ 5.0 mL/L. The feeding medium used in glucose fed-batch cultivation contained 50% (w/v) glucose supplemented with 12 mL/L PTM1 solution. The feeding medium used in methanol fed-batch phase was the pure methanol with 12 mL/L PTM1 solution.

2.3. Fermentation Conditions

The seed culture was prepared from a 600 µL frozen cell stock vial and cultured for 24 h in 50 mL YPD medium in a 500-mL shake flask at 30°C and 220 r/min. 10% (v/v) of inoculum was inoculated into the basal salts medium in a 5-L fermentor (Nanjing Highke Bioengineering Equipment Co., Ltd. China) with 2 L basal salts medium. The pH of the medium was adjusted and controlled at 5.0 with the addition of 25% ammonium hydroxide and 30% phosphoric acid. The temperature was controlled at 30°C, and dissolved oxygen (DO) level was maintained over 20% of air saturation by a cascaded control of agitation rate from 500 r/min to 1000 r/min and aeration rate from 3.5 L/min to 6 L/min. When glucose was exhausted, indicated by a rapid increase of DO, fed-batch was started by feeding 50% (w/v) glucose plus 12 mL/L PTM1. Before starting methanol induction phase, the glucose feeding was stopped for 4 h to avoid repression of AOX promoter. When dissolved oxygen level rose up to 80%, the induction culture was initiated by feeding methanol plus 12 mL/L PTM1. The glucose fed-batch culture and induction culture were performed in different strategies as follows.

2.3.1. Different Cell Concentration at the Beginning of Induction

The study used regular feeding scheme. The glucose fed-batch mode was started by adding a 50% glucose solution at a constant flow rate of 25 mL/h till cell concentration reached 45 g/L, 65 g/L and 85 g/L,

respectively. During the post-induction phase, the methanol feeding rate was at 5 mL/h, and was adjusted off-line by gas chromatography every hour to maintain the residual methanol concentration at 5 g/L.

2.3.2. Methanol Feeding Scheme to Enhance Heterologous Phytase Production

With the purpose to maintain the ratio of methanol to cell concentration during induction phase, methanol fed-batch cultivation was started as follows: (1) gradually increase the feeding rate of methanol from 5 mL/h to 12 mL/h in induction prophase (0-10 h), (2) adjust the methanol feeding rate to 15 mL/h to maintain the methanol concentration at 13 g/L in induction mid-phase (10-96 h), and (3) decrease the feeding rate of methanol to 5 mL/h upon the increase of DO in later stage (after 96 h).

2.4. Cell Concentration Determination

Cell concentration was measured using a spectrophotometer (Biospe-1601; Shimadzu Co., Kyoto, Japan) at 600 nm after an appropriate dilution. The optical density (OD₆₀₀) value was converted to dry cell weight (DCW) according to a predetermined calibration line [OD₆₀₀: DCW (g/L) = 1: 0.19]. 10 mL sample was collected and centrifuged at 10,000× g for 10 min, and the supernatant was frozen for determination.

2.5. Phytase Activity Assay

One unit of phytase activity (U) was defined as the amount of enzyme that catalyzes the release of 1.0 µmol of inorganic phosphate per minute from 5.0 mM sodium phytate in pH 5.5 buffer at 37°C. The fermentation broth samples were properly diluted by the acetate buffer (0.25 M, pH 5.50) containing Triton X-100 and BSA, until the absorbance below could be linearly quantified by a UV-visible spectrophotometer (UVmini-1240, Shimadzu, Japan). Draw 0.2 mL of the diluted enzyme solution and 1.8 mL of the acetate buffer without Triton X-100 and BSA (0.25 M, pH 5.50) mixture, and were first pre-heated at 37°C for 5 min, then 4.0 mL of substrate composed of 5.0 mM sodium phytate (P8810, Sigma Co. Ltd., USA) was added. The mixture was reacted at 37°C for 30 min and the reaction was stopped by adding 4 mL freshly prepared color reagent (a concentration of 100 g/L of ammonium molybdate solution, 2.35 g/L solution of ammonium metavanadate and nitric acid solution (65%) are mixed in the ratio of 1:1:2) and cooled to room temperature (the color reagent was first added and the substrate solution was added at last in the blank group), then the solution was measured spectrophotometrically at 415 nm. The standard curve was determined by adding color reagent to the different concentration of potassium phosphate monobasic solution at 37°C for 30 min, followed by addition of substrate solution with the same procedure as mentioned above. Finally, according to the standard curve to calculate the linear regression equation of the amount of inorganic phosphorus, and then calculate the activity.

2.6. Methanol Concentrations

The residual methanol concentration was determined by gas chromatography (GC) with a flame-ionized detector

(Shimadzu GC2010). A glass column (30 m×0.32 mm 0.50μm) packed with PEG-20M was used. Injection, detector, and column temperature were 200°C, 220°C, and 170°C, respectively. The gas flow rate of nitrogen, hydrogen, and air were 40 mL/min, 40 mL/min, and 450 mL /min, respectively. The chromatographic quantitative analysis was performed by internal standard method.

3. Results and Discussion

3.1. Effects of Initial Cell Concentration in the Beginning of Induction Phase on Phytase Expression

Figure 1 showed that cell concentration increased quickly and the phytase production raised continuously with initial cell concentration at 45 g/L by the regular methanol feeding strategy, and both reached their maximum values at 12965 U/mL and 98.5 g/L, respectively (Figure 1-1). It was observed that cell slow growth till the end of cultivation, which was potentially due to the limited space when cell concentration reached 85 g/L. On the other side, phytase production was 120.9% and 28.2% higher than those when the initial cell concentrations were at 45 g/L and 65 g/L, respectively (Figure 1-3). Table 1 presents that the related fermentation process parameters of different initial cell concentrations at the beginning of induction phase.

Figure 1. Time courses of the cell concentration, phytase production and methanol concentrations at different initial cell concentrations controlled to 45g/L (Figure 1-1), 65 g/L (Figure 1-2) and 85 g/L (Figure 1-3) at the beginning of induction phase

Table 1. Comparison of fermentation process parameters for phytase production under different modes of induction

Fermentation process parameters	Mode of cultivation				
	I	II	III	IV	V
Initial cell concentration (g/L)	45	65	85	85	85
Average methanol concentration (g/L)	5.0	5.0	5.0	10.0	15.0
Ratio of methanol to initial cell concentration (g/g)	0.111	0.077	0.059	0.118	0.176
Maximum cell concentration (g/L)	98.5	97.6	99.5	100.3	101
Maximum phytase (U/mL)	12965	22335	28643	40582	18235
Total consumed methanol (g/L)	230	240	223	234	276
Average specific phytase production rate (U/g/h)	113	217	287	419	173
Yield of DCW on methanol (g/g)	0.261	0.132	0.003	0.002	0.004
Yield of phytase on methanol (U/g)	55	93	139	194	69
Phytase productivity (U/mL/h)	102.9	102.9	341	398	190

Due to there is almost no difference between three experiments in DO concentration, we assumed that the initial concentration was probably the prime reason that resulted in such an obvious difference of phytase production. With lower initial cell concentration, a higher percentage of assimilation of methanol into biomass while methanol oxidized to obtain the required energy for phytase production was reduced, in theory, make it possible. As demonstrated in Table 1, the yield of DCW on methanol with cell concentration at 45 g/L was higher than that at cell concentration of 85 g/L, but the yield of phytase on methanol was much lower than that at cell concentration of 85 g/L. In addition, if the initial cells reached high level, they would contain high intracellular concentration of ribosome, which could contribute to synthesize a lot of key enzymes and recombinant foreign protein for the subsequent methanol metabolism when the carbon source was switched from glucose to methanol [22,23].

3.2. Effects of Methanol Concentration during Induction Phase on Phytase Production

Phytase biosynthesis is controlled by the same promoter responsible for the synthesis of the methanol oxidase, thus methanol concentration was a key factor to be optimized. In our research, different concentrations of methanol for phytase expression were investigated via maintaining the initial cell concentration at 85 g/L during induction phase. Table 1 presents the comparison of related fermentation process parameters at different methanol concentrations. In this study, we found that the *P. pastoris* strain had the ability to tolerant to high concentration of methanol. Moreover, it worked massive effectively under the non-restricted methanol concentration. As illustrated in Figure 2, at the methanol concentration of 10 g/L, the phytase production was enhanced to 11944 U/mL, which was 41.7% higher than that of methanol concentration at 5 g/L. When methanol concentration was controlled too high (15 g/L), phytase production was inhibited and the yield decreased. It was possibly due to the metabolic stress when the cell machinery was overburdened in high methanol concentration, which may give rise to physiological changes that negatively affected process performance [24,25,26].

Figure 2. Time courses of phytase production at different methanol concentrations in the induction phase. The methanol concentration was controlled at 5 g/L, 10 g/L and 15 g/L.

3.3. Effects of the Ratio of Methanol to Cell Concentration on Phytase Production

A further study was implemented to research the impact of the ratio of methanol to cell concentration on phytase production when the initial cell concentration reached 85 g/L at the beginning of induction phase. As shown in Figure 3, phytase production increased with the increment of the ratio from 0.063 g/g to 0.173 g/g and declined at a higher ratio of 0.228 g/g. As far as we know, it was the first time suggested that the application of the ratio of methanol to cell concentration for controlling methanol feeding to produce phytase in *P. pastoris*. Due to which has one big advantage over conventional feeding scheme. Whatever the cell concentration is, the methanol concentration could be adjusted timely to ensure that the phytase could be effectively induced in the induction phase.

Figure 3. Effects of the ratio of methanol to cell concentration on phytase production (×10000), phytase productivity (×100) and yield of phytase on cell (×100).

3.4. Enhancement of Phytase Production in Recombinant *P. pastoris* by Controlling the Ratio of Methanol to Cell Concentration

For purpose of the high-efficient production of phytase, an optimization method was suggested to obtain the maximum phytase production. In the methanol induction phase, the methanol feeding strategy was adopted to keep the optimum ratio of methanol to cell concentration.

At the start of methanol induction phase, cells were in the transition stage from glucose to methanol metabolism and the specific methanol uptake rate increased gradually, the methanol feeding rate was increasingly fed step by step as described by 2.3 [27,28,29]. After the adaptation state, non-growth cells of *P. pastoris* maintained the specific methanol uptake rate constant with DO level at a range of 20–30%. The feeding rate was maintained constant. Cellular metabolism ability start to decrease after 96 h, which was reflected by the increase of DO concentration, and the specific methanol uptake rate also reduced. So, methanol feeding rate was decreased so as to reduce metabolic stress on *P. pastoris* cells. At last, results shows that by controlling the ratio between 0.063 g/g and 0.132 g/g, phytase activity and productivity reached their maximal values of 53984 U/mL and 529.25 U/(mL·h), respectively, which were 1.33-fold and 1.3-fold higher than those of the regular feeding scheme (Figure 4).

Figure 4. Effects of cell concentration, phytase production and methanol concentration by controlling the ratio of methanol to cell concentration

4. Conclusions

To our knowledge, it is the newly study the fed-batch application in phytase production from *P. pastoris*. It is no doubt that the results had presented that phytase production could be increased obviously with optimized production methods via controlling the ratio of methanol to cell concentration in the methanol fed-batch cultivations. High production of recombinant phytase may benefit to characterization of the molecules and their modes of action, and may broaden the application of phytase in industry. Meanwhile, it maybe provides an alternative strategy to enhance other heterologous protein production with *P. pastoris*.

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