Process Biochemistry 59 (2017) 159–166

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Transcriptional analysis for carbon metabolism and kinetic modeling for heterologous proteins productions by *Pichia pastoris* in induction process with methanol/sorbitol co-feeding

Luqiang Jia[a], Enock Mpofu[b], Tingyong Tu[a], Qiangqiang Huai[a], Jiaowen Sun[a], Shanshan Chen[a], Jian Ding[a,*], Zhongping Shi[a,*]

[a] The Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, 214122 Wuxi, China
[b] Department of Food Processing Technology, Harare Institute of Technology, Harare, Zimbabwe

ARTICLE INFO

Keywords:
P. pastoris
Methanol/sorbitol co-feeding
Methanol concentration
Dissolved oxygen
Transcriptome analysis
Kinetic model

ABSTRACT

It is difficult to control concentrations of methanol/dissolved oxygen at high levels simultaneously in heterologous proteins productions by *Pichia pastoris* during induction phase. Two strains, a methanol utilization slow (Mut^S) type and a plus (Mut^+) type were used with methanol/sorbitol co-feeding strategy to induce porcine interferon-α and human serum albumin-human granulocyte colony stimulating factor respectively, under the conditions of "methanol sufficient-oxygen limited (MS-OL)" and "methanol limited-oxygen sufficient (ML-OS)". For the Mut^S/Mut^+ strains, the target proteins titers under "MS-OL" were 6-fold/19.2% of those under "ML-OS". The key genes in methanol metabolism of the Mut^S strain were up-regulated under "MS-OL", but they were not differently expressed in the Mut^+ strain. Methanol utilization rate (r_{MeOH}) of the Mut^S strain reduced when decreasing methanol concentration, but r_{MeOH} of the Mut^+ strain unchanged unless methanol concentration decreased to a low-limit of 0.6 g/L. Finally, kinetic models were designed to describe the methanol/sorbitol co-feeding process.

1. Introduction

The methylotrophic *Pichia pastoris*, the most effective and available systems for the expression of heterologous proteins, has the following advantages: it can be easily manipulated at the molecular genetic level; it can easily grow to high cells density and express proteins at high levels [1,2]. Heterologous protein production by fed-batch culture with recombinant *P. pastoris* is basically divided into two phases: a growth phase to accumulate a large amount of functional cells, and an induction phase with methanol as inducer to produce heterologous protein [3]. Methanol/sorbitol co-feeding is an effective method in inducing heterologous protein by *P. pastoris*. Recently, it was reported that the addition of sorbitol could supply extra energy to cell and enhance both of cell growth and target protein productivity [4,5]. In the induction with methanol/sorbitol co-feeding, no matter for Mut^S or Mut^+ strain, simultaneously controlling concentrations of methanol and dissolved oxygen (DO) at high (proper) levels is very difficult, because metabolisms of methanol and oxygen couple with each other. However, controlling only one of them at high (proper) level is available. Methanol utilization slow (Mut^S) and methanol utilization plus (Mut^+) are the two typical phenotypes of methylotrophic *Pichia pastoris*. In Mut^+ strain, alcohol oxidase activity is extremely high, because two functional genes (*aox*1 and *aox*2) encode alcohol oxidase. In Mut^S strain, only *aox*2 genes exists, leading to the comparatively low alcohol oxidase activity. This is the most obvious difference between Mut^S and Mut^+ strains. In previous research, it was found that Mut^S and Mut^+ strains had different responses to the levels of methanol and oxygen supply: Mut^+ strain effectively produced recombinant protein under "methanol limited and oxygen sufficient (ML-OS)" condition, but decreasingly produced recombinant protein under "methanol sufficient and oxygen limited (MS-OL)" condition [6]. Oppositely, high recombinant protein yield could be achieved under MS-OL condition for Mut^S strain [7]. However, except the apparent induction performances, the physiological states in molecular level of Mut^S and Mut^+ strains under the two standard conditions have seldom been concerned.

Gene expression is a key event determining responses to environmental stimuli. Studying the whole genome at the transcriptional level may facilitate the elucidation of the molecular mechanisms of physiological processes in *P. pastoris* cell under ML-OS and MS-OL conditions. The transcriptome is the overall set of transcribed regions of the

* Corresponding authors.
E-mail addresses: dingjian@jiangnan.edu.cn (J. Ding), jnbioprocess@163.com (Z. Shi).

http://dx.doi.org/10.1016/j.procbio.2017.05.011
Received 23 January 2017; Received in revised form 6 May 2017; Accepted 10 May 2017
Available online 11 May 2017
1359-5113/

genome. Next-generation sequencing technologies are powerful strategies for identifying and quantifying gene expression at genome-wide level in unprecedented perspective [8,9]. RNA sequencing (RNA-Seq) based on next-generation deep sequencing is the most powerful tool available for comparative transcriptome profiling [10,11]. The analysis of transcriptional changes of *P. pastoris* may reveal specific genes involved in the regulation of physiological events under the aforementioned two induction conditions.

In this work, the transcriptome analysis was used to compare cell physiological states under the MS-OL and the ML-OS conditions. Based on the transcriptional analysis for carbon metabolism of *P. pastoris*, the Mut^S and Mut^+ strains' characteristics in utilizing methanol and sorbitol were speculated and verified. Finally, two kinetic models were designed to describe the methanol/sorbitol co-feeding processes of Mut^S and Mut^+ *P. pastoris*.

2. Materials and methods

2.1. Microorganism

A recombinant Mut^S strain (KM71), provided by Shanghai Academy of Agricultural Science, was used to express poircine interferon-α (pIFN-α). A recombinant Mut^+ strain (GS115), provided by School of Pharmaceutical Sciences, Jiangnan University, was used to express human serum albumin-human granulocyte colony stimulating factor ($HSA\text{-}GCSF^m$).

2.2. Media

The composition of the media for seed culture, fed-batch culture, feeding and induction was reported in our previous work [4].

2.3. Heterologous protein expression by P. pastoris fed-batch cultivations

The fed-batch culture was implemented in a 5 L bench-scaled fermenter (BLBIO-5GJ-3-H, Bailun Bio Co. China), with the initial batch media of 2.3 L. Inoculation and aeration rate were 13% (v/v) and 4 vvm. Temperature and pH were controlled at 30°C and 6.0 during cell growth stage. The previously proposed improved DO-stat method [7] was used for feeding glycerol during growth stage, allowing cell density to reach 100–130 g-DWC/L. The induction phase was initiated by feeding methanol and sorbitol after glycerol was depleted. When fermentation was shifted into induction period, MS-OL and ML-OS conditions were achieved by the following "Strategy I" and "strategy II", respectively.

Strategy I: based on the on-line measurement of methanol electrode (FC-2002, Subo Co., China), the methanol concentration was maintained at 5 g/L with ON-OFF control manner, meanwhile sorbitol was co-fed with methanol/sorbitol feed ratio of 4:1 (g/g). Strategy II: DO was maintained at 10% (OD_{set} = 10%) by regulating methanol feeding rate (F) with the equation: $F = F^* + K_c \times (DO\text{-}DO_{set})$. The standard methanol feeding rate (F^*) and the control parameter (K_c) were set at 0.7 mL min^{-1} and 0.05, respectively. Sorbitol was also co-fed with methanol/sorbitol feed ratio of 4:1 (g/g).

2.4. Analytical methods

Cell density, concentrations of methanol and target proteins were off-line determined with the previously reported methods [12]. Formate concentration in cell was determined with the previously reported methods [4]. The transcriptome analysis based on RNA-seq was entrusted to Novogene Co., and its steps are the same with the report [13]. The differences of the genes expressed levels are described with the regulated ratio (R) defined by Eq. (1), where L_1 and L_2 are the expressed levels of the specific gene under the MS-OL and ML-OS conditions respectively. The R value higher than 0.5850 indicates that the specific gene is up-regulated under MS-OL condition; the R value lower than −0.5850 indicates that the specific gene is down-regulated under MS-OL condition; the R value in the range of −0.5850–0.5850 indicates that the specific gene is not differently expressed.

$$R = \log_2 \frac{L_1}{L_2} \quad (1)$$

3. Results and discussion

3.1. Induction performances of the Mut^S and Mut^+ strains under MS-OL and ML-OS conditions

Methanol and oxygen are the most significant factors in the heterologous protein production by *P. pastoris*. In the induction processes with methanol/sorbitol co-feeding, simultaneously controlling concentrations of methanol and DO at high (proper) levels is very difficult, but controlling only one of them, meaning MS-OL or ML-OS condition, is available. In the induction with methanol/sorbitol co-feeding, target protein could be effectively expressed when methanol concentration was higher than 5 g/L [7]. Therefore, the condition with a methanol concentration higher than 5 g/L was considered as methanol sufficient (MS). DO is a crucial factor for foreign expression by *P. pastoris*. However, there are no clear classifications regarding to high (fully aerobic) and low (anaerobic) DO concentrations in foreign protein expression related literatures. In general, in most of heterologous protein production by *P. pastoris*, DO is kept between 10% and 30% to maintain aerobic environment [14,15]. Thus, in this study, we considered that 10% DO could be categorized to be the "aerobic classification", meaning the oxygen sufficient (OS) condition.

pIFN-α production by the Mut^S strain was implemented with MS-OL and ML-OS conditions respectively. Under MS-OL condition, methanol concentration was controlled at 5–10 g/L with DO at 0% in most induction phase; under ML-OS condition, OD was controlled at 10 15% with methanol concentration lower than 1 g/L in most induction phase (Fig. 1A). In the two induction processes, sorbitol was not excessively accumulated in broth, and its concentrations were still lower than 3 g/L (Fig. 1C). The final pIFN-α yield under MS-OL condition was 6-fold of that under ML-OS condition (Fig. 2A and C). Meanwhile, $HSA\text{-}GCSF^m$ production by the Mut+ strain was also implemented with the two conditions. The MS-OL and the ML-OS conditions were achieved (Fig. 1B), and almost no sorbitol was accumulated in broth (Fig. 1D). The final $HSA\text{-}GCSF^m$ yield under ML-OS condition was 5.2-fold of that under MS-OL condition (Fig. 2B and D). The results indicate that the ML-OS condition is superior for the protein production of Mut^+ strain, and the MS-OL condition is superior for the protein production of Mut^S strain. It is widely known that the formate accumulated in *P.pastoris* cell may reduce cell viability and repress protein expression. The formate accumulated in cell and secreted into supernatant of each runs were actually measured. The former was at very low level of 0.05 g/g-DCW (Fig. 1E and F) while the latter could not be detected.

Besides the MS-OL and the ML-OS, "methanol-sufficient with oxygen-sufficient condition" could be considered as another optional condition for induction with methanol/sorbitol co-feeding. This condition was not investigated due to the following reasons: 1) Oxygen consumption couples with methanol consumption, simultaneously controlling methanol concentration and DO at each individual "suboptimal" level is very difficult. 2) "methanol-sufficient with oxygen-sufficient condition" could be realized when aerating pure oxygen. Because of the high sensitivity of DO in response to aeration and agitation changes when pure oxygen is used, severe fluctuation of DO can not be avoided [6], which deteriorates the expression of target protein [16,17].