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Enhanced porcine interferon-α production by recombinant *Pichia pastoris* with a combinational control strategy of low induction temperature and high dissolved oxygen concentration

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ABSTRACT

Porcine interferon-α (pIFN-α) production by recombinant *Pichia pastoris* with standard induction strategy at 30 °C often suffers problems such as low antiviral activity, long cells adaptation period, etc. To solve these problems, a combinational induction strategy by simultaneously controlling induction temperature at 20 °C and dissolved oxygen concentration (DO) over 50% was proposed and the relevant fermentation runs were conducted in a 5 l bioreactor. With this control strategy, pIFN-α antiviral activity could be continuously enhanced and eventually reached a level of 3.62×10^7 IU/ml, which was about 16-fold of the maximum obtained when induction was done at 20 °C but without DO control, and more than 100-fold of the maximum obtained with the standard induction strategy at 30 °C. The novel control strategy could enhance alcohol oxidase (AOX) activity and relieve oxygen supply limitation in oxidative phosphorylation reaction to accelerate ATP regeneration simultaneously. As a result, the metabolic flux towards pIFN-α synthesis was enhanced and the adaptation period was shortened, enabling the entire system to be operated in a most efficient way.

1. Introduction

The methylotrophic yeast *Pichia pastoris*, an ideal recombinant protein expression system, has been widely used for both large-scaled industrial production and bench-scaled researches [1,2]. The *P. pastoris* expression system has the following excellent features: the simplicity of manipulation on the inducible alcohol oxidase (AOX) promoter to drive expression of foreign gene, the potential of realizing high cell density, and the ideal heterologous proteins secretion nature. As an example, the commercially important porcine interferon-α (pIFN-α), a vaccine adjuvant capable of attenuating occurrence of porcine foot-and-mouth disease, reducing the porcine reproductive and respiratory syndrome [PRRSV], can be effectively produced with the expression system [3,4].

Heterologous proteins 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 with glycerol as the carbon source, and an induction phase by feeding methanol to switch on the heterologous protein expression system [5]. Research works concerning glycerol feeding strategy optimization for growth phase to achieve high cell density and less inhibitory by-products have been extensively reported [6,7]. In the previous studies, we proposed a generalized, artificial neural network pattern recognition model and DO/pH measurements based on-line adaptive feeding control strategy (ANNPR-Ctrl) for realizing high cell density in growth phase [8,9].

The standard operation for heterologous proteins expression including pIFN-α production by *P. pastoris* is basically the adequate control of substrates concentrations during the two phases, while maintaining temperature at 30 °C throughout the entire fermentation period. With this mode, glycerol feeding is shifted to methanol feeding when cell concentration reaches a pre-determined level, and then methanol concentration is maintained at an adequate level to initiate heterologous proteins expression. The heterologous proteins productions under the standard operation at 30 °C often encounter the following problems: first, the long cells adaptation period (5–10 h) after shifting into the induction environment which deteriorates the fermentation productivity; second, effective induction period cannot continue for long time and accumulation of targeted foreign protein even stops at 10–20 h after induction [10–12]. As a result, recombinant protein titers cannot be enhanced with the increase of induction period as expected; third,

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the relatively low heterologous protein activity. As a result, process optimization for the induction phase is actually more difficult than that for the growth phase. Induction intensity (methanol concentration), DO, pH, temperature, etc. are the operation variables in induction phase, among them methanol concentration is recognized as the most important one. Therefore, extensive studies have been performed concerning the determination of optimal methanol feeding strategy by controlling either methanol concentration or specific growth rate during induction phase [13,14].

Recently, some researchers reported that reducing induction temperature to 20 °C or even lower levels is beneficial for the efficient expression of heterologous proteins. A common opinion accounting for this effect lies in the fact that, lowering temperature could at least help to relieve cell skeleton lysis and protease secretion when cultivated on methanol [15–17]. On the other hand, dissolved oxygen concentration (DO), another important parameter affecting recombinant *P. pastoris* expression, was also studied by a couple of researchers. Khatri and Hoffmann [18] suggested that in antibody fragment (scFv) production, high methanol concentrations were required to compensate the lack of oxygen and to fully induce recombinant protein production. Lee et al. [19] indicated that in induction phase, maintaining a higher DO set point and adopting more frequent feeding could significantly enhance elastase inhibiting peptide (EIP) expression. However, the DO effects on heterologous protein expression by *P. pastoris* shown in several reports were discrepant: some suggested a positive effect and others indicated conflicting results. In those reports, mechanism of their results was not interpreted. In addition, the combinational effects of induction temperature and DO level on heterologous protein expression with *P. pastoris* were seldom reported.

Aiming at solving the above mentioned problems and further improving heterologous proteins expression performance by *P. pastoris*, in this study, fermentation performance under various operation environments (temperature, DO, and the combination of both) was evaluated in terms of protein activity and apparent dynamic parameters such as specific AOX activity, specific rates of methanol consumption and oxygen uptake, etc., with the pIFN-α producing strain as prototype.

2. Materials and methods

2.1. Strain

Expression plasmid pPICZ-αIFN was constructed by ligation of pINF-α gene into pPICZα (Invitrogen, Carlsbad, CA, USA) at downstream of the promoter AOX1. pPICZ-αIFN was linearly integrated into the chromosome DNA of the host *P. pastoris* KM71 (Muts his-, PAOXII, Invitrogen, Carlsbad, CA, USA) before transformation. The construction of the recombinant *P. pastoris* KM71H (IFNα-pPICZαA) was implemented at Animal Husbandry and Veterinary Research Institute, Shanghai Academy of Agricultural Science, China. With this strain, a maximal pIFN-α antiviral activity of 2.1×10^4 IU/ml could be achieved in shaking flask cultivation.

2.2. Fermentation medium

Seed medium (in g/l, unless otherwise specified): Glucose 20, Peptone 20, Yeast extract 10. Batch medium for jar fermentor: glycerol 20, $(NH_4)_2SO_4$ 5, H_3PO_4 2 (%, v/v), $MgSO_4$ 1, $CaSO_4$ 0.1, K_2SO_4 1; PTM_1 10 (ml/l), pH 6.0. Feeding medium for growth: glycerol 500, $(NH_4)_2SO_4$ 0.5, KH_2PO_4 0.5, $MgSO_4$ 0.03; PTM_1 10 (ml/l), pH 6.0. Feeding medium for induction: methanol 500, $(NH_4)_2SO_4$ 0.5, KH_2PO_4 0.5, $MgSO_4$ 0.03; PTM_1 10 (ml/l), pH 6.0. Mixed feeding medium for transition phase: growth medium/induction medium volumetric ratio of 25:1.

2.3. Analytical methods

2.3.1. Measurements of cell density and methanol concentration

The cell concentration was determined by measuring the optical density at 600 nm (OD_{600}), and then dry cell weight (DCW) was calculated by a consistent calibration curve of DCW versus OD_{600} (g-DWC/l = $0.25 \times OD_{600}$). Methanol was detected by a gas chromatography (GC112A, FID detector, Shanghai Precision & Scientific Instrument Co., China) with an Alpha-Col AC20 capillary column (SGE Int'l Pty. Ltd., Australia). A penetrative polymer-membrane type's on-line methanol electrode (FC-2002, Subo Co., China) capable of inserting into the bioreactor directly and supporting high temperature sterility, was used for on-line methanol measurement. The on-line measured methanol concentration data were collected into a PC via a multi-channels A/D converter (PCL-812PG, Advantech Co., Taiwan).

2.3.2. Measurements of pIFN-α antiviral activity and pIFN-α concentration

The samples were centrifuged at 11,000 rpm for 10 min prior to the measurement. The pIFN-α antiviral activity was determined according to Chinese pharmacopoeia [20], using human amniotic cells WISH and vesicular stomatitis virus (VSV) (Wanxing Bio-Pharmaceutical Co., China). The commercially available human interferon was used as the standard sample. Before measurement, the standard and fermentation supernatant samples were properly diluted according to specific requirements. The pIFN-α antiviral activity was defined as the reciprocal of the maximal dilution rate, and under this rate, 50% cytopathic inhibition or 50% viruses plaque formation could be reached. IU referred to the abbreviation of "International Unit". As for pIFN-α concentration measurement, an amount of 20 μl sample was placed in each cell of the electrophoretic plate. The SDS-PAGE electrophoresis (15% resolving gel) was performed with the molecular weight standards until the bromophenol blue marker had reached the bottom of the gel. After SDS-PAGE analysis, the pIFN-α concentration was quantified with a G:Box Bio Imaging System and GeneTools software (SynGene Co., Cambridge, UK), each band was scanned in triplicate and an average of three readings was obtained.

2.3.3. AOX activity assay

Alcohol oxidase (AOX) activity was determined using the method described by Suye et al. [21]. Samples taken from the bioreactor with accurate volume were centrifuged at 11,000 rpm for 20 min. The cells were collected and washed twice with 50 mM phosphate buffer (pH 7.5), re-centrifuged, and then re-suspended in the same buffer with the volume ratio of 1:1 (buffer: sample). The suspension was sonicated by a sonifier (JY92-II, Scientz Biotechnology Co., China) at 0 °C for a total period of 10 min (pulse on, 5 s; pulse off, 10 s). The cell debris was removed by centrifugation at 5,000 rpm for 20 min, and the supernatant was used as the cell-free extract. The cell-free extract was then properly diluted by phosphate buffer (pH 7.0) to prepare the assay solution before activity measurement. AOX activity was assayed with a spectrophotometer (UV-2100, Unico, China) at 500 nm by measuring the optical increase within 10 min at 37 °C, with 3 ml colorized reaction mixture consisting of 100 μmol phosphate buffer (pH 7.0), 1 μmol 4-aminoantipyrine, 4.3 μmol phenol, 10 units of peroxidase, and 200 μmol methanol, and the crude enzyme solution (diluted extract, supplemented until the mixture volume reached exactly 3 ml). One enzyme unit is