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Enhanced Production of Human Serum Albumin by Fed-Batch Culture of *Hansenula polymorpha* with High-Purity Oxygen

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Fed-batch cultures of *Hansenula polymorpha* were studied to develop an efficient biosystem to produce recombinant human serum albumin (HSA). To comply with this purpose, we used a high-purity oxygen-supplying strategy to increase the viable cell density in a bioreactor and enhance the production of target protein. A mutant strain, *H. polymorpha* GOT7, was utilized in this study as a host strain in both 5-l and 30-l scale fermentors. To supply high-purity oxygen into a bioreactor, nearly 100% high-purity oxygen from a commercial bomb or higher than 93% oxygen available *in situ* from a pressure swing adsorption (PSA) oxygen generator was employed. Under the optimal fermentation of *H. polymorpha* with high-purity oxygen, the final cell densities and produced HSA concentrations were 24.6 g/l and 5.1 g/l in the 5-l fermentor, and 24.8 g/l and 4.5 g/l in the 30-l fermentor, respectively. These were about 2–10 times higher than those obtained in air-based fed-batch fermentations. The discrepancies between the 5-l and 30-l fermentors with air supply were presumably due to the higher contribution of surface aeration over submerged aeration in the 5-l fermentor. This study, therefore, proved the positive effect of high-purity oxygen in enhancing viable cell density as well as target recombinant protein production in microbial fermentations.

Keywords: Fed-batch culture, high cell density, high-purity oxygen fermentation, human serum albumin, *Hansenula polymorpha*

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Human serum albumin (HSA) is an excellent substitute for human plasma and one of the most abundant extracellular proteins. Owing to its pharmaceutical importance, the expression of HSA in several yeast systems, including *Saccharomyces cerevisiae*, *Kluyveromyces* sp., and *Pichia pastoris*, has been attempted to produce large quantities of recombinant HSA that is free from contamination by human pathogens [5]. The major form of HSA expressed by these yeasts has been the mature form of 67 kDa, although 45 kDa degradation products were also detected as a minor fraction [3, 12, 14]. The degraded 45 kDa product has been observed in all yeast systems employed so far for HSA production.

Because of its great clinical demand as a replacement fluid of human plasma or compensator for blood loss, there has been much effort to develop efficient biosystems to produce a large amount of HSA through engineered microbial hosts. Several yeast systems such as *Saccharomyces cerevisiae* [6, 12], *Kluyveromyces lactis* [3, 8], and *Pichia pastoris* [14] have been used. In particular, yeast-derived recombinant proteins have lower levels of structural heterogeneity than the blood-derived protein, suggesting that yeast could be an excellent choice as a host system to produce HSA for pharmaceutical purposes. Among them, *H. polymorpha* has been recognized as a promising host organism for the production of heterologous proteins including HSA, owing to its several advantageous features such as excellent expression capacity derived from the methanol-inducible genes and stable multimeric integration of the expression cassette for constructing efficient expression vectors [4]. To improve the fermentation performance, the high cell density culture of *H. polymorpha* by using fed-batch culture was also studied previously [7].

For development of efficient biosystems to produce recombinant protein, the increase of viable cell density has been recognized as being essential. A great deal of effort has been made to improve the efficiencies of microbial fermentation by tuning the characteristics of the cultural environment, such as the media, strains, and operating conditions. Recently, we reported several papers showing high cell density culture is indeed possible with high-purity oxygen supply in many occasions of aerobic microbial fermentations [10, 11]. High-purity oxygen supply was also recognized as an efficient method to make high cell density culture even in a large-scale fermentation [11].

In this report, *H. polymorpha* GOT7 was used, which is a mutant of *H. polymorpha*, to secrete HSA to the culture broth. The effects of supplying high-purity oxygen on HSA synthesis, cell growth, and by-product formation were investigated in a 5-l as well as large-scale (30-l) fermentor. This study, using a vacuum pressure swing adsorption (VPSA) oxygen generator to supply enough oxygen for high cell density culture, showed a model of the high-purity oxygen fermentation process for mass production of recombinant proteins by large-scale high cell density cultures of recombinant *H. polymorpha.*

Materials and Methods

Microorganism

The yeast strain used in this study was *Hansenula polymorpha* GOT7 (KCTC-0502BP), which was previously prepared [7]. This strain is a mutant of *H. polymorpha* DL1-L (leu2, ATCC 26012) to secrete human serum albumin to the culture broth. To construct the HSA expression cassette, the HSA cDNA lacking its 5'-UTR was directly fused with the methanol oxidase (MOX) promoter by fusion PCR technology. The cassette was subsequently cloned into the integration vector. The expression vector was then transformed into *H. polymorpha* and stabilized according to the DMSO–lithium acetate method as previously described [13].

Culture Conditions

Batch cultures were conducted in a 5-l fermentor (Best Korea Fermentor) containing 1.9 l of the culture medium and 100 ml of the seed cultures, which were precultivated to exponential growth phase at 37°C, pH 6.0, and 1 vvm of gas flow-rate. In this study, culture medium was changed according to the expression (constitutive/inducible) system. YPM medium containing 1% yeast extract, 2% bacto peptone, and 2% methanol was used for the constitutive expression system. For the inducible expression system, two different media were used, the YPG medium (1% yeast extract, 2% bacto peptone, and 2% dextrose) and the YPD medium (1% yeast extract, 2% bacto peptone, and 2% glycerol). Fed-batch cultures were conducted in both 5-l and 30-l fermentors. The gas flow-rate was maintained at 1 vvm based on the initial culture volume, and the pH and temperature were maintained at 6.0 and 37°C, respectively. As a feeding solution, 20 g/l glycerol solution was continuously fed until the cell growth rate became slow to maintain at $\mu=0.1\ h^{-1}$ with a peristaltic pump. After then, μ was regulated manually to decrease stepwise to $0.01\ h^{-1}$. As a substitute for glycerol and an induction material for recombinant HSA, 20 g/l methanol was added to meet the demand of carbon source. During this time, glycerol was fed together with methanol. After the μ was decreased lower than $0.01\ h^{-1}$, only methanol was continuously added. The concentration of methanol was kept in the range of 8~13 g/l, as previously proved to be optimal for cell growth and HSA expression or antidecomposition [6, 7].

High-purity oxygen was supplied through an oxygen bomb or by vacuum pressure swing adsorption (VPSA). The agitation speed was controlled to 400~500 rpm in small-scale (5 l) fermentation and 150 rpm in large-scale (30 l) fermentation to maintain the dissolved oxygen concentration as high as possible.

Analytical Methods

Dry cell weight (DCW), pH, optical density (OD_{600}), and other basic values were measured using methods described elsewhere.

Protein samples were analyzed by electrophoresis on SDS–PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). Raw samples were centrifuged at 12,000 rpm for 6 min. Only supernatants were collected to analyze with SDS–PAGE, where 8% of separating gel and 5% of stacking gel were used. After fractionation, the gel was stained by Coomassie blue and destained by destaining solution (methanol 10%, acetic acid 10%). Protein yields were determined using PRO-MEASURE Protein Measurement Solution (Intron Biotech, Korea), which employs the calorimetric Bradford method [2]. The HSA concentration was measured by using the specific affinity of bromocresol green (BCG) to albumin. After titrating fermentation broth containing HSA with acids to pH 4.2, albumin becomes cationic, and the anionic dye of BCG can be tightly bounded on them. This HSA–BCG complex exhibits a strong absorbance at 628 nm [1]. Productivity was calculated by the produced HSA concentration divided by culture time. The analysis of methanol in the culture broth was performed by gas chromatography (Varian 3300, CA, USA).

Results and Discussion

Expression of HSA

pH and temperature are key factors that should be optimized for improving cell growth and recombinant protein production of many cultivating microorganisms. Through several arrays of flask cultures to find the optimal condition for HSA expression, pH 6.0 and 37°C were selected in this work. We also tested different expression systems, including constitutive expression using YPM medium and inducible expression using YPD or YPG media; the inducible system yielded about 1.5 times higher HSA (data not shown). Therefore, we employed the inducible system using methanol as the induction material in further fed-batch cultures.

Fed-Batch Cultures in Small-Scale (5 l) Fermentor

To develop an efficient biosystem to produce HSA, fed-batch cultures of *H. polymorpha* GOT7 were performed. Both YPD and YPM media were used as basal media to

compare their effects on the growth of *H. polymorpha* and HSA production. To maximize HSA production, a gradual feeding strategy was selected. In this way, after the exhaustion of dextrose and glycerol in the early medium of batch culture, 20 g/l glycerol solution was fed. The glycerol concentration in the feeding solution was slowly phased down, and the methanol concentration in the feeding medium was increased for achieving a high adaptability of cells on the new feeding medium (glycerol/methanol). Finally, after μ decreased below 0.01 h^{-1}, methanol was fed solely into the fermentor.

We first performed fed-batch cultures with the YPD medium using air and high-purity oxygen supply. As shown in Fig. 1A, the glycerol solution was fed to maintain μ=0.1 h^{-1} until 15 h (OD_{600}=50), with a peristaltic pump. From 15 h to 25 h, both glycerol and methanol were fed into the bioreactor to make sure that cells could adapt methanol as the carbon source and an inducer for HSA expressions. The μ was also decreased stepwise to about 0.01 h^{-1}. After that, only methanol was fed to effectively induce HSA expressions. In the experiment, the final HSA concentration and DCW were 2.6 g/l and 16.2 g/l, respectively. With the same cultural condition and feeding strategy described above, a fed-batch culture with high-purity oxygen gas supply rather than air was performed (shown in Fig. 1B). It was found that the final HSA concentration and DCW reached 5.1 g/l and 24.6 g/l, respectively. Both of these increased to 2 and 1.5 times, respectively, by only changing the supplying gas from air to high-purity oxygen. The dissolved oxygen (DO), which was monitored at stabilized cultural conditions including agitation speed and temperature, was maintained higher than 20% in both cases; however, a higher DO of over 60% was monitored by using high-purity oxygen supply, indicating sufficient feeding to eliminate oxygen limitation. In the same manner, the productivity of HSA increased from 39.7 to 85.4 mg/l·h. These all reveal the affirmative effect of high-purity oxygen for increasing cell density and recombinant protein production.

YPG-based fed-batch cultures were also performed with air and high-purity oxygen supply, respectively. The feeding strategy was similar to that with YPD medium. Up to 16 h, glycerol solution was fed to maintain μ=0.1 h^{-1}. After that, the glycerol solution feeding rate was slowly decreased to regulate μ to 0.01 h^{-1}. As a substitute for glycerol, methanol was added from 16 h. After 32 h, only methanol was added into the bioreactor. From Fig. 2, it can been seen that the final HSA concentration and DCW

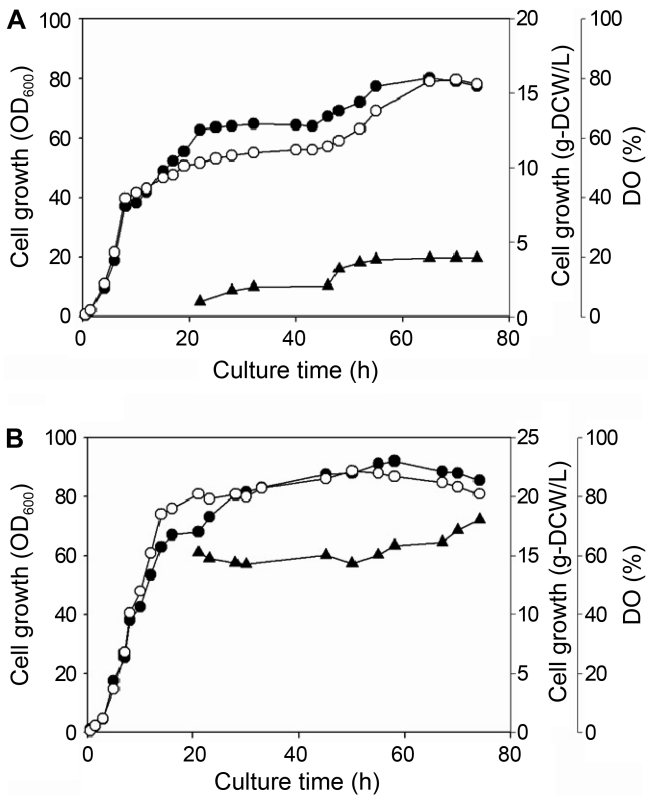

Fig. 1. Five-liter scale fed-batch cultures with air (**A**) and high-purity oxygen (**B**) supply using YPD medium.
Symbols are OD_{600} (●), DO (▲), and DCW (○).

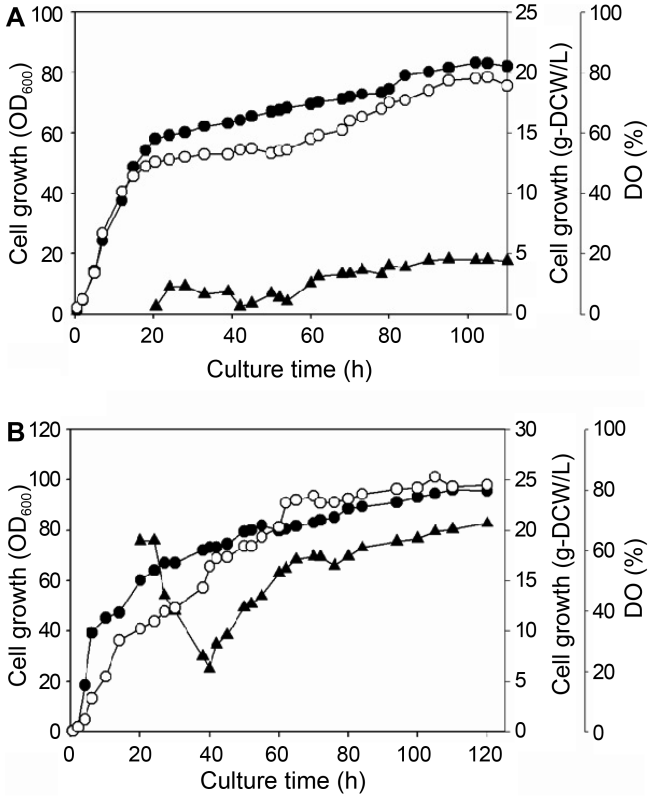

Fig. 2. Five-liter scale fed-batch culture with air (**A**) and high-purity oxygen (**B**) supply using YPG medium.
Symbols are OD_{600} (●), DO (▲), and DCW (○).

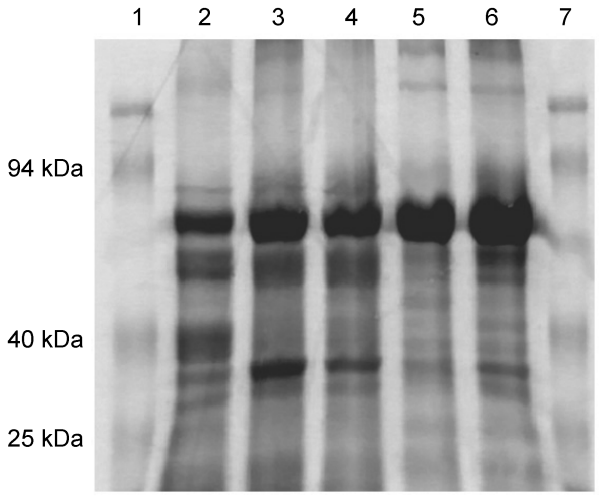

Fig. 3. SDS–PAGE analysis of HSA expression in 5-l fed-batch cultures.
The same amount (20 μg) of samples was loaded from lane 2 to lane 6. Lane 1 and 7, MW markers; Lanes 2 and 3, 75 h fermentation using YPD with air and high-purity oxygen, respectively; Lane 4, 110 h fermentation using YPG with air; Lanes 5 and 6, 120 h and 130 h fermentation using YPG with high-purity oxygen, respectively.

reached 2.7 g/l and 19.6 g/l, respectively, in the case of air supply (Fig. 2A). The final HSA concentration and DCW using high-purity oxygen supply reached 5.8 g/l and 25.3 g/l, respectively (Fig. 2B). Similar to YPD-based fed-batch cultures, YPG-based fermentation also proved the enhanced effect of high-purity oxygen on HSA production and cell growth. Both the HSA concentration and DCW increased to 2.2 and 1.3 times, respectively. The DO level monitored during fermentation in both cases further confirmed that high-purity oxygen supply could eliminate oxygen limitation, and apparently did not affect the degradation of HSA.

The expressed HSA in both YPD/YPG media was confirmed *via* SDS–PAGE analysis. As shown in Fig. 3, the major band was placed at around 67 kDa, indicating the right HSA expression, although weak degraded HSA bands were also shown at around 45 kDa. Similar to the BCG analysis of determining HSA concentration, thicker and high density bands were shown with high-purity oxygen supply than with just air. This also indicates that the HSA expression could be enhanced *via* a high-purity oxygen supply strategy in fed-batch cultures.

Fed-Batch Cultures in Large-Scale (30 l) Fermentor

High-purity oxygen supply was effective to enhance viable cell density and subsequent recombinant protein production in smaller scale fermentations [10, 11]. We also expected that high-purity oxygen produced from VPSA may provide an effective method for the large-scale industrial production of recombinant proteins. Along this line, large-scale fed-batch cultures of up to 30 l were performed with both air and high-purity oxygen supply produced by VPSA.

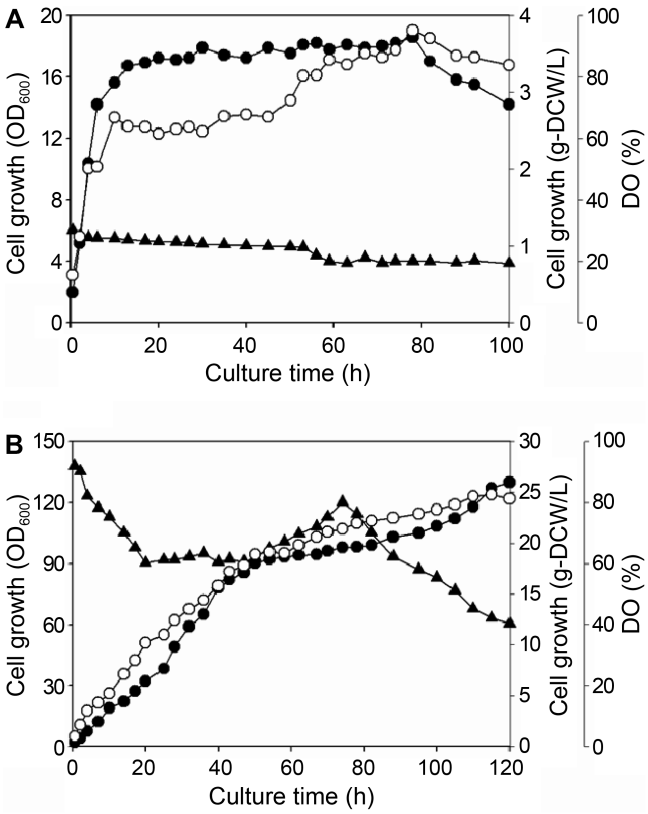

Fig. 4. Thirty-liter scale fed-batch cultures with air (**A**) and high-purity oxygen (**B**) supply using YPD medium.
Symbols are OD_{600} (●), DO (▲), and DCW (○).

As shown in Fig. 4A, when the fed-batch cultures based on YPD medium using the same fermentation strategy described above for small-scale fermentation was carried out in a 30-l fermentor with air supply, the final HSA concentration and DCW decreased significantly to 0.4 g/l and 3.8 g/l, respectively. This poor scale-up efficiency was attributed to the limited oxygen transfer by a poor mixing quality in the large-scale fermentor compared with that in a small-scale fermentor [9], which was further confirmed by the observed DO level during fermentation (Fig. 4). The enhancement of increasing cell density and HSA production by using high-purity oxygen was much more vivid in the large-scale fed-batch fermentation (Fig. 4B), although there was a relatively high discrepancy observed between DCW and OD_{600}, presumably due to the cell lysis after the long culture time of over 80 h. With high-purity oxygen generated by PSA, the final produced HSA concentration and DCW reached 4.5 g/l and 24.8 g/l, respectively. Compared with the large-scale fed-batch culture with air supply, these increased 11 and 7 times, respectively, in the case of high-purity oxygen supply. Additionally, these parameter values

Table 1. Production of HSA by fed-batch cultures with air or high-purity oxygen supply in small (5 l) and large (30 l) scale bioreactors.

Medium (Scale)	Gas supply	OD_{600}	DCW (g/l)	r-HSA (g/l)	Productivity (mg/l·h)
YPD (5 l)	Air	81.00	16.2	2.6	39.7
	O_2	94.00	24.6	5.1	85.4
YPG (5 l)	Air	83.20	19.6	2.7	27.0
	O_2	96.10	25.3	5.8	55.9
YPD (30 l)	Air	18.6	3.8	0.4	6.7
	O_2	130	24.8	4.5	45.0

were nearly similar to those of the small-scale fermentation with high-purity oxygen supply under the same cultural conditions. This suggests that the method of high-purity oxygen supply can be effective to increase the fermentation efficiency even in a large-scale industrial process (Table 1).

Industrialization of the fermentation process generally needs contamination-proof, strain stability, and reliable cell retention methods. Among these, the most urgent technology is how to retain cells that lead to high cell density culture, whereas the issues of strain stability and contamination-free environment depend mainly on the on-site experience. In this viewpoint, the high-purity oxygen supplying strategy described here can be recognized as one of the most effective ways to increase a viable cell density and target protein production, with further development of VPSA to produce a large amount of oxygen effectively and cheaply.

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