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Implementing Chemostat Fermentation of *Pichia Pastoris* Producing Recombinant HBsAg to Optimize Cell Density Affected by Methanol Rate

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Abstract: High cell density fed-batch fermentation is the main strategy for recombinant hepatitis B surface antigen (rHBsAg) production. In this study, we employed short-term continuous fermentation to optimize the cell density of recombinant *Pichia pastoris* (*P. pastoris*). After reaching the maximum specified broth volume of 5 L in the fed-batch fermentation process, the operation mode was altered into the continuous mode with a dilution rate of 0.009 1/h. We used various values of methanol inflow to examine its impact as a limiting nutrient on cell density. After reaching the steady-state point, the continuous fermentation was stopped. The process's performance was evaluated based on titer, yield, productivity, and ease of process control. According to the results, the optimal methanol inflow in the pilot-scale fermentation process was 39.9 ml/h as the cell density increased from 363 g/l wet cell weight (WCW) in the fed-batch stage to 450 g/l WCW. We could successfully scale up the fermentation process with the biomass concentration of 450 g/l without having any major issues such as excessive heat dissipation or insufficient oxygen supply. This approach is a simple method for enhancing rHBsAg production efficiency in *P. pastoris* without requiring any new and complex facility.

Keywords: recombinant hepatitis B surface antigen; methylotrophic yeast; cell density; short-period chemostat; yield; titer; productivity.

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1. Introduction

Recombinant DNA technology is used in the biopharmaceutical industry to produce a wide range of products, including therapeutic proteins, enzymes, amino acids, and antibiotics [1-4]. These recombinant products are produced using genetic engineering in host cells such as bacteria, yeast, plants, mammalian, and insect cells [5-8]. Among these host cells, *Pichia pastoris* (*P. pastoris*), as a member of methylotrophic yeasts, has become a widely used host cell to express heterologous proteins [9-11]. This lower eukaryotic cell can be cultivated in simple and inexpensive media with high cell density [12,13]. Moreover, with numerous features, including easy genetic manipulation, high levels of extracellular and intracellular proteins' expression, and the ability to perform post-translational modifications, *P.pastoris* is suitable for the expression of complex recombinant proteins on a commercial-scale [14,15]. The presence of two tightly regulated promotors, i.e., AOX1 and AOX2 is one reason making

the expression of foreign genes on a large-scale in *P. pastoris* more favorable than the other host cells [14,16]. The AOX1 and AOX2 promoters can be induced strongly and weakly by methanol as a sole carbon and energy source, respectively [17].

Recombinant hepatitis B surface antigen (rHBsAg), the main component of the hepatitis B vaccine, is one of the main recombinant proteins which has been produced commercially by *P. pastoris* [18]. This antigen is in the form of a virus-like particle (VLP) consisting of about 100 monomers and lipids [19,20]. In *P. pastoris*, the rHBsAg is expressed intracellularly and accumulated in an extended endoplasmic reticulum, necessitating cell disruption for extracting the product [19,21].

High cell density fed-batch fermentation is the main approach for recombinant protein production with high yields in *P. pastoris* [10,22,23]. Many studies have investigated the possibility of maximizing the cell density of *P. pastoris* in the fed-batch process via optimizing media culture, applying different feeding strategies, and using different strains of *P. pastoris* [24,25]. Although enhancing the cell density can lead to a higher concentration of the recombinant protein, it is not necessarily the optimal process considering productivity, yield, and the process scale.

Compared to the batch and fed-batch fermentation, continuous fermentation is another type of operation to manipulate and control the cell density [26,27] simply. Thus, the optimum operating condition in terms of titer, yield, and productivity can be easily investigated [27,28]. In the current study, we applied short-term chemostat fermentation to examine the process's efficiency in different cell densities of *P. pastoris*. For this purpose, we have tested and compared the impact of varying methanol feeding rates in short-term chemostat fermentation on biomass concentration, titer, productivity, and yield of the process. Finally, after obtaining the optimal cell density on the pilot-scale, we performed the fermentation process on a large-scale.

2. Materials and Methods

2.1. Materials.

All buffers and culture media were prepared with analytical grade chemicals purchased from Merck Company (Germany) and water for injection (WFI) from the Pasteur Institute (Iran). 10 L fermenter (model Winpact FS01-V-B -L) and 300 L fermenter (AROKO, Iran) were used for the fermentation process. For cell disruption, Spiral Mill Kit (CELLCRUSHERTM) was employed.

2.2. Microorganism and culture medium.

The Mut+ strain of *P. pastoris* GS115 (His4) was used to express HBsAg under the control of the inducible AOX1 promoter. The obtained recombinant *P. pastoris* from master cell bank was inoculated into 1 L Erlenmeyer with 200 mL standard medium [1% (w/v) yeast extract, 2% (w/v) peptone, 1.34% (w/v) yeast nitrogen base, 0.4 mg/l biotin and 100 mM potassium phosphate (pH 6.0)] containing 1% (w/v) glycerol (BMGY). The inoculums were placed in a shaker incubator at 29°C with a stirring speed of 500 rpm for 36-38 h until wet cell weight (WCW) reached 30 g/l.

2.3. Inoculation of microorganisms.

The prepared pre-induction growth was used to inoculate in a 10 L fermenter containing 2300 ml of filter-sterilized fermentation media. Each liter of fermentation culture media contained 40 ml glycerol, 5.0 ml H_2SO_4, basal salt medium (26.7 ml 85% H_3PO_4, 0.93 g $CaSO_4$, 18.2 g K_2SO_4, 14.9 g $MgSO_4 \cdot 7H_2O$, 4.13 g KOH), trace mineral mix (6.0 g $CuSO_4 \cdot 5H_2O$, 0.08 g NaI, 3.0 g $MnSO_4 \cdot H_2O$, 0.2 g $Na_2MoO_4 \cdot 2H_2O$, 0.02 g H_3BO_3, 0.5 g $CoCl_2$, 20.0 g $ZnCl_2$, 65.0 g $FeSO_4 \cdot 7H_2O$) and vitamin mix.

2.4. Batch and fed-batch fermentation.

Before starting continuous fermentation, four conventional stages, i.e., shake flask cultivation, glycerol batch fermentation, methanol adaptation, and methanol fed-batch fermentation, were applied[1,14]. The bioreactor's fermentation process was first carried out in the batch mode at 29 ℃ and pH around 4.7-5 by adding 20% w/v ammonia solution. The impeller rotational speed was 500-800 rpm while maintaining the dissolved oxygen (DO) at (1.5-2.2 mg/l) 20-30% saturation[20]. The first step, glycerol batch cultivation, was conducted for about 12 h until reaching the biomass yield of 110 g/l WCW.

During the second stage, the methanol induction as the sole carbon source for HBsAg expression began when glycerol was completely exhausted as the DO level reached 100% saturation and remained constant. In the beginning, we injected methanol into the media with a flow rate of 17 mL/h for an hour. After a one-hour pause, the methanol injection was repeated with the same flow rate for 3 hours (h). Finally, the methanol flow was stopped for approximately 6 h until the yeast cells adapted entirely to the methanol, which was detectable by a drastic decrease of DO.

In the third stage, after methanol adaptation, the fed-batch fermentation with methanol induction was initiated. The operational condition in the pilot-scale fed-batch process (10 L) was determined by scaling down the well-established protocol of large-scale (600 L) production of rHBsAg from *P. pastoris* in Pasteur Institute of Iran[1,14,19]. After setting the optimum parameters, the fed-batch fermentation process was carried out at 29 ℃ and pH around 4.7-5 via 20% v/v ammonia under DO stat condition (20%-30%) 1-1.2 VVM inlet air with DO cascade, using methanol as the limiting nutrient.

2.5. Short-term continuous fermentation.

As shown in Figure1, continuous fermentation was initiated after reaching the maximum possible cell density of 330 mg/l in 5-liter broth volume in fed-batch fermentation.

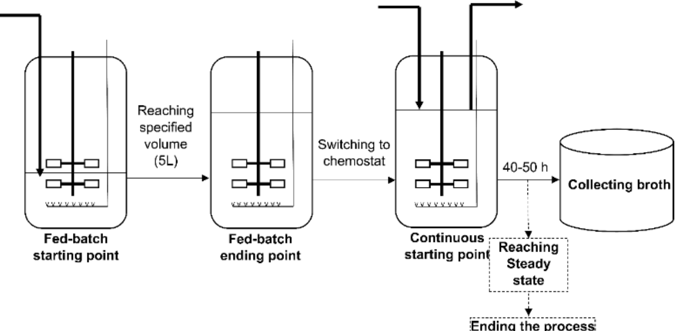

Figure 1. Schematic diagram of the experimental setup of 10 L fermenter for production of rHBsAg in *P. pastoris* in pilot-scale. At the final stage of the conventional fed-batch process, a short-period continuous fermentation process was applied until reaching the steady-state point.

The fermentation process was carried out in a specific growth rate equal to fed-batch fermentation, i.e., 0.009 1/h. The DO-stat continuous fermentation operated with a dilution rate of 0.009 1/h at 29 ℃ and pH around 4.7-5 until reaching the steady-state point. Then, the process stopped. Table 1 presents the investigated feeding rates of methanol in this study.

Table 1. The values of methanol inflow and culture inflow in each experiment of short-period chemostat fermentation at a dilution rate of 0.009 1/h.

Experiment No.	1	2	3	4	5
Methanol inflow, ml/h	31.9	34.6	37.3	39.9	42.6
Culture inflow, ml/h	13.1	10.4	7.7	5.1	2.4
Outflow, ml/h	45	45	45	45	45

As shown in Table 1, the culture media inflow was reduced by increasing methanol inflow to keep the total dilution rate constant. To provide sufficient nutrients in the fermenter for yeast cells in high cell densities, the amount of each nutrient in the culture media source increased while methanol remained as a limiting nutrient in the continuous fermentation process (Table 2).

Table 2. Amounts of nutrients in each experiment of short-period chemostat fermentation.

Experiment No.	1	2	3	4	5
Ammonium sulfate, g/h	0.80	0.87	0.94	1.00	1.07
Magnesium sulfate.17 H2O, g/h	0.27	0.30	0.32	0.34	0.36
Dipotassium hydrogen phosphate, g/h	0.66	0.72	0.77	0.83	0.88
Calcium chloride.2 H2O, g/h	0.02	0.02	0.02	0.02	0.02
EDTA, g/h	0.02	0.02	0.02	0.02	0.02
Vitamin A, ml/h	0.65	0.70	0.76	0.81	0.86
Vitamin B, ml/h	0.65	0.70	0.76	0.81	0.86
Orthophosphoric acid 35%, ml/h	0.35	0.38	0.41	0.43	0.46
Trace elements, ml/h	0.33	0.36	0.39	0.41	0.43

2.6. Analytical essays.

Samples were taken at regular intervals for analysis during fermentation. WCW was also measured after separation of biomasses (cell pellets) via centrifugation at 3000g for 15 min. After determination of wet biomass amount, cells were disrupted to release rHBsAg inside the cells. For this purpose, separated cell pellets were washed with phosphate buffer saline (PBS) and centrifuged twice at 3000 g for 10 minutes. After discarding the supernatants, the cell pellets were frozen at – 20 ℃, and after defrosting, they were suspended in 0.5 ml of disruption buffer (pH 8, Tris 20 mM, NaCl 250 mM, EDTA 5 mM, DTT 2.5 mM and 1 mM PMSF dissolved in isopropanol). Subsequently, 5.75 g of glass beads with 0.50 mm diameters were added to the tube apparatus's disruption buffer. The cells were disrupted by Spiral Mill Kit for 7 min. Intracellular protein was measured in the supernatant of cell-free lysate using the Coomassie blue method. We quantified rHBsAg concentration by sandwich ELISA where sheep polyclonal antibodies against HBsAg were coated on the plate and conjugated with horseradish peroxidase [23].

3. Results and Discussion

We examined the impact of the chemostat fermentation process on final cell density with five different methanol feeding rates (Table 1) with a constant broth volume of 5 L.

Approximately 40-50 hours were required for the fermentation process reach the steady-state level. Figure 2 illustrates the results of the cell density and rHBsAg concentration with various methanol feeding rates.

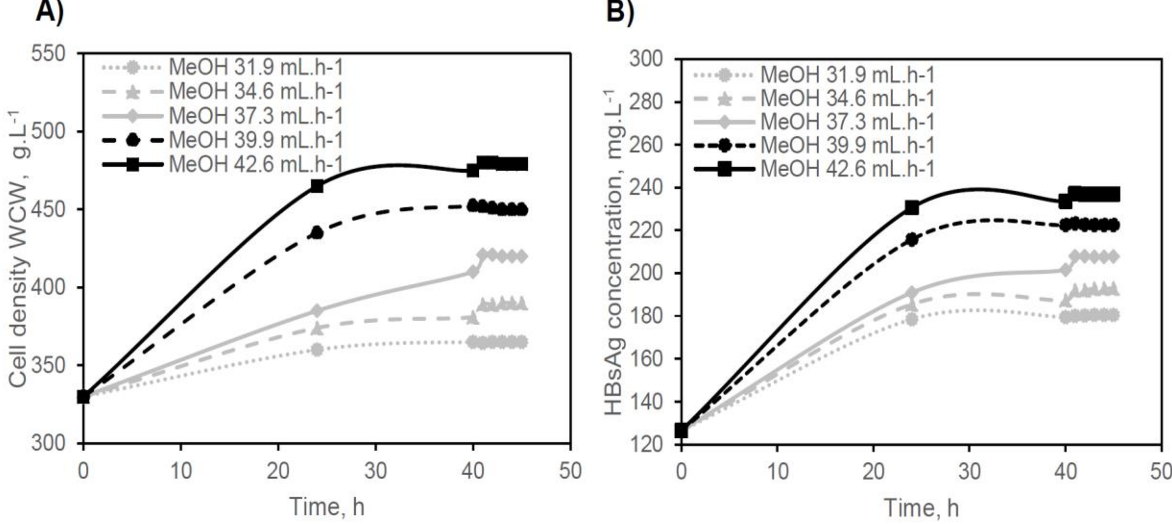

Figure 2. Fermentation profile of *P. pastoris* in the fed-batch process with different methanol inflow rates. (**a**) The biomass concentration considering the fermentation time; (**b**) Concentration of rHBsAg considering the fermentation time.

According to the obtained results, it is estimated that the maximum achievable cell density in the chemostat fermentation with a dilution rate of 0.009 1/h would be about 500 g/l WCW. Obtaining a higher value is not possible without increasing the total flow and dilution rate. In our experiments, using a methanol inlet flow of 42.6 ml/h, the maximum cell density was 480 g/l WCW using a methanol inlet flow of 42.6 ml/h. The main reason for not increasing the methanol feed rate further to reach 500 g/l was that performing the fermentation process would be more difficult keeping the concentration of nutrients in culture media close to saturation point. As shown in Figure 2(B), the amount of rHBsAg increased by enhancing cell density. Comparing Figure 2(A) and Figure 2(B), shows that the rate of enhancing biomass concentration and rHBsAg concentration was nearly the same. This result suggests that we could perform the fermentation process using different methanol flow rates nearly under the same operational condition. Since the dilution rate- specific growth rate- was the same during all the experiments, the enhancement of methanol inlet flow has only influenced the amount of biomass and rHBsAg without affecting their ratios in the fermenter.

The total product titer, yield, specific productivity, and volumetric productivity with 5 different methanol inlet flows are shown in Figure 3. These values were calculated based on total broth amount, broth in the fermenter, and collected outflow during chemostat fermentation. As shown in Figure 3 (A), the rHBsAg concentration and titer have increased by enhancing the methanol feeding rate. This was expected since increasing methanol concentration, as a limiting nutrient, must lead to a higher cell density. As the fermentation process's duration was similar for all experiments, the volumetric productivity demonstrated the same trend (Figure 3(B)). As shown in Figure 3(C), the obtained values for specific productivity were close to each other.

No major difference was detected in the specific productivity since the specific growth rate, which correlates with productivity, was constant during our experiments. The reason for minor differences might be related to the small changes in rHBsAg concentration in the collected outflow before reaching the steady-state condition. We evaluated the yield (mg

product/mg substrate) of each experiment by measuring the total consumed methanol and produced rHBsAg during the same fermentation period.

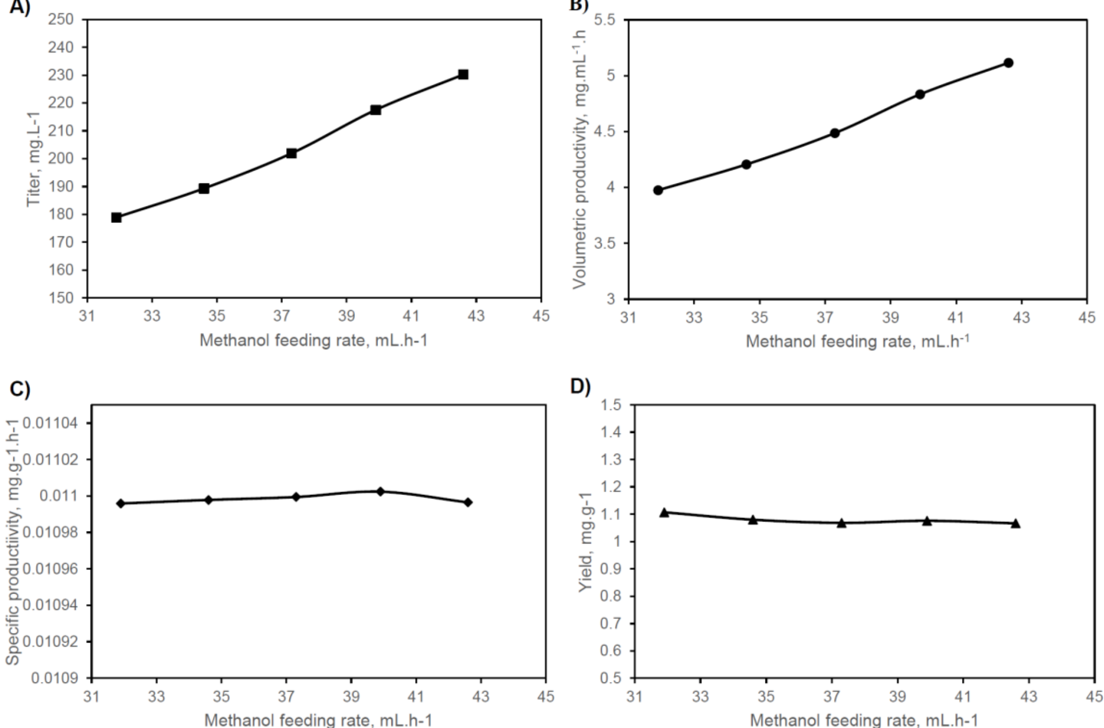

Figure 3. Comparison of (**a**) titer; (**b**) volumetric productivity; (**c**) specific productivity; (**d**) yield of rHBsAg in the continuous fermentation process of *P. pastoris* using various methanol inlet flows.

The calculated ratio of total produced rHBsAg to total consumed methanol demonstrates that the yield was nearly the same in different methanol flow rates. The slight difference between various cell densities is related to the impact of continuous fermentation in the non-steady stage. Thus, by increasing the time of chemostat fermentation, these minor differences would decrease. Considering titer, yield, and productivity together, cell densities of 450 g/l and 480 g/l showed the best performance in our study for the pilot-scale.

In the large-scale production process, transport phenomena, specifically heat dissipation and dissolved oxygen concentration, are the major limiting factors for high cell density fermentation. Thus, after evaluating the performance of short-term chemostat fermentation in different cell densities, we scaled-up the process in 300 L fermentation. Thus, we could examine the feasibility of performing the fermentation process in the large-scale fermenter for high cell density under acquired optimal cell densities. For this purpose, the short-term continuous fermentation process was carried out for cell densities of 480 g/l and 450 g/l, respectively. The first experiment using the cell density of 480 g/l was stopped due to extreme heat dissipation during the fermentation process. The results demonstrated that controlling the process condition for large-scale fermentation is not possible for this cell density using the usual fermenters. On the contrary, the process condition was controlled easily using the cell-density of 450 g/l. We could obtain the rHBsAg concentration nearly close to the pilot-scale process. Considering both experiments in large-scale and pilot-scale, it can be concluded that the cell density of 450 g/l is the optimum condition for the production of rHBsAg using the short-term chemostat fermentation.

Generally, the short-period continuous fermentation three main advantages against fed-batch fermentation. Firstly, it is easier to manipulate cell density in this operation without requiring any cost-intensive facilities and changing broth volume in the fermenter. Secondly,

in this type of operation, we can produce a higher amount of HBsAg by producing and collecting the broth during a short-term fermentation process. Finally, the industry's implementation seems to be straightforward and might not encounter major challenges considering scaling up and product quality.

4. Conclusions

Our research's primary purpose was to establish a short-term continuous fermentation process for optimization of cell density of *P. pastoris* producing rHBsAg. This study compared the titer, yield, and productivity of chemostat fermentation in various methanol feeding rates. The obtained results indicated that the cell density of 450 g/l in chemostat fermentation with a dilution rate of 0.009 1/h is an optimal point for the production of the rHBsAg derived from *P. pastoris*, both in pilot-scale and large-scale without common drawbacks such as heat dissipation and insufficient oxygen supply. By implementing this short-period chemostat method, we can optimize the cell density and produce and collect a higher amount of broth, which leads to a higher amount of product in each process operation.

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Conflicts of Interest

The authors certify no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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