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ORIGINAL PAPER

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Optimization of cell density and dilution rate in *Pichia pastoris* continuous fermentations for production of recombinant proteins

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Abstract This paper provides an approach for optimizing the cell density (X_c) and dilution rate (D) in a chemostat for a *Pichia pastoris* continuous fermentation for the extracellular production of a recombinant protein, interferon τ (INF-τ). The objective was to maximize the volumetric productivity (Q, mg INF-τ l^{-1} h^{-1}), which was accomplished using response surface methodology (RSM) to model the response of Q as a function of X_c and D within the ranges $150 \leq X_c \leq 450$ g cells (wet weight) l^{-1} and $0.1\ \mu_m \leq D \leq 0.9\ \mu_m$ ($\mu_m = 0.0678$ h^{-1}, the maximum specific growth rate obtained from a fed-batch phase controlled with a methanol sensor). The methanol and medium feed rates that resulted in the desired X_c and D were determined based on the mass balance. From the RSM model, the optimal X_c and D were 328.9 g l^{-1} and 0.0333 h^{-1} for a maximum Q of 2.73 mg l^{-1} h^{-1}. The model of specific production rate (ρ, mg INF-τ g^{-1} cells h^{-1}) was also established and showed the optimal $X_c = 287.7$ g l^{-1} and $D = 0.0361$ h^{-1} for the maximum ρ (predicted to be 8.92×10^{-3} mg^{-1} g^{-1} h^{-1}). The methanol specific consumption rate (v, g methanol g^{-1} cells h^{-1}) was calculated and shown to be independent of the cell density. The relationship between v and μ (specific growth rate) was the same as that discovered from fed-batch fermentations of the same strain. The approach developed in this study is expected to be applicable to the optimization of continuous fermentations by other microorganisms.

Keywords *Pichia pastoris* · Continuous fermentation · Optimization · Model · Interferon τ · Response surface methodology

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Introduction

Known as an efficient host for the production of recombinant proteins, *Pichia pastoris* is widely used. While most of the literature describes *P. pastoris* fermentations using a fed-batch mode, several successful continuous fermentations have also been reported. Digan et al. [3] described a continuous fermentation with a *P. pastoris* methanol utilization-plus phenotype (Mut^+) for the extracellular production of bovine lysozyme c2. A cell density of 120 g l^{-1} dry cell weight (DCW) was achieved at a dilution rate of 0.05 h^{-1}. Bovine lysozyme c2 concentration and cell density in the chemostat were approximately 350 mgl^{-1} and 100 g l^{-1} DCW, respectively. Goodrick et al. [4] reported a continuous fermentation constitutively expressing human chitinase under the control of the pGAP promoter. A feed medium containing 30% glucose (the limiting carbon source) was fed in the continuous phase. A cell density of 400 g l^{-1} wet cell weight (WCW) and a rh-chitinase concentration of 360 mg l^{-1} were maintained in the chemostat at a dilution rate of 0.83 day^{-1} (0.0346 h^{-1}) that continued for 30 days. Curvers et al. [2] ran a *P. pastoris* Mut^+ continuous fermentation that expressed human chymotrypsinogen B by feeding a medium containing 197.5 g l^{-1} methanol (25% by vol.). The feed rate was controlled by maintaining the methanol concentration at a constant level of 4 g l^{-1} and resulted in a cell density of 65 g l^{-1} DCW and volumetric productivity of 25 mg l^{-1} h^{-1}.

The advantage of the *P. pastoris* expression system is its high cell density (up to 130 g l^{-1} DCW) on a simple

defined basal salts medium and the ability to produce grams per liter quantities of recombinant proteins. However, proteases can accumulate and have an adverse impact on protein production and quality. Thus, it is necessary to optimize the cell density and dilution rate in a continuous fermentation. Such an investigation with *P. pastoris* has not been reported to date. The objective of this paper was to model the response of productivity to various cell densities and dilution rates, using an experimental design based on the response surface methodology (RSM). A Mut^+ *P. pastoris* that extracellularly expresses the recombinant ovine interferon-τ (IFN-τ) was used in this study. This strain has been successfully used to produce IFN-τ in fed-batch fermentations [8]. IFN-τ consists of 172 amino acids (about 20 kDa) and has a wide range of antiviral, antiproliferative and immunomodulatory activities; and it might be used as a therapeutic agent for treating human diseases like viral infections and autoimmune disorders [1, 6].

Materials and methods

Strain and shake-flask culture

A X-33 *P. pastoris* strain, which has the Mut^+ phenotype and extracellularly expresses the recombinant ovine protein IFN-τ, was obtained from Pepgen Corp. (Alameda, Calif., USA). It has been described elsewhere [5]. Frozen stock (1 ml) was used to inoculate a 1-l Erlenmyer flask containing 200 ml of buffered minimal glycerol yeast extract medium, containing 1% yeast extract (with amino acids but without ammonium sulfate), 2% peptone, 1.34% yeast nitrogen base, 4×10^{-5}% biotin, 1% glycerol, 100 mM potassium phosphate, pH 6.0). The flask was incubated for 20–24 h at 30°C and 300 rpm (shake rate) and reached an optical density at 600 nm of 10–20.

Batch and fed-batch phase on glycerol

The entire 200 ml of propagated shake-flask culture was used to inoculate a 5-l fermentor (Bioflo 3000; New Brunswick Scientific Co., Edison, N.J., USA) containing 3–4 l of basal salts medium that contains (per liter): 42.9 g KH_2PO_4, 5.0 g $(NH_4)_2SO_4$, 1.0 g $CaSO_4{\cdot}2H_2O$, 11.7 g $MgSO_4{\cdot}7H_2O$, 14.3 g K_2SO_4, 1.92 g citric acid, 40.0 g glycerol and 2.0 ml PTM4 trace salts solution (filter-sterilized, added separately). The PTM4 solution contains (per liter): 2.0 g $CuSO_4{\cdot}5H_2O$, 7.0 g $ZnCl_2$, 0.08 g NaI, 22.0 g $FeSO_4{\cdot}7H_2O$, 3.0 g $MnSO_4{\cdot}H_2O$, 0.2 g Biotin, 0.2 g $Na_2MoO_4{\cdot}2H_2O$, 0.02 g boric acid, 0.5 g $CoCl_2$ and 2.0 ml H_2SO_4. The pH was maintained at 5.0 using undiluted (about 28%) ammonium hydroxide, the dissolved oxygen (DO) was maintained above 20% saturation by adjusting the agitation rate and using a pure oxygen supply (oxygen sparging was initiated at maximum agitation speed) and the temperature was maintained at 30°C. The cells grew to approximately 100 g l^{-1} WCW during the glycerol batch-phase and the cell density was increased to the desired level (150–450 g l^{-1} WCW) using a 63% (w/v) glycerol solution containing 0.2% (v/v) PTM4 that was fed at 20 g l^{-1} h^{-1}. During the glycerol fed-batch phase, growth was limited by the glycerol feed rate and no glycerol accumulation occurred.

Continuous phase on methanol

To adaptively switch the substrate from glycerol to methanol, 2 ml l^{-1} methanol was injected into the fermentor, and simultaneously, the glycerol feed rate was programmed to decrease linearly from 20 g l^{-1} h^{-1} to zero over a period of 3 h. This 3-h period is considered a transition phase that is important for cells to adapt the methanol efficiently and completely [9]. After the transition phase, the continuous phase was initiated by feeding 100% methanol containing 0.1–0.2% (w/v) antifoam (KFO 673; KABO Chemicals, Wyo., USA) and basal salts medium (without glycerol, pH adjusted to 6.0). The broth pH was adjusted to and maintained at 6.0, while temperature and DO were still kept at 30°C and >20%, respectively. During the continuous phase, growth was limited by methanol. Figure 1 shows the schematic diagram of the continuous fermentation system. The substrate feed rates followed the experimental design.

Analysis

Time-course samples were centrifuged at 2,000 *g* for 10 min to determine the cell density (g WCW l^{-1} broth)

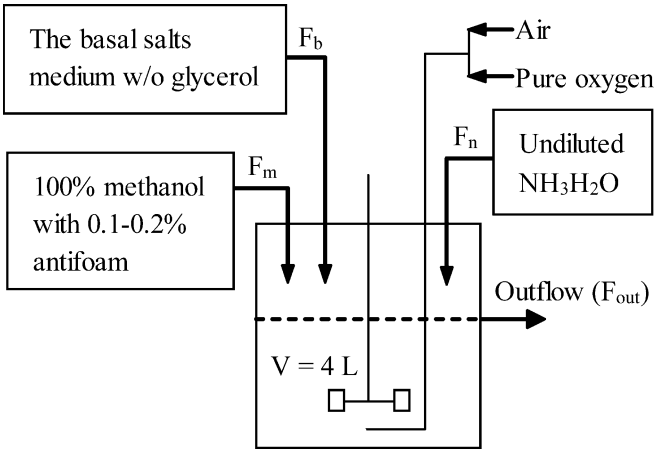

Fig. 1 Schematic diagram of the continuous fermentation. The feed rates of methanol (F_m) and basal salts medium without glycerol (F_b) were set based on the experimental design to result in the desired cell density and dilution rate in the chemostat; and the feed rate of ammonium hydroxide (F_n) was automatically controlled to maintain the pH at 6.0. Total inflow rate $F_{in}=F_b+F_m+F_n$. In chemostat, $F_{out}=F_{in}$; and the volume (V) was maintained at 4 l constantly by pumping the outflow through an outlet set at the same level

and measure the IFN-τ concentration in the supernatant with the method described elsewhere [8].

Experimental design

Mass balance

The pH of the basal salts medium feed solution (without glycerol) was adjusted to 6.0 with concentrated ammonium hydroxide; and thus the amount of ammonium hydroxide consumed (F_n) during chemostat is very small compared with F_b and F_m. The total flow into the bioreactor (F_{in}, l h^{-1}) and the dilution rate (D, h^{-1}) can be approximated as:

$$F_{in} = F_b + F_m \quad (1)$$

$$D = F_{in}/V = \mu \quad (2)$$

where μ is the specific growth rate and $V = 4$ l. In our previous work [9], we established a growth model for *P. pastoris* describing the relationship between the methanol concentration S(g l^{-1}) and μ (h^{-1}):

$$\mu = \frac{0.146S}{1.5 + S + S^2/8.86} \quad (3)$$

Here, we use this model to estimate the methanol concentration in the chemostat (S_c) for a given dilution rate (D). Equation 3 shows that as S increases so does μ, up to a methanol concentration of 3.65 g l^{-1}, at which point substrate inhibition occurs, resulting in a decrease in μ. In this study, we do not exceed 3.65 g l^{-1}. Using the curve-fitting software Table Curve 2D (SPSS, Chicago, Ill., USA) we can solve Eq. 3 for S_c as a function of D:

$$S_c = \frac{0.071 + 6.1D - 85.7D^2}{1 - 21.8D + 117.1D^2} \quad (4)$$

According to the mass balance, the cell density in the chemostat (X_c) is:

$$X_c = Y_{X/S}(S_{in} - S_c) \quad (5)$$

$$S_{in} = 790F_m/F_{in} \quad (6)$$

where S_{in} (g l^{-1}) is the methanol concentration in the feed, 790 g l^{-1} is the methanol density, $Y_{X/S}$ is the apparent cell yield on methanol (g WCW g^{-1} methanol) and varies with μ [9]. $Y_{X/S}$ can be estimated based on the maximum growth rate ($\mu_m = 0.0678$ h^{-1}) and the maximum methanol specific consumption rate ($v_m = 0.0564$ g methanol g^{-1} WCW h^{-1}) determined previously for *P. pastoris* expressing IFN-τ [8]:

$$Y_{X/S} = \mu_m/v_m = 1.2\,\text{g WCW/g methanol} \quad (7)$$

The production of IFN-τ under continuous culture was optimized using a central composite design [7] based on the variables X_c and D. Using Eqs. 1, 2, 3, 4, 5, 6 and 7, it was possible to determine the feed rates F_m and F_b that would result in the desired X_c and D. Because $Y_{X/S}$ is determined based on μ_m and v_m, the feed rates were an approximation of the desired feed rate for the desired X_c and D, but this is sufficient for the central composite design.

Results

The range of X_c was set at 150–450 and the range of D at $0.1\ \mu_m \le D \le 0.9\ \mu_m$ with $\alpha = 1.41421$ (the distance from the center of the design space to a star or axial point) and the center points = 2. Table 1 shows the ten different X_c and D, based on the design and the actual X_c and D. The difference between the design and actual X_c is caused by using a $Y_{X/S}$ estimated from the μ_m and v_m (Eq. 7) used to calculate the F_m and F_b for all the runs. The deviation of D is caused by foam production and an instability in the aeration rate that caused the broth volume to vary. The INF-τ concentration (P_c, mg l^{-1}) was determined for each condition and used to calculate the volumetric productivity Q (mg l^{-1} h^{-1}) and specific production rate ρ (mg g^{-1} WCW h^{-1}), based on the actual X_c and D:

$$Q = DP_c \quad (8)$$

$$\rho = DP_c/X_c \quad (9)$$

A quadratic model was used to regress Q and ρ ($P < 0.05$ for both) as a function of X_c and D; and the results of the analysis of variance (ANOVA) are presented in Tables 2 and 3 [7]:

$$Q = -9.85 + 0.0576X_c + 186.44D - 8.14 \times 10^{-5}X_c^2 - 2191.83D^2 - 0.123X_cD \quad (10)$$

$$\rho = -0.0294 + 1.726 \times 10^{-4}X_c + 0.746D - 2.51 \times 10^{-7}X_c^2 - 7.205D^2 - 7.857 \times 10^{-4}X_cD \quad (11)$$

Figures 2 and 3 show the response surface plots of Q and ρ based on Eqs. 10 and 11, respectively. From these

Table 1 Central composite design for the two variables X_c and D, the feed rates derived from Eqs. 1, 2, 3, 4, 5, 6, 7, the actual X_c and D obtained and the ITF-τ concentration (P_c, mg l^{-1}) achieved in the chemostat

Design			Feed rate		Result		
Run number (randomized)	X_c	D	F_m	F_b	X_c	D	P_c
1	300	0.0068	0.009	0.019	243.7	0.0076	45.4
2	406	0.0531	0.091	0.121	323.2	0.0355	85.2
3	300	0.0339	0.043	0.093	232.7	0.0420	49.8
4	450	0.0339	0.064	0.071	415.5	0.0268	75.0
5	300	0.0339	0.043	0.093	285.7	0.0310	68.5
6	300	0.0610	0.078	0.166	363.3	0.0500	39.0
7	150	0.0339	0.022	0.114	201.3	0.0344	47.0
8	194	0.0531	0.044	0.169	216.2	0.0427	32.0
9	406	0.0147	0.025	0.034	329.1	0.0136	148.5
10	194	0.0147	0.012	0.047	203.0	0.0170	37.0

Table 2 ANOVA for significance of regression of Q with Eq. 10 [9]

Source of variation	Sum of squares	Degrees of freedom	Mean square	F_0	P value
Regression	4.981	5	0.996	8.8	0.0279
Error	0.453	4	0.113		
Total	5.434	9			

Table 3 ANOVA for significance of regression of ρ with Eq. 11 [9]

Source of variation	Sum of squares	Degrees of freedom	Mean square	F_0	P value
Regression	5.09×10^{-5}	5	1.02×10^{-5}	6.3	0.0497
Error	6.49×10^{-6}	4	1.62×10^{-6}		
Total	5.74×10^{-5}	9			

models, we obtain both the optimal $X_c = 328.9$ g l^{-1} and $D = 0.0333$ h^{-1} for the maximum Q (predicted to be 2.73 mg l^{-1} h^{-1}) and the optimal $X_c = 287.7$ g l^{-1} and $D = 0.0361$ h^{-1} for the maximum ρ (predicted to be 8.92×10^{-3} mg g^{-1} h^{-1}).

The specific methanol consumption rate v (g methanol g^{-1} WCW h^{-1}) is:

$$v = (S_{in} - S_c)D/X_c \quad (12)$$

With the results of X_c and D in Table 1, we calculated v and investigated different models of v as a function of X_c and D. The ANOVA showed that X_c is not a significant model term (data not shown). The regression obtained from various trials is a linear model:

$$v = 0.754D + 0.003 \quad (13)$$

This equation describes the relationship between v and μ (at chemostat $D = \mu$) and is about the same as what Sinha et al. [8] discovered from the fed-batch fermentations of the same strain ($v = 0.75\mu + 0.0055$).

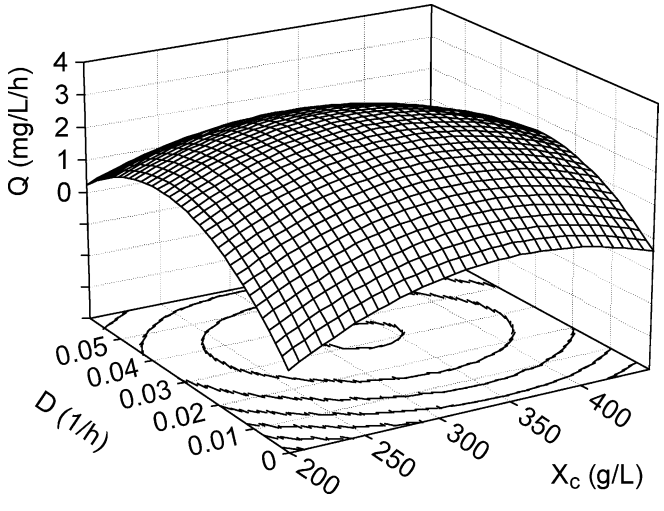

Fig. 2 Response surface of volumetric productivity (Q) to the dilution rate (D) and cell density (X_c) at the chemostat based on Eq. 10

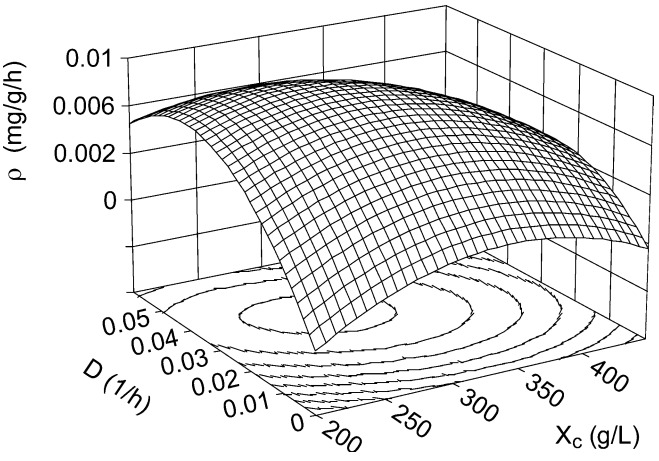

Fig. 3 Response surface of specific production rate (ρ) to the dilution rate (D) and cell density (X_c) at the chemostat based on Eq. 11

Discussion

The objective of process optimization for a continuous fermentation is to maximize volumetric productivity (Q), which for this study was dependent on cell density (X_c) and dilution rate (D). RSM [7] provides a solution to the experimental design with these two factors varied over multi-levels. However, X_c is a dependent on the feeding strategy, rather than an independent variable (such as temperature) that can be simply set to a desired value. Thus, the challenge is how to determine the feed rate of the limiting substrate (methanol) to obtain the desired X_c in the experimental design. Based on the mass balance, we solved this problem through the estimation of S_c with the growth model (Eq. 3) developed previously [9]. Within the ranges $0.1\ \mu_m \le D \le 0.9\ \mu_m$ and $150 \le X_c \le 450$, we found that the S_c calculated from Eq. 4 was much less than the S_{in} determined from the mass balance. Thus, S_c in Eqs. 5 and 12 can be approximated to zero, which simplifies the mass balance calculation, eliminating the need for a growth model.

The relationship between μ and v from Eq. 13 is about the same as that obtained from fed-batch fermentations of the same strain [8] and also quite similar to other *P. pastoris* strains [9–11]. Based on these results, we can express the relationship as $v = a\mu + m$, with $a = 0.73$–0.84 and $m = 0.003$–0.013. Taking average values for a and m, $Y_{X/S}$ can be estimated as $Y_{X/S\text{-AVG}} = \mu/(0.785\mu + 0.008)$, which takes into account the maintenance coefficient (m) and is closer to the actual $Y_{X/S}$ than that estimated by Eq. 7. The deviation of the actual X_c from the desired X_c can also be reduced using $Y_{X/S\text{-AVG}}$ for calculating the feed rates, F_m and F_b. For this study, the factors affecting the deviation of the actual D from the desired D are the inflow rates, agitation speed, aeration rate (gas hold-up) and foaming. The first two factors are easy to maintain constant, while latter two need closer monitoring and control. A proper amount of

antifoam is required in the feed solutions to inhibit foam production during the whole continuous phase.

Although the actual X_c and D obtained from the runs did not exactly match the design due to the reasons discussed above, the models of Q and ρ corresponding to X_c and D were successfully established (Eqs. 10, 11) through the ANOVA significance test of the regressions [7]. From the models, we found the optimal X_c (328.9 g l^{-1}) for the maximum Q was higher than the optimal X_c (287.7 g l^{-1}) for the maximum ρ, while the optimal D(0.0333 h^{-1}) for the maximum Q was about the same as the optimal D (0.0361 h^{-1}) for the maximum ρ. This indicates that, in a continuous fermentation, the optimal conditions for maximizing ρ may not necessarily be the optimal conditions for maximizing Q. We also noticed that the optimal μ ($=D$) for the maximum ρ obtained was different from that (0.025 h^{-1}) obtained from the fed-batch fermentations [8]. This is because the fed-batch phase, with a constant μ, is a quasi-steady state (cell density is not constant) and the optimal μ obtained corresponds to a range of cell densities (200–415 g l^{-1}). The optimal μ obtained from the chemostat corresponds to the optimal cell density (287.7 g l^{-1}). For methanol consumption capability, the result showed v was independent of cell density within the range 200–415 g l^{-1}. This discovery supports that a constant μ can be realized in a fed-batch phase by simply carrying out the feeding strategy that is based on the model $v = a\mu + m$, as observed previously [9–11]. The approach developed in this study is expected to be applicable to the optimization of continuous fermentations by other microorganisms.

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