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# Effects of Glycerol Concentration and pH on Growth of Recombinant *Pichia pastoris* Yeast†

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## ABSTRACT

Batch fermentations were used to study the effect of different glycerol concentrations and pH conditions on growth of recombinant *Pichia pastoris*. Two strains of *P. pastoris* were used: a wild-type in methanol utilization ($Mut^+$) and a mutant defective in methanol utilization ($Mut^-$). Under constant pH conditions of 5.0, glycerol concentrations up to 12% were efficiently utilized. Cell yield ($Y_{X/S}$) of about 0.8 and a final cell density of about 95 g/L (dry cell) were achieved. However, there were significant differences (probability [Pr] > F 0.0351) in specific growth rates between the initial glycerol concentrations of 2, 7, and 12%. When fermentations were conducted without pH control, growth continued until the pH had decreased to about 2.5. Growth stopped at pH 2.2 with uncontrolled pH, and residual glycerol concentrations were greater than 2%. As a result, $Y_{X/S}$ decreased to about 0.3. There were no differences between $Mut^+$ and $Mut^-$ strains during cell growth on glycerol.

**Index Entries:** *Pichia pastoris;* alcohol oxidase; $Mut^+$; $Mut^-$; specific growth rate.

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## INTRODUCTION

In recent years, methylotrophic yeast, *Pichia pastoris*, has proven to be an excellent host for expression of heterologous proteins *(1)*. This organism can grow to a cell density of (120 g/L dry wt) and has the potential for high-level expression and efficient secretion of recombinant proteins *(2–4)*. The promoter used to drive the expression system is from the methanol-induced alcohol oxidase gene, *AOX1*. The strength of this promoter is demonstrated by the observation that alcohol oxidase is undetectable in cells grown in glucose, glycerol, or ethanol, but comprises up to 30% of the total soluble protein in methanol-grown cells *(5)*.

For the production of recombinant proteins in fermentor cultures of *P. pastoris*, a two-stage scheme is usually employed *(6)*. In the first stage, a large cell mass is generated in a batch cultivation with glycerol as the carbon (C) source. On glycerol, growth is rapid, with a generation time of 3.0 h, and foreign gene expression is repressed. However, high nutrient concentration can sometimes inhibit cell growth *(7)*. In *Saccharomyces cerevisiae* production medium containing excess glucose results in repression of the oxidative pathway and formation of inhibitory concentrations of ethanol. This is known as the Crabtree effect *(8)*. A similar problem occurs in *Escherichia coli* cultivations with excess glucose caused by acetate production *(9)*. Many yeast species, under aerobic conditions, can utilize glycerol as a C source *(10)*. The glycerol catabolic pathway involves a passive diffusion of glycerol across the cell membrane, followed by phosphorylation by a glycerol kinase and oxidation by a mitochondrial glycerol phosphate-ubiquinone oxidoreductase *(11,12)*. Even though glycerol is not fermentable, the effect of excess glycerol on the growth and formation of metabolic by-products are not fully understood. This information is useful for improving growth conditions and therefore productivity.

In the second phase of recombinant *P. pastoris* fermentations, protein production is initiated by a continuous feed of methanol. Although the concentration of the recombinant protein increases with the cell density, the final protein yield is sometimes reduced because of proteolytic degradation *(13,14)*. One of the methods of minimizing proteolytic degradation is to allow the pH to drop from 5.0 in the growth phase to 3.0 in the induction phase *(15)*.

As part of a process development campaign, the authors were interested in optimizing the growth and protein production phases of recombinant *P. pastoris*. For this purpose, *P. pastoris* containing *AOX1-lacZ* gene was chosen as the model system. The first step was to understand the effects of glycerol concentration and changing pH on the growth of recombinant *P. pastoris*. This work describes the effect of different glycerol concentrations on the growth of recombinant $Mut^+$ and $Mut^-$ strains of *P. pastoris* in shake

flasks and 1-L batch fermentations. The effect of constant pH and decreasing pH on the growth of these two strains was also determined.

## MATERIALS AND METHODS

### Strains

*P. pastoris* strains PPF1(pSAOH5) and MC100-3(pSAOH5) were provided by J. Cregg, Oregon Graduate Institute of Science and Technology, Portland, OR. The $Mut^+$-PPF1(pSAOH5) is a wild-type strain with a single integrated copy of *AOX1-lacZ* containing fusion vector, pSAOH5. The $Mut^-$-MC100-3(pSAOH5) is isogenic to PPF1(pSAOH5), except for the defective *AOX1* and *AOX2* genes. Details of vector construction, strain transformation, and difference between $Mut^+$ and $Mut^-$ strains are described elsewhere *(16–18)*.

### Media

For fermentation studies in shake flasks and 1-L fermentors, basal salts medium (BSM), containing 33% 10X basal salts (42 mL/L 85% phosphoric acid, 1.8 g/L $CaSO_4 \cdot 2H_2O$, 28.6 g/L $K_2SO_4$, 23.4 g/L $MgSO_4 \cdot 7H_2O$ and 6.5 g/L KOH), was used. The fermentors were inoculated with 100 mL of cultures grown overnight. In this study, two basal media, a BSM and a yeast nitrogen base (YNB; 13.4 g/L without amino acids in 0.1 *M* phosphate buffer, pH 6.0) *(6)* were used for growing the inoculum. After sterilization a $YTM_4$ trace salt solution (5.0 mL/L $H_2SO_4$, 65 g/L $FeSO_4 \cdot 7H_2O$, 6.0 g/L $CuSO_4 \cdot 5H_2O$, 20.0 g/L $ZnSO_4 \cdot 7H_2O$, 3.0 g/L $MnSO_4 \cdot H_2O$, and 0.1 g/L biotin), at a concentration of 5 mL/L of medium, was added to the shake flasks and the fermentors.

### Cell Concentration

The cell concentrations were followed by measuring the optical densities (OD) with a spectrophotometer (DU-70, Beckman, Fullerton, CA) at 600 nm. Dry wt of the cell suspensions were determined by centrifugation of 1 mL cell broth in a preweighed 1.5-mL Eppendorf centrifuge tube, followed by drying to constant weight at 80°C in a oven. The correlation between dry wt cell concentration and $OD_{600nm}$ was found as dry wt cell (g/L) = $-0.21 + 0.42 * OD_{600nm}$.

### Analytical Techniques

Glycerol concentrations were measured by HPLC using an ICE-ASI, Dionex column, Sunnyvale, CA and Dionex Pulsed Amperometric Detector (Platinum electrode, E1, 0.20[60]; E2, 1.25[60]; E3, −0.1[240]). The solvent

was 100 m*M* perchloric acid at a flow rate of 1 mL/min. Ethanol concentrations were determined with a Hewlett Packard 5880A GC chromatograph using a column packed with chromosorb W (AW) (80/100; 10% carbowax 20M-TPA + 0.1% $H_3PO_4$ (Supelco, Bellefonte, PA) and a flame ionization detector.

## Specific Growth Rate

Specific growth rate ($\mu$) was determined from the slope of ln (OD) vs time. The measured specific growth rates (MS) were analyzed by ANOVA for any significant differences using SAS (SAS Institute, NC).

## Shake Flask Studies

Shake-flask experiments were conducted in baffled 500-mL capacity shake-flasks. Three glycerol levels (2, 7, and 12%) and two strains ($Mut^+$ and $Mut^-$) were used in a $2 \times 3$ factorial treatment arrangement. Duplicate flasks containing 200 mL BSM were randomly assigned one of the six. The media in the flasks were adjusted to pH 5.0 with a 30% ammonium hydroxide solution, and autoclaved. After sterilization, 1 mL $YTM_4$ trace salts solution was added, and each flask was inoculated with 20 mL overnight-grown culture and incubated at 30°C and 200 rpm in a rotary shaker-incubator (NBS Series 25D, New Brunswick Scientific, Edison, NJ). Samples were taken every 6 h, and assayed for cell density and pH. At the end of the fermentation, the glycerol concentration in each flask was determined by HPLC.

## Batch Fermentations

The batch fermentation experiments were designed by using a randomized complete-block design (RCBD) procedure. Two strains ($Mut^+$ and $Mut^-$), two pH levels (controlled at 5.0 and uncontrolled), and three initial glycerol concentrations (2, 7, and 12%) were used in a $2^2 \times 3$ factorial treatment arrangement. Each fermentation was randomly assigned to a treatment combination. Fermentations were carried out using a Bioflo 3000 (New Brunswick Scientific) 2-L vessel with 1-L working volume. The dissolved oxygen concentration in the fermentor was maintained at 40% of air saturation by automatic adjustment of the agitation rate through dissolved oxygen control. For all fermentations, the initial pHs of the autoclaved media were adjusted to 5.0 with 30% $NH_4OH$ solution. The $NH_4OH$ also served as the nitrogen source in the medium. For experiments with constant pH, the pH during fermentation was maintained at 5.0 using 5 *N* NaOH solution, because further addition of $NH_4OH$ may have improved the growth conditions of the media relative to the media used in the uncontrolled pH fermentations. Foaming was controlled by using 5%

(v/v) Struktol J673 antifoam (Kabo Chemical, Jackson Hole, WY). Two different types of media, BSM and YNB, one for each replication, were used to grow the inoculum. The inoculum medium type was used as the blocking factor in the experimental design. It was assumed that there was no interaction between the medium (blocking variable) used in the inoculum and any of the treatment factors used in these experiments. After inoculation of the fermentors, growth was monitored by measuring the OD at 600 nm at regular time intervals. The fermentations were stopped when there was no further increase in cell density. At the end of the fermentations, the glycerol concentrations in the fermentors were determined by HPLC.

## RESULTS AND DISCUSSION

### Shake-Flask Experiments

Preliminary experiments were conducted in shake flasks to determine the effect of initial glycerol concentration on the growth of recombinant *P. pastoris*. Strains $Mut^+$ and $Mut^-$ were grown at glycerol concentrations of 2, 7, and 12% in BSM. In all the experiments, the cultures reached maximum cell density (approx 9.0 g/L dry cell wt) after 100–120 h. The specific growth rates, final cell densities, pH, and cell yields ($Y_{X/S}$) for the $Mut^+$ and the $Mut^-$ strains are given in Table 1. The rapid decrease in pH from an initial value of 5.0 to a final pH of about 3.0 showed that the buffering capacity of BSM was limited. Lower-than-expected final cell mass was probably caused by the decreasing pH and/or poor agitation and oxygen solubility. The final $Y_{X/S}$ (g dry cell mass/g glycerol consumed) achieved for different glycerol concentrations were similar for the $Mut^+$ and the $Mut^-$. The $Y_{X/S}$ was around 0.6 for fermentations with 2% as starting glycerol concentration; it was about 0.2 for higher glycerol concentrations, indicating that the C source was more efficiently utilized for cell generation at lower glycerol concentration.

Based on ANOVA, there was no significant difference ($Pr > F$ 0.966) between the growths of the $Mut^+$ and the $Mut^-$ strains. The average biomass and pH profiles observed for the two strains ($Mut^+$ and $Mut^-$) are shown in Fig. 1. However, there was a significant difference among the specific growth rates in shake flasks with 2, 7, and 12% initial glycerol concentration ($Pr > F$ 0.009). Whether this observed difference is enhanced by the limiting dissolved oxygen found in shake-flask conditions is unclear.

### Batch Fermentations

The problems of agitation and dissolved oxygen limitation in the shake-flask experiments were addressed by conducting the fermentations

Table 1
Final pH, Yield, and Specific Growth Rate in Shake-Flask Fermentations

| Strain | Mut$^+$ | | | Mut$^-$ | | |
|---|---|---|---|---|---|---|
| Initial glycerol (%) | 2 | 7 | 12 | 2 | 7 | 12 |
| Final pH | 3.03 | 3.09 | 3.16 | 3.06 | 3.08 | 3.17 |
| Final dry cell mass (g/L) | 9.27 | 8.92 | 7.50 | 8.34 | 8.85 | 7.13 |
| Final glycerol (%) | 0.5 | 2.3 | 8.85 | 0.6 | 3.0 | 8.8 |
| $Y_{x/s}$ | 0.618 | 0.190 | 0.238 | 0.596 | 0.221 | 0.223 |
| Specific growth rate/h | 0.26 | 0.22 | 0.15 | 0.20 | 0.29 | 0.15 |

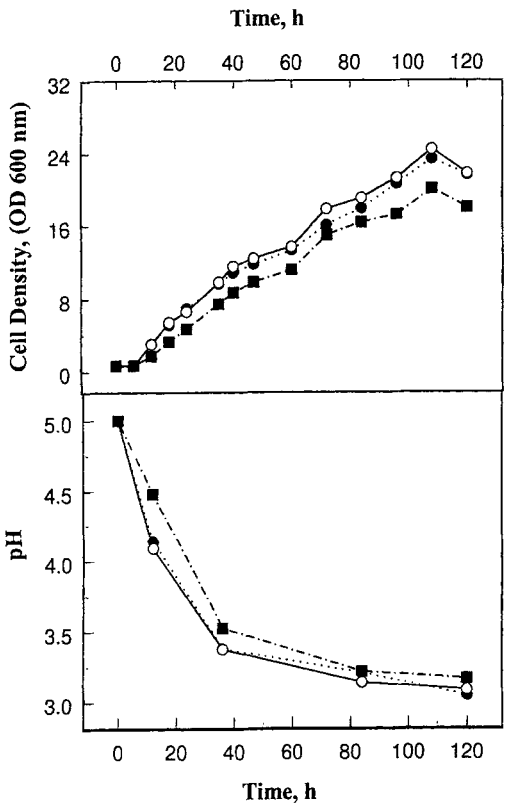


Fig. 1. Average cell densities and pHs for Mut$^+$ and Mut$^-$ on 2% (●), 7% (○), and 12% (■) glycerol concentrations in shake-flask fermentations.

in 1-L Bioflo fermentors equipped with pH, agitation, and dissolved-oxygen controls. In these fermentations, the dissolved oxygen was maintained above 40% saturation.

In a typical fermentation with recombinant *P. pastoris,* the cells are grown at a pH of 5.0 but are permitted to fall to 3.0 during the induction

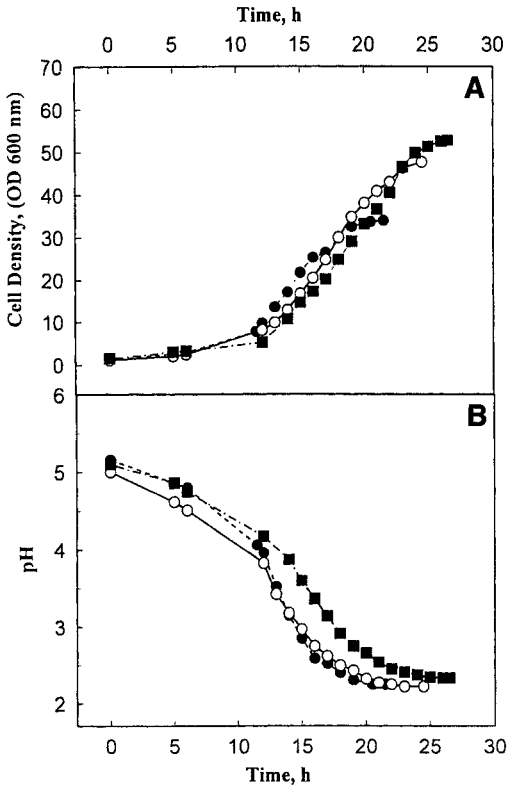


Fig. 2. Effect of glycerol concentrations, 2% (●), 7% (○), and 12% (■) on cell density **(A)** and pH **(B)** for $Mut^+$ in uncontrolled-pH fermentations.

of recombinant protein *(15)*. The low pH during induction helps to limit bacterial contamination and to protect the protein of interest from possible proteolytic degradation *(13,14)*. In this fermentation study, pH was included as a variable. To study the effect of decreasing pH on growth, fermentations were conducted without pH control.

## Uncontrolled pH Conditions

When batch fermentations were conducted without pH control, the pH decreased rapidly from its initial value of 5.0. Growth continued until the final pH was near 2.2. This is illustrated in Fig. 2 for the $Mut^+$ strain and in Fig. 3 for the $Mut^-$ strain. The final pH, cell density, residual glycerol, and $Y_{X/S}$ appear in Table 2. As was observed in shake-flask experiments (Table 1), the $Y_{X/S}$ from the fermentations decreased with increasing glycerol concentration. There may be two reasons for inefficient use of C for cell production. One could be that yeasts are known to accumulate storage carbohydrates like glycogen and trehalose during the onset of stationary phase *(19–21)*. The other possibility may be the energy spent to

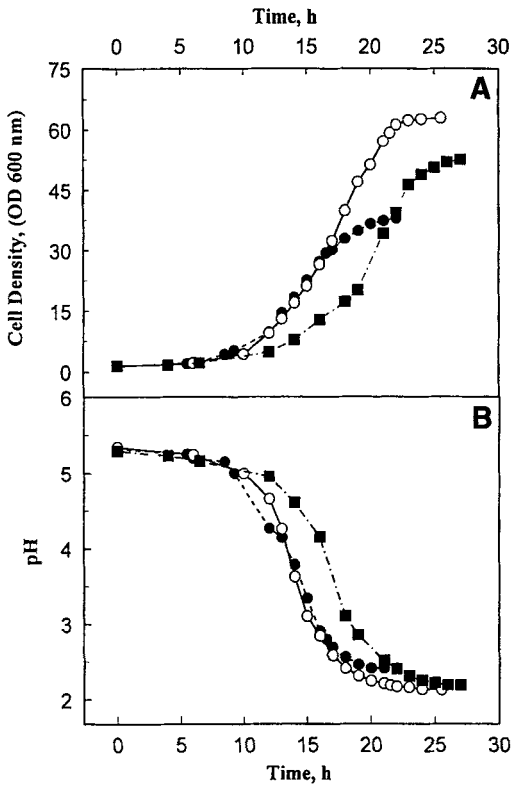


Fig. 3. Effect of glycerol concentrations, 2% (●), 7% (○), and 12% (■) on cell density **(A)** and pH **(B)** for Mut⁻ in uncontrolled-pH fermentations.

Table 2
Final pH and Yield for Uncontrolled and Constant pH Fermentations

| Strain | Mut⁺ | | | Mut⁻ | | |
|---|---|---|---|---|---|---|
| Initial glycerol (%) | 2 | 7 | 12 | 2 | 7 | 12 |
| Uncontrolled pH | | | | | | |
| Final pH | 2.25 | 2.22 | 2.33 | 2.4 | 2.13 | 2.19 |
| Final dry cell mass (g/L) | 13.99 | 19.70 | 21.84 | 15.68 | 26.12 | 21.75 |
| Final glycerol (%) | 0 | 2.18 | 5.09 | 0 | 1.62 | 4.89 |
| $Y_{x/s}$ | 0.700 | 0.409 | 0.316 | 0.784 | 0.486 | 0.306 |
| Constant pH | | | | | | |
| Final dry cell mass (g/L) | 16.36 | 51.42 | 96.71 | 17.25 | 53.89 | 92.96 |
| $Y_{x/s}$ | 0.818 | 0.735 | 0.806 | 0.863 | 0.770 | 0.775 |

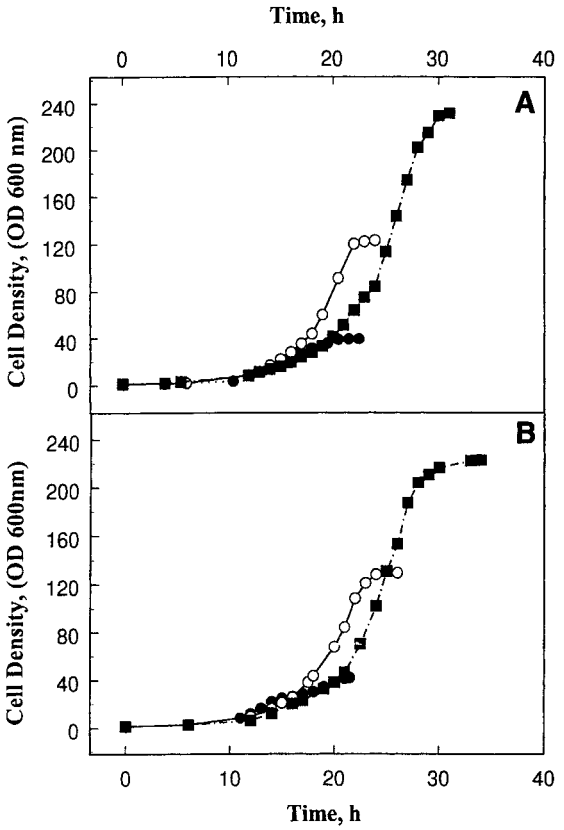


Fig. 4. Effect of glycerol concentrations, 2% (●), 7% (○), and 12% (■) on cell densities of $Mut^+$ **(A)** and $Mut^-$ **(B)** in constant-pH fermentations.

overcome the large difference between intracellular pH (around 6.0) and external pH of the medium *(22,23)*.

## Controlled pH

To determine if a constant pH would optimize C utilization for biomass at different substrate concentrations, the authors repeated fermentations at constant pH of 5.0 by adding 5 *N* NaOH solution. Growth curves for these constant pH fermentations are shown in Fig. 4A and 4B for $Mut^+$ and $Mut^-$ strains, respectively, and the results are summarized in Table 2.

In these batch fermentations, the initial glycerol concentration in the fermentor effected the growth rate of *P. pastoris* significantly (Pr > F 0.0003). Average specific growth rates for $Mut^+$ and $Mut^-$ strains, at 2% glycerol, was 0.24/h; at 7% glycerol it was 0.229/h; and at 12% glycerol, it was estimated to be about 0.191/h. The authors noticed that total consumption and efficient utilization ($Y_{X/S}$ = 0.80) of glycerol, even at a high level of 12%. The authors also observed a significant difference (Pr > F

Table 3
Ethanol Production (g/L) at Different Initial Glycerol Concentrations and pH Conditions in 1-L Fermentations

| Glycerol concentration (%) | 2 | 7 | 12 |
|---|---|---|---|
| Constant pH (5.0) | 0.0308 | 0.5320 | 2.389 |
| Uncontrolled pH | ND[a] | 0.0306 | 0.258 |

[a] ND, not detected.

0.003) between growth under uncontrolled pH ($\mu$ = 0.207/h) and that at a constant pH of 5.0 ($\mu$ = 0.234/h). However, there was no significant difference (Pr > F 0.1095) between the $Mut^+$ and the $Mut^-$ strains on glycerol. Thus, it is clear from these experiments that high cell densities of up to 100 g/L (dry cell wt) can be achieved with glycerol as a C source. It is also evident, under the conditions tested, that growth stopped only after complete utilization of available glycerol.

### Ethanol Production

Ethanol is the most commonly produced byproduct in yeast fermentation. In *S. cerevisiae* cultivations, residual ethanol in the medium can reduce and sometimes even inhibit the growth. In recombinant *P. pastoris* fermentations, a low external ethanol concentration is also paramount, because of its strong repressing effect on the *AOX1*-promoter. Ethanol is also responsible for posttranslational effects like inactivation of enzyme alcohol oxidase and breakdown of the peroxisomal matrix in *P. pastoris (24)*. The authors analyzed the culture broth from uncontrolled and constant pH fermentations for ethanol production, and the results are given in Table 3. It is interesting to note that the final ethanol concentration for uncontrolled pH fermentations was much lower than those for constant pH 5.0 fermentations. This may be because the pH optima for ethanol production in yeast is about 6.0 *(25)*.

## CONCLUSIONS

The methylotrophic yeast *P. pastoris* has proven to be a good host for production of foreign proteins of commercial interest. The fermentation scheme involves a batch-growth phase on glycerol and a fed-batch on methanol for protein production. Based on findings from this study, it is clear that the final cell density obtained was a function of the glycerol concentration. Under constant pH conditions, even 12% glycerol is fully utilized. However, the specific growth rate varied with the initial glycerol

concentration. When pH was not controlled, the pH dropped to a final value of 2.2, and glycerol in excess of 2% remained unutilized with a decreasing yield coefficient ($Y_{X/S}$). It was clear that growth of the two strains $Mut^+$ and $Mut^-$ on glycerol was similar. High initial glycerol and a pH controlled at 5.0 resulted in accumulation of about 2 g/L ethanol.

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