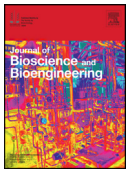

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Improvement of recombinant endoglucanase produced in *Pichia pastoris* KM71 through the use of synthetic medium for inoculum and pH control of proteolysis

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The long lag time in basal salts medium (BSM) and an occurrence of proteolysis are major problems for recombinant protein production in *Pichia pastoris* KM71. In this study, optimal conditions were explored for fed-batch cultivation of recombinant fungal endoglucanase in *P. pastoris* KM71. It was found that lag and process times were much reduced when the synthetic FM22 medium was used for the inoculum compared with enriched buffered glycerol complex (BMGY) medium. The highest endoglucanase activity was obtained at 30°C which was more than 10 fold higher than that produced from shake flask. At 30°C, the specific endoglucanase activity was dependent on culture pH and a higher specific activity was observed at pH 5.0 than at pH 6.0. The higher activity was likely due to lower rate of proteolysis, since a truncated protein species was apparent at pH 6.0, but not pH 5.0. Thus, production of endoglucanase at 30°C and pH 5.0 is the optimal condition suitable for economical production in large scale. The combination of using synthetic FM22 medium for inoculum and proteolysis control by growth at lower pH could be applied for production of other recombinant proteins in *P. pastoris*.

[**Key words:** Inoculum development; Lag time; *Pichia pastoris*; Endoglucanase; Proteolysis]

Pichia pastoris is a widely utilized yeast species for the production of recombinant proteins in research and industry. Heterologous protein can be produced in large-scale from recombinant *P. pastoris* more cost-effectively in fermenters using cheap synthetic medium compared to traditional shake-flask cultivation using enriched complex medium (1). Moreover, cultivation parameters that affect protein expression levels such as temperature, pH, agitation, and aeration can be controlled more easily in fermenters (2,3).

Recombinant protein production in *P. pastoris* typically exploits the inducible alcohol oxidase 1 (*AOX1*) expression system in the fed-batch cultivation, which is separated into two major phases. These are growth phase using glycerol as a carbon source to promote cell growth and induction or production phase using methanol as a substrate to induce expression of the target genes (3–5). During the lag phase of microbial growth in batch culture, the cells adapt to their new environment and thus no net growth or heterologous protein production occurs (6). Hence, for efficient protein production, the fermenter culture must pass through the lag phase rapidly, and the time taken for this step is known as the lag time. The lag time is related to the size of inoculum and medium composition for inoculum and fermenter cultivation. The inoculum size is typically 5–10% (v/v) and the medium used for inoculum cultivation should be the same or similar in composition to the fermenter cultivation medium to minimize the cell adaptation period (7).

Generally, to produce recombinant proteins in *P. pastoris*, the inoculum is cultivated in enriched medium such as yeast extract–peptone–dextrose (YPD) and/or buffered glycerol complex (BMGY) before it is transferred to the basal salts medium (BSM) which is a synthetic defined medium commonly used for high cell density cultivation in fermenter (Invitrogen Corp.). In our laboratory, *P. pastoris* KM71 has been exploited to produce several recombinant thermostable enzymes. The *P. pastoris* KM71 strain has methanol utilization slow (Mut^S) phenotype and is advantageous during cultivation. During production of recombinant proteins (e.g., endoglucanase, exoglucanase, β-glucosidase) in *P. pastoris* KM71, however, we encountered a major problem of a long lag time when the inoculum cultivated in enriched medium was transferred to BSM medium. The long lag time is likely due to the requirement for cell adaptation to different media. Inoculum cultivation in the BSM medium may help solve this problem, but it is not practical since the pH of medium in shake flask has to be adjusted from 2.0 to 5.0 by addition of ammonia solution after autoclaving (Invitrogen Corp.). One possible solution to this problem is to use the synthetic FM22 medium, which is another medium commonly used in the fermenter. A major advantage for this medium is that it does not require pH adjustment after sterilization and its composition is similar to BSM medium (8,9).

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Another significant problem for high cell density cultivation of some proteins is proteolysis due to secretion of proteases from *P. pastoris* host and death cell lysis. Several strategies have been applied to reduce the degree of proteolysis. These include the control of cultivation conditions and culture medium composition, the use of protease-deficient strains, and modification of the protein sequence to remove target sites of native proteases (10). Among these strategies, controlling cultivation conditions such as changing pH and temperature is a simple and cost effective method to limit the protease activity (11). Furthermore, the decrease of temperature has been shown to improve cell viability and reduce the release of proteases from death cell (11,12).

In our laboratory, one of the thermostable enzymes produced in *P. pastoris* KM71 was endoglucanase from fungal *Syncephalastrum racemosum* BCC18080, which has been shown to be one of potent enzymes used for biomass conversion to monosaccharides for organic acid and alcohol production (13–15). However, when this enzyme was produced from *P. pastoris* KM71 using shake flask cultivation in BMGY and BMMY medium at pH 6.0, it was expressed suboptimally since substantial level of truncated product (30 kDa) was observed in addition to full-length protein (50 kDa). This strongly suggested the presence of active proteases in the culture medium (15). Thus, methods that reduce proteolysis during cultivation will be beneficial.

In this study, the combination of inoculum development and proteolysis control was investigated for improving the production of recombinant endoglucanase in *P. pastoris* KM71. It was shown that the use of synthetic FM22 medium for inoculum cultivation was highly advantageous for batch fermentation as it gave a shorter lag time and a higher specific growth rate compared to that using enriched BMGY medium. Moreover, temperature and pH for cultivation were found to have significant effects on proteolysis of endoglucanase.

MATERIALS AND METHODS

Strain *P. pastoris* KM71 expressing extracellular endoglucanase was constructed previously and deposited in the BIOTEC Culture Collection under number BMGC129 (15). This strain is Mut^S and histidine auxotroph (His^-) and was used throughout this study. It was maintained on YPD (yeast extract 10 g/L, peptone 20 g/L, and dextrose 20 g/L) agar containing 100 μg/mL zeocin at 4°C. Expression of endoglucanase gene was controlled under the methanol inducible *AOX1* promoter.

Inoculum preparation using enriched medium The primary inoculum was prepared by inoculating a colony of recombinant *P. pastoris* KM71 into a 125 mL shake flask containing 20 mL YPD broth. The culture was incubated at 30°C with 250 rpm shaking for 24 h.

The secondary inoculum was prepared by transferring the diluted 20 mL of the primary inoculum into a 500 mL shake flask containing 80 mL BMGY medium (yeast extract 10 g/L, peptone 20 g/L, and glycerol 20 g/L dissolved in 100 mM potassium phosphate buffer, pH 6.0). The culture with an initial cell density of approximately 0.5 g/L was incubated under the same conditions as the primary inoculum for 52 h.

Inoculum preparation using synthetic medium The primary inoculum was prepared by inoculating a colony of recombinant *P. pastoris* KM71 into a 125 mL shake flask containing 20 mL of synthetic FM22 medium (8,9) with slight modification. The synthetic FM22 medium in 1 L contained KH_2PO_4 42.9 g, $CaSO_4$ 0.93 g, K_2SO_4 14.3 g, $MgSO_4 \cdot 7H_2O$ 11.7 g, $(NH_4)_2SO_4$ 5.0 g, glycerol 20.0 g, histidine 2.0 g and PTM1 4.35 mL/L. The pH was adjusted to 5.5 with 1.0 M KOH. The PTM1 trace salts in 1 L contained $CuSO_4 \cdot 5H_2O$ 6.0 g, KI 0.08 g, $MnSO_4 \cdot H_2O$ 3.0 g, $Na_2MoO_4 \cdot 2H_2O$ 0.2 g, H_3BO_3 0.02 g, $ZnCl_2$ 20.0 g, $FeCl_3$ 13.7 g, $CoCl_2 \cdot 6H_2O$ 0.9 g, H_2SO_4 5.0 mL, and biotin 0.2 g. The culture was incubated at 30°C with 250 rpm shaking for 48 h. The secondary inoculum was prepared by transferring the diluted 20 mL of primary inoculum into a 500 mL shake flask containing 80 mL FM22 medium supplemented with 2.0 g/L histidine. The culture with an initial cell density of approximately 0.5 g/L was incubated under the same conditions as the primary inoculum for 54 h.

Fed-batch cultivation In this study, high-cell density fed-batch cultivation of the recombinant *P. pastoris* KM71 was carried out in a 5 L twin fermenter (Biostat B plus twin, Sartorius Stedim Biotech, Germany) using 2 L of BSM medium (85% H_3PO_4 26.7 mL/L, $CaSO_4$ 0.93 g/L, K_2SO_4 18.2 g/L, $MgSO_4 \cdot 7H_2O$ 14.9 g/L, KOH 4.13 g/L, glycerol 40.0 g/L) with 4.35 mL/L PTM1 trace salts and 2.0 g/L histidine. The fed-batch cultivation was divided into four stages: (i) glycerol batch stage, (ii) glycerol fed-batch stage, (iii) methanol induction stage, and (iv) production stage as described previously (16). In the glycerol batch stage, the inoculum in the deceleration phase (100 mL) was transferred into a fermenter. The fermentation condition was controlled at 1vvm aeration, 1000 rpm agitation, 30°C, pH 5.0. NH_4OH (25% v/v) was used as the nitrogen source and pH control reagent. The batch culture was grown until the glycerol was exhausted, which was indicated by a sharp increase in the dissolved oxygen tension (DOT). The final cell concentration at the end of glycerol batch stage was about 20 g/L. For the glycerol fed-batch stage, the glycerol feed medium (GF: glycerol 500 g/L with 12 mL/L PTM1) was added to the fermenter with an exponential feed rate of $\mu_{set} = 0.18\ h^{-1}$ according to Eq. 1:

$$F(t) = \frac{\mu_{set} V_0 X_0}{S_0 Y_{\frac{X}{S}}} \exp^{\mu_{set} t} \quad (1)$$

where μ_{set} is the desired specific growth rate, V_0 is initial volume, X_0 is initial cell concentration, S_0 is concentration of substrate in feed medium, and $Y_{x/s}$ is biomass yield from substrate. When the cell density reached about 40 g/L after 3.5 h of GF feed, the process was switched to the methanol induction stage. The methanol feed medium (MF: 12 mL/L PTM1 in methanol) was fed intermittently (at 0, 1.5, 2.5, and 3 h) to obtain a 1 g/L final concentration of methanol. Then, the MF medium was fed continuously starting at a rate of about 1.30 g/h and gradually increasing to 2.80 g/h after 24 h in the production stage. Samples were taken and analyzed for cell growth, total protein concentration, and endoglucanase activity.

Analysis of inoculum from enriched and synthetic media The quality of inoculum cultivated in enriched BMGY medium or synthetic FM22 medium was investigated by monitoring the growth profile during the glycerol batch stage of the fed-batch cultivation. The culture broth was collected every 4–6 h and dry cell weight (DCW) was analyzed. The data of DCW were used to estimate lag time and specific growth rate (μ).

Optimization of cultivation conditions for mitigation of proteolysis The cultivation conditions in the first three stages were controlled at 30°C, pH 5.0, 1 vvm aeration, and 1000 rpm agitation. Four different conditions of production stage were tested for mitigation of proteolysis. These are (i) 25°C, pH 5.0; (ii) 25°C, pH 6.0; (iii) 30°C, pH 5.0; and (iv) 30°C, pH 6.0. Samples were collected at an appropriate time and DCW, total protein concentration, endoglucanase activity, and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were analyzed.

Cell concentration determination Cell concentration was determined by measuring the optical density (OD) using a spectrophotometer (UV-1201V Spectrophotometer, Shimadzu, Japan) at 600 nm. The DCW was determined by centrifugation (2500 ×*g* for 10 min) of 5 mL culture broth and cells were washed with 1% phosphoric acid to remove precipitated salts followed by distilled water. Cells were dried at 105°C for 24 h and DCW was calculated.

Endoglucanase activity, total protein concentration, SDS-PAGE, and non-denaturing-PAGE The endoglucanase activity was assayed by measuring the reducing sugars liberated as D-glucose equivalents using carboxymethyl cellulose (CMC) as substrate. The amount of reducing sugar released was measured using the dinitrosalicylic acid (DNS) assay (17). One unit of enzyme is defined as amount of enzyme producing 1 μmol of reducing sugar in 1 min at 50°C, pH 5.5. The total protein concentration was determined according to Bradford method (18) using bovine serum albumin as a standard protein. SDS-PAGE was performed with 15% polyacrylamide gel and loaded with denaturing sample. For non-denaturing-PAGE, 10% polyacrylamide gel was applied without sample denaturation. The gel was then stained with Coomassie Brilliant Blue R-250 (19). Recombinant protein was deglycosylated using PNGaseF according to the manufacturer's instructions (New England Biolabs).

Zymogram analysis Zymogram analysis was performed according to Karnchanatat et al. (20). The non-denaturing-PAGE after electrophoresis was transferred to substrate solution (1% CMC in 50 mM sodium acetate buffer pH 5.5) and incubated at 50°C for 1 h. The gel was then stained in 0.1% (w/v) Congo-red solution for 10 min and then destained with 2 M NaCl. The endoglucanase activity was observed as a clear zone against red background.

RESULTS

Inoculum development The inoculum preparation for the recombinant *P. pastoris* KM71 in this work was divided into two steps, the primary and secondary inocula as described in Materials and methods. The growth of primary inoculum cultivated in enriched YPD medium and synthetic FM22 medium was slow but continuously increased and reached the deceleration phase at 24 h and 48 h, respectively (data not shown). In order to increase the inoculum size, secondary inoculum was further performed. For the secondary inoculum cultivated in enriched BMGY medium, the culture reached the deceleration phase at 24 h with