High-level Production of Bovine Enterokinase Light Chain Using Fed-batches by Recombinant *Pichia pastoris*


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A recombinant *Pichia pastoris* containing enterokinase light chain gene from bovine was cultured in flasks firstly at different pH, methanol addition, and cell mass concentration for enterokinase production. Activity of enterokinase increased as cell mass concentration increased at more than $\varphi = 6\%$ methanol added. No significant change of activity of enterokinase was observed when methanol added was less than $\varphi = 4\%$. No activity of enterokinase lost after 120 h conservation at different pH. Secondly, high-level enterokinase production was achieved in 3.7 L bioreactor at pH 4.0, $\varphi = 0.6\%$ methanol, $p = 52.5$ kPa. Activity of enterokinase was not cell mass concentration dependent at $\varphi = 0.6\%$ methanol in bioreactor. Yield of enterokinase was 479.99 mg L$^{-1}$ after 97.5 h induction. Maintenance coefficient and methanol consumed were calculated to analyse the enterokinase production.

**Key words:** High cell mass concentration fermentation, *Pichia pastoris*, enterokinase, amount of methanol consumption

**Introduction**

Enterokinase (EK, EC 3.4.21.9), discovered in 1984, was a protease of the intestinal brush border that specifically cleaved the acidic hexapeptide (Val-(Asp)$_4$-Lys) from trypsinogen to yield active trypsin. This cleavage initiated a cascade of proteolytic reactions leading to the activation of many pancreaticzymogens. Enterokinase had been found from porcine, bovine, human, and ostrich intestine. Enterokinase was reported as a disulfide-linked heterodimer with a heavy chain of 82–140 kDa and a light chain of 35–63 kDa. The heavy chain was postulated to mediate association with the intestinal brush border membrane, the light chain contained catalytic center with high specificity and tolerance to wide range of cleaves condition. The broad application of enterokinase to cleave hexapeptide regardless of amino acid sequence of down-stream is known worldwide. Many heterologous hosts were used including *E. coli*, filamentous fungus, *Pichia pastoris*, and COS cells to produce enterokinase. However, much work focused on high expression of enterokinase at molecules manipulation and recombinant host cultivation in flasks. Therefore, yield of enterokinase was low and price of enterokinase was high although enterokinase was used widely. In our study, high-level production of bovine enterokinase light chain (EK$_L$) was achieved in recombinant *Pichia pastoris* by fed-batches.

**Materials and methods**

**Strain**

*Pichia pastoris* GS115-PAO815-EKL

**Medium**

YPD (L): yeast extract 10 g, peptone 20 g, glucose 20 g, agar 15 g (if necessary).

BMGY (L): yeast extract 10 g, peptone 20 g, potassium phosphate buffer 0.1 mol, pH 7.0, YNB 13.4 g, biotin 4 · 10$^{-4}$ g, glycerol 20 g.

Basal salts medium (BSM) (L): $H_3PO_4$ 26.7 mL, CaSO$_4$ 0.93 g, K$_2$SO$_4$ 18.2 g, MgSO$_4$ · 7H$_2$O 14.9 g, KOH 4.13 g, glycerol 40 g, PTM 4.35 mL, pH was adjusted by $\varphi = 28–30\%$NH$_4$OH to 5.0.

PTM1 (L): CuSO$_4$ · 5H$_2$O 6.0 g, KI 0.08 g, MnSO$_4$ · H$_2$O 3.0 g, Na$_2$MoO$_4$ · 2H$_2$O 0.2 g, H$_3$BO$_3$ 0.02 g, ZnSO$_4$ · 7H$_2$O 20 g, FeSO$_4$ · 7H$_2$O 65.0 g, CoCl$_2$ · 6H$_2$O 0.5 g, biotin 0.2 g, H$_2$SO$_4$ 5.0 mL.

**Flask cultivation**

Cells were transferred from YPD agar plates into the 250 mL flasks, which contained 25 mL BMGY and grew at 30 °C 250 rpm for 18–20 h. Cell was used as inoculum for flask experiments.
Flask experiments were performed in the 250 mL flask containing 25 mL BMGY medium at 30 °C, 250 rpm with ϕ = 4 % YPD inoculums. A ϕ = 2 % methanol was added into BMGY after 24 h cultivation to induce EKL every 24 h.

Bioreactor cultivation

A V = 3.7 L bioreactor (KLF2000 3.7 L, Bioengineering AG, Switzerland) equipped with a pH electrode, a dissolved oxygen electrode and two peristaltic pumps were used to scale up. Each fed-batch began with ϕ = 4 % inoculums in 2.0 L BSM at 30 °C, 500 rpm, 100 L h⁻¹ air. The pH value was adjusted by ammonium hydroxide and maintained automatically through fed-batches. The oxygen was supplied by air flow (Q = 100–300 L h⁻¹) and agitation (500 to 1000 rpm). After depletion of the γ = 40 g L⁻¹ glycerol, ϕ = 50 % glycerol containing 12 mL L⁻¹ PTM1 was fed for cell growth. In the second phase, methanol feed rate was 1.0 mL L⁻¹ h⁻¹ after 0.5–2 h of glycerol depleted. The methanol feed rate was increased by 1.0 mL L⁻¹ h⁻¹ until settled methanol volume fraction was reached. Antifoam was injected manually throughout the fermentation to avoid excess foam.

Analytical procedures

Biomass

Cells were harvested by centrifugation at 6000 rpm for 3 min, and then washed twice with deionized water, and dried to a constant mass at 90 °C. The cell optical density was positively related to the dry cell mass (DCM) with an experimentally determined calibration curve. The cell optical density of the sample (properly diluted) was monitored by a spectrophotometer (U-1100, Hitachi Ltd., Tokyo, Japan) at λ = 600 nm.

Methanol fraction

Cell supernatant liquid was mixed with isopyknic mixture of 1-butanol and 1-propanol (Ψ = 4:1) to extract methanol. Sodium sulfite was used to pull out water from the mixture. Methanol fraction was determined by gas chromatography (GC 6890N, Agilent, USA) with a DB-WAX column. Temperatures of injection and detector were θ = 200, and 250 °C, respectively. Column temperature profile was 100 °C for 1 min, and increased at a rate of 20 °C min⁻¹ until the temperature reached 200 °C.

Yield and activity of EKL

In flask experiments, activity of EKL was determined by percentage of cleaving a fusing protein (Trx-PTH) using supernatant after centrifugation. Reaction system (40 μL) was following: γ = 40 g L⁻¹ Trx-PTH, pH 8.0, c = 50 mmol L⁻¹ Tris-Cl, 1 mmol L⁻¹ CaCl₂, 1 μL supertant. SDS-PAGE was done according to protein protocol after cleaving reaction, which was preformed at 37 °C for 20 min. The protein brands were analyzed by Smartview (FR200A Shanghai Furi Science & Technology Co. Ltd., Shanghai). The activity of EKL was quantified according to the method of Invitrogen Ltd (CA, USA). In bioreactor experiments, yield of EKL was determined by SDS-PAGE after centrifugation of broth. The protein brand was analyzed as stated previously. EKL activity was also determined by using the fluorogenic enterokinase substrate Gly-Asp-Asp-Asp-Asp-Lys-naphthylamide.

Maintenance coefficient analysis

Maintenance coefficient was analyzed according to the mass balance. As EKL was secreted from the cell and yield of EKL was too low compared with biomass, mass balance equation can be described as eq. (1). Specific methanol consumption rate can be depicted as eq. (2). Eq. (3) can be expressed by combining eq. (1) with eq. (2). YXS and “μ” were calculated from data of bioprocess. “km” were obtained by curve fitting.

\[
\frac{ds}{dt} = \frac{\mu Y_X}{Y_G} + k_m Y_X
\] (1)

\[
q_s = \frac{1}{\gamma_X} \frac{ds}{dt} = \frac{\mu Y_X}{Y_{X/S}}
\] (2)

\[
1 = \frac{1}{Y_{X/S}} = \frac{1}{\mu G} + k_m
\] (3)

Results and discussion

Effect of air pressure on yield of EKL in bioreactor

Known from Henry formula, oxygen solution was improved when air pressure increased. High air pressure was used as a treatment to avoid oxygen limitation during high cell mass concentration fermentation. On the other hand, too much air pressure accelerated cell lysis. For avoiding the influence of methanol fraction on yield of EKL. No methanol was detected during induction phase using Do-stat strategy fermentation. Air pressure was increased to 52.5 kPa in this study for EKL expression. Fig. 1a showed the curves of cell growth with the DCM of γ = 37.96 g L⁻¹ and γ = 68.60 g L⁻¹ after 90 h cultivation (63 h induction) under 10.5 kPa and 96 h cultivation (62.5 h induction) under 52.5 kPa respectively. No significant difference of biomass was observed between the two
curves during cell growth at cost of glycerol. During the induction phase, the yield coefficients of biomass per methanol were 0.07233 g mL⁻¹ and 0.09458 g mL⁻¹ under 10.5 kPa and 52.5 kPa respectively. The methanol consumption rates were 4.4293 mL L⁻¹ h⁻¹ and 4.8699 mL L⁻¹ h⁻¹, therefore, the yield coefficient of biomass per methanol and methanol consumption rate were improved under 52.5 kPa compared with that under 10.5 kPa. DO was enhanced under 52.5 kPa as the result of higher oxygen solution than that under 10.5 kPa (Fig. 1b). The final yield of EKL in the culture under 10.5 kPa was 62.63 g L⁻¹, whereas that in the culture under 52.5 kPa was 142.24 g L⁻¹ (Fig. 1c). Another effect of air pressure on cell metabolism was described by maintenance coefficient. Maintenance coefficient during induction phase under 52.5 kPa (0.0724 mL g⁻¹ h⁻¹) was lower than that under 10.5 kPa (0.1312 mL g⁻¹ h⁻¹), which benefited cell metabolism for EKL expression. The yield coefficients of EKL per methanol were 0.4350 and 0.4673 mg mL⁻¹.

**Effect of methanol added on activity of EKL in flask experiments**

In flask experiments, different fraction of methanol (φ = 0.5, 1.0, 2.0, 4.0, and 6.0 %) was added every 24 h to induce EKL after 24 h cultivation. Fig. 2 showed the biomass, activity of EKL and methanol volume fraction after 144 h cultivation (120 h induction). Little inhibition was observed on cell growth as methanol added increased. The highest activity of EKL (13619.14 U L⁻¹) was obtained as φ = 2 % methanol was added. Methanol scarcity might be the reason why activity of EKL was low at φ = 0.5 % and φ = 1.0 % methanol added. Activity of EKL decreased when φ = 4.0 % or φ = 6.0 % methanol was added, this may be due to the influence of methanol toxicity.¹⁷ Therefore, φ = 2 % methanol was added to induce EKL in following flask experiments. Methanol volume fraction should be investigated in bioreactor for EKL expression.

**Effect of pH on the activity of EKL in flask experiments**

Recombinant EKL was secreted to broth during induction phase by φ = 2 % methanol added per 24 h at different pH. Therefore, different pH in medium was prepared by potassium phosphate buffer, phosphate acid or potassium hydroxide. The pH value during the cultivation was maintained by addition of potassium hydroxide every 12 h. The bell shape of the biomass suggested that it was beneficial to cell growth at the range of pH 5.0–7.0. At the point of activity of EKL, pH 4.0–6.0 gave high relative activity of EKL. Specific activity of EKL at pH 4.0 was the highest among the different pH. Fermentation broth containing EKL at different pH was lay at 37 °C for 120 h to investigate the stability of EKL. The activity of EKL did not decrease during the period of conservation (Fig. 3). There-
fore, it was considered that activity of EKL was not lost during fed-batches.

**Effect of pH on yield of EKL in 3.7 L bioreactor**

As EKL was stable in broth at different pH, three fed-batches with different pH (4.0, 5.0, and 6.0) were performed to clarify the influence of pH on EKL expression at the condition of 52.5 kPa. Methanol was added after 10 h glycerol fed almost by DO-stat strategy. Thirty percent DO was maintained during induction phases without methanol detected. Table 1 showed the summary of parameters of three fed-batches. No distinguished difference of biomass was observed during fermentation, however, the yield and specific yield of EKL increased as pH decreased from 6.0 to 4.0. The methanol consumption rate increased when pH decrease from 6.0 to 4.0. This may be because of metabolism condition improved at pH 4.0, as maintenance coefficient was decreased from 0.0964 to 0.0724 mL g–1 h–1. The maximal yield and specific yield of EKL were 277.15 mg L–1 and 3.28 mg g–1 at pH 4.0 after 136.5 h cultivation (90 h induction).

**Effect of biomass and amount of methanol added on the yield of EKL in flask experiments**

Methanol volume fraction was known as an important factor during *Pichia pastoris* fermentation for heterogeneous protein expression. Yield of EKL was reported as a cell concentration dependent by Tan et al. Effect of different amount of methanol added and cell concentration on activity of EKL was investigated in flasks to optimize the yield of EKL (Fig. 4). Activity of EKL decreased significantly when amount of methanol added increased, especially higher than φ = 6.0 %. Activity of EKL did not change much when the amount of methanol was lower than φ = 4.0 % regardless of cell concentration. Activity of EKL increased as cell concentration increased as the amount of methanol added increased, especially higher than φ = 6.0 %. This result was confirmed by Tan’s work. However, methanol fraction was maintained between φ = 1.0–3.0 % in Tan’s report which was too high for EKL expression.

**Effect of methanol fraction on yield of EKL in 3.7 L bioreactor**

Three grades of methanol fractions (φ = 0, 0.6, and 1.0 %) were performed during induction phase to improve the yield of EKL. Table 2 is the summary of parameters of three fed-batches. Biomass (86.36 g L–1) at φ = 0 % methanol was higher than that at φ = 0.6 and 1.0 % methanol. This might be

<table>
<thead>
<tr>
<th>pH</th>
<th>Biomass γx/g L–1</th>
<th>Induction time t/h</th>
<th>EKL γ/mg L–1</th>
<th>EKL fraction w/mg g–1</th>
<th>Methanol consumption rate/mL L–1 h–1</th>
<th>Maintenance coefficient km/mL g–1 h–1</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.0</td>
<td>84.52</td>
<td>90</td>
<td>277.15</td>
<td>3.28</td>
<td>5.52</td>
<td>0.0724</td>
</tr>
<tr>
<td>5.0</td>
<td>73.09</td>
<td>105</td>
<td>174.68</td>
<td>2.39</td>
<td>5.18</td>
<td>0.0899</td>
</tr>
<tr>
<td>6.0</td>
<td>76.36</td>
<td>117</td>
<td>156.09</td>
<td>2.04</td>
<td>4.04</td>
<td>0.0964</td>
</tr>
</tbody>
</table>

**Table 1 – Effect of pH on the cell growth and yield of EKL in fed-batches**

**Table 2 – Effect of methanol volume fractions on biomass and yield of enterokinase in fed-batches**

<table>
<thead>
<tr>
<th>Methanol volume fraction φ/%</th>
<th>Biomass concentration γx/g L–1</th>
<th>Induction time t/h</th>
<th>EKL γ/mg L–1</th>
<th>EKL mass fraction w/mg g–1</th>
<th>Methanol consumption rate/mL L–1 h–1</th>
<th>Maintenance coefficient km/mL g–1 h–1</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>86.36</td>
<td>90</td>
<td>277.15</td>
<td>3.21</td>
<td>5.52</td>
<td>0.0724</td>
</tr>
<tr>
<td>0.6</td>
<td>68.19</td>
<td>95.5</td>
<td>323.98</td>
<td>4.75</td>
<td>5.63</td>
<td>0.1219</td>
</tr>
<tr>
<td>1.0</td>
<td>37.97</td>
<td>60</td>
<td>124.46</td>
<td>3.28</td>
<td>4.27</td>
<td>0.1629</td>
</tr>
</tbody>
</table>
that methanol was oxidized efficiently when methanol fraction was \( \varphi = 0 \% \) because DO at \( \varphi = 0 \% \) methanol was higher than that at \( \varphi = 0.6 \% \) methanol (Fig. 5). At the condition of \( \varphi = 1.0 \% \) methanol, DO was higher than that of other grades because of methanol toxicity. Yield of \( \text{EK}_\text{l} \) was 323.98 mg L\(^{-1} \) at \( \varphi = 0.6 \% \) methanol, which was the results of higher intensity of methanol induction than \( \varphi = 0 \% \) methanol. Disadvantageous effect of methanol toxicity on yield of \( \text{EK}_\text{l} \) was the lowest methanol consumption rate and maintenance coefficient increasing.

**Effect of cell mass concentration on yield of \( \text{EK}_\text{l} \) from \textit{Pichia pastoris} in 3.7 L bioreactor**

Scale up of effect of cell concentration on yield of \( \text{EK}_\text{l} \) was performed in 3.7 L bioreactor by three cell concentration grades (50, 80, 100 g L\(^{-1} \)). Fermentation conditions were pH 4.0, \( \varphi = 0.6 \% \) methanol fraction and 52.5 kPa. Fig. 6a shows the curves of cell growth during cultivation. Fig. 6b shows the curves of \( \text{EK}_\text{l} \) yield during induction phase. Yields of \( \text{EK}_\text{l} \) increased without changes significantly regardless of cell concentration which was confirmed by flask experiments. The methanol fraction was too high for \( \text{EK}_\text{l} \) expression in Tan’s work which gave lower activity of \( \text{EK}_\text{l} \) than that of \( \varphi = 0.6 \% \) methanol in this work. Disadvantageous effect of methanol toxicity on yield of \( \text{EK}_\text{l} \) was the lowest methanol consumption rate and maintenance coefficient increasing.

Yield of recombinant \( \text{EK}_\text{l} \) in \textit{Pichia pastoris} was optimized by fed-batches. DO was improved for yield of \( \text{EK}_\text{l} \) enhancement (46.19 \%) at 52.5 kPa air pressure. In flask experiments, good stability of \( \text{EK}_\text{l} \) was known at different pH. The amount of methanol added, not the cell concentration at low amount of methanol added, played an important role in high \( \text{EK}_\text{l} \) expression by \textit{Pichia pastoris}. Scaled up in 3.7 L bioreactor, pH 4.0, \( \varphi = 0.6 \% \) methanol, and 100 g L\(^{-1} \) cell concentration were the final conditions for \( \text{EK}_\text{l} \) expression by \textit{Pichia pastoris}. The yield of \( \text{EK}_\text{l} \) was 479.99 mg L\(^{-1} \) after optimization. Maintenance coefficient had the low value when high yield of \( \text{EK}_\text{l} \) was obtained.

**Conclusion**

Yield of recombinant \( \text{EK}_\text{l} \) in \textit{Pichia pastoris} was optimized by fed-batches. DO was improved for yield of \( \text{EK}_\text{l} \) enhancement (46.19 \%) at 52.5 kPa air pressure. In flask experiments, good stability of \( \text{EK}_\text{l} \) was known at different pH. The amount of methanol added, not the cell concentration at low amount of methanol added, played an important role in high \( \text{EK}_\text{l} \) expression by \textit{Pichia pastoris}. Scaled up in 3.7 L bioreactor, pH 4.0, \( \varphi = 0.6 \% \) methanol, and 100 g L\(^{-1} \) cell concentration were the final conditions for \( \text{EK}_\text{l} \) expression by \textit{Pichia pastoris}. The yield of \( \text{EK}_\text{l} \) was 479.99 mg L\(^{-1} \) after optimization. Maintenance coefficient had the low value when high yield of \( \text{EK}_\text{l} \) was obtained.

**List of symbols**

\[ a \quad \text{EK}_\text{l} \text{ activity, U L}^{-1} \]
\[ c \quad \text{concentration, mol L}^{-1} \]
\[ k_m \quad \text{maintenance coefficient, mL g}^{-1} \text{ h}^{-1} \]
\[ Y_G \quad \text{yield biomass per substrate, g g}^{-1} \]
$Y_{XS}$ – observed yield biomass per substrate, g g\(^{-1}\)

$p$ – pressure, kPa

$Q$ – volume flow rate, L h\(^{-1}\)

$q_s$ – substrate consumption rate, h\(^{-1}\)

$s$ – substrate, g

$t$ – time, h

$V$ – volume, mL, L

$w$ – mass fraction, %

$\gamma$ – mass concentration, g L\(^{-1}\)

$\gamma_s$ – biomass concentration, g L\(^{-1}\)

$\theta$ – temperature, °C

$\lambda$ – wave length, nm

$\mu$ – specific cell growth rate, h\(^{-1}\)

$\phi$ – volume fraction, %

$\Psi$ – volume ratio

References