Two-Step Fed-Batch Culture of Recombinant Escherichia coli for Production of Bacillus licheniformis Maltogenic Amylase

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Abstract Two-step fed-batch fermentations were carried out to overproduce Bacillus licheniformis maltogenic amylase (BLMA) in recombinant Escherichia coli. The first step was to increase the cell mass by controlling the feeding of a glucose solution, while the second step was designed to improve the amylase expression efficiency by supplementing organic nitrogen sources. The linear gradient feeding method was successfully adopted to maintain the glucose concentration below 0.2 g/l during the fed-batch mode, as effectively minimizing acetic acid formation. When the dissolved oxygen (DO) level became limiting, an accumulation of acetic acid and drastic decrease in specific BLMA productivity were observed. Glucose and organic nitrogen sources consisting of yeast extract and casein hydrolysate were simultaneously supplied in the pH-stat mode to further increase the specific BLMA expression efficiency. An organic nitrogen source consisting of 200 g/l yeast extract and 100 g/l casein hydrolysate was found to be the best among the various combinations tested. The feeding of an organic nitrogen source in the second-step fed-batch period was highly beneficial in enhancing the BLMA production. The optimized two-step fed-batch culture resulted in 78 g/l maximum dry cell mass and 443 U/ml maximum BLMA activity, corresponding to 1.5-fold increase in the dry cell mass and 3.7-fold enhancement in BLMA production, compared with the simple fed-batch fermentation.

Key words: Bacillus licheniformis maltogenic amylase, recombinant E. coli, acetic acid, fed-batch, gradient feeding, organic nitrogen

Large-scale cultivation of recombinant E. coli often employs fed-batch processes [16, 28]. Such fed-batch schemes can relieve inhibition by acetic acid in recombinant E. coli cultures, through the controlled feeding of a glucose solution. A number of feeding strategies are being employed to reduce acetic acid formation, including regulation of the specific growth rate [4, 5] or recycling of the culture broth [14].

In addition to minimizing acetic acid formation by process monitoring and control, it is also essential to consider physiology of the host at high cell densities. With an increase in cell density, the intracellular concentration of the desired protein often decreases as a result of nutrient depletion [30]. However, the current lack of knowledge on the physiological requirements of recombinant cells in a high cell density forces us to use various nutrient supplementation methods, such as constant feeding [25, 27-28], pH-stat [29], or a linear change in the feeding rates [6] during the production phase. Consequently, the application of a well-designed fermentation strategy to minimize inhibitory acetic acid formation and the use of a balanced medium to fulfill the physiological requirements of E. coli are considered to be critical to achieve a high cell density and high cloned protein yield.

A new type of amylase gene was recently cloned from Bacillus licheniformis and expressed in recombinant E. coli [11]. The enzyme, BLMA, degrades soluble starch by preferentially cleaving maltosyl units and/or transferring it to acceptor molecules, such as glucose or maltose, by linking mainly the α-1,6-glycosyl bond, thereby forming functional oligosaccharides, including panose and isopanose. In addition, BLMA also has the ability to hydrolyze pullulan and cyclodextrins.

In the current study, two-step fed-batch fermentations were performed to overproduce BLMA in high cell density cultures of recombinant E. coli. A linear gradient feeding...
method was chosen to allow the recombinant cells to grow while reducing acetic acid formation during the first fed-batch mode. The second fed-batch culture was initiated in the pH-stat mode when the DO level became limited. Special emphasis was placed on the effects of organic nitrogen sources in the second fed-batch mode in an attempt to enhance specific BLMA productivity.

**MATERIALS AND METHODS**

**Bacterial Strain and Plasmid**

Recombinant *E. coli* TG1 (*supE hsdS Δ5 thi Δ(lac-proAB) F’ [traD36 proAB’ lacI lacZ ΔM15])* harboring the pUCIJ119 plasmid was used in this study. Plasmid pUCIJ119 contains the structural gene encoding *B. licheniformis* amylase under the control of its own promoter from *B. licheniformis* [11].

**Media and Culture Conditions**

One loop of a colony on an LB [10 g tryptone (Difco, Detroit, MI, U.S.A.), 5 g yeast extract (Difco), and 50 mg ampicillin per liter]-agar plate was transferred into 5 ml of an LB medium and incubated overnight at 28°C. The cultured cells were transferred into shake flasks containing 100 ml of a fresh synthetic medium (Table 1), and further incubated for 16 h in a rotary shaking incubator at an agitation speed of 200 rpm. Trace metal solution and thiamine-HCl were prepared through filter sterilization with 0.45 µm filter. MgSO₄ and glucose were added after separate sterilization. For the fermenter cultures, 50–100 ml of the shake flask-cultured cells were transferred into a fermenter (KoBiotech Co., Inchon, Korea) equipped with temperature and pH control units and operated with a 2.5-l working-volume. Temperature was maintained at 28°C, while pH was controlled at 6.8±0.1 by the addition of 1.0 N HCl or 28% NH₄OH. The agitation speed was maintained at 700 rpm during the batch phase and 1,000–1,300 rpm during the fed-batch phase.

**Fed-Batch Cultures**

Cells were grown batchwise in the fermenter until the initially added glucose was completely exhausted. The feeding of the growth medium (Table 1) started when the cells reached the late-exponential growth phase in the batch fermentation. Before starting the fed-batch mode, both the glucose and acetic acid concentrations were measured. The glucose concentrations and their feed rate (F) during the first fed-batch mode were determined using the mass balance equations for the cell mass and glucose in the fermenter.

Cell mass (X) was determined from the dry cell mass increase divided by the amount of glucose consumed in the batch phase, was assumed to remain constant throughout the cultivation. µₒ is the specific growth in the fed-batch mode, the feed rate (F) was then estimated using the above equations [17]. The cellular yield coefficient (Yₓ/s), determined from the dry cell mass increase divided by the amount of glucose consumed in the batch phase, was assumed to remain constant throughout the cultivation. µₒ is the specific growth in the fed-batch mode, the feed rate (F) was then estimated using the above equations [17]. The parameters in the above equations were estimated from the batch cultures and Xₒ is the maximum dry cell mass that can be obtained in a batch phase with a given initial glucose concentration. Considering the maximum volumetric capacity of the fermenter and the dry cell mass increase in the fed-batch mode, the feed rate (F) was then estimated using the above equations [17]. The cell mass (X) was determined from the dry cell mass increase divided by the amount of glucose consumed in the batch phase, was assumed to remain constant throughout the cultivation. The cell mass (X) was determined from the dry cell mass increase divided by the amount of glucose consumed in the batch phase, was assumed to remain constant throughout the cultivation. µₒ is the specific growth in the fed-batch mode, the feed rate (F) was then estimated using the above equations [17]. The parameters in the above equations were estimated from the batch cultures and Xₒ is the maximum dry cell mass that can be obtained in a batch phase with a given initial glucose concentration. Considering the maximum volumetric capacity of the fermenter and the dry cell mass increase in the fed-batch mode, the feed rate (F) was then estimated using the above equations [17]. The parameters in the above equations were estimated from the batch cultures and Xₒ is the maximum dry cell mass that can be obtained in a batch phase with a given initial glucose concentration. Considering the maximum volumetric capacity of the fermenter and the dry cell mass increase in the fed-batch mode, the feed rate (F) was then estimated using the above equations [17]. The parameters in the above equations were estimated from the batch cultures and Xₒ is the maximum dry cell mass that can be obtained in a batch phase with a given initial glucose concentration. Considering the maximum volumetric capacity of the fermenter and the dry cell mass increase in the fed-batch mode, the feed rate (F) was then estimated using the above equations [17].

### Table 1. Composition of synthetic media used for batch and fed-batch cultures.

<table>
<thead>
<tr>
<th>Component</th>
<th>Batch (g/l)</th>
<th>Dilute reservoir (g/l)</th>
<th>Concentrate reservoir (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K H₂PO₄</td>
<td>13.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(NH₄)₂HPO₄</td>
<td>4.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citric acid</td>
<td>1.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>1.2</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Thiamine-HCl</td>
<td>0.005</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>Glucose</td>
<td>30</td>
<td>Various¹</td>
<td>575</td>
</tr>
<tr>
<td>Trace metal solution²</td>
<td>10 ml</td>
<td>30 ml</td>
<td>30 ml</td>
</tr>
</tbody>
</table>

¹The glucose concentration in the dilute medium reservoir linearly increased as a function of the culture time, which was determined from the balance equations described in Materials and Methods.

²The trace metal solution contained per liter: 6 g Fe (III) citrate, 1.5 g MnCl₂·H₂O, 0.8 g Zn (CH₃COO)₂·2H₂O, 0.3 g H₃BO₃, 0.25 g Na₂MoO₄·2H₂O, 0.25 g CoCl₂·6H₂O, 0.15 g CuCl₂·2H₂O, 0.84 g Na₂EDTA·2H₂O.
the aeration rate. For the pH-stat mode in the second fed-batch culture, glucose (770 g/l) and organic nitrogen sources were supplied to maintain pH within 6.8±0.1. Various combinations of yeast extract and casein hydrolysate (Difco) in the feed solution were tested to obtain an optimum concentration for the organic nitrogen sources.

### Analytical Methods

The dry cell mass was monitored by measuring the optical density at 660 nm using a spectrophotometer (Shimadzu UV2201, Tokyo, Japan) and estimated as the mean of at least duplicate measurements against a predetermined calibration curve. The culture broth was withdrawn from the fermenter at 2–5 h intervals and centrifuged (Vision Scientific 15000CF, Inchon, Korea) at 6,000 ×g for 10 min. The glucose concentration was determined using either an enzymatic kit (Sigma Chemical Co., St. Louis, MO, U.S.A.) or a glucose analyzer (YSI 1500G, Yellow Springs, OH, U.S.A.). The acetic acid was determined using a Roche enzymatic kit (Mannheim, Germany). The harvested cells were washed twice with ice-cold distilled water, resuspended in 50 mM Tris-HCl buffer (pH 7.5), and stored at 4°C until further analysis. The cells were disrupted using an ultrasonic processor (Cole-Parmer 40W, Vernon Hills, IL, U.S.A.) and then centrifuged at 12,000 xg for 10 min. The supernatant was used for measuring the BLMA activity [11]. One unit of BLMA activity was defined as the amount of enzyme that produced one mmole of reducing sugars per minute at 50°C and pH 6.8.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli [13] using a slab gel kit (Hoefer Scientific Instruments SE600, San Francisco, CA, U.S.A.) with 12.5% acrylamide running gel and 4% stacking gel. The relative quantity of BLMA to the total cellular proteins of recombinant *E. coli* was estimated using a densitometer (Bio-Rad GS700, Hercules, CA, U.S.A.).

### RESULTS AND DISCUSSION

#### Batch and Fed-Batch Cultures

A batch cultivation of recombinant *E. coli* TG1/pUCIJ119 was carried out in a 5-l fermenter using a defined medium. The initially added glucose was exhausted within 12 h. A final dry cell mass of 10 g/l and 22 U/ml BLMA activity were obtained. The experimental results clearly show that BLMA was expressed in a growth-associated pattern. Moreover, no acetic acid was produced throughout the cultivation, thereby indicating the effectiveness of the synthetic medium for a high cell density of recombinant *E. coli* TG1.

A fed-batch fermentation with *E. coli* TG1/pUCIJ199 was also performed to enhance the volumetric productivity of BLMA by adopting the linear gradient feeding method. When the initial glucose was completely exhausted, the linear gradient fed-batch mode was started. The glucose concentration in the feed solution linearly increased in proportion to the increase of the dry cell mass relative to the cultivation time. The acetic acid concentration remained below 0.2 g/l, and the specific BLMA productivity also remained fairly constant in the fed-batch mode (Fig. 2). However, when the dry cell mass reached approximately 40 g/l, the DO became limited with concomitant accumulation of acetic acid and glucose. As the acetic acid concentration increased, the BLMA expression became severely inhibited, in good agreement with other reports [2-3, 12, 16]. Finally, the conventional one-step fed-batch cultivation resulted in a 52 g/l dry cell mass and 119 U/ml BLMA activity, which corresponded to a 5.2-fold increase in the dry cell...
mass and 5.4-fold enhancement in BLMA activity, compared with the simple batch cultivation of \textit{E. coli} TG1/pUCIJ119 in the same medium.

In cases where high level of substrate or byproduct inhibit cell growth and cloned gene expression, medium feeding strategies are often used in order to maintain the substrate concentration below a critical level to minimize byproduct formation and retain the metabolic activities of the host cells. Feeding methods based on DO-stat [18], pH-stat [9], or on-line monitoring and controlling the glucose concentration [7] are often critical in achieving a high level of cell mass and product yield. Relatively simple feed-forward strategies such as constant-rate feeding and exponential feeding have also been successfully applied for obtaining high cell densities of \textit{E. coli} [16, 28]. The linear gradient feeding method used in the current study is simple to implement and more useful than other strategies, such as intermittent or continuous feeding. However, it has limitations in that the parameters necessary for determining the feed rate profiles must be carefully selected and estimated from \textit{a priori} batch or fed-batch cultivations.

### Effects of Organic Nitrogen Source in Fed-Batch Culture

A two-step fed-batch fermentation that could overcome limitations of conventional fed-batch fermentations for recombinant \textit{E. coli} TG1/pUCIJ199 was attempted to enhance BLMA productivity. Therefore, the cultivation was shifted to the fed-batch mode after complete exhaustion of the glucose as indicated by a sudden increase in the DO. As in the previous fed-batch cultivation, the linear gradient feeding method was then employed to control the glucose concentration in the first phase of the fed-batch fermentation and thereby increase the cell mass until DO-limitation occurred. The parameters used in the two-step fed-batch fermentations are summarized in Table 2. The final dry cell mass in the linear gradient fed-batch mode was set at 60 g/l. The DO was more tightly controlled by increasing the agitation speed or aeration rate to prevent any excessive acetic acid formation. Nonetheless, it was still very difficult to maintain the DO level at 10–20% air saturation in high cell densities. For the second stage of the two-step fed-batch fermentations, the supply of glucose and organic nitrogen started when the dry cell mass reached the set value of 60 g/l. Various concentrations of yeast extract and casein hydrolysate in the feed solution were tested to obtain an optimum combination of organic nitrogen sources.

The maximum dry cell mass and BLMA activity obtained from the series of two-step fed-batch fermentations are summarized in Table 3. No acetic acid formation or decrease in specific BLMA productivity were observed in any of the two-step fermentations. Although there was a significant scattering in the maximum dry cell masses of the run 2 and run 5, almost the same final BLMA activity was recorded for the given casein hydrolysate concentration in the feed solution. This indicated that 100 g/l yeast extract in the feed was sufficient to enhance the BLMA productivity. Furthermore, no dramatic increase in BLMA productivity was observed even with 200 g/l yeast extract and casein hydrolysate (run 2 and run 3). These results were also compatible with those for the run 2 and run 5. In contrast, casein hydrolysate by itself exerted no significant effect on the BLMA expression when sufficient amount of yeast extract was present in the feed (run 3 and run 5). However, the run 1 and run 2 showed prominent effects of the yeast extract on the BLMA production, which increased almost three-fold when compared to the same casein hydrolysate concentration in the feed solution. Accordingly, it appeared that the biosynthetic precursors and nutrients present in the yeast extract were more directed toward BLMA production rather than cell growth. In the run 1, it would appear that the carbon flux into the central metabolic pathway exceeded the cellular capacity under oxygen-limited conditions, which inevitably resulted in acetic acid accumulation.

### Table 2. Parameters used to determine glucose concentrations and feed rate for the linear gradient feeding of fed-batch run 5.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Description</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>( V_0 )</td>
<td>Initial reactor volume in fed-batch culture</td>
<td>2 l</td>
</tr>
<tr>
<td>( V_f )</td>
<td>Final reactor volume in fed-batch culture</td>
<td>3.5 l</td>
</tr>
<tr>
<td>( Y_c )</td>
<td>Cell yield based on glucose</td>
<td>0.4 g/g</td>
</tr>
<tr>
<td>( \mu )</td>
<td>Specific growth rate in fed-batch mode</td>
<td>0.3/h</td>
</tr>
<tr>
<td>( X_c )</td>
<td>Initial dry cell mass in fed-batch mode</td>
<td>8 g/l</td>
</tr>
<tr>
<td>( X_f )</td>
<td>Final dry cell mass in fed-batch mode</td>
<td>60 g/l</td>
</tr>
<tr>
<td>( S_s )</td>
<td>Glucose concentration in concentrate reservoir</td>
<td>575 g/l</td>
</tr>
<tr>
<td>( S_d )</td>
<td>Glucose concentration in dilute reservoir</td>
<td>71 g/l</td>
</tr>
<tr>
<td>( V_{d0} )</td>
<td>Initial volume of dilute reservoir</td>
<td>0.75 l</td>
</tr>
</tbody>
</table>

### Table 3. Effects of concentrations of yeast extract and casein hydrolysate in the feed solution on maximum dry cell mass, BLMA activity, and acetic acid in the second phase of fed-batch fermentations of recombinant \textit{E. coli} at pH 6.8 and 28°C.

<table>
<thead>
<tr>
<th>Run</th>
<th>Yeast extract (g/l)</th>
<th>Casein hydrolysate (g/l)</th>
<th>Dry cell mass (g/l)</th>
<th>BLMA activity (U/ml)</th>
<th>Final acetic acid (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50</td>
<td>100</td>
<td>70</td>
<td>151</td>
<td>13</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>100</td>
<td>63</td>
<td>444</td>
<td>&lt;2.0</td>
</tr>
<tr>
<td>3</td>
<td>200</td>
<td>200</td>
<td>70</td>
<td>417</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>4</td>
<td>200</td>
<td>-</td>
<td>68</td>
<td>315</td>
<td>&lt;2.0</td>
</tr>
<tr>
<td>5</td>
<td>200</td>
<td>100</td>
<td>78</td>
<td>443</td>
<td>&lt;0.2</td>
</tr>
</tbody>
</table>
Profiles of the dry cell mass and BLMA production for the run 5 are presented in Fig. 3. The cell mass and specific BLMA production continued to increase in the pH-stat mode when fed glucose and organic nitrogen. The acetic acid and glucose were kept below 0.2 g/l throughout the cultivation. SDS-PAGE analysis and densitometry also confirmed an increase of specific BLMA productivity in the second step. The optimized two-step fed-batch cultivation (run 5) resulted in a maximum dry cell mass of 78 g/l and BLMA activity of 443 U/ml, corresponding to 1.5-fold increase in the dry cell mass and 3.7-fold enhancement in BLMA activity, compared to the simple fed-batch cultivation performed with the same strain.

It is clear from the above fed-batch cultivations that a combination of controlled glucose feeding and supplementation of organic nitrogen sources consisting of 200 g/l yeast extract and 100 g/l casein hydrolysate was supplied in the second phase of the fed-batch fermentation.

In the current study, an organic nitrogen source consisting of yeast extract and casein hydrolysate was simultaneously supplied along with glucose in the pH-stat mode to retard any sudden DO change. Although glucose was limited in the fed-batch fermentations, the DO change due to the medium pulse in the pH-stat mode was not so drastic, because the cells continued to consume the organic substrates. There are several previous research reports to demonstrate positive effects of organic nitrogen sources on cell growth and cloned protein production [24, 26, 30]. An elevated level of specific BLMA expression in the second step in the present study indicated that yeast extract and casein hydrolysate provided important nutrients for BLMA production. Earlier studies on the production of glyceraldehyde-3-phosphate dehydrogenase and a recombinant human insulin-like growth factor also clearly show that supplementation of organic nitrogen sources, such as yeast extract and peptone, not only assists cell growth but also stabilizes the cloned protein expression [4, 19, 26]. Together with the above consideration, it is suggested that concomitant application of the pH-stat technique and organic nitrogen supplementation is highly efficient in achieving high cloned protein yields, through enhancing or at least retaining the biological activity of recombinant E. coli cells in a high cell density.

Acknowledgments

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