

High cell density and high expression of recombinant human ApoA-I_{Milano} in *Escherichia coli* by twice temperature-shifted induction

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Abstract The effect of temperature on the formation of recombinant protein, apolipoprotein A-I_{Milano} was investigated in the present study. The temperature of the initial growth phase was set at 30°C, while temperature variation in induction phase was arranged in three modes. High cell-density culture of *Escherichia coli* and high expression of recombinant human by twice temperature-shifted induction were carried out. Experimental results showed that ApoA-I_{Milano} reached 4.8 g/L with the final cell density of OD₆₀₀, 150. It was found that twice temperature-shifted induction could successfully avoid the effect of acetic acid on cell density and the expression of the product. The present study provides a basic procedure for the production of recombinant ApoA-I_{Milano}.

Keywords recombinant *Escherichia coli*, ApoA-I_{Milano}, high density, high expression, twice temperature-shifted induction

1 Introduction

According to the type of promoter, the mode of the expression of heterogenous protein in *Escherichia coli* is divided into constitutional and induced types. The first type of expression system is more convenient and economic. Premature expression of a product often leads to the detriment of a living cell, which eventually results in the decrease of cell density and expression level. Thus, an induced type of expression is frequently used in fermentation of engineered strain (Sun et al., 1995). In large-scale production of engineered protein, temperature and

isopropylthiogalactoside (IPTG) induction are applied extensively. However, as for the large-scale production of clinically used protein, IPTG is unsuitable to serve as an inducer due to its toxicity and high cost. Therefore, in the production of medicine, temperature induction is always the priority (Wu, 2001). Traditional approach on temperature induction is usually conducted by a single-step raising of temperature. In the present study, twice temperature-shifted induction was used for the expression of recombinant human ApoA-I_{Milano} in *E. coli*, which showed to be more favorable for cell growth and product expression. As far as we know, no report on the application of twice temperature-shifted induction for the production of recombinant medicine has been made.

2 Materials and methods

2.1 Recombinant strain

The recombinant strain DH5 α /pBV220-ApoA-I_{Milano} was constructed and preserved in our laboratory. The strain with P_{LR} dial promoter was capable of inducing the expression of protein ApoA-I_{Milano} at 42°C, and possessed the resistance to ampicillin.

2.2 Media

- 1) Seed medium: Luria broth (LB) medium was used.
- 2) Basal medium no. 1: peptone 8 g/L, glucose 3 g/L, yeast extract 8 g/L, K₂HPO₄·12H₂O 8 g/L, KH₂PO₄ 4 g/L, MgSO₄·7H₂O 0.25 g/L, CaCl₂ 0.11 g/L, trace elements 4 ml/L.
- 3) Feeding medium no. 1: peptone 8 g/L, glucose 3 g/L, yeast extract 8 g/L, MgSO₄·7H₂O 0.25 g/L.

Translated from *Microbiology*, 2005, 32(2): 54–59 [译自: 微生物学通报, 2005, 32(2): 54–59]

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4) Basal medium no. 2: glucose 4 g/L, $K_2HPO_4 \cdot 12H_2O$ 6 g/L, KH_2PO_4 6 g/L, $(NH_4)SO_4$ 3 g/L, NH_4Cl 0.5 g/L, NaCl 0.5 g/L, $MgSO_4 \cdot 7H_2O$ 0.25 g/L, ammonium citrate 0.8 g/L, ethylenediaminetetraacetic acid (EDTA) 0.0084 g/L, biotin 0.001 g/L, thiamine 0.02 g/L, trace elements 4 ml/L.

5) Feeding medium no. 2 : glucose 500 g/L, ammonium citrate 0.8 g/L, $MgSO_4 \cdot 7H_2O$ 0.25 g/L.

6) Remark: trace element stock solution: $MgSO_4 \cdot 7H_2O$ 1 g/L, $MnSO_4 \cdot H_2O$ 1 g/L, $ZnSO_4 \cdot 7H_2O$ 2.78 g/L, $CoCl_2 \cdot 6H_2O$ 2 g/L, molybdic acid dihydrate 2 g/L, $CuSO_4 \cdot 5H_2O$ 1.58 g/L, boric acid 0.5 g/L. In the above media, glucose, magnesium sulfate and trace elements were sterilized separately.

2.3 Control of fermentation

2.3.1 Procedure for fed-batch fermentation

In a 5-L jar fermenter, 2.5 L basal medium for fed-batch fermentation was added. After sterilization, 200 mL inoculum from shake flask was added into the fermenter. During fermentation, feeding was controlled at 0.1 g/L residual glucose. In the growth phase, pH and temperature were controlled at 7.0 and 30°C, respectively, and then increased to 7.4 and 42°C after induction, respectively. Dissolved Oxygen (DO) was controlled at over 30% air saturation by the regulation of the agitator speed in the course of fermentation.

2.3.2 Procedure for high cell density and high-expression fed-batch fermentation (twice-induction)

The procedure for high cell density and high-expression fed-batch fermentation is basically the same as the above method except that the control of temperature was somewhat different. In the initial growth phase, it was controlled at 30°C. Twice induction was initiated when the growth entered into the middle-stage of the logarithmic phase. For the first induction, the temperature was increased to 42°C for 3–4 hours and then decreased to around 37°C for about 7 hours, and followed by next induction, by increasing of the temperature to 42°C for 5–6 hours, at which the fermentation was terminated.

2.4 Assay method

- 1) Cell density: OD_{600} value was measured with spectrophotometer (Yang et al., 2001).
- 2) Glucose concentration: Fehling's reagent method (Tianjing Institute of Light Industry et al., 1980) was used.
- 3) Acetic acid: gas chromatography (Martin, 1985) was applied.
- 4) Target protein: the expression of the protein was determined with antibody sandwich enzyme-linked immunosorbent assay (ELISA) (Li and Zhao, 1998).

3 Results and discussion

3.1 Effects of the mode of induction with different temperatures on the growth and expression

There are two modes of conventional induction. In the initial growth phase, the temperature was controlled at 30°C until the cell density reached a certain value, then raised to 42°C and maintained at the end of induction phase. Another method was almost the same as the previous one except that the temperature decreased after the induction has been carried out for the same time. In present study, twice temperature-shifted induction was conducted.

The results of the first mode of induction showed that the maximum expression level of ApoA-I_{Milano} was attained after the induction at 42°C for 5–6 hours, while the cell density apparently declined (Fig. 1).

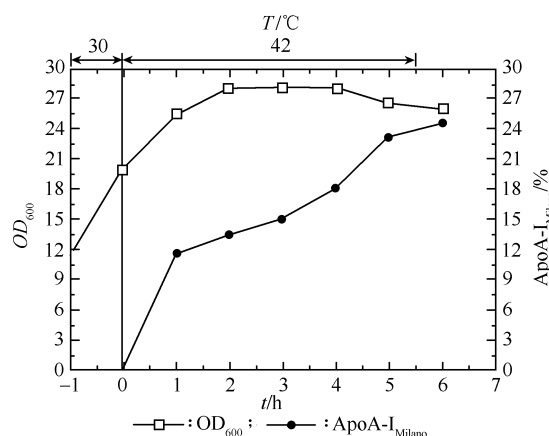


Fig. 1 Effect of thermostatic induction on the growth and expression of target protein

In the second mode of induction, the temperature was decreased to 30°C after induction, which resulted in the increase of biomass concentration again and the decreasing of the synthesis of ApoA-I_{Milano} (Fig. 2). This may be attributed to the depression of the formation of inclusion bodies, which led to the reduction of target protein production (Lee, 1997).

In the third mode of induction using twice increase of temperature, cell growth increased continuously, and the maximum cell density OD_{600} attained 150, which was far higher than that using the first mode of induction ($OD_{600} = 28$). The expression ratio of ApoA-I_{Milano} achieved 24.13%, although it was the same as compared with the first mode, but its total production level attained 4.8 g/L, 4.27 times as that of the control (Fig. 3).

3.2 Analysis on the physiological parameters during the induction using twice-increase of temperature

The relations between specific growth rate (μ) and specific

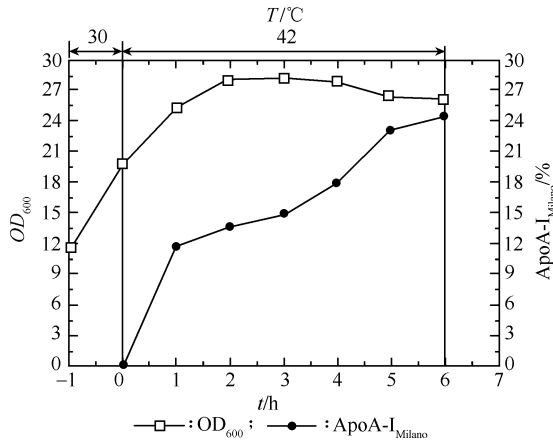


Fig. 2 Effect of single induction followed by temperature decrease on the growth and expression of the product

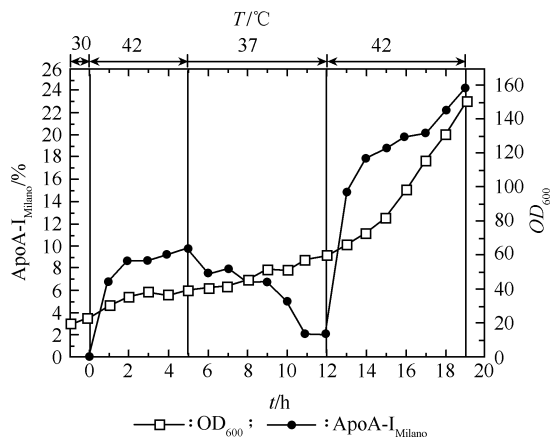


Fig. 3 Effect of the induction with twice temperature increase on cell growth and expression of the protein

product formation rate (Q_p) or specific substrate consumption rate (Q_s) after the induction using twice-increase of temperature were shown in Figure 4. Within 1 hour of induction, the growth rate and the consumption rate, Q_s were sped up, followed by the increase of Q_p after 2–3 hours of induction. If the cell was still subjected to high temperature (42°C), it was liable to aggravate the metabolic burden of the cell with the accumulation of the product, while the substantial accumulation of protease would lead to the decreasing of μ , Q_s , Q_p and the product. At this point, the decrease of the temperature to 37°C was likely to alleviate the disadvantages when growing at high temperature. Results showed that in spite of the unfavorable effect of the decreasing of temperature on the formation of the product at this period, it enhanced the values of μ and Q_s . When the induced temperature increased to 42°C again after being cultivated at 37°C for 7 hours, Q_p started to increase again and the expression ratio of the target protein was nearly two times as that produced after the first induction. Thus, it could be seen that present mode of induction was favorable to the physiological balance of the producing strain, which seemed to be a key factor to the high cell-density growth and the high expression of the heterogenetic protein.

Good physiological balance was thus achieved primarily by varying the temperature and control of the processing time, and these have been confirmed in our experiments.

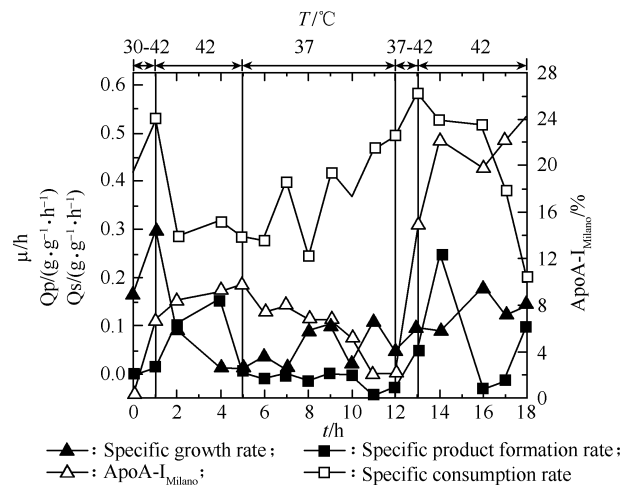


Fig. 4 Relationship among the specific growth rate (μ), specific product formation rate (Q_p), specific consumption rate (Q_s) and expression ratio of ApoA-I_{Milano} in the course of induction

3.3 Effect of acetic acid on growth and expression of ApoA-I_{Milano} during induction with twice temperature variation

The formation of acetic acid was the nature of glucose fermentation with *E. coli*, and it was produced continuously along with the fermentation (Shiloach et al., 1996). Single induction was unable to realize high cell density and high expression, which might be attributed to the inhibition of the synthesis of macromolecular, such as DNA, RNA, protein and lipid by the high concentration of acetic acid (Cherrington et al., 1990). In contrast, the result using twice induction was evidently better than that with single induction.

Figure 5 showed that after the first and second inductions, the average concentrations of acetic acid were 4.56 and 4.2 g/L, the specific acetic acid formation rates were 7.05 and 1.45 ($\text{g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$), maximum OD_{600} values attained 39 and 150, maximum ApoA-I_{Milano} expression ratio were 9.7% and 24.1% and the maximum productivity of target protein reached 0.5 and 4.8 ($\text{g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$), respectively, suggesting that twice induction was apparently superior to the single induction in terms of growth and product expression.

The reason for this might be that twice induction could recover the physiological activity of the strain, in contrast to the single induction. And the demand for energy by the cell was lower, which led to the reduction of specific acetic acid formation rate. Consequently, twice temperature-shifted induction was successful in alleviating the occurrence of the negative effect of acetic acid.

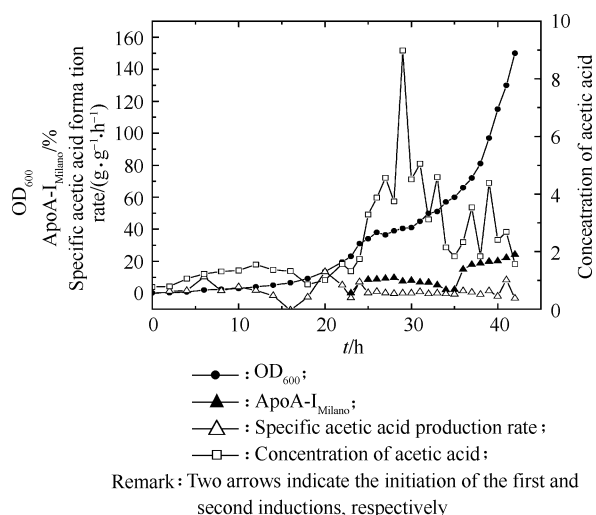


Fig. 5 Relationship among the cell density, expression ratio of target protein, specific acetic acid production rate and concentration of acetic acid in the course of twice temperature induction

4 Conclusion

The twice temperature-shifted induction approach has been confirmed through optimization process for the engineered strain DH5 α /pBV220, which leads to the realization of the high cell-density and high product expression in a 5-L fermenter using entirely synthetic medium. Results show that the expression level has risen to 4.8 g/L, which is 9.1% higher than that reported in the literature, using complex medium in a 3.5-L fermenter. Since the medium employed in this study is completely synthetic, it is more favorable for large-scale production at lower cost and higher efficiency.

Acknowledgements This work was funded by the National Research Project for High-Tech Development (No. 2002AA217021) and supported by a Grant-aid for the Special Project of State Key Science and Technology.

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