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Full Length Research Paper

Optimization of fed-batch fermentation for a staphylokinase-hirudin fusion protein in *Escherichia coli* BL21

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In this study, the fed-batch fermentation technique was applied to improve the yield of STH, a chimeric protein composed of staphylokinase (SAK) and hirudin (HV) with thrombin recognition peptide as a linker peptide, produced by *Escherichia coli* BL21. Three kinds of feeding mediums, GM (glucose + magnesium sulfate), GMY (GM + yeast extract) and GMYT (GMY + tryptone), were investigated with R-medium as an initial culture medium in a 5 L fermentor. The results showed that the dry cell weight were similar in the three feeding mediums, but the yield of total protein and STH were significantly different (total protein: GM, GMY, GMYT = 1, 4.72, 7.36; STH: GM, GMY, GMYT = 1, 1.93, 3.23). Then we replace the R-medium with the complex medium which contains yeast extract and tryptone in fed-batch fermentation based on the GMYT as feeding medium. The results showed that the total protein and STH in the complex medium were 6.29 and 7.76 fold of those in R-medium culturing condition, respectively. Under optimal conditions (GMYT and complex medium), a final STH expression of 1.48 g/l fermentation broth was achieved after 20 h cultivation. The efficacy of STH production was 37 times more than that before technique optimization. Furthermore, the optimal conditions were successfully scaled up to 40 L fermentor. This may prompt STH to develop into a new thrombolytic agent. It is interesting to know that for the harvest, the yeast extract and tryptone can significantly contribute to the protein expression.

Key words: Complex medium, fed-batch fermentation, feeding medium, R-medium.

INTRODUCTION

Cardiovascular and cerebrovascular diseases have become a major threat to human health. Thrombosis is an important incentive for such diseases. Thrombolytic therapy is among the most important means of these diseases' treatment. However, in the course of thrombolytic therapy, embolization often occurs again after thrombolysis. In order to prevent reocclusion, thrombolytic and anti-coagulant agents in combination have become an available therapeutic schedule. To this end many fusion proteins with both thrombolytic and anticoagulant activity have been designed, such as staphylokinase-hirudin1 (SAK-HV1), HV1-SAK (Szarka et al. 1999), PLATSAK (van Zyl et al., 1997, 2000), SAK-RGD-K2-Hir (Szemraj et al., 2005, 2007) and so on. However, these fusion proteins are not satisfactory, because they may have a more severe bleeding risk because both SAK and HV can evoke haemorrhage.

To reduce the bleeding risk that often accompanies high thrombolytic efficacy in the combination treatment of thromboembolic diseases, a novel chimeric protein, STH,

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Abbreviations: SAK, Staphylokinase; **HV,** hirudin; **STH,** staphylokinase and hirudin fusion protein linked by thrombin recognition peptide; **GM,** glucose + magnesium sulphate; **GMY,** glucose + magnesium sulphate + yeast extract; **GMYT,** glucose + magnesium sulphate + yeast extract + tryptone; **LB,** lysogeny broth; **DO,** dissolved oxygen; **SDS-PAGE,** sodium dodecyl sulfate polyacrylamide gel electrophoresis; **DCW,** dry cell weight.

composed of SAK and HV2 with thrombin recognition peptide as a linker peptide, has been constructed and expressed in *Escherichia coli* BL21 by our laboratory (Wang et al., 2009). *In vitro* studies show that STH has a high thrombus affinity and may release its anticoagulant activity in a thrombus-targeting way. As a result, STH not only shows improved thrombolytic effect, but also effectively reduces bleeding side effects *in vivo* (Wang et al., 2009). Thus, STH is thought to be a promising candidate for effective and safe therapy for thrombosis in clinic. However, the yield of STH expressed in *E. coli* is less than 0.1 g/l under batch fermentation (unpublished data). This may limit the potential of STH to become a thrombolytic agent. Therefore, it is necessary to improve the yield of STH to meet the demand of pre-clinical and clinical study.

In this report, we investigated STH expression under different fed-batch fermentation strategies. Three feeding mediums and two initial culture mediums were under estimate. And the optimized conditions were scaled up to 40 L fermentor.

MATERIALS AND METHODS

Strain and plasmid

E. coli BL21 was used as the host strain to express the fusion protein STH. The construction of the temperature-sensitive expression recombinant plasmid pBV220-STH was described in our previous report (Wang et al., 2009).

Medium composition

The R-medium (modified from Riesenberg et al., 1991) used for STH production contained the following materials (per L): Sucrose 20 g, KH_2PO_4 13.5 g, $(NH_4)_2HPO_4$ 4.0 g, $MgSO_4 \cdot 7H_2O$ 1.4 g, citric acid 1.7 g, ampicillin 0.1 g, antifoam (Sigma) 0.1 ml and trace metal solution 10.0 ml. The complex medium contained the following (per L): KH_2PO_4 3.0 g, K_2HPO_4 6.0 g, $(NH_4)_2SO_4$ 2.0 g, yeast extract (Merck) 5.0 g, tryptone (Merck) 10 g, glucose 6.0 g, $MgSO_4 \cdot 7H_2O$ 1.0 g, ampicillin 0.1 g, antifoam (Sigma) 0.1 ml and trace metal solution 2.0 ml. The trace metal solution consisted of the following (per L): $MnSO_4 \cdot 4H_2O$ 3.0 g, $CuSO_4 \cdot 5H_2O$ 6.0 g, KI 0.08 g, $Na_2MoO_4 \cdot 2H_2O$ 0.2 g, H_3BO_3 0.02 g, $CoCl_2$ 0.5 g, $ZnCl_2$ 20 g, $FeSO_4 \cdot 7H_2O$ 65.0 g, Biotin 0.2 g and H_2SO_4 5.0 ml.

Three types of feeding medium GM (glucose + magnesium sulfate), GMY (GM + yeast extract) and GMYT (GMY + tryptone) consisted of the following (per L): Glucose 500 g, magnesium sulfate 15 g; glucose 500 g, magnesium sulfate 15 g, yeast extract 100 g and glucose 500 g, magnesium sulfate 15 g, yeast extract 100 g, tryptone 200 g, respectively.

Seed culture

The engineered strain *E. coli* BL21/pBV-STH was streaked on lysogeny broth (LB)-agar plate containing ampicillin at a final concentration of 100 µg/ml and cultivated for 16 h at 30℃. A single colony was transferred to 5 ml LB medium in a 25 ml test tube and incubated at 30℃ for 12 h on a rotary shaker (220 rpm). Then, 0.2 ml of the above seed culture was transferred to 200 ml LB medium in a 1000 ml Erlenmeyer flask and incubated at 30℃ for 6 h on a rotary shaker (220 rpm). The above mentioned LB medium contained 100 µg/ml ampicillin.

Fed-batch fermentation

Optimization of feeding medium

The final seed culture was inoculated into a 5 L bioreactor (Bioflo, NBS, USA) containing 2.2 L R-medium. The maximum values of agitation speed and flow rate of aeration were set at 800 rpm and 4.5 vvm, respectively, on which the dissolved oxygen (DO) was set as 100% air saturation. Fermentation was carried out at 30℃. The initial agitation speed and flow rate of the aeration was 250 rpm and 1.0 vvm, respectively. The pH of the medium was maintained at 7.0 through automatic addition of aqueous ammonia (25% v/v). Feeding was initiated with a supply of one of the three feeding medium (GM, GMY and GMYT), when the DO was decreased first and then increased dramatically. The feeding speed was increased stepwise based on the DO fluctuation, which was maintained above 20% air saturation by adjusting the agitation speed and the flow rate of the aeration. When the agitation speed and flow rate of the aeration reached the maximum of the setting, adjustment of the feeding speed became the major means to maintain the DO above 20% air saturation. When the OD600 of the culture reached 60, the protein expression was induced by a temperature shift from 30 to 42℃ for another 3 h. Then the *E. coli* bodies were harvested by centrifugation at 5000 g, 4℃ for 15 min.

Optimization of initial culture medium

On the basis of the optimized feeding medium, the R-medium was replaced with complex medium. The feeding medium was GMYT and the feeding was started when a DO sparge was observed. STH expression was induced when OD600 > 60. The other fermentation conditions were the same as the earlier mentioned conditions.

Scale up of fermentation with the optimized initial culture medium and feeding medium

The optimized fermentation condition was scaled up to 40 L bioreactor (B. Braun, Germany) and repeated for three times.

Protein extraction and determination

The fermentation broth was centrifuged at 5000 g, 4℃ for 15 min and the cell pellet were washed with 20 mM Tris-HCl, pH 8.0 for two times. Protein was extracted from the cell pellet by 6 freeze-thaw cycles in 20 mM Tris-HCl buffer, pH 8.0. The supernatant was applied to 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and STH content was determined with gel scanning system. The total protein concentration of the supernatant was determined with Lowry et al. (1951) method.

Fibrinolytic activity assay of STH

Fibrinolytic activities of STH was determined on a fibrin plate containing 0.5% (w/v) agar, 0.5 NIHU/ml of human thrombin (Sigma), 10 nM of human plasminogen (Roche) and 0.5 mg/ml of bovine fibrinogen (Sigma). Ten microliters of samples were added to the precast wells on the plate and incubated at 37℃ for 16 h. A standard curve was made with serially diluted SAK (1×10^4 IU/mg), by plotting the logarithm of the lyzed zone diameter on the abscissa (X axis) against the logarithm of the specific activity on the ordinate

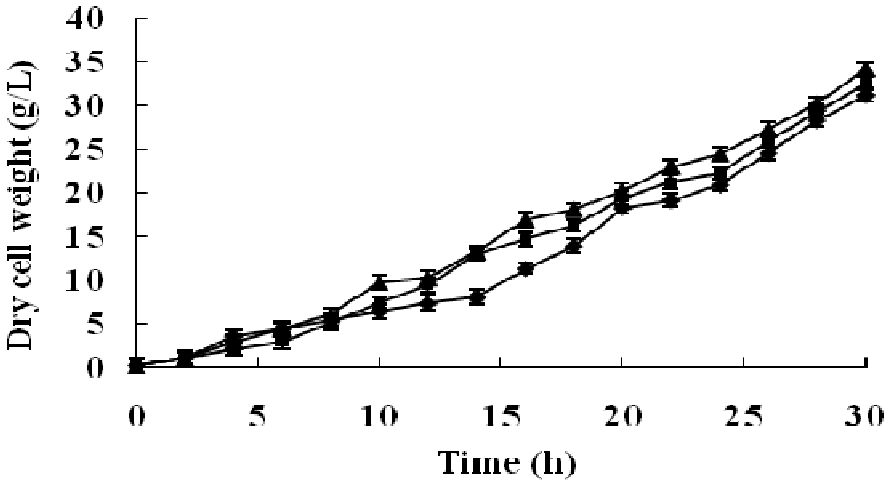

Figure 1. Dry cell weights of the recombinant *E. coli* in different feeding mediums. (Filled rhombus) GM, (filled square) GMY, (filled triangle) GMYT.

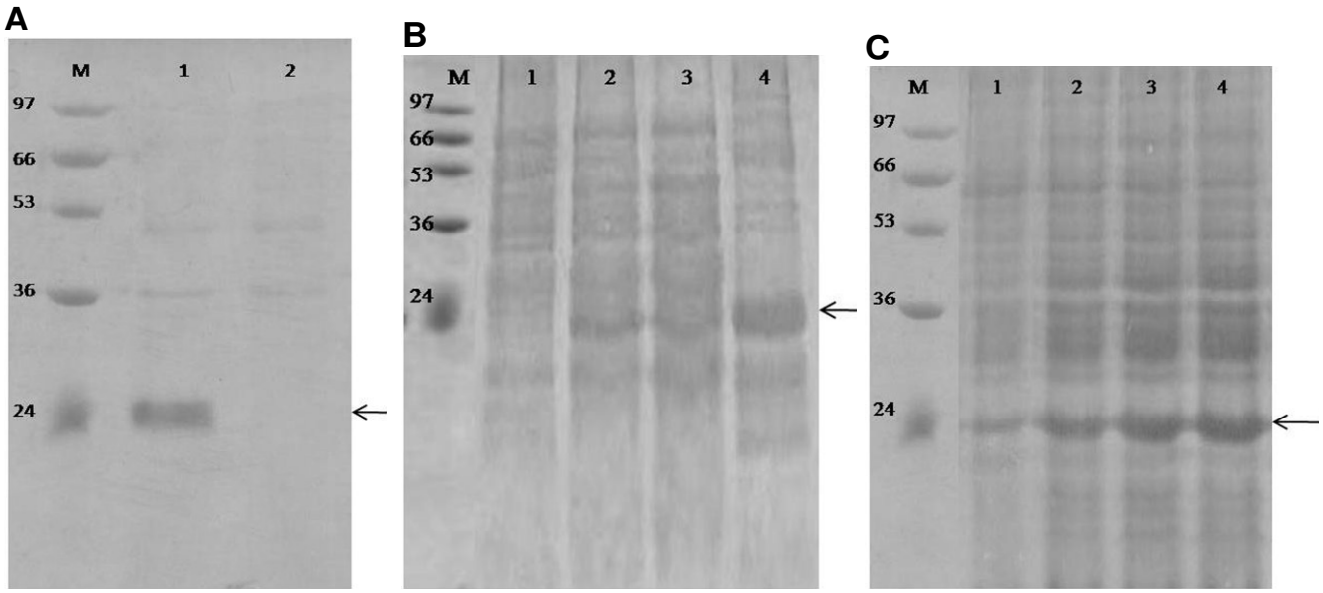

Figure 2. Effects of feeding mediums on the total protein and STH productions. To find the optimal feeding medium for STH production by *E. coli* BL21, three types of feeding mediums, GM, GMY and GMYT were fed to the fermentaion medium (R-medium) in 5 L fermentor, respectively. Protein productions was further analyzed by SDS-PAGE. The position of STH was marked by arrow in the gel; (A) GM as feeding medium; line M: protein maker; line 1: after heat induction; line 2: before heat induction. (B) GMY as feeding medium; line M: protein maker; line 1: before heat induction; lines 2 - 4: heat induction 1, 2 and 3 h. (C) GMYT as feeding medium; Line M: protein maker; line 1 - 4: heat induction 0.5, 1, 2 and 3 h.

(Y axis). Specific activities of STH were obtained from the standard curve.

Analytical methods

Dry cell weight (DCW) was gravimetrically determined using the pellet fraction from 20 ml samples. After centrifugation at 16,000 g for 10 min and washed twice with distilled water, the biomass was poured into preweighed aluminum cups and placed in a ventilating oven at 80℃ overnight until constant weights were obtained.

RESULTS

Effects of feeding medium on STH production

In order to find the optimal feeding medium for STH production by *E. coli* BL21, three types of feeding mediums, including GM, GMY and GMYT were fed to the fermentation media (R-medium) using 5 L fermentor, respectively. The DCW is shown in Figure 1, and the yield of total protein and STH in each case in Figure 2.

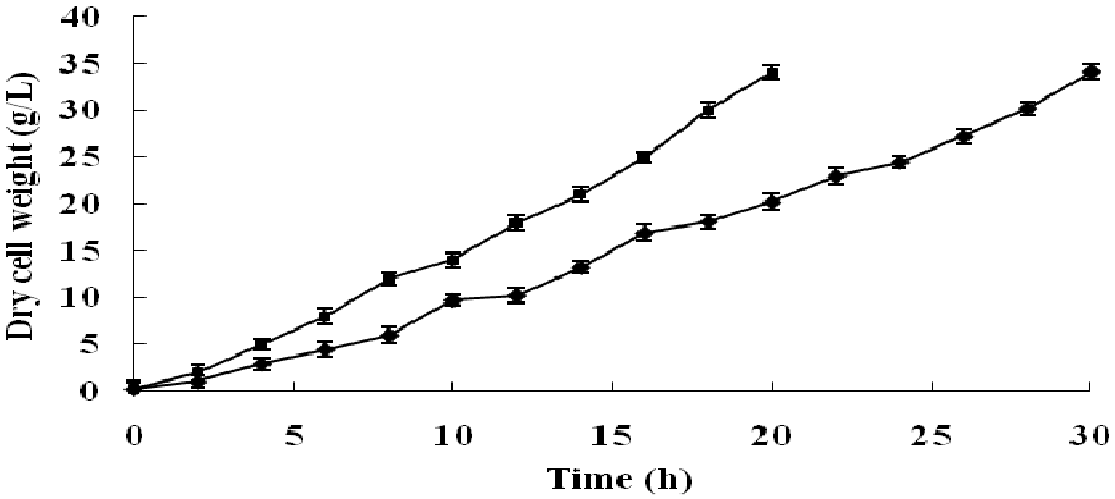

Figure 3. Dry cell weights of the recombinant *E. coli* in different initial culture mediums. (Filled rhombus) R-medium, (filled square) complex medium.

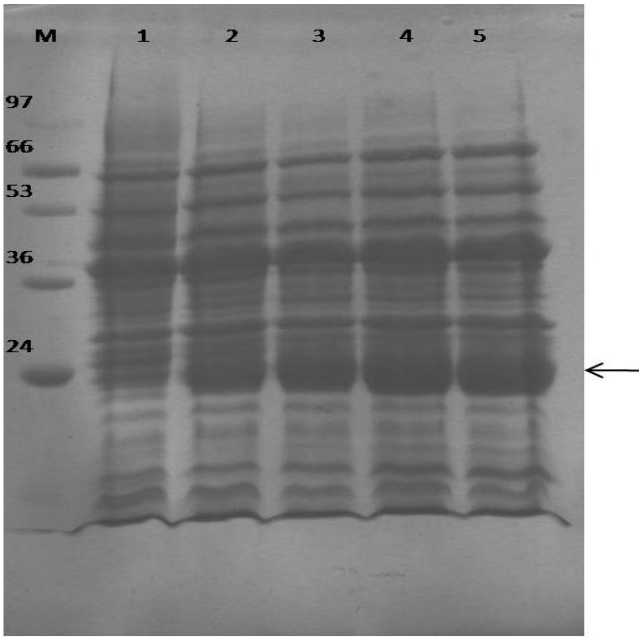

Figure 4. The total protein and STH expression by *E. coli* BL21 with complex medium. Protein productions was further analyzed by SDS-PAGE. The position of STH was marked by arrow in the gel; Line M: protein maker; line 1: before heat induction; lines 2 - 5: heat induction 0.5, 1, 2 and 3 h.

As shown in Figure 1, with different feeding mediums, *E. coli* BL21 exhibited a similar cell growth rate. The DCW were similar in the three feeding strategies. However, the yield of total protein and STH with GM and GMY as feeding medium were relatively lower than that with GMYT as feeding medium (Figure 2). The yield of total protein was: GM, GMY, GMYT = 1, 4.72, 7.36; the yield of STH: GM, GMY, GMYT = 1, 1.83, 3.16).

Effects of initial culture medium on STH production

The higher yield of STH under GMYT feeding medium prompted us to replace the initial R-medium with complex medium. Figure 3 shows that engineered *E. coli* grew faster in complex medium than in R-medium. The DO sparge was observed after 5 h of fermentation, which was shorter than that in R-medium fermentation (8 h). When complex medium was used, it took 17 h for the cells to achieve OD600>60 and the fermentation was completed at 20 h. However, the fermentation time in R-medium was as long as 30 h.

The yields of total protein and STH versus different initial medium were plotted in Figures 3 and 4. The total protein and STH in complex medium was 6.29 and 7.94 fold of those in R-medium culturing condition, respectively.

Pilot-scale cultivation in 40 L fermentor

In order to study whether the optimal conditions (complex medium as the initial medium and GMYT as the feeding medium) in 5 L fermenter could be scaled up, experiments in 40 L fermentor was carried out in three batches. The results (Figures 5 and 6) show that the reproducibility of the cell growth and the protein expression were well. The average of DCW reached 33.20 g/l, average total protein was 4.99 g/l and the average expression of target protein STH was 1.48 g/l.

DISCUSSION

In this study, we investigated the effects of feeding mediums and initial culture mediums to cell growth and

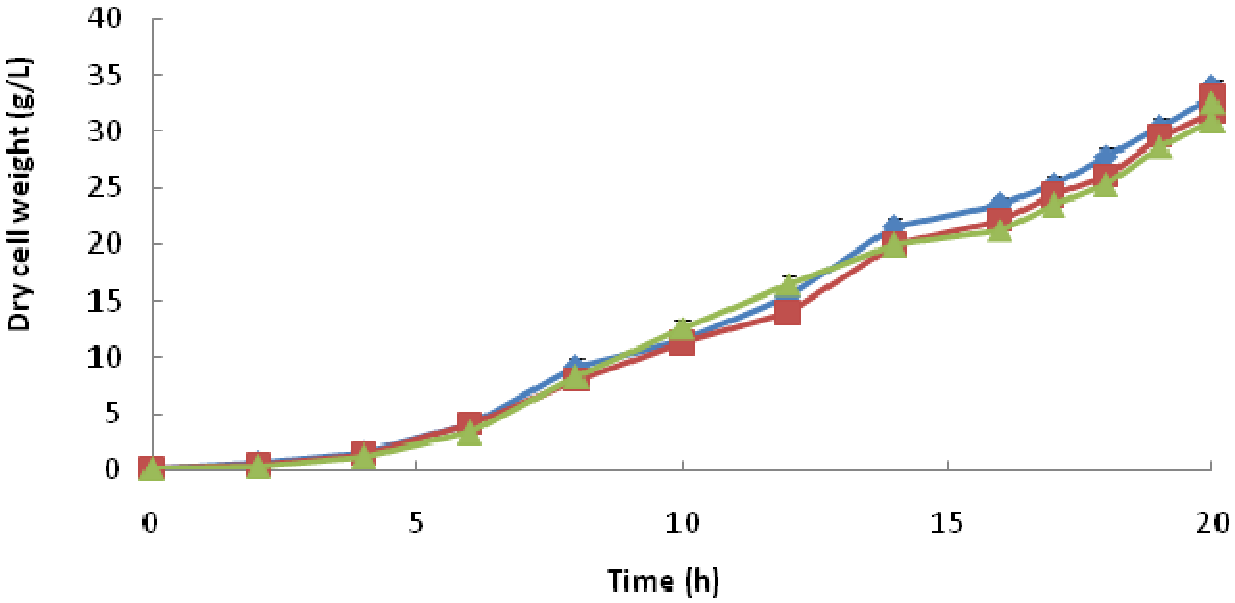

Figure 5. Dry cell weights of the recombinant *E.coli* at three batchs in 40 fermentor. (Filled rhombus) batch I, (filled square) batch II, (filled triangle) batch III.

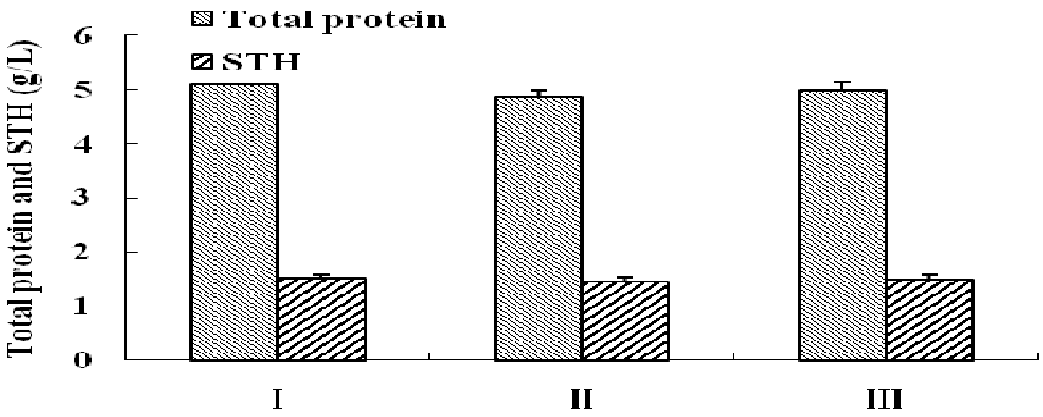

Figure 6. Total protein and STH productions at three batchs in 40 fermentor.

protein expression. As an initial culture medium, R-medium is widely used to achieve high cell density in *E. coli* (Lee and Chang, 1993; Riesenberg et al., 1991). The composition of R-medium is definitive. This makes the large-scale culture and the following purification of the target protein relatively easy.

In this paper, we selected R-medium as the initial medium during optimization of the feeding medium. Although the DCW at the end of fermentation in the three feeding strategies were similar, the total protein and STH expression were significantly different. This indicates that the high cell density may not infer to high protein expression. Since the GMYT feeding medium can increase the expression of STH (Figure 2), we hypothesize that the yeast extract and tryptone may contribute to the protein expression. Therefore, we replaced the R-medium with the complex medium which contains yeast extract and tryptone in fed-batch fermentation based on the optimal feeding strategy. The results (Figures 3 and 4) show that the complex medium cannot only support the high cell growth but also contribute to STH expression. The fermentation period of the optimized technology shortens 1/3 (20 h in complex medium, 30 h in R-medium). However, the protein expression increases 24.7 times (0.06 g/l STH in R-medium and GM feeding medium, 1.48 g/l STH in complex medium and GMYT feeding medium). As a whole, the efficacy of STH production increases 37 times after technology optimization. Moreover, the optimal fermentation technology can be successfully scaled up (Figures 5 and 6). This is very

Table 1. Summary of fed-batch fermentation for STH in *E. coli* BL21.

Initial medium	Feeding medium	Volume of fermentor (L)	Total time (h)	DCW (g/L)	Yield of total protein (g/L)	Yield of STH (g/L)	Specific activity (×10^5IU/L)
R- medium	GM	5	30	31.13	0.11	0.06	0.06
	GMY	5	30	32.42	0.52	0.11	0.11
	GMYT	5	30	34.12	0.81	0.19	0.19
Complex medium	GMYT	5	20	34.30	5.10	1.51	1.56
		40	20	33.21	4.99	1.48	1.53

important for us to fuel the industrialization of STH.

In summary, through optimization of the feeding media and the initial culture media, we successfully improved the production efficacy of STH. This may supply sufficient STH to meet the pre-clinical and clinical study and fundamentally support STH to develop into a thrombolytic agent (Table 1). However, it is a pity, due to the lack of pure oxygen equipment, that the final biomass is not very satisfying, as it only reached 33.20 g/l. Many reports have proved the important role of oxygen supply in *E. coli* high-density fermentation (Bauer and Shiloach, 1974; Herbert et al., 1965; Shiloach and Fass, 2005). Therefore, pure oxygen supply will be tried in our further study.

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