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Fermentation optimization of maltose-binding protein fused to neutrophil-activating protein from *Escherichia coli* TB1

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ABSTRACT

Background: The fermentation conditions of recombinant maltose-binding protein fused to neutrophil-activating protein (rMBP-NAP) of *Helicobacter pylori* were optimized from *Escherichia coli* TB1 with varying medium, inoculum age and size, time, inducer, pH and temperature in batch fermentation.
Results: It was revealed that the optimal conditions for the production of rMBP-NAP in shake flask were as follows: M9 medium (with 3% yeast extract powder added), inoculum age of 19 h, inoculum size of 6%, initial pH of 6.6, temperature of 37°C, and 0.7 mmoL/L IPTG induced 21 h in a 50 mL/250 mL shake flask. The recombinant protein yield was increased from 59 to 592 mg/L after optimization. Fermentation process conducted in a 10 L fermenter with similar conditions could get 30 g/L wet cell and 1.738 g/L soluble protein with the rMBP-NAP expression level of 11.9%.
Conclusion: The results improve the expression level of rMBP-NAP, and it is expected that these optimized conditions can be well applied for large scale production of rMBP-NAP.

1. Introduction

Helicobacter pylori, a typical gram-negative bacterium, which survives in an acid environment, infects about half of the whole human population [1,2,3]. *H. pylori* neutrophil-activated protein (HP-NAP) is a virulence factor that raises the neutrophils to the inflamed mucosal tissue during *H. pylori* infection and the purified recombinant protein has been shown to be chemotactic for human neutrophils and monocytes [4,5,6,7]. HP-NAP is a 150 kD oligomer extracted from the water extract of *H. pylori*, and crystal structure analysis shows that HP-NAP is a spherical dodecameric protein consisting of twelve identical monomers with a central iron-binding cavity, whose monomer is a 16.9 kD protein with a four-helix bundle structure [8].

HP-NAP is susceptible of elaborating an anti-tumor activity because of its capacity of creating an IL-12/IFN-γ-enriched milieu by acting on innate immune cells [4]. It is hard to get purified HP-NAP from *H. pylori* water extracts, since the amount of HP-NAP from water extraction is very small. *Escherichia coli* has been proven to be an appropriate host for large-scale production because it can grow in simple, inexpensive medium, and it is relatively easy to scale-up owing to a short fermentation cycle [9]. Fusion tag is employed to enhance the expression and productivity of soluble fusion protein [10], and maltose-binding protein (MBP) is frequently used to improve the solubility of its fusion partners [11]. The expression system of HP-NAP fused with MBP has been successfully established in our lab [12]. Recombinant plasmid pMAL-c2x-napA has been constructed, and a high expression of fusion protein can be obtained when induced by isopropyl-β-D-thiogalactoside (IPTG) [12]. However, the process yield was low, with only 5 g/L wet cell and 106 mg/L soluble rMBP-NAP obtained. So it was urgent to optimize the fermentation conditions of this fusion protein for the following pharmacological tests.

In order to enhance the production of rMBP-NAP, our study mainly focused on the optimization of the fermentation process for this fusion protein expressed in *E. coli*. The fermentation conditions in shake flask were optimized, followed by experiments in 10 L fermenter.

2. Materials and methods

2.1. Strain and chemicals

E. coli TB1 (pMAL-c2x-napA) was constructed and preserved in our lab [12]. IPTG was purchased from Merck, Germany. Protein Marker (12–120 kD) was obtained from Trans Gen Biotech Co., Ltd. (Beijing, China). Tryptone and yeast extract were purchased from Oxoid Ltd.

Table 1
Components of the medium screened.

Medium	Components (g/L)
LB	Tryptone 10, yeast extract 5, NaCl 10
TB	Tryptone 12, yeast extract 24, glycerol 4 mL, 17 mmol KH_2PO_4, 72 mmoL K_2HPO_4
$2\times YT$	Tryptone 16, yeast extract 10, NaCl 5
GYT	10% (v/v) glycerol, 0.125% (m/v) yeast extract, 0.25% (m/v) tryptone
MBL	Glucose 5, peptone 20, yeast extract powder 10, KH_2PO_4 3.5, K_2HPO_4 5, $(NH_4)_2HPO_4$ 3.5, NaCl 5, $MgSO_4 \cdot 7H_2O$ 1, trace element mixture* 3 mL
M9	5·M9** 200 mL/L, 20% glucose solution 20 mL, 3% (m/v) yeast extract powder

* Trace element mixture (g/L): $FeCl_3 \cdot 6H_2O$ 3.24, $ZnCl_2$ 0.22, $CoCl_2 \cdot 6H_2O$ 0.24, $NaMoO_4 \cdot 2H_2O$ 0.24, $CaCl_2 \cdot 2H_2O$ 0.12, $CuSO_4 \cdot 5H_2O$ 0.20, H_3BO_3 1.0, $MgSO_4$ 0.74.
** 5·M9 (g/L): $Na_2HPO_4 \cdot 12H_2O$ 85.48, KH_2PO_4 15, NaCl 2.5, NH_4Cl 5.0

(Hampshire, England). Peptone and yeast extract powder were obtained from Aobox Biotechnology Co., Ltd. (Beijing, China). BCA protein assay kit was purchased from Solarbio (Beijing, China). All other reagents used in this study were of analytical grade.

2.2. Optimization of the fermentation in shake flask

The fermentation was carried out in 250 mL Erlenmeyer flasks, which were incubated in a shaker at 200 rpm. The fermentation conditions include: the medium (the components are shown in Table 1), inoculum age, inoculum size, volume of the medium, induction time, inducer concentration, initial pH of the medium, and induction temperature [13,14,15,16]. Only one parameter was varied at a time during the optimization process. Single factor optimization is a useful tool to optimize fermentation conditions for its convenience though it ignores the interaction between factors.

2.3. Fermentation in 10 L fermenter

The fermentation process was scaled up in a 10 L fermenter (Zhenjiang East Biotech Equipment and Technology Co., Ltd., Zhenjiang, China) based on the above optimized conditions in flask as follows: 6 L M9 medium (with 3% yeast extract powder added), inoculum age 19 h, inoculum size 6%, the initial pH of the medium 6.6, temperature at 37°C and 0.7 mmoL/L IPTG inducted 21 h. The entire process was continuous without feeding and speed of agitator was kept at 150 rpm. Dissolved oxygen and pH value were monitored online.
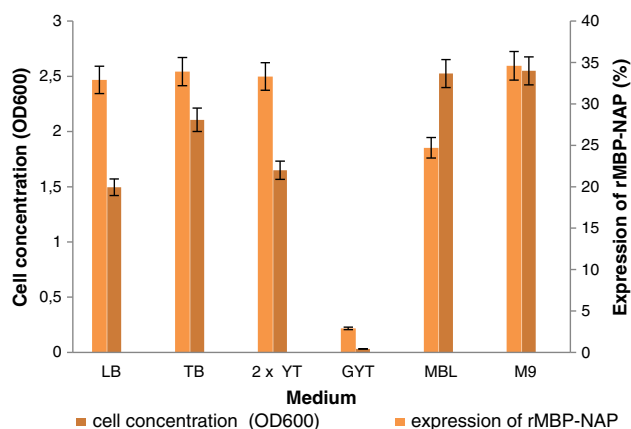

Fig. 1. Effect of the medium on the cell growth and rMBP-NAP production. The culture conditions were as follows: inoculum age of 12 h, inoculum size of 1%, initial medium pH of 6.6, temperature of 37°C and 0.3 mmoL/L IPTG inducted 3 h in a 150 mL/250 mL shake flask. M9 (with 3% yeast extract powder added) is represented by M9.
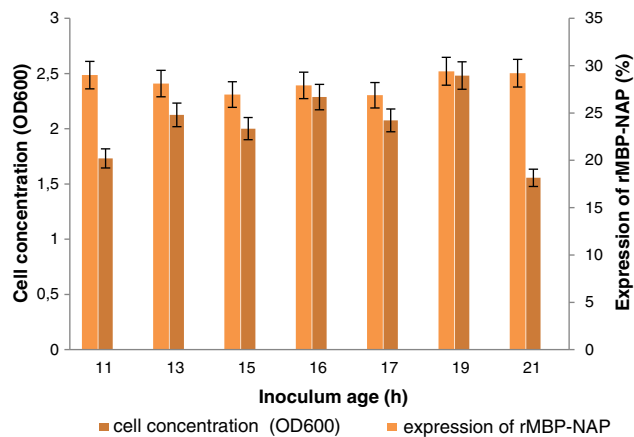

Fig. 2. Effect of inoculum age on the cell growth and rMBP-NAP production. M9 medium (with 3% yeast extract powder added) was employed; the other culture conditions were the same as those in Fig. 1.

2.4. Analysis methods

The optical density determined cell density at 600 nm (OD_{600}). A total of 4 mL fermentation broth was taken at predetermined time, followed by centrifugation at $4000 \times g$ for 5 min at 4°C, then 2 mL supernatant was collected for the analysis of cell concentration by a UV–VIS Spectrophotometer (UV-1700 Pharmaspec, Shimadzu, Japan).

The expression of induced fusion protein was identified by SDS-PAGE with Image J2X. The harvested cells were sonicated in an ice-water bath, followed by centrifugation at $9000 \times g$ for 30 min at 4°C to obtain the supernatant for SDS-PAGE [12]. The expression level was evaluated by the percentage of rMBP-NAP accounted for total protein by the gel image analysis system (Healforce, Shanghai, China) together with the program Image J2X (National Institutes of Health, USA). The protein amount was determined by BCA kit following the manufacturer's instructions.

3. Results and discussion

3.1. Optimization in Erlenmeyer flask

The medium formulation is an important factor influencing the final cell growth, which in turn affects the recombinant protein expression for *E. coli* and other fermentation systems [17]. Here medium including LB, M9 medium, TB, MBL, 2xYT, and GYT were screened, and the results are shown in Fig. 1. It could be concluded from Fig. 1 that
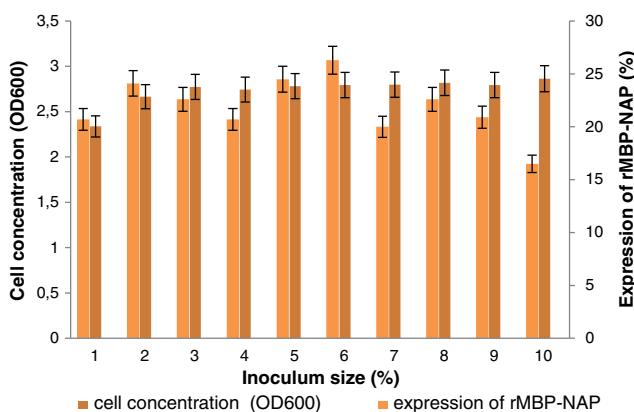

Fig. 3. Effect of inoculum size on the cell growth and rMBP-NAP production. The inoculum age was 19 h, and the other culture conditions were the same as those in Fig. 2.
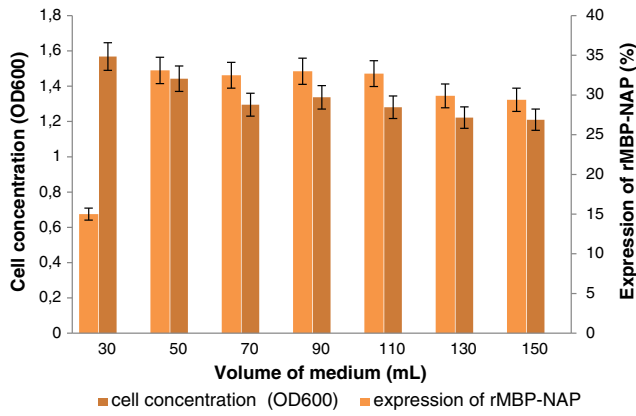
Fig. 4. Effect of medium volume on the cell growth and rMBP-NAP production. The inoculum size was 6%; the other culture conditions were the same as those in Fig. 3.

Fig. 6. Effect of inducer on the cell growth and rMBP-NAP production. The induction time was 21 h; the other culture conditions were the same as those in Fig. 5.

the final cell concentration of the recombinant *E. coli* TB1 and the expression level of rMBP-NAP were dependent upon the medium employed. The recombinant *E. coli* TB1 did not grow in GYT medium. M9 medium also did not support the cell growth (data not shown) due to the absence of yeast extract [18]. Yeast extract is a known source of trace components, which has been proved to increase both the growth rate of recombinant *E. coli* and the production of the target protein [19]. Compared with other medium, M9 with 3% yeast extract powder added achieved the maximum final cell concentration ($OD_{600} = 2.549$) and expression level of rMBP-NAP (34.6%). So M9 (with 3% yeast extract powder added) was chosen as the medium for further study.

Inoculum age and size are important variables for recombinant protein production from *E. coli* [20]. Effect of inoculum age on cell growth and rMBP-NAP expression was investigated, and the result is depicted in Fig. 2. The logarithmic growth phase of *E. coli* TB1 was between 10 h and 22 h. The optimum inoculum age was 19 h, with OD_{600} of 2.482 and rMBP-NAP expression level of 29.4% (Fig. 2). So the initial seed should be cultured for 19 h. As for the inoculum size, the results are shown in Fig. 3. When the inoculum size ranged from 3% to 10%, OD_{600} was higher than 2.7. The highest rMBP-NAP expression level (26.3%) was obtained when the inoculum size was 6%. So the optimum inoculum size was 6%.

During *E. coli* fermentation, dissolved oxygen level affected by medium volume usually influences cell growth and protein expression in flasks [21]. Effect of medium volume in 250 mL flask is illustrated in Fig. 4. Higher OD_{600} was obtained when the flask was filled with 30 or 50 mL medium, and higher expression (33.1%) was obtained for

50 mL medium. Then, 50 mL was chosen as the optimum medium volume in 250 mL flask.

Induction period also plays important roles in recombinant protein expression, and the proteins may degrade over a long induction time [22]. Effect of time can be concluded from the time profile of the fermentation, which is shown in Fig. 5. The final cell concentration increased with time, and then decreased after a peak at 17 h. Considering that the rMBP-NAP expression level at 21 h (34.9%) was higher than that at 17 h and the comparable OD_{600} at the two times, the optimum fermentation process should be continued for 21 h.

Both IPTG and lactose were usually employed as inducer for the fusion protein expression [23,24]. Here lactose and IPTG were compared, and the results are shown in Fig. 6. The expression level of rMBP-NAP induced by lactose was much lower than that by IPTG at the same concentration. Effect of IPTG concentration showed that 0.7 mmoL/L IPTG was optimum inducer concentration, which led to an OD_{600} of 1.982 and a protein expression level of 28.8%.

Effect of initial pH of the medium was shown in Fig. 7. All the OD_{600} were higher than 3 when the pH value of the medium was 6.2, 6.6 and 6.7, indicating that *E. coli* TB1 could grow up quickly in acid environments, while neutral (pH 7.0) or alkaline environment (pH 7.2) might not be suitable for the growth of *E. coli* TB1 (Fig. 7). Highest rMBP-NAP expression level (16.9%) was obtained when the pH of the medium is 6.6. Moreover, this pH value can be obtained by solution preparation from deionized water without extra acid or alkali added. Consequently, the optimum initial pH of the medium was 6.6.

The temperature could significantly influence protein denaturation, promotion or inhibition of the production of particular metabolites and
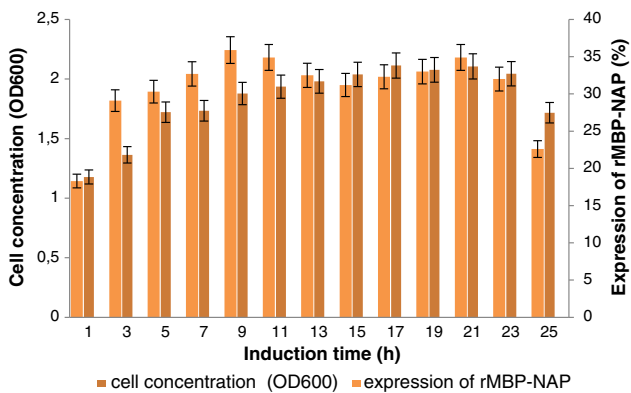

Fig. 5. Effect of induction time on the cell growth and rMBP-NAP production. The medium volume was 50 mL in a 250 mL flask; the other culture conditions were the same as those in Fig. 4.
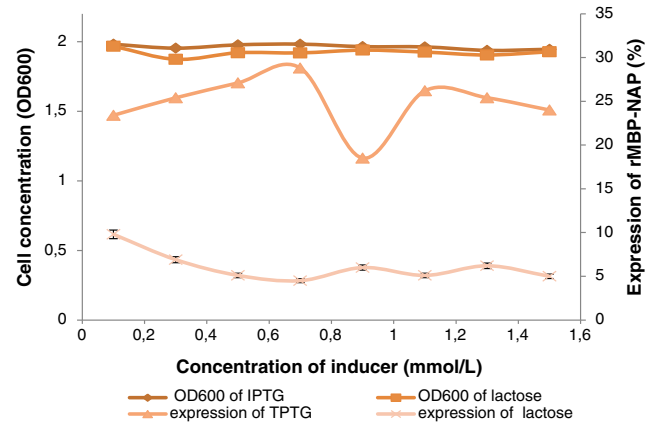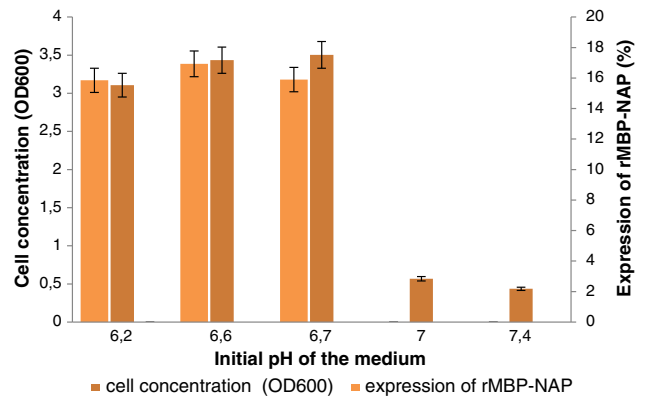

Fig. 7. Effect of initial pH of the medium on the cell growth and rMBP-NAP production. 0.7 mMIPTG was used as inducer; the other culture conditions were the same as those in Fig. 6.

Table 2
Comparison of fermentation before and after optimization.

	Not optimized	Optimized
Final cell concentration (OD_{600})	1.242	4.420
Expression level of rMBP-NAP	0.263	0.335
Yield of rMBP-NAP (mg/L)	59	592

cell death [25]. The performance of *E. coli* TB1 at varied temperatures showed similar cell growth and rMBP-NAP expression level (data not shown), so the temperature was controlled at 37°C.

Comparison of fermentation before and after optimization is summarized in Table 2. The final cell concentration increased more than three times (from 1.242 to 4.420) after optimization, while the yield of rMBP-NAP increased for about ten folds, from 59 mg/L to 592 mg/L. The optimization conditions could lay a foundation for the fermentation process scale up.

3.2. Batch fermentation in 10 L fermenter

Based on the optimized conditions in shaker flask, the fermentation was conducted in a 10 L fermenter, and the results were shown in Fig. 8. The logarithmic growth phase was from 0 h to 3 h. When IPTG was added at 3 h, rMBP-NAP could be detected. A total of 30 g/L wet cell and 1.738 g/L soluble protein were obtained after the fermentation at 21 h. The protein expression level was 11.9%. The above results were obtained without optimization, and no nutrients were added during the fermentation. Optimization in fermentor should be investigated further to improve the productivity and the expression of rMBP-NAP.

4. Conclusions

In the present study, the effects of different fermentation conditions on the rMBP-NAP production from *E. coli* TB1 were investigated. The yield of rMBP-NAP increased from 59 mg/L to 592 mg/L after optimization in the flask. The optimum conditions in 10 L fermenter could get 30 g/L wet cell and 1.738 g/L soluble protein with the rMBP-NAP expression level of 11.9%.
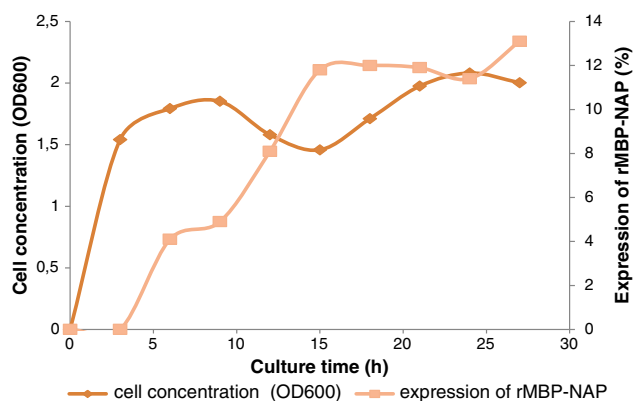
Financial support

Fig. 8. Time profile of the rMBP-NAP fermentation in a 10 L fermenter. 6 L M9 medium (with 3% yeast extract powder added), inoculum age 19 h, inoculum size 6%, initial pH of the medium 6.6, temperature at 37°C, shaking speed 150 rpm, and 0.7 mmoL/L IPTG inducted 21 h.

Author contributions

Proposed the theoretical frame: ZJ, QZ; Conceived and designed the experiments: QS, JL; Contributed reagents/materials/analysis tools: XL, TW; Wrote the paper: QS, JL; Performed the experiments: QS, XL, TW; Analyzed the data: ZJ, QZ, QS, JL.

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