

## Original Article

# A study of high cell density cultivation process of recombinant *Helicobacter pylori* multi-epitope vaccine engineering bacteria

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Received October 19, 2014; Accepted January 7, 2015; Epub January 15, 2015; Published January 30, 2015

**Abstract:** Objective: To establish high cell density cultivation process of recombinant *Helicobacter pylori* multi-epitope vaccine engineering bacteria BIB. Methods: Based on the results of shake flask fermentation, the process was magnified into volume of a 50 L fermenter to optimize and verify the factors affecting the yield of the target protein, such as the fermentation medium, working seed inoculation amount, inducer concentration, induction starting time, induction duration, inducer adding mode and feeding strategy. Results: After activated in modified TB medium at 37 °C for 8 h, the BIB working seed was inoculated at 5% (v/v) and was induced for expression for another 11 h by the final concentration of 5 mmol/L lactose. In growth phase, glucose at rate of 80 ml/h was used as carbon source, and in induction phase, glycerol at rate of 40 ml/h was used as carbon source; ammonia water was added dropwise to control pH at about 7.0, and revolution speed is adjusted to control the dissolved oxygen at above 30%; ultimately the output of bacterial body was 70 g/L and protein expression amount was about 32%. Conclusion: After high cell density cultivation of the recombinant engineering bacteria, expression and yield of the target protein rBIB significantly increased.

**Keywords:** *Helicobacter pylori*, Hp, multi-epitope vaccine, engineering bacteria, high cell density cultivation, HCDC

## Introduction

In 1983, Warren and Marshall isolated *Helicobacter pylori* (the abbreviation is Hp) from gastric mucosa biopsies of patients with chronic active gastritis; a large number of studies have confirmed that Hp is closely related to chronic gastritis, peptic ulcer and gastric cancer [1] and in 1994 Hp was listed as Class I carcinogenic factor by World Health Organization [2]. Hp is a pathogen infecting humans worldwide, with high infection rate in the population. In recent years, studies have found that among several recommended antibiotic drugs for eradicating Hp infection, metronidazole resistance rate reaches 60% to 70%, clarithromycin resistance rate reaches 20% to 38% and levofloxacin resistance rate reaches 30% to 38% [3], therefore, antibiotic therapy obviously

affects eradication rate of Hp infection. In order to completely eradicate Hp infection and reduce the use of antibiotics, immunizing effective Hp vaccine is an important prophylactic strategy in prevention and treatment of Hp.

Domestic and foreign scholars use bioinformatics technology to screen antigens which are related to Hp infection, and currently the relatively confirmative antigens include urease, adhesin, heat shock protein and neutrophil-activating protein [4] and so on. This project group has screened out dominant epitope gene sequences of its T cells and B cells from above genes, performed tandem fusion of gene sequences with cholera toxin B subunit (CTB) [5] to construct recombinant Hp multi-epitope vaccine (the abbreviation is BIB) engineering bacteria with intramolecular adjuvant, and has

confirmed that the recombinant protein (the abbreviation is rBIB) expressed by the BIB engineering bacteria has good immunogenicity and immune protection by a large number of experiments in vivo and in vitro. In this study, based on the results of shake flask fermentation, the process was magnified into volume of a 50 L fermenter and the factors affecting the yield of the target protein, such as the fermentation medium, working seed inoculation amount, inducer concentration, induction starting time, induction duration, inducer adding mode and feeding strategy, were further optimized and verified. This study has initially established high cell density cultivation (the abbreviation is HCDC) process of recombinant Hp multi-epitope vaccine engineering bacteria and has laid the foundation for further research rBIB protein and its large-scale production.

### Materials and methods

#### Materials

**Bacterial seed:** BIB engineered bacteria were constructed and maintained by this project group, and bacterial seed identification was in line with Recombinant Hp Multi-epitope Vaccine Engineering Bacteria Quality Standard.

**Instruments and main reagents:** A fermenter came from Shanghai Baoxing Biological Equipment Engineering Co., Ltd., model: BIOTECH-50JS 50 L; peptone and yeast extract were purchased from OXOID Company; specific anti-CTB monoclonal antibody was provided by Sichuan Vaccine Technology Co., Ltd.; other reagents were imported or domestic analytical reagents. LB, SOC, TB, SOB, 2×YT, Φb×broth media were prepared according to this project group' *Standard Operating Procedure on Medium Preparation* by referring to the method of "Molecular Cloning".

#### Methods

**Engineering bacteria seed screening and seed bank establishment:** BIB colonies with the highest expression amount and stable plasmids were selected to expand culture. After transferred at 1% volume ratio and then cultured to logarithmic growth phase, the bacteria were harvested; lyoprotectant was added for vacuum lyophilization; batch was written and they were stored at -80°C. The original seed bank, main seed bank and working seed bank were established by order.

**Activation of the working bacterial seed:** 1 item of working bacterial seed at -80°C (G20130514) was taken; after resuscitated with 500 μL LB liquid medium, it was inoculated into 10 mL LB liquid medium and shaken at 240 r/min at 37°C overnight, and then primary seed was obtained; the primary seed was taken at 1% ratio for inoculation in a 1 L shake flask and then cultured under the same conditions for 6 h, and then the secondary seed was obtained which was used as fermentation working seed and was placed at 4°C for backup.

**Screening of medium:** The medium most suitable for fermentation was screened from LB, TB and other three kinds of media. Fermentation working seed was inoculated into above media at 1% ratio; after growing at 37°C for 4 h, 5.0 mmol/L lactose was used to induce expression for 7 h and then bacterial body wet weight and protein expression amount were measured respectively.

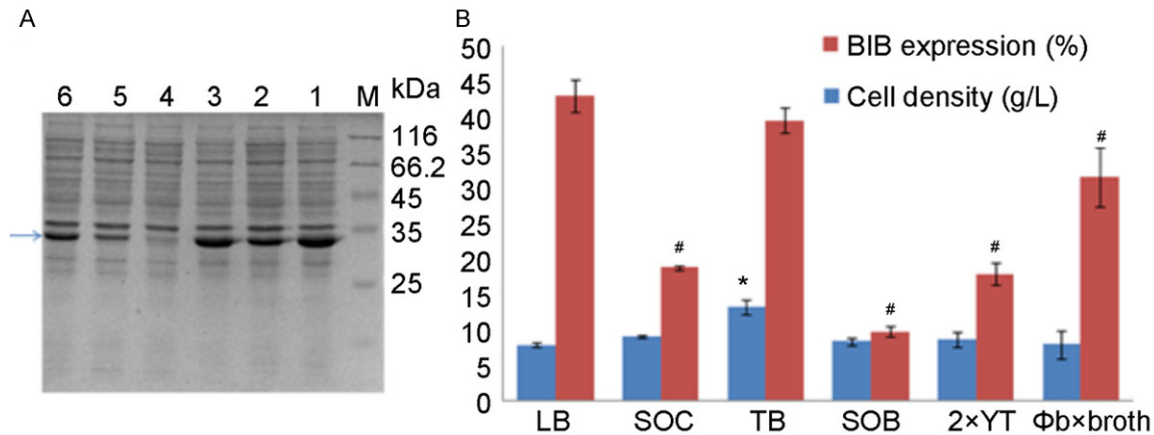
**The optimal concentration of inducer:** Fermentation working seed was inoculated into medium at 1% volume ratio; after growing at 37°C for 4 h, a final concentrations of 0, 0.5, 1.0, 2.0, 3.0, 4.0, 8.0 mmol/L lactose were added to induce expression for 7 h respectively, and with 7 h expression induced by 0.5 mmol/L of IPTG being used as positive control, bacterial body wet weight and protein expression amount were measured.

**The optimal induction starting time:** Fermentation working seed was inoculated into medium at 1% volume ratio; after cultured for 1, 2, 3, 4, 5, 6 h respectively at 37°C, a final concentration of 5.0 mmol/L lactose was added to induce expression for 7 h respectively, and 7 h expression induced by 0.5 mmol/L of IPTG was used as positive control; then, bacterial body wet weight and protein expression amount were measured.

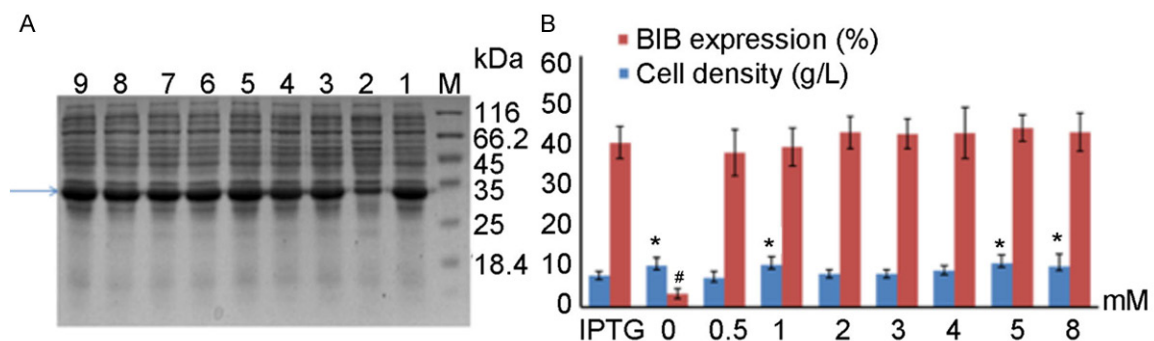
**The optimal induction time:** Fermentation working seed was inoculated into medium at 1% volume ratio; after cultured for 4 h at 37°C, a final concentration of 5.0 mmol/L lactose was added to induce expression for 2, 4, 5, 6, 7, 8, 9 h respectively, and 7 h expression induced by 0.5 mmol/L of IPTG was used as positive control; then, bacterial body wet weight and protein expression amount were measured.

**The inducing adding mode:** Fermentation working seed was inoculated into medium at 1% vol-

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**Figure 1.** Effects of different medium on BIB expression and cell density. A. SDS-PAGE analysis of the expression of BIB in different culture media. Lane M: Protein marker, lanes 1-6: the proteins of *E. coli* BL21 (DE3) expressing BIB (33 kDa, blue arrow) in different culture media (LB, SOC, TB, SOB, 2xYT, Φb×broth). B. Data are mean ± S.D.  $P < 0.05$  was considered as statistically significant,  $n = 3$ . Cell density: \* $P < 0.05$ , compared with the group of LB; expression level of BIB: # $P < 0.05$ , compared with the group of LB.



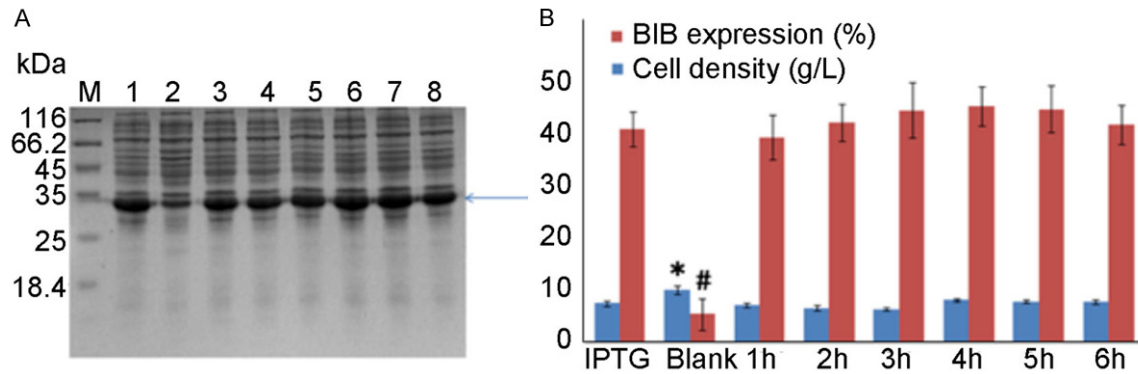
**Figure 2.** Effects of different concentrations of the lactose on BIB expression and cell density. A. SDS-PAGE analysis of BIB during lactose as inducer with different concentrations. Lane M: Protein marker, lanes 1: the proteins of *E. coli* BL21 (DE3) expressing BIB (33 kDa) after induced by IPTG; lanes 2-9: the proteins of *E. coli* BL21 (DE3) expressing BIB (33 kDa, blue arrow) after induced by different concentrations of lactose (0, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 8.0 mM). B. Data are mean ± S.D.  $P < 0.05$  was considered as statistically significant,  $n = 3$ . Cell density: \* $P < 0.05$ , compared with the group of IPTG; expression level of BIB: # $P < 0.05$ , compared with the group of IPTG.

ume ratio; after cultured for 4 h at 37°C, a final concentration of 5.0 mmol/L lactose was added once to induce expression for 7 h; 2.5 mmol/L lactose was added twice at 0 h and 3 h respectively, and the total induction time was 7 h; 1.25 mmol/L lactose was added four times at 0, 1, 2, 3 h respectively, and the total induction time was 7 h. Seven h expression induced by 0.5 mmol/L of IPTG was used as positive control. Then, bacterial body wet weight and protein expression amount were measured.

**The optimal inoculation amount of engineering bacteria:** Fermentation working seed was inoculated into medium at 0.1%, 0.5%, 1.0%, 1.5%, 2.0%, 4.0%, 8.0%, 10.0% volume ratio respec-

tively; after cultured for 4 h at 37°C, a final concentration of 5.0 mmol/L lactose was added to induce expression for 7 h, and 7 h expression induced by 0.5 mmol/L of IPTG was used as positive control; then, bacterial body wet weight and protein expression amount were measured.

**HCDC conditions of a fermenter:** According to the explored conditions from the shaking flask and the relevant literature, the medium was further optimized [6-10]. Thereafter, the temperature was set to 37°C, ammonia water was added to control pH at 7.0 in the fermenter, stirring speed was regulated (200~500 r/min) and the dissolved oxygen index was controlled at



**Figure 3.** Effects of the induction period on BIB expression and cell density. A. SDS-PAGE analysis of BIB during different induction period. Lane M: Protein marker, lanes 1: the proteins of *E. coli* BL21(DE3) expressing BIB (33 kDa, blue arrow) after induced by IPTG; lanes 2-8: the proteins of *E. coli* BL21(DE3) expressing BIB (33 kDa, blue arrow) during different induction period (1, 2, 3, 4, 5, 6 h); B. Data are mean  $\pm$  S.D.  $P < 0.05$  was considered as statistically significant,  $n = 3$ . Cell density: \* $P < 0.05$ , compared with the group of IPTG; expression level of BIB: # $P < 0.05$ , compared with the group of IPTG.

above 30% during the fermentation process. After the bacteria grew for 2 h, flowing feeding began at the speed of 80 ml/h; after induction, the speed was adjusted to 40 mL/h. After growing for 10 h, induce expression of the engineering bacteria with 5.0 mmol/L lactose, and sampling was performed once every 2 h for measuring fermentation liquid absorbance OD<sub>578</sub> value, bacteria body wet weight and protein expression amount.

**Analytic method:** After diluted 20 $\times$  by culture medium, 200  $\mu$ L of the fermentation liquid was taken into an ELISA analyzer to measure the absorbance value at 578 nm. Additionally, 100 ml bacterial liquid was taken for centrifugation at 13000 g at 4 $^{\circ}$ C for 10 min; after the supernatant was discarded, bacterial body wet weight was measured. After SDS-PAGE electrophoresis of the protein sample, a gel scanning analyzer was used to scan and Quantity-One software was used to analyze the expression amount of the target protein.

**Statistical method:** Measurement data were expressed as  $\pm$  s, and SPSS 19.0 software was used to perform univariate analysis of variance between various data groups.

## Results

### Screening of fermentation medium

Seen from **Figure 1A** and **1B**, in LB medium, the protein expression amount was the highest but the bacterial body wet weight was the low-

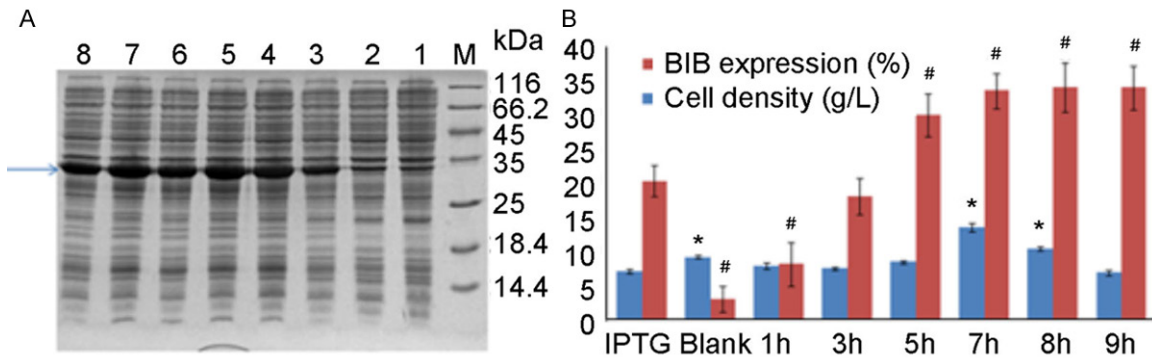
est, while in TB medium, the bacterial body wet weight was the highest and the protein expression amount was also higher; since LB medium was the most common *Escherichia coli* (the abbreviation is *E. coli*) medium, it was selected as the seed medium and TB medium was used as HCDC medium.

### The optimal induction concentration of lactose

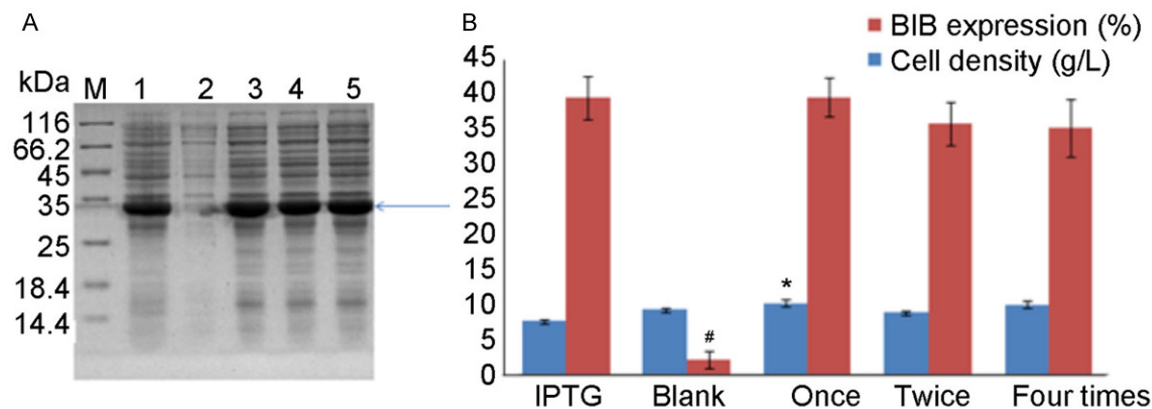
Seen from **Figure 2A** and **2B**, when concentration of lactose was between 0.5-5 mmol/L, bacterial body wet weight and protein expression amount increased with increase of lactose concentration. However, when lactose concentration was more than 5 mmol/L, bacterial body wet weight continue to increase, but protein expression amount decreased. Protein expression amount reached the highest level 42.7% when Lactose concentration was 5 mmol/L, therefore, 5 mmol/L was selected as the optimal induction concentration of lactose.

### The optimal induction starting time of engineering bacteria

Seen from **Figure 3A** and **3B**, when the induction starting time was between 3~6 h, bacterial body wet weight and protein expression amount were at higher level and the engineering bacteria growth curve confirmed that the bacteria were in logarithmic phase. At 4 h, both bacterial body wet weight (7.88 g/L) and protein expression amount (43.6%) were the highest, so induction was selected to start 4 h after inoculation.



**Figure 4.** Cell density and expression level of BIB at different stages after induced in fed-batch culture. A. SDS-PAGE analysis of BIB at different stages after induced. Lane M: Protein marker, lanes 1: the proteins of *E. coli* BL21 (DE3), lanes 2-7: expression level of BIB at different stages after induced in fed-batch culture (1, 3, 5, 7, 8, 9 h); lanes 8: the proteins of *E. coli* BL21 (DE3) expressing BIB (33 kDa, blue arrow) after induced by IPTG; B. Data are mean  $\pm$  S.D.  $P < 0.05$  was considered as statistically significant,  $n = 3$ . Cell density: \* $P < 0.05$ , compared with the group of IPTG; expression level of BIB: # $P < 0.05$ , compared with the group of IPTG.



**Figure 5.** Effects of different lactose Fed-method on BIB expression and cell density. A. SDS-PAGE analysis of BIB during different lactose Fed-method. Lane M: Protein marker, lanes 1: the proteins of *E. coli* BL21 (DE3) expressing BIB (33 kDa, blue arrow) after induced by IPTG; lanes 2: the proteins of *E. coli* BL21 (DE3), lanes 3-5: the proteins of *E. coli* BL21 (DE3) expressing BIB (33 kDa, blue arrow) during different lactose Fed-method (5 mM at one times; 2.5 mM for twice; 1.25 mM for four times); B. Data are mean  $\pm$  S.D.  $P < 0.05$  was considered as statistically significant,  $n = 3$ . Cell density: \* $P < 0.05$ , compared with the group of IPTG; expression level of BIB: # $P < 0.05$ , compared with the group of IPTG.

*The optimal induction time of engineering bacteria*

Seen from **Figure 4A** and **4B**, when the induction time was 7 h, bacterial body wet weight was the highest (13.66 g/L) and protein expression amount was 33.6% and when the induction time was over 8 h and 9 h, bacterial body wet weight decreased with increase of induction time. Therefore, the induction time of 7 h was selected.

*The lactose adding mode*

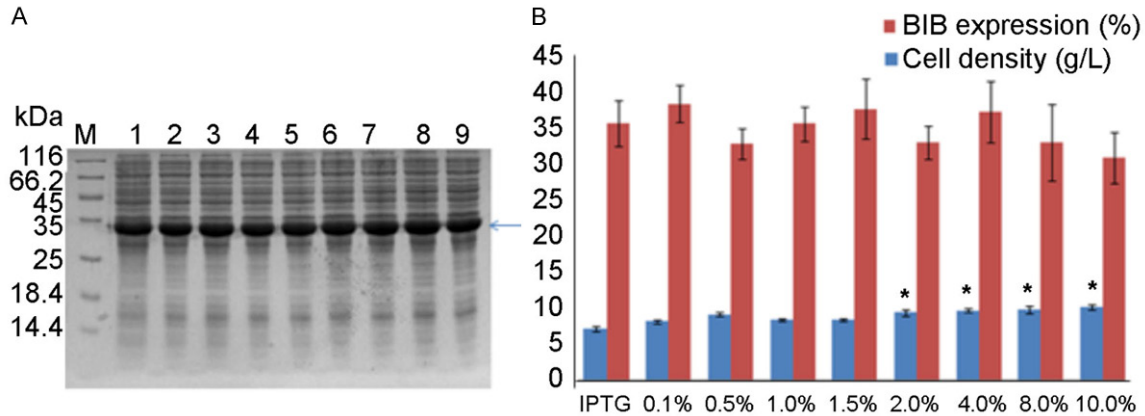
Seen from **Figure 5A** and **5B**, when lactose was added once, bacterial body wet weight (10.21

g/L) and protein expression amount (39.4%) were the highest, and when lactose was added twice and 4 times, BIB bacterial body wet weight decreased and rBIB expression amount did not increase. Therefore, lactose addition once was selected.

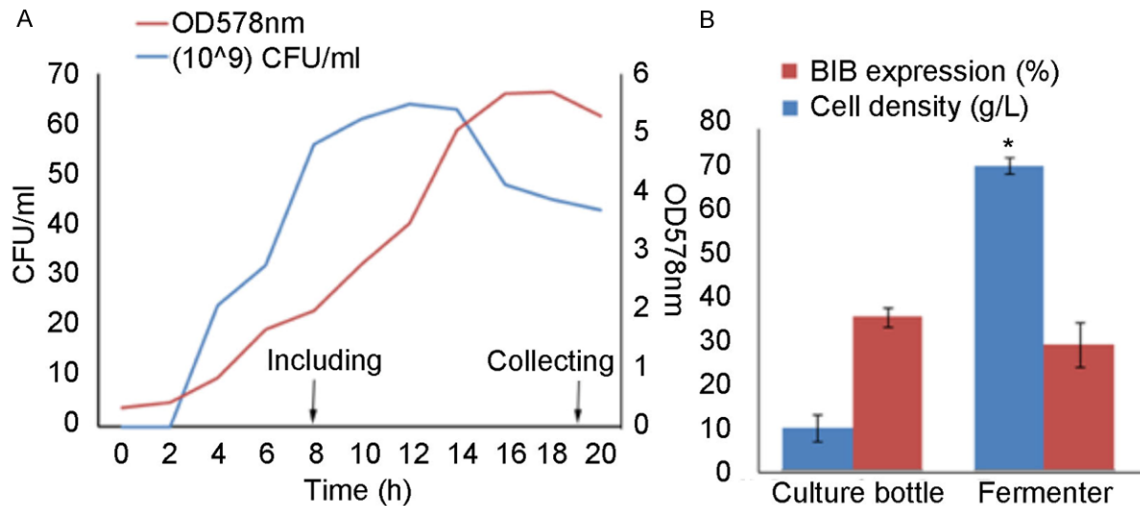
*The optimal inoculation amount of engineering bacteria*

Seen from **Figure 6A** and **6B**, when the engineering bacteria inoculation amount gradually increased, bacterial body wet weight gradually increased, but protein expression amount did not increase. It was taken into account that if the inoculation amount was too small, ferment-

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**Figure 6.** Effects of seed volume on BIB expression and cell density. A. SDS-PAGE analysis of BIB during different seed volume. Lane M: Protein marker, lanes 1: the proteins of *E. coli* BL21 (DE3) expressing BIB (33 kDa, blue arrow) after induced by IPTG; lanes 2-9: the proteins of *E. coli* BL21 (DE3) expressing BIB (33 kDa, blue arrow) during different seed volume (0.1%, 0.5%, 1.0%, 1.5%, 2.0%, 4.0%, 8.0%, 10.0%); B. Data are mean  $\pm$  S.D.  $P < 0.05$  was considered as statistically significant,  $n = 3$ . Cell density: \* $P < 0.05$ , compared with the group of IPTG.



**Figure 7.** Comparison of expression level of BIB produced in shake flask and in fermentor. A. The growth of *BIB* in the Fermenter. B. Data are mean  $\pm$  S.D.  $P < 0.05$  was considered as statistically significant,  $n = 3$ . Cell density: \* $P < 0.05$ , compared with the group of culture bottle.

tation time needed to be extended and if inoculation amount was too much, bacterial body growth in middle and late phases would be affected, therefore, 1% inoculation amount was selected for seed liquid (bacterial body wet weight: 8.33 g/L; protein expression amount: 35.3%) and 4% inoculation amount was selected for fermentation in a fermenter (bacterial body wet weight: 9.70 g/L; protein expression amount: 36.9%).

### HCDC conditions in a fermenter

Seen from **Figure 7A**, after activated at 37°C for 10 h, the *BIB* engineering bacteria were

inoculated into TB medium for fermentation at 4% volume ratio, and 5 mmol/L lactose was used to induce expression for 11 h. 4 h-5 h after inoculation was the logarithmic growth phase of the *BIB* engineering bacteria, when 80 ml/h glucose was used as supplemental carbon source; 5 h later, after the *BIB* engineering bacteria went into induction period, 40 ml/h glycerol was used as flowing feeding carbon source. During the whole process, ammonia water was added dropwise to control pH at about 7.0 and revolution speed was adjusted to control the dissolved oxygen at about 30%; ultimately, bacterial body wet weight output was 70 g/L and recombinant protein expression

amount was about 32%. The expression amount of BIB engineering bacteria increased with increasing induction time, and reactivity of the recombinant protein and the specific identification antibody (CTB mAb) increased with increasing protein expression amount. The fermentation experiment showed that compared with fermenter fermentation with shaking flask fermentation, high-density fermenter fermentation process could greatly enhance bacterial body wet weight ratio of BIB engineering bacteria, but the difference in expression amount of recombinant protein was not significant, which greatly enhanced the yield of rBIB protein. The detailed data were shown in **Figure 7B**.

### Discussion

When expression products of *E. coli* or its genetically engineered bacteria were produced by using general fermentation process, *E. coli*'s biomass, protein expression amount, metabolite concentrations in the bacterial body and in fermentation liquid are all relatively low, so it is difficult to achieve large-scale production. While high cell density cultivation (HCDC) is that under certain conditions and training system, the most number of engineering bacterial cells were obtained, thus the target product can be more efficiently obtained. That is, a certain culture technology and equipment are used to greatly enhance fermentation density of bacterial bodies and increase bacterial body density compared with general cultivation, thus ultimately improving the specific production rate of the product [11]. In addition, because *Escherichia coli* (*E. coli*) have simple structure, clear genetic background, short growth cycle and clear growth conditions, they have become the most commonly used host bacteria [12].

Lactose operon (*lac*) is an inducible negatively regulative operon that is currently most studied and widely used [13]. Inducer IPTG (Isopropyl beta-D-thiogalactopyranoside) is a highly-efficient inducer of *lac* promoter and non-metabolic inducer that can directly enter *E. coli* cells to exert induction effect. However, its potential toxicity has certain inhibitory effect on bacterial body growth, thus some countries have expressly stipulated that it cannot be used in production process of recombinant proteins for human use [14, 15]. Lactose is a natural inducer of *lac* promoter. Compared with IPTG, lactose is non-toxic, inexpensive and can be used as carbon and nitrogen sources, with its cost

being less than 1% of IPTG. Therefore, it is suitable for large-scale production [16]. Despite this, transport and conversion of lactose are more complex than IPTG and it can be metabolized by bacterial body, and affect physiology and metabolism of the bacterial body to certain degree. Therefore, the conditions of lactose used as the inducer need to be explored. This study found that when lactose concentration was between 0.5-5 mmol/L, recombinant protein expression amounts induced by IPTG and by lactose were similar; with increase of lactose concentration, bacterial body wet weight and protein expression amount all increased, however, when lactose concentration exceeded 5 mmol/L, bacterial body wet weight still increased while protein expression amount did not increase. The results have suggested that expression amount of recombinant protein is the highest when lactose concentration is 5 mmol/L.

High biomass obtained by HCDC of recombinant *Escherichia coli* requires very strict fermentation conditions and there are many factors affecting HCDC. When lactose is used as inducer, it is necessary to finely study and optimize bacterial body growth and induction conditions. Therefore, in order to improve the yield of the target protein rBIB, engineering bacteria growth conditions and expression conditions need to be optimized. This experiment first optimized various individual factors such as fermentation medium, working seed inoculation amount, inducer concentration, induction starting time, induction duration and inducer adding mode, and by using 50 L fermenter validation and relevant literature verification, continued to optimize fermentation conditions and ultimately obtained the optimal conditions for HCDC of the engineering bacteria. HCDC process increased biomass of the engineering bacteria by 7 times than shaking flask fermentation and had a similar expression amount of the target protein, thus the yield of the target protein also increased by nearly 7 times.

The BIB working seed was activated in TB medium at 37°C for 8 h; next, it was inoculated at 5% (v/v) and then was induced for another 11 h by the final concentration of 5 mmol/L lactose. In growth phase of the engineering bacteria, glucose at rate of 80 ml/h was used as carbon source, and in induction phase, glycerol at rate of 40 ml/h was used as carbon source; ammonia water was added dropwise to control pH at

about 7.0, and revolution speed was adjusted to control the dissolved oxygen at above 30%; ultimately output of the bacterial body was 70 g/L and the protein expression amount was about 32%. The study of this process lays a solid foundation for industrial production of recombinant Hp multi-epitope vaccine. However, the yield of rBIB needs to be further improved and deeper and systematic research and verification are needed.

### Disclosure of conflict of interest

None.

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