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ORIGINAL PAPER

High level expression of recombinant BoNT/A-Hc by high cell density cultivation of *Escherichia coli*

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Abstract The carboxylic domain of the *Clostridium botulinum* neurotoxin heavy chain (BoNT/A-HC), which has been reported as a vaccine candidate, contains the principle protective antigenic determinants. In this study, the high level expression of the BoNT/A-Hc was achieved by high cell density cultivation of recombinant *Escherichia coli* in a 2-l batch stirred-tank bioreactor. In order to maximize protein expression, post-induction time and IPTG inducer concentration were optimized by the Taguchi statistical design method. Results showed that the middle of the logarithmic phase and an IPTG concentration of 1 mM presented the optimum conditions for the maximum expression of BoNT/A-HC. High cell density cultivation was subsequently carried out as an effective strategy for the high level expression of recombinant BoNT/A-Hc. Consequently, soluble BoNT/A-Hc was produced at the maximum level of 486 mg l^{-1}, at 3 h post-induction, which was approximately 9.3 and 7.8 times higher than the levels produced by the shake flask and batch culturing methods, respectively.

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Introduction

The botulinum neurotoxins (BoNTs), synthesized by a family of seven toxinotypes (A–G), are responsible for potent food intoxication known as botulism, which is a severe neuroparalytic disease [1]. BoNTs possess similar structures but are immunologically distinct, each of which is expressed as the single polypeptide chain of 150 kDa [2]. These poorly active chains are proteolytically cleaved into the active di-chain domain, which consists of a 50-kDa light chain and a 100-kDa heavy chain (HC), linked by a disulfide bond [3]. The 50-kDa C-terminal fragment of the HC, known as the binding domain, recognizes surface receptors on target neurons, thus provoking the transmembrane internalization of the whole toxin [4, 5]. Botulism can be prevented by the presence of neutralizing antibodies (or vaccination) against the botulinum neurotoxins [1]. The current formalin-inactivated pentavalent vaccine for botulism is effective, but this vaccine requires large scale production of the highly toxic material, which involves extensive treatment with formalin [6]. Indeed, recombinant BoNT/A-Hc has been shown to be non-toxic and antigenic, and capable of eliciting a protective immunity in animals challenged with homologous BoNTs, because it contains the principal protective antigenic determinants. Therefore, this fragment has been used as an appropriate recombinant vaccine candidate for botulism [7, 8].

The *Escherichia coli* expression system with its ability of rapid growth, high expression levels, and well-characterized genetic background, is routinely used as an

ideal host system for the production of heterologous proteins [9, 10]. Recently, BoNT-Hc fragments of serotypes A, C, D and F were cloned and expressed in *E. coli*, but because of the relatively high AT content (%) of the *E. coli* genome, the expression levels of these fragments were found to be average or low [2, 3, 8, 11]. Therefore, the development of a simple and cost-effective strategy to obtain high yields of BoNTs fragments in *E. coli* is highly crucial and necessary.

The high cell density cultivation (HCDC) technique has been developed for growing recombinant *E. coli* in order to maximize volumetric productivity and specific yields of recombinant proteins [12]. HCDC offers several additional advantages, such as reduced culture volume, enhanced downstream processing, reduction in fermentation effluents, lower production costs and reduction of investment in equipments [13]. Fed-batch cultivation is a useful method for obtaining high cell densities within a short cultivation time [14].

In fed-batch fermentation, feeding strategy is the most important factor in having a successful HCDC process. Various feeding strategies, such as constant feeding, increasing feeding and the exponential feeding rate have been developed. The exponential feeding strategy in fed-batch fermentation is one of the most widely used methods for HCDC, because it is easy to implement the process and manipulate the specific growth rate. Also, the exponential feeding method has been developed to allow cells to grow at constant or variable specific growth rates; it also provides the advantage that acetate production, a serious problem associated with the process, can be minimized by controlling the specific growth rate below the critical value of acetate formation [12, 14, 20].

Moreover, in the HCDC method, in order to achieve high cell densities and productivity, optimization of the medium composition and physical conditions are also important factors to be considered [12]. In the previous study, we reported the optimization of the medium composition and operational variables using the Taguchi statistical design method for BoNT/A-Hc expression in recombinant *Escherichia coli* in shake flask conditions [11].

The aim of this research is to use the HCDC method for the high level expression of the recombinant soluble BoNT/A-Hc in *E. coli*. To this end we focused on the following three main aspects: (1) selection of the best strain based on three main factors; growth, expression level and plasmid stability, (2) optimization of IPTG concentration and OD of induction in batch fermentation using the Taguchi statistical design, and (3) using an exponential feeding rate for HCDC of recombinant *E. coli* to produce BoNT/A-Hc.

Materials and methods

Bacterial strain and plasmid construction

The *Escherichia coli* strain BL21 (DE3) as an expression system, and the pET28a vector with the histidine tag and the kanamycin resistant gene as a selection marker representing the expression plasmid, were both purchased from Novagen. The *bont/a-hc* gene was subcloned into the pET28a vector under the control of T7/lac promoter. The recombinant plasmid was then transformed into the *E. coli* strain BL21 (DE3) by the heat shock temperature method [16] and bacterial cells harboring the recombinant pET28a vector were stored in 30% (v/v) glycerol and 1% (w/v) peptone, at −70 °C.

Media and solutions

The Luria–Bertani medium (LB) composed of yeast extract (5 g l^{-1}), NaCl (10 g l^{-1}) and tryptone (10 g l^{-1}), was used for culturing recombinant *E. coli* in shake flask, and LB agar (LB medium containing 15 g l^{-1} agar) was used for bacterial plate cultivations. The pH values of the media were adjusted to 7.1 and then sterilized by autoclaving at 121 °C for 20 min.

The simple modified M9 medium that was optimized in the previous study [11] was used for flask, batch and fed-batch cultures. Optimum compositions of the modified M9 medium and feeding solution are shown in Table 1. The pH of the modified M9 medium was adjusted to 7.1 and the medium was sterilized by autoclaving as mentioned above. Glucose and $MgSO_4 \cdot 7H_2O$ in the media were sterilized separately. Based on the pET28a structure, kanamycin was added as a selection marker to the media after sterilization. Batch and fed-batch fermentations were carried out in a 5-l

Table 1 Composition of the modified M9 medium for batch culture and feeding solution for fed-batch culture

Composition	M9 modified medium	Feeding solution
Glucose (g l^{-1})	15	500
$MgSO_4 \cdot 7H_2O$ (g l^{-1})	4	30
$(NH4)_2SO_4$ (g l^{-1})	3.5	–
K_2HPO_4 (g l^{-1})	30	–
KH_2PO_4 (g l^{-1})	15	–
Citric acid (g l^{-1})	2	–
NaCl	0.5	–
Trace elements solution (ml l^{-1})[a]	1.5	–

[a] (g l^{-1} in 1 M HCl): $FeSO_4 \cdot 7H_2O$, 2.8; $MnCl_2 \cdot 4H_2O$, 2; $COSO_4 \cdot 7H_2O$, 2.8; $CaCl_2 \cdot 2H_2O$, 1.5; $CuCl_2 \cdot 2H_2O$, 0.2; $ZnSO_4 \cdot 7H_2O$, 0.3 g

fermentor (Bioflo III New Brunswick scientific, USA) with a 2-l working volume.

Selection of the best recombinant strain

The recombinant bacterial cells were spread onto LB agar plates containing 30 μg ml^{-1} of kanamycin as the selection marker. In order to select the best strain, 10 single colonies were picked from the LB agar plates and were each cultivated in LB medium for 15 h at 37 °C, with shaking at 150 rpm. Then, from among the cultivated clones, the best clone was selected by evaluation of three main factors: expression, growth and plasmid stability. The selected clone was then cultivated on LB agar plate supplemented with the appropriate antibiotic. After 24 h of cultivation, the resulting cultures were collected with a sterile loop into eppendorf tubes containing 30% (v/v) glycerol, 1% (w/v) peptone, and stored at −70 °C until further use.

Bacterial growth and expression of recombinant BoNT/A-Hc in shake flask

An inoculum was prepared by transferring a single colony from an LB agar plate into 5 ml of LB medium, which was then incubated on a shaker incubator (150 rpm) at 37 °C for 15 h. A 5-ml sample of the inoculum culture was used to inoculate a 250-ml flask containing 50 ml of LB medium supplemented with 30 μg ml^{-1} of kanamycin, and the cells were then cultivated at 37 °C and 150 rpm for 15 h. For the determination of cell growth, the culture was appropriately diluted with distilled water, and the optical densities were measured at 600 nm using a UV–vis spectrophotometer (Beckman DU530; Beckman Coulter, Inc., Fullerton, CA, USA). One OD unit corresponds to 0.5 g of dry cell weight (DCW) per liter. An uninoculated medium was used as a blank.

For protein expression, 5 ml of the overnight-cultivated culture was added to a 250-ml flask containing 50 ml of LB medium containing 30 μg ml^{-1} of kanamycin, and incubated at 37 °C on a shaker incubator (150 rpm) until an OD_{600} of 1.2 was achieved. The culture was then induced with IPTG (0.7 mM) and cultivated overnight at 30 °C (150 rpm). The bacterial cells were collected by centrifugation (5,000 rpm, 10 min) and protein expression was evaluated by SDS-PAGE. All experiments for the recombinant protein expression in shake flask were repeated twice.

Plasmid stability

The plasmid stability of recombinant cells was evaluated during 6 days of subculturing. A 5-ml inoculum culture was grown in a 50-ml flask under the same conditions as mentioned above. After 24 h of growth, subculturing was carried over a 6-day period by transferring 5 ml of the inoculum into 50 ml of LB in a 250-ml flask, and the culture was then incubated for 24 h as mentioned above. Subsequently, colony counts within a range of 100–150 were obtained by spreading 100 μl of a diluted sample (in 0.85 (w/v) NaCl) of the incubated culture onto LB plates without the antibiotic. After 24 h of incubation, 100 colonies were selected and transferred to LB plates supplemented with kanamycin and incubated under the same conditions mentioned above. The percentage plasmid stability was calculated from the number of plasmid-containing cells that were able to grow on LB agar plates containing kanamycin [15].

Western blot analysis

The recombinant BoNT/A-Hc was verified by the Western blotting technique. Following SDS–PAGE analysis of the recombinant BoNT/A-Hc, the expressed product was transferred from gel to a nitrocellulose membrane using transfer buffer (2.93 g l^{-1} glycine, 5.81 g l^{-1} Tris-base, 0.37 g l^{-1} SDS and 200 ml l^{-1} methanol, pH 8.3). The membrane was then suspended in the blocking buffer (1.25% (w/v) non-fat dried skimmed milk powder) and shaken for 2 h at room temperature (25 °C). After three washes with washing buffer (2.42 g l^{-1} Tris-base, 18.1 g l^{-1} NaCl, and 3.87 ml HCl at 6 N), the membrane was incubated in a 1:100 dilution of rabbit anti-BoNT/A-Hc [6], with gentle shaking for 1 h. Subsequent to washing, the blots were immersed in a 1:1,000 dilution of the polyclonal anti-rabbit conjugate [6], then washed three times in washing buffer and incubated in 2 ml of substrate solution (the substrate solution contained 30 mg of 4-chloro-1-naphthol and 10 ml of methanol, in 10 ml of washing buffer supplemented with 8 μl of 35% (v/v) H_2O_2). Once a protein band was visualized, the reaction was stopped with distilled water.

BoNT/A-Hc assay

Soluble cytoplasmic proteins from recombinant *E. coli* were extracted as follows. Cells were harvested as above and then resuspended in sonication buffer (fivefold of the sample weight) containing 50 mM Na–phosphate, 300 mM NaCl and 5 mM imidazole (pH 7.6). The cell suspension was then lysed with four short bursts of sonication (on ice). Phenylmethylsulphonyl fluoride (PMSF) as the inhibitor of serine protease was added to the suspension only during the initial sonication. The lysate was thereafter centrifuged (14,000 *g*, 10 min) to remove cell debris and non-soluble proteins. The total soluble protein concentration in the resulting supernatant was quantified using the Bradford dye method with BSA as a standard [16], and separated and

visualized by electrophoresis of the protein sample on a 13% (w/v) polyacrylamide gel containing sodium dodecyl sulfate (SDS). The gel was subsequently stained with the Coomassie Blue solution and destained with methanol and acetic acid. After destaining, the relative abundance of BoNT/A-Hc was determined by densitometry of the bands on the gel using an UltraScan XL Gel Doc densitometer (Pharmacia, Bromma, Sweden) [11, 17].

Statistical design

In order to maximize protein expression during batch fermentation, the optimized M9 medium [11] was selected as a simple medium, and the OD of induction and IPTG concentration were optimized using the Taguchi statistical method. The L4 orthogonal array was used to optimize the OD of induction and IPTG concentration at two levels in the batch fermentation (Table 2). The Qualitek-4 software was used for automatic design and evaluation of the standard analysis of variance (ANOVA) of the Taguchi experiments. ANOVA revealed and identified the main factor effects, optimum conditions, and also estimated the performance of the optimum conditions.

Batch fermentation

Batch fermentation was performed in a 5-l Bioflo III New Brunswick scientific fermentor (USA) with a working volume of 2 l that was equipped with a digital control system.

Seed culture was prepared by inoculating a 1-l flask containing 200 ml of optimized M9 medium with a single colony. The culture was then incubated at 37 °C overnight with shaking at 150 rpm [11]. Batch culture was initiated by the addition of 200 ml of seed culture to the bioreactor containing 1,800 ml of M9 medium. The batch conditions included a pH of 7.1 controlled by the addition of 25% (v/v) aqueous ammonia, a temperature of 37 °C which was maintained throughout the fermentation, an agitation speed of 900 rpm and air flow rate of 1.5 vvm. Dissolved oxygen (DO) was monitored by a DO electrode and maintained above 40% air saturation by controlling the agitation rate, air flow rate, and automatic mixing of air and oxygen. Foam was controlled by addition of sterilized silicon oil as an anti-foam reagent.

During batch fermentation, aseptic sampling was carried out at different cultivation times, in order to evaluate plasmid stability, OD and glucose concentration. All experiments in batch fermentations were repeated twice.

Table 2 L4 orthogonal array for studying the effect of OD of induction and IPTG concentration in batch culture

Trial no.	OD of induction	IPTG concentration (mM)
1	4.5	1
2	4.5	0.5
3	10	0.5
4	10	1

Fed-batch fermentation

After depletion of glucose levels and an increase in the dissolved oxygen content during batch fermentation, the fed-batch phase was initiated using an exponential feeding strategy based on the following equation (Eq. 1):

$$\mathrm{Ms(t)} = \left[\mu_{\mathrm{set}}.X_{\mathrm{F}}.V_{\mathrm{F}}/Y_{\mathrm{x/s}}.S + m\,(X_{\mathrm{F}}.V_{\mathrm{F}}/S)\right] \cdot \exp(\mu_{\mathrm{set}} \cdot t) \quad (1)$$

where Ms(t) is the mass feeding rate (g h^{-1}); μ_{Set}, the specific growth rate ($h^{-1)}$; $Y_{\mathrm{x/s}}$, the yield of biomass (g DCW g^{-1} glucose); m, the specific maintenance coefficient (g h^{-1}); X_{F}, the biomass concentration at the start of feeding (g_{DCW} l^{-1}); V_{F}, the volume of the medium in the bioreactor (l); t, the time (h) and S the substrate concentration (g l^{-1}).

During fed-batch cultivation, specific growth rate was fixed at a value of 0.2 h^{-1} or less and induction was performed by the addition of 1 mM IPTG when OD had reached 90 at 600 nm. Fermentation was terminated 4 h after induction. In the batch and fed-batch fermentations, glucose (as the sole carbon source) and acetate concentrations were analyzed by an enzymatic kit (Chem Enzyme, Co., Tehran, Iran).

Results

Selection of the best clone

Among the 10 single *E. coli* colonies that were grown following transformation with recombinant pET28a, the most suitable recombinant strain was selected using three criteria: expression level, growth rate and plasmid stability. The results indicated that the growth for the cells in shake flask was the same for all 10 clones (data not shown), whereby the logarithmic phase of growth started after 1 h of incubation at 37 °C, and growth finished after 8 h when an OD and DCW of 3.6 and 1.8 g l^{-1} were obtained, respectively (data not shown). Expression of BoNT/A-Hc by the recombinant cells before and after induction with IPTG was evaluated by SDS–PAGE [13% (w/v)] analysis (Fig. 1) and its identity was then validated using the Western blotting technique (Fig. 2). Figure 1 shows bands corresponding to a 50-kDa BoNT/A-Hc following SDS–

PAGE of total soluble proteins extracted from the selected clones. Results of gel densitometry showed densities of 16, 16, 14, 16, 17, 15, 15, 10, 14 and 15% with regard to the BoN/A-Hc bands relative to the total soluble protein for each of the 10 colonies. Therefore, clone 5 was considered for further evaluations. Figure 3 shows plasmid stability of the best clone (clone 5) during 8 days of subculturing in antibiotic-free LB medium and shows 80% plasmid stability after 8 days of subculturing. Thus according to the above findings, clone 5 (lane 7 in Fig. 1) was selected and stored at −70 °C, and used as a suitable recombinant strain for further BoNT/A-Hc production in the shake flask, batch and fed-batch fermentations.

Batch fermentation

In order to investigate the growth of recombinant *E. coli*, BoNT/A-Hc expression and plasmid stability, batch fermentation was carried out in a 5-l fermentor containing the modified M9 medium [11]. Bacterial growth during batch fermentation indicated that the logarithmic phase of growth started after 4.5 h of cultivation, when an OD of 4.5 was reached. Also, when an OD of approximately 20 (8 h of fermentation) was reached, glucose levels were depleted resulting in the termination of growth. Furthermore, plasmid stability was found out to be less than 90% stable during the fermentation process (data not shown).

An L4 orthogonal array was used to identify the optimum induction time and IPTG concentration variables (Table 2).

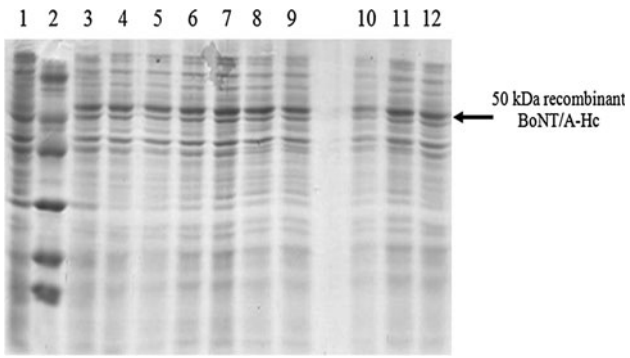

Fig. 1 SDS-PAGE (13% w/v) analysis of recombinant BoNT/A-Hc expression in 10 *E. coli* strains before and after induction with IPTG in shake flask cultures. *Lane 1* before induction, *lane 2* protein marker, *lane 3–12* recombinant strains designated 1–10, respectively

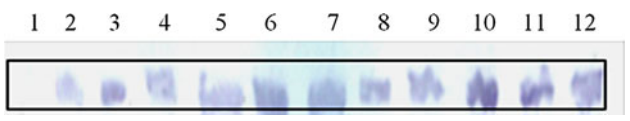

Fig. 2 Western blot analysis of BoNT/A-Hc recombinant expression for 10 strains with anti-BoNT/A-Hc. *Lane 1* before induction, *lane 2* purified BoNT/A-Hc; *lane 3–12* recombinant strains 1–10

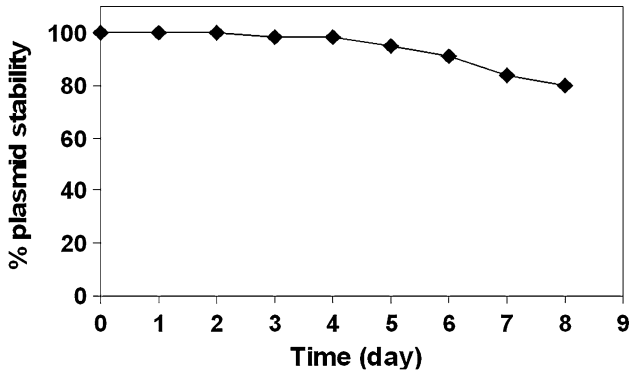

Fig. 3 The plasmid stability of the selected recombinant *E. coli* strain in grown LB medium during 8 days of subculturing

Results were then analyzed by standard ANOVA for determination of the optimum points of the variables. These data showed that an optimum induction time at the middle of the logarithmic phase (OD 10) and 1 mM IPTG led to a better yield of BoNT/A-Hc (Table 3). Profiles of OD, glucose and recombinant soluble BoNT/A-Hc concentrations during the course of the batch fermentation under optimum conditions are shown in Fig. 4. Under the optimized conditions of the batch fermentation, the maximum OD, recombinant soluble BoNT/A-Hc concentration and productivity, at 4 h after induction, were 13, 62 mg l^{-1} and 5.1 mg l^{-1} h^{-1}, respectively.

Fed-batch culture

Fed-batch process was carried out as a robust strategy to achieve a maximum recombinant BoNT/A-Hc yield. In this study, exponential feeding strategy was used for fed-batch cultivation of the recombinant *E. coli* strain to obtain a high biomass and therefore, a high recombinant BoNT/A-Hc concentration.

Glucose concentration, feeding rate and BoNT/A-Hc profiles during the batch and fed-batch phases are shown in Fig. 5. In the batch phase, after 6 h, the initial glucose was consumed and the fed-batch phase was initiated by exponential feeding with a fixed specific growth rate of 0.2 h^{-1}. The fed-batch fermentation was induced by 1 mM IPTG, when an OD of 90 was reached, and continued for another 4 h after induction.

As shown in Fig. 5, glucose concentration during fed-batch fermentation was insignificant. Also, after induction (with 1 mM IPTG), there was a reduction in the rate of growth as glucose concentrations were increasing.

Figure 6 shows SDS–PAGE analysis of total soluble proteins before and 4 h after induction in fed- batch cultivation. BoNT/A-Hc concentration assay indicated that the specific product reached its peak value at 3 h after induction. The maximum OD and concentration of recombinant

Table 3 ANOVA with regard to the effects of OD of induction and IPTG concentration on BoNT/A-Hc expression in batch fermentation

Factor	DOF	F-ratio	Optimized level	Percentage contribution (%)
OD of induction	1	7,562,499	10	43.2
IPTG concentration	1	9,922,499	1 mM	56.8

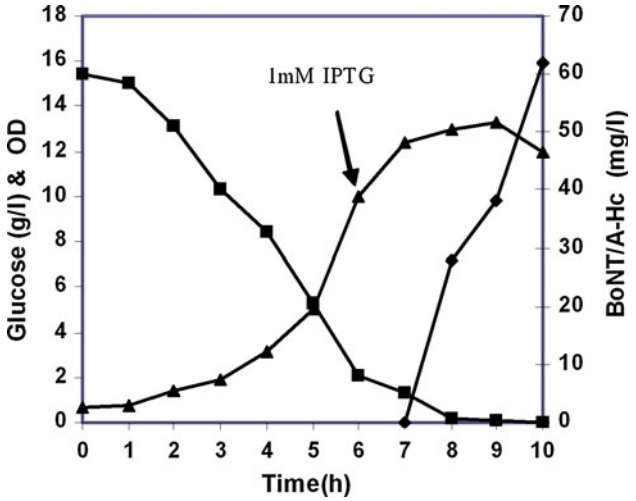

Fig. 4 OD (*closed triangles*); glucose concentration (*closed squares*) and soluble cytoplasmic BoNT/A-Hc concentration (*closed diamonds*) profiles of the recombinant *E. coli* strain during batch fermentation

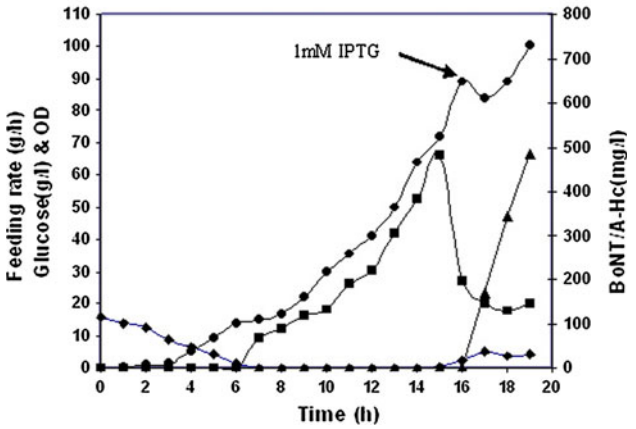

Fig. 5 Feeding rate (*closed squares*); glucose concentration in the medium (*closed diamonds*); OD (*closed circles*); Soluble cytoplasmic BoNT/A-Hc concentration (*closed triangles*) profiles during high cell density cultivation (HCHC) of the recombinant *E. coli* strain with an exponential feeding strategy

soluble BoNT/A-Hc obtained at 3 h after induction were 100 and 486 mg l^{-1}, respectively.

The results of fed-batch cultivation in the 2-l fermentor showed that maximum level of soluble BoNT/A-Hc was 486 mg l^{-1} at 3 h of induction, which had increased by approximately 9.3- and 7.8-fold when compared to the shake flask and batch fermentations, respectively.

Discussion

The carboxylic domain of the *C. botulinum* type A neurotoxin heavy chain (BoNT/A-Hc) has been reported as a vaccine candidate for the prevention of botulism [8]. Recently efforts have been made to produce recombinant BoNT/A-Hc in *E. coli* or yeast, because the recombinant protein is less expensive and less hazardous to produce than the traditional toxin-inactivated vaccines [9]. As mentioned previously, the high AT content (%) in the *E. coli* genome has been found to lead to low level expression of the BoNT fragments [3, 8, 10]. This was reflected in the previous study where operational and M9 medium composition variables were optimized using the Taguchi statistical method for the expression of recombinant BoNT/A-Hc in *E. coli* BL21, but the yield of the soluble recombinant BoNT/A-Hc under the optimized

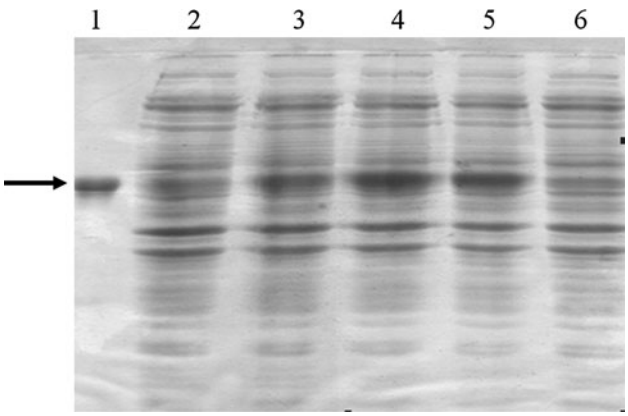

Fig. 6 SDS-PAGE analysis of HCDC before and 4 h after induction (15 h from the start of fermentation). *Lane 1* standard BoNT/A-Hc, *lanes 2–5* total bacterial proteins at 1–4 h after induction, respectively, *lane 6* total bacterial proteins before the induction

conditions in the shake flask culture was only 52.1 mg l^{-1} [11]. Hence, a suitable method for the high level expression of the soluble BoNT/A-Hc in *E. coli* as a vaccine candidate is highly crucial and of vital importance.

Because most proteins are accumulated intracellularly in recombinant *E. coli*, productivity of the recombinant protein is proportional to the final cell density and high density or high biomass can be achieved by the HCDC method [12].

Table 4 Comparison of the analysis of parameters in shake flask, batch and fed-batch cultivations of the recombinant *E. coli* during BoNT/A-Hc expression

Parameters	Shake flask	Batch culture	Fed-batch culture
Cultivation time	18	12	21
Final OD	3.5	13	100
BonT/A-Hc (mg l^{-1})	52.1	62	486
Productivity of BoNT/A-Hc (mg l^{-1} h^{-1})	2.8	5.1	23.1

Moreover, HCDC enables researchers to achieve higher product concentrations, which is not possible by the shake flask and batch culture methods [12]. The first step for recombinant protein production by HCDC is selection of a suitable strain [18]. Therefore, in this study the Hc part of BoNT/A was subcloned into the pET28a vector and expression of recombinant BoNT/A-Hc was evaluated by the Western blot technique. Then from among the 10 cultivated clones, the best recombinant strain was selected based on three main factors; expression level, growth rate and plasmid stability. The results showed that the difference in the growth of recombinant *E. coli* among the selected strains was not highly significant. Therefore, it is clear that all 10 stains are the same regarding the growth criteria, but SDS–PAGE and gel scanning densitometry indicated that the highest BoNT/A-Hc levels were expressed in strain 5 with a 17% density relative to the total bacterial proteins. Hence this strain was considered as suitable for the expression of recombinant BoNT/A-Hc. With regard to plasmid stability, the replica plating method showed that the selected *E. coli* strain (strain 5) had 90% stability after 6 days of subculturing. Therefore, strain 5 with an ability to express protein at levels higher than the other strains, and with a plasmid stability of 90% was selected for HCDC and large scale production of recombinant protein.

After the selection of the suitable recombinant strain, batch fermentation with the modified M9 medium [11] was carried out in a 2-l fermentor. The growth kinetics of *E. coli* in batch fermentation and the stability of the recombinant plasmid pET28a were determined and evaluated. The Taguchi statistical method was then used for the design of experiments and optimization of the OD of induction and IPTG concentration. The results were analyzed by the Qualitek-4 software and standard ANOVA. Consequently, the percentage contribution of each variable and its optimum level were then obtained (Table 3). The results indicated that induction at the middle of the logarithmic phase of growth (OD of 10), and an IPTG concentration of 1 mM represented the optimum values. In the batch fermentation and under optimum conditions, the maximum level of recombinant soluble BoNT/A-Hc that was obtained at 3 h after induction was 62 mg l^{-1} in batch fermentation, which is higher than its expression value in the shake flask culture.

Finally, HCDC was carried out as a strategy to improve the recombinant BoNT/A-Hc expression yield and productivity. The fed-batch culture process involving an exponential feeding rate was applied to achieve HCDC. In the fed-batch culture, higher levels of soluble recombinant BoNT/A-Hc were expressed 3 h after induction, with 1 mM IPTG as an inducer. Acetate, a well-known growth inhibitory metabolite for *E. coli*, is overproduced under anaerobic or oxygen-limiting conditions [19]. Therefore, the DO concentration was maintained above 40% saturation and specific growth rate was fixed at below or 0.2 h^{-1} during the fed-batch fermentation. Thus, acetate in the fed-batch fermentation was lower than 5 g l^{-1}, which has been reported as the inhibitory growth limit [20]. Glucose concentration was also controlled, without any starvation or accumulation of glucose during the process.

Table 4 summarizes the comparison of results achieved by shake flask, batch and fed-batch fermentations. As shown in this Table, optical densities in the final process of the shake flask, batch and fed-batch cultures were 3.5, 13 and 100, respectively, which shows that the OD of the recombinant *E. coli* strain increased significantly from 3.5 in the shake flask to 100 in the fed-batch fermentation. Also, soluble BoNT/A-Hc concentrations increased from 52.1 in the shake flask to 62 in batch and 486 mg l^{-1} in the fed-batch cultivations; the latter showing a 9.3- and 7.8-fold increase over the shake flask and batch fermentations, respectively. Therefore, the increase in volumetric productivities of the batch and fed-batch fermentations in comparison with the shake flask culture was 1.8- and 4.5-fold, respectively. Liu et al. [19] reported that the expression of hepatopoietin Cn in recombinant *E. coli* BL21 increased from 5% in the shake flask culture to 12% in HCDC, which resulted in a final biomass concentration of 16 g l^{-1} DCW; however, in this case the cells were grown in a 40-l bioreactor containing complex medium. In contrast, the HCDC results of this study (50 g l^{-1} final DCW in a 5-l bioreactor with simple modified M9 medium) are much higher than that reported by Liu et al. [19].

With regard to other researches involving the maximum production of BoNTs fragments, Zhang et al. [21] only managed to achieve 3 mg of BoNT/C-Hc/g wet cells, following fed-batch fermentation by *Pichia pastoris* utilizing methanol and also mixed feeds of glycerol and methanol. Potter et al. [22] showed that the glycerol-batch, glycerol-fed-batch and methanol-fed-batch fermentations for the production of BoNT/A-Hc in the yeast *Pichia pastoris* gave 450 mg kg^{-1} of wet cell mass, based on the Bradford protein assay.

Based on the high AT content (%) of the *C. botulinum* genome, Chen et al. [8] and Yu et al. [2] have used a synthetic BoNT/F-Hc gene with reduced AT content to improve the expression level of the gene in *E. coli*, and have reported the production of the recombinant synthetic BoNT/A-Hc gene in *E. coli* at a level lower than 70 mg l^{-1}, however, in these investigations, the recombinant protein has been expressed in shake flask culture containing LB medium and purified through one-step affinity chromatography.

In conclusion, this research is the first report of BoNT/A-Hc expression in recombinant *E. coli* by HCDC. Consequently, a significantly high cell density of recombinant *E. coli* was obtained based on the exponential feeding rate and optimized procedure, which improved the productivity of the recombinant BoNT/A-Hc by the HCDC method. Hence, the results of this research could be highly applicable and useful in the overexpression of recombinant BoNT/A-Hc as a safe and efficacious vaccine for botulism. This study can also provide appropriate conditions for the production of other nontoxic subunits of the *C. botulinum* neurotoxins.

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