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Optimization of the BoNT/A-Hc expression in recombinant *Escherichia coli* using the Taguchi statistical method

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The use of the recombinant BoNT/A-Hc (carboxylic domain of the *Clostridium botulinum* neurotoxin heavy chain) has been proposed as a vaccine candidate for botulism. This fragment contains the principle protective antigenic determinant. In the present study, in order to maximize recombinant protein expression, after verification of recombinant BoNT/A-Hc by Western blotting, modified M9 medium was selected as a simple medium, and the operational and medium-composition variables together with their interactions were optimized by using the Taguchi statistical method. ANOVA for the obtained data indicated that 3.5 g, 15 g, 30 g, 15 g, 4 g, 0.7 mM, 1.5 ml per litre of medium, 30 °C and 15 h represented optimum values of $(NH_4)_2SO_4$, glucose, K_2HPO_4, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, isopropyl β-D-thiogalactoside concentration, trace-elements solution, temperature and post-induction time respectively. Consequently, under these optimum conditions, 52.1 mg/l of soluble BoNT/A-Hc was obtained in shake flask culture.

Introduction

BoNTs (botulinum neurotoxins) are produced by various strains of the anaerobic bacterium *Clostridium botulinum* and are classified into seven serotypes based on their distinct antigenicity (designated as serotypes A–G) [1]. BoNTs, the causative agents of botulism, are the most potent toxins known to humans and animals [2]. They affect nerve cells that control the action of muscles by preventing them from releasing synaptic vesicles filled with the neurotransmitter acetylcholine, thus inducing muscle paralysis [3]. The botulinum toxins are all naturally synthesized as the single-chain 150 kDa polypeptide, requiring post-translational proteolysis to the 50 kDa Lc (light chain), containing the catalytic activity, and the 100 kDa Hc (heavy chain) [4]. The C-terminal fragment of the Hc recognizes surface receptors on the neuronal membrane and then provokes the internalization of the whole toxin into endocytic vesicles [5,6]. The current pentavalent toxoid vaccine for botulism is composed of formalin-inactivated holotoxin derived from serotypes of *C. botulinum*. However, this vaccine requires large-scale production of the highly toxic material, which also involves difficult and extensive detoxification treatment with formalin. In order to reduce costs and increase safety conditions, the recombinant immunogenic BoNT/A-Hc (carboxylic domain of the *Clostridium botulinum* neurotoxin heavy chain) has been used as an efficient antigen to generate neutralization antibodies for immunotherapy and also as an appropriate vaccine candidate [7,8].

Optimum conditions are the basis for economical and high-yield production in chemical engineering and biotechnological processes, therefore the optimization of medium components and operational variables is of vital importance to the fermentation process [9]. Statistical methods are crucial to the improvement of productivity because they play an important role in experimental design, evaluation of factors and optimization of medium composition and operational variables. Several statistical methods are widely used in biological processes [10–13]. Among these, the Taguchi method serves as a screening filter which examines the effects of process variables and identifies those factors that have major effects on the process through the use of a single trial with a few experiments [14–16].

In the present study, in order to increase the rate of growth of *Escherichia coli* and obtain large quantities of recombinant BoNT/A-Hc, the Taguchi statistical method was used to optimize medium composition and operational variables in shake flask culture.

Key words: *Clostridium botulinum*, *Escherichia coli*, protein production, Taguchi method, vaccine.

Abbreviations used: BoNT/A-Hc, carboxylic domain of the *Clostridium botulinum* neurotoxin heavy chain; BoNT, botulinum neurotoxin; Hc, heavy chain; IPTG, isopropyl β-D-thiogalactoside; Lc, light chain; NIGEB, National Institute of Genetic Engineering and Biotechnology; Ni-NTA, Ni^{2+}-nitrilotriacetate.

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Table 1 Composition of modified M9 medium

Reagent	Amount
Glucose (g/l)	15
$MgSO_4 \cdot 7\,H_2O$ (g/l)	4
$(NH4)_2SO_4$ (g/l)	3.5
K_2HPO_4 (g/l)	30
KH_2PO_4 (g/l)	15
Citric acid (g/l)	2
Trace-elements solution (ml per litre)[a]	1.5

[a](g/l in 1M 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.

Materials and Methods

Bacterial and plasmid construction

The *bont/a-hc* gene with the His_6-tag at the C-terminal end was subcloned into the pET28a vector (Novagen). The recombinant plasmids were used to transform *Escherichia coli* BL21 (DE3) and the *bont/a-hc* gene was chemically expressed using IPTG (isopropyl β-D-thiogalactoside) under the control of the *T7/lac* promoter.

Media and culture conditions

Culture medium Simple modified M9 medium [17] was selected and optimized for flask culture experiments. Optimum composition of the M9 medium is shown in Table 1. The medium was sterilized by autoclaving at 121 °C for 20 min. Glucose and $MgSO_4 \cdot 7H_2O$ were sterilized separately. Kanamycin was filter sterilized (30 μg/ml) and then added to the sterile M9 medium.

Seed culture medium Seed culture was prepared by inoculating 50 ml of sterile M9 into a 250 ml Erlenmeyer flask with *E. coli* previously grown on Luria–Bertani agar. The inoculated medium was incubated at 37 °C for 24 h with shaking at 200 rev./min. Subsequent M9 media were then inoculated with 10% (v/v) of the resulting seed culture.

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

The recombinant cells were cultivated in medium supplemented with 30 μg/ml of kanamycin at 37 °C (150 rev./min) up to a D_{600} of 1.2, then induced with IPTG (0.7 mM) and incubated overnight at 30 °C. The bacterial cells were collected by centrifugation at 5000 ***g*** for 10 min at 4 °C. Growth was monitored by measuring D_{600} using a UV–visible spectrophotometer (Beckman DU530; Beckman Coulter, Inc., Fullerton, CA, U.S.A.). One attenuance unit corresponds to 0.5 g of cells per litre. An un-inoculated medium was used as a blank. Protein expression was evaluated by SDS/PAGE.

Western blotting

The recombinant protein was detected by Western blotting. After SDS/PAGE, expressed proteins on the gel were transferred to a nitrocellulose membrane using transfer buffer (2.93 g/l glycine, 5.81 g/l Tris base, 0.37 g/l SDS and 200 ml of methanol per litre, pH 8.3). The membrane was then suspended in blocking buffer [1.25% (w/v) non-fat dried skimmed milk powder] with shaking for 2 h at room temperature (25 °C). After three washes with washing buffer (2.42 g/l Tris-base, 18.1 g/l NaCl and 3.87 ml of 6 M HCl), the membrane was incubated in a 1:100 dilution of rabbit anti-BoNT/A-Hc antibody [7], with gentle shaking for 1 h. Subsequent to washing, the blots were suspended in a 1:1000 dilution of the polyclonal anti-rabbit conjugate [7], 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% H_2O_2). Once the protein band was visualized, the reaction was stopped with water.

BoNT/A-Hc assay

Soluble cytoplasmic proteins from recombinant *E. coli* were extracted following cell lysis. Cells were harvested as above and then resuspended in sonication buffer (5-fold of the sample weight) containing 50 mM sodium 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). PMSF serine protease was added to the suspension only during the initial sonication. The lysate was thereafter centrifuged (14000 ***g*** for 10 min) to remove cell debris and insoluble proteins. The total soluble protein concentration in the resulting supernatant was quantified using the Bradford dye method with BSA as a standard [25] and proteins were resolved by SDS/13% PAGE. The gel was stained with a Coomassie Blue solution and destained with methanol and acetic acid. After destaining, the relative abundance of BoNT/A-Hc was determined by densitometry of gel bands using an UltraScan XL Gel Doc densitometer (Pharmacia, Bromma, Sweden) [26–29].

Statistical design of experiments

In order to maximize protein expression in shake flask culture, the simple modified M9 medium (Table 1) was selected and the effect of operational variables (D_{600} of induction, temperature, time after induction and IPTG concentration), medium composition [glucose, $MgSO_4 \cdot 7H_2O$, $(NH4)_2SO_4$, K_2HPO_4/KH_2PO_4 ratio and trace-elements solution] and also their significant interactions were optimized by using the Taguchi statistical method.

The L16 orthogonal array was selected for screening of the above factors and interaction between three factors: OD of induction, glucose and time after induction at two levels

Table 2 L16 orthogonal array of the Taguchi design for screening of selected factors

A, time after induction (h); B, attenuance of induction; C, $(NH4)_2SO_4$ (g/l); D, temperature (°C); E, glucose (g/l); F, $MgSO_4 \cdot 7H_2O$ (g/l); G, K_2HPO_4/KH_2PO_4 ratio; H, trace-elements solution (ml); I, IPTG concentration (mM).

	Variables								
Trial no.	A	B	C	D	E	F	G	H	I
1	1	1	1	1	1	1	1	1	1
2	1	1	1	1	2	2	2	2	2
3	1	1	2	2	1	1	2	2	2
4	1	1	2	2	2	2	1	1	1
5	1	2	1	2	1	2	1	2	2
6	1	2	1	2	2	1	2	1	1
7	1	2	2	1	1	2	2	1	1
8	1	2	2	1	2	1	1	2	2
9	2	1	1	2	1	2	2	1	2
10	2	1	1	2	2	1	1	2	1
11	2	1	2	1	1	2	1	2	1
12	2	1	2	1	2	1	2	1	2
13	2	2	1	1	1	1	2	2	1
14	2	2	1	1	2	2	1	1	2
15	2	2	2	2	1	1	1	1	2
16	2	2	2	2	2	2	2	2	1

Table 3 Selected variables and their levels for the screening stage by the Taguchi experimental design method

Variable	Low level	High level
Time after induction (h)	7	15
D_{600} of induction	0.6	1.2
$(NH4)_2SO_4$ (g/l)	1.5	3.5
Temperature (°C)	30	37
Glucose (g/l)	5	15
$MgSO_4 \cdot 7H_2O$ (g/l)	1	3
K_2HPO_4/KH_2PO_4 ratio	0.5	1.5
Trace-elements solution (ml)	0.5	1.5
IPTG concentration (mM)	0.5	1

(Tables 2 and 3). After screening, more effective factors and their interactions were optimized using L9 (Tables 4 and 5) and L4 (Tables 6 and 7) orthogonal arrays.

Qualitek-4 software was used for automatic design and standard ANOVA of Taguchi experiments. ANOVA revealed and identified the effect of each factor and the optimum condition and estimated the performance of the optimum condition.

BoNT/A-Hc purification

The recombinant induced cells were harvested by centrifugation (5000 ***g*** for 10 min). The pellet was resuspended in lysis buffer (5-fold of the sample weight) containing 50 mM sodium phosphate, 300 mM NaCl and 5 mM imidazole (pH 7.6). The cells were then lysed with four short bursts of sonication (on ice). PMSF serine protease was then added only during the initial sonication, and then centrifuged for 30 min at 18400 ***g***. The supernatant was

Table 4 L9 orthogonal array of the Taguchi design for optimization of significant factors

Trial no.	Time after induction (h)	$MgSO_4 \cdot 7H_2O$ (g/l)	K_2HPO_4/KH_2PO_4 ratio	IPTG concentration (mM)
1	1	1	1	1
2	1	2	2	2
3	1	3	3	3
4	2	1	2	3
5	2	2	3	1
6	2	3	1	2
7	3	1	3	2
8	3	2	1	3
9	3	3	2	1

Table 5 Significant variables and their levels employed for optimization experiments using the L9 Taguchi design

Variable	Medium level	High level	Low level
Time after induction (h)	13	15	11
$MgSO_4 \cdot 7H_2O$ (g/l)	3	4	2
K_2HPO_4/KH_2PO_4 ratio	1.5	2	1
IPTG concentration (mM)	0.5	0.7	0.3

Table 6 L4 orthogonal array for studying the interaction between time after induction and D_{600} of induction

Trial no.	Time after induction (h)	D_{600} of induction
1	5	1.2
2	5	0.6
3	15	0.6
4	15	1.2

Table 7 L4 orthogonal array for evaluating the interaction effects between glucose and D_{600} of induction

Trial no.	Glucose (g/l)	D_{600} of induction
1	5	0.6
2	15	0.6
3	5	1.2
4	15	1.2

loaded onto an Ni-NTA (Ni^{2+}-nitrilotriacetate) resin, the column was washed with washing buffer (50 mM sodium phosphate, 300 mM NaCl and 5 mM imidazole) and the flow-through fraction was collected from the column. The BoNT/A-Hc was then eluted with elution buffer (500 mM sodium phosphate, 300 mM NaCl and 150 mM imidazole). Aliquots of the entire fraction were analysed by SDS/13% PAGE.

Results

SDS/PAGE and Western blot analysis

The expression of recombinant BoNT/A-Hc in the cells, before and after induction with IPTG, was validated by SDS/13% PAGE analysis. A band corresponding to a 50 kDa BoNT/A-Hc was observed by SDS/PAGE of total bacterial

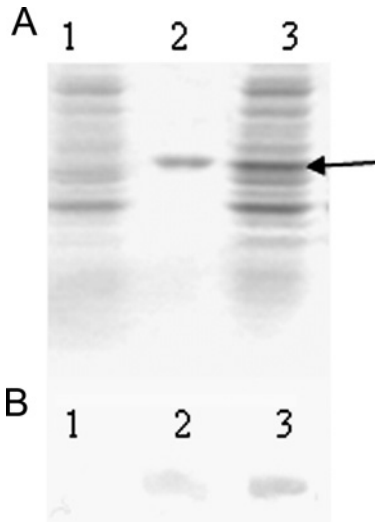

Figure 1 SDS/PAGE and Western blot analysis for evaluation of recombinant BoNT/A-Hc

(**A**) SDS/PAGE of BoNT/A-Hc in the recombinant *E. coli* strain before and after induction. The arrow shows a 50 kDa BoNT/A-Hc. (**B**) Western blot of the total soluble proteins from recombinant *E. coli* that were detected with anti-BoNT/A-Hc antibody. Lane 1, before induction; lane 2, standard BoNT/A-Hc; lane 3, recombinant BoNT/A-Hc after induction.

proteins (Figure 1a). The identity of BoNT/A-Hc was then verified using Western blotting (Figure 1b).

Taguchi experimental design for screening of selected factors

In order to optimize the operational conditions and medium composition for the rapid growth of *E. coli* and maximum expression of recombinant BoNT/A-Hc in shake flask culture, the Taguchi statistical design was applied. Firstly, the standard L16 orthogonal array was used for the screening of nine variables containing five medium-composition factors [$MgSO_4 \cdot 7H_2O$, glucose, $(NH4)_2SO_4$, K_2HPO_4/KH_2PO_4 ratio and trace-elements solution] and four operational variables (D_{600} of induction, temperature, time after induction and IPTG concentration). Interactions between D_{600} of induction, time after induction, glucose and $(NH4)_2SO_4$ were also considered. Results were then analysed by standard ANOVA for determination of significant variables (Table 8). These data showed that recombinant BoNT/A-Hc expression was affected by the IPTG concentration, K_2HPO_4/KH_2PO_4 ratio, time after induction and $MgSO_4 \cdot 7H_2O$ variables. However, glucose, $(NH4)_2SO_4$, trace-elements solution, D_{600} of induction and temperature each had negligible effects on the yield of BoNT/A-Hc expression. Evaluation of the interactions demonstrated that interaction between D_{600} of induction with glucose and D_{600} of induction with time after induction had significant contributions, showing BoNT/A-Hc expressions of 16.8% and 13.8% respectively (Table 8). Thus the effects of $(NH4)_2SO_4$, glucose, temperature, trace elements solution and D_{600} of induction were pooled together (Figure 2).

L4 orthogonal array for evaluating effects of interaction

While keeping other variables at the suggested level of the initial screening design, interactions between D_{600} of induction with glucose and time after induction were studied by the L4 orthogonal array separately.

The data were analysed by standard ANOVA, and the percentage contribution of each variable and the optimum level were then obtained (Tables 9 and 10). Hence, the optimum levels obtained for D_{600} of induction, glucose concentration and time after induction with regard to bacterial growth and BoNT/A-Hc expression were 1.2, 15 g/l and 15 h respectively.

Table 8 ANOVA of the effects of assigned variables and interaction on BoNT/A-Hc expression

N. a., not applicable.

Trial no.	Factor	Degrees of freedom	*F* ratio	Contribution (%)	Optimized levels
1	Time after induction (A)	1	189 293.067	12.47	1
2	D_{600} of induction (B)	1	591.382	0.03	1
3	A×B	1	206 338.868	13.81	N. a.
4	$(NH4)_2SO_4$ (C)	1	46 086.428	3.08	2
5	A×C	1	25 901.080	1.73	N. a.
6	B×C	1	15 558.657	1.04	N. a.
7	Temperature (D)	1	34 953.358	2.34	1
8	Glucose (E)	1	1076.428	0.07	1
9	A×E	1	4085.793	0.27	N. a.
10	B×E	1	251 766.306	16.82	N. a.
11	$MgSO_4 \cdot 7H_2O$ (F)	1	198 197.752	13.2	2
12	C×E	1	27 942.602	1.87	N. a.
13	K_2HPO_4/KH_2PO_4 ratio (G)	1	230 927.750	15.04	2
14	Trace-elements solution (H)	1	950.419	0.06	2
15	IPTG concentration (I)	1	262 377.822	17.5	1

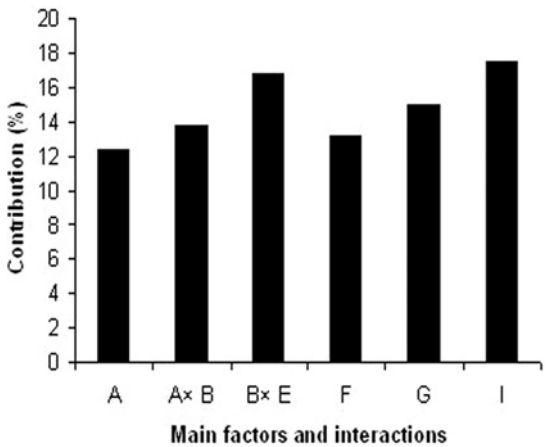

Figure 2 Percentage contribution of factors and interactions selected after ANOVA, as main factors and main interactions after pooling negligible trials

A, time after induction; B, D_{600} of induction; E, glucose; F, $MgSO_4 \cdot 7H_2O$; G, K_2HPO_4/KH_2PO_4 ratio; I, IPTG concentration.

Table 9 ANOVA of the results for evaluation of the time after induction–D_{600} of induction interaction effect

Variables	Degrees of freedom	*F* ratio	Contribution (%)	Optimized level
Time after induction (h)	1	4950624	81	15
OD of induction	1	1155625	19	1.2

Table 10 ANOVA of the results for evaluation of the D_{600} of induction–glucose interaction effect

Variables	DOF	F-ratio	Contribution (%)	Optimized level
D_{600} of induction	1	15626	4	1.2
Glucose (g/l)	1	429023	96	15

Taguchi experimental design for optimization of significant factors

After screening the variables and evaluating the effects of interactions between them, the most effective factors were then selected for the optimization stage, which involved further levels of evaluation.

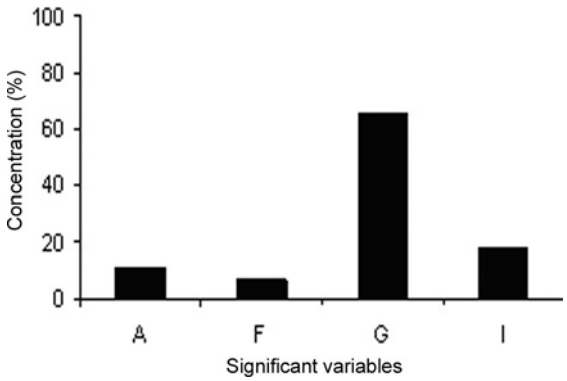

Figure 3 Percentage contribution of significant variables on BoNT/A-Hc expression using ANOVA of results

A, time after induction; F, $MgSO_4 \cdot 7H_2O$; G, K_2HPO_4/KH_2PO_4 ratio; I, IPTG concentration.

The L9 orthogonal array was used to investigate the effects of four main factors, K_2HPO_4/KH_2PO_4 ratio, IPTG concentration, time after induction and $MgSO_4 \cdot 7H_2O$ concentrations, at three levels while other variables were kept constant at optimum levels. The ANOVA of the obtained results indicated BoNT/A-Hc expressions of 65.5%, 18%, 6.3% and 10.9% with respect to K_2HPO_4/KH_2PO_4 ratio, IPTG concentration, time after induction and $MgSO_4 \cdot 7H_2O$ concentration respectively (Table 11 and Figure 3). Therefore, after screening and optimization of all considered variables and interaction between their factors, the optimal conditions for bacterial growth and BoNT/A-Hc expression consisted of 3.5 g, 15 g, 30 g, 15 g, 4 g and 1.5 ml per litre of medium, and 30 °C, 0.7 mM and 15 h with regard to $(NH_4)_2SO_4$, glucose, K_2HPO_4, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, trace elements solution, temperature, IPTG concentration and post-induction time respectively.

BoNT/A-HC purification

After the optimization of conditions for bacterial growth and BoNT/A-Hc expression, the purification of recombinant BoNT/A-Hc was accomplished by Ni-NTA affinity chromatography (Qiagen, Hilden, Germany). BoNT/A-Hc was allowed to bind selectively to the Ni-NTA resin through the His_6-tag. The target recombinant

Table 11 ANOVA of the results of the significant-factors-optimization experiment in shake flasks

Trial no.	Factor	Degrees of freedom	*F* ratio	Contribution (%)	Optimized level
1	Time after induction	2	546248.68	10/9	15
2	$MgSO_4 \cdot 7H_2O$ (g/l)	2	60124765	6.3	4
3	IPTG concentration	2	548749.02	18	0.7
4	K_2HPO_4/KH_2PO_4 ratio	2	548671.20	65.5	2

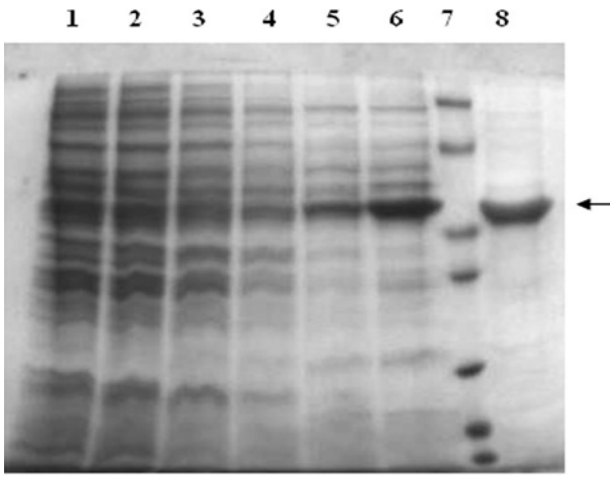

Figure 4 SDS/PAGE analysis of recombinant BoNT/A-Hc purified with the Ni-NTA resin column

Lanes 1–4, wash fractions; lane 5, elution with 50 mM imidazole; lane 6, elution with 100 mM imidazole; lane 7, protein marker; lane 8, elution with 150 mM imidazole. The arrow indicates purified BoNT/A-Hc.

protein was eluted at an imidazole concentration of 150 mM (Figure 4), and BoNT/A-Hc expression was subsequently analysed as mentioned above.

Verification of expected Taguchi results

The expected Taguchi results indicated a soluble BoNT/A-Hc expression level of 54 mg/l under optimal conditions in shake flask culture. A value of 52 mg/l was obtained experimentally for BoNT/A-Hc expression under optimum conditions in shake flask.

Discussion

BoNT/A-Hc has been reported as a vaccine candidate. Therefore, in the present study, the Hc portion of BoNT/A was subcloned into the pET28a vector and then expressed in *E. coli* BL21 (DE3). The best recombinant clone was selected based on three main factors; expression level, growth rate and plasmid stability (results not reported).

In the present study, the selection of the best strain and simple medium and also optimization of effective variables were crucial in maximizing cytoplasmic recombinant BoNT/A-Hc production. After selection of modified M9 medium as a simple medium, the Taguchi statistical method was used to identify significant effective variables and their interactions for the purpose of recombinant BoNT/A-Hc expression. The Taguchi approach of orthogonal-array experimental design for process optimization involves the study of a given system by a set of independent factors over a specific range of levels in order to identify the influence of individual factors and establish relationships between the variables and also performance at the optimum levels obtained [18]. As shown in Table 8, K_2HPO_4/KH_2PO_4 ratio, IPTG concentration, time after induction and $MgSO_4 \cdot 7H_2O$ concentration have significant effects on the yield of BoNT/A-Hc expression. In addition, interactions of D_{600} of the induction with glucose concentration and time after induction have significant effects on BoNT/A-Hc expression. In the next step, significant interactions were studied by orthogonal arrays ($2 \times L4$). Based on the Qualitek-4 software and standard ANOVA, glucose and time after induction at high levels and D_{600} of induction at low levels are suggested to be significant, while in the screening step there are no significant differences between the high and low levels for glucose and attenuance of induction; however, their interactions are important. Finally, by using the L9 orthogonal array, the optimum levels of significant variables such as K_2HPO_4/KH_2PO_4 ratio, IPTG concentration, time after induction and $MgSO_4 \cdot 7H_2O$ concentration at three levels were 2, 0.7, 15 h and 4 mg per litre of medium respectively. Consideration of the main effects of the significant variables on BoNT/A-Hc expression shows that the K_2HPO_4/KH_2PO_4 ratio plays a relatively more critical and influential role than the other selected variables (Figure 3).

Table 12 Verification of results obtained at optimal conditions and comparison with expected results

Condition	Maximum soluble BoNT/A-Hc produced in shake flask culture (mg/l)
Basal (control)	12.6
Optimum	52.1
Expected value under optimum conditions	54

The maximum level of recombinant soluble BoNT/A-Hc obtained under optimum conditions was 52.1 mg per litre of culture medium in a shake flask, which is 4.1-fold higher than that of the control condition (Table 12). Therefore, optimization of conditions using the Taguchi statistical method has made it possible to obtain culture conditions that allow for rapid growth of the strain and a high level of recombinant BoNT/A-Hc expression in shake flask culture.

The effect of the temperature variable on recombinant protein expression by *E. coli* has been widely investigated. Decreasing the temperature usually results in an enhancement of the solubility of the protein expressed [16,19]. In the present study, however, the change in temperature (30 or 37 °C) did not have any significant effect on the yield of the BoNT/A-Hc protein expressed in shake flask culture, but ANOVA for the obtained data indicated that

cell cultivation at 30°C was better than that at 37°C. Apparently, the indication is that decreasing the temperature becomes important for the enhancement of the solubility of recombinant protein for the purpose of large-scale production, using *E. coli* as a host cell. Previous studies suggest that the optimal concentration of inducer for the expression of heterologous genes is product specific [17]. Da. *et al.* [20] have reported the saturation concentration of IPTG (1 mM) for expression of the P450 2C9 protein in *E. coli* DH5α by using a statistically based experimental design. In the present study, an IPTG concentration of 0.7 mM was obtained as an optimum value for the expression of recombinant protein under the control of the *T7*/*lac* promoter.

Two studies have reported glucose to be an effective factor in submerged culture [21,22], when compared with the present study where both glucose concentration and D_{600} of induction interactions are significant. Additionally, another study indicates that $MgSO_4 \cdot 7H_2O$ is also important; however, in this case the cells have been grown for the purpose of TaqI endonuclease production [23].

Some researchers have successfully applied Taguchi statistical design for the optimization of medium composition and production of recombinant proteins in flask culture and fermentation processes. The optimized culture conditions of the present study are similar to those of others [12–17,22,23]. Expression of BoNT/A-Hc or Lc in *E. coli* has remained a difficult problem because of the relatively high AT content of the *C. botulinum* genome (70%) [24]. The yield of soluble recombinant BoNT/A-Hc (52.1 mg/l) obtained in the present study is higher than those reported by other researchers [1–7,24].

The work presented here is a first report that has used the Taguchi statistical design method for the expression and production of BoNT/A-Hc in recombinant *E. coli*. These results, derived at the laboratory scale, provides an improved set of conditions and a basis for further investigations involving the larger-scale production of recombinant BoNT/A-Hc by the high-cell-density cultivation method.

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