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Using Taguchi Statistical Method for the Determination of Suitable Conditions for Cloning and Over Expression of N-terminal region of *ipad* in *Escherichia coli*

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IpaD is one of the most important virulence factors in *Shigella dysentriae*. Recent studies showed that N-terminal region of IpaD has a major role in bacterial invasion and it could be considered as a suitable candidate for vaccine development against *S. dysentriae*. In this study, the gene was cloned into Ecoli in order to have sufficient amount of protein and subsequently the recombinant protein was purified. So as to optimize protein expression, Taguchi statistical method was used. After optimization of the method, Protein expression was increased by 2.2 fold compared to control. This study showed that the K_2HPO_4/KH_2PO_4 ratio and OD_{600nm} of Induction are the main factors in the expression of the recombinant IpaD by *E.coli* Bl21 DE3, while other variables have slight effects on the expression procedure.

Key words: IpaD, Optimization, *S. dysentriae*, Taguchi Statistical Method,

Shigella strains specially *Shigella dysentriae* considers as a main cause of dysentery during the last century with various mortality rates. Several antigens in shigella are encoded by a 220 kb plasmid. Among them, IpaD (Invasion plasmid antigen D) with 332 residues and molecular weight 37 kDa is the essential virulence factor of *S. dysentriae*. In addition to having effector function that is necessary for invasion and intracellular survival, IpaD also regulates the secretion and translocation of other invasion effectors into the host cells[2-3].

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Laboratory techniques and calculation analysis of the obtained data indicated that the N-terminal region of IpaD has an essential role in bacterial invasion[4]. The reaction between bile salts, especially deoxycolate with N-terminal portion of IpaD may recruit and employ other proteins to locate on cell membrane of the bacterium and makes it ready to invade[4]. Based on recent studies the ability of microorganisms in making pores in erythrocyte membranes was blocked by the antibody that detects the surface exposed N-terminus of IpaD[2]. Moreover, the production of an effective vaccine against *shigella* was has been reported, which is based on IpaD and its functional derivatives[5]. Therefore, IpaD could be considered as a potential candidate, but antigenicity of IpaD needs further investigation. In this study in order to reduce costs and increase safety conditions,

recombinant immunogenic N-terminus region IpaD peptide has been used as an efficient antigen. Great amount of N-terminal IpaD peptide is needed for the production of poly and monoclonal antibodies with the aim of bacterial detection and immunotherapy, construction of Immunochromatography column for purification of antibodies and also research on the IpaD as an appropriate vaccine candidate. The use of statistical methods has been proposed for determination of suitable conditions in genetic engineering and biotechnological processes. Statistical methods are crucial to the improvement of efficiency because they play an important role in experimental design, evaluation of factors, optimization of medium composition and operational variables. Several statistical methods are widely used in biological processes. Among them, the Taguchi method serves as a screening filter, which examines the effects of process variables and identifies those factors which have major effects on the process through the use of single trial with a few experiments [6]. Taguchi statistical method via ANOVA (analysis of variances) offers the best model to obtain the optimized condition for high yield production. ANOVA is a table of information that displays relative influence of factor and interactions assigned to the column of an orthogonal array. In this study, Taguchi statistical method was used in order to determine the most suitable conditions for cloning and expression of the N-terminal region of *ipad* in *Escherichia coli.*

MATERIALS AND METHODS

Vector pET-28a(+) (Novagen USA), *Hind*III and *EcoR*I enzymes (Fermentas, Lithuania.), *Pfu* DNA polymerase (2.5U/µl, Fermentas, Lithuania), IPTG (Vivantis, Malesia), The Luria-Bertani medium (LB) composed of yeast extract (5 g/l), NaCl (10 g/l), tryptone (10 g/l) and modified M9 medium [6] and [7] (Table 1). SOC medium contains Bacto-tryptone (20 g/l), Bacto-yeast extract (5 g/l), NaCl (0.5 g/l), 1M KCl (2.5 ml) and ddH_2O to 1000 ml. The media was sterilized then 10ml of filter steriled $MgCl_2$ (1M), 10ml $MgSO_4$ (1M) and 20ml glucose (1M) was added. *Shigella dysenteria*, was prepared from Milad hospital and confirmed by biochemical and serological tests. The genomic DNA of *shigella dysenteria* was Extracted and used as a template in PCR experiment. *E. coli* BL21 (DE3) plysS was used for cloning and expression experiments.

Preparation of N-terminal region of ipaD and vector construction by PCR

N-termial region of *ipad* was amplified by using of forward primer "IpaDF :5′ TCATGAATTC AGAACAACAAATCAG 3′ with an *EcoR*I endonuclease site and reverse primer "IpaDR : 5′TCTTAAGCTTTTAAGTATATGAACTAACG3′ with *Hind*III endonuclease site. Amplification was made in 35 cycles of PCR with a total volume of 50 µl of reaction mixture containing 1µl of *S. dysenteria* extracted genomic DNA (0.5µg/µl), 5 µl of 10X *Pfu* buffer with 20mM $MgSo_4$, 5 µl dNTP Mix (2mM each), 2 µl of each primer (10 pmol) and 0.25 µl of *Pfu* DNA polymerase (2.5 unit/µl). By adding double-distilled water, the reaction mixture reached the final volume. The PCR products of N-terminus region of *ipad* were digested by *EcoR*I and *Hind*III enzymes. Digested *ipad* was inserted into the *EcoR*I/*Hind*III site of expression vector pET-28a(+), through enzyme digestion and ligation reactions. After transformation the recombinant plasmid pET-28a(+)-*ipad* was confirmed by PCR and restriction enzyme digestion.

Vector electrotransportation into E. coli, using Taguchi statistical method

Previous study results on optimization of electroporation conditions [8] was used to transfer recombinant pET-28a(+)-*ipad* construct into *E. coli* BL21 (DE3) plysS.

Competent cell preparation

To prepare the competent bacterial cells, the pellet was collected when the OD_{600nm} of the culture medium reached 0.7 and re-suspended in 50ml of sterile ice cold 10% glycerol. The cells were centrifuged and re-suspended in 5ml sterile ice cold 10% glycerol. After second centrifugation the pellet was re-suspended in 1ml sterile ice cold 10% glycerol.

Plasmid Transformation (optimum conditions)

Two microliters of pET-28a (+)-*ipad* (50 ng/µl) was mixed with 25µl of competent cell suspension (OD_{600nm} = 0.7). After 10 min incubation in ice, suspension was transferred into cold cuvette with 0.1cm inter-electrode gap. Electroporation procedure performed with 12 kV/cm and 50 µF of field strength and capacitance respectively.

Thereafter, 1ml of SOC medium was added to the mixture and then it was incubated at 37°C for 120 minutes. Afterwards, the cultures were centrifuged and the pellets were re-suspended by 100µl of LB medium. About 10µl of each culture was plated on LB agar containing 40µg/ml kanamycin. The resulting transformant colonies usually appeared after 15h incubation at 37°C. Cloning procedure was confirmed by restriction enzymes and sequencing of the inserted fragment, through purified recombinant plasmid. The transformation efficiency (Transformants/µg DNA) was calculated[9].

High level expression of recombinant N-ter IpaD peptide in *E. coli* using the Taguchi statistical method

Statistical design of expression procedure

To increase the expression of IpaD in flask culture, the minimum modified M9 medium was selected and the effect of important operational variables (OD_{600nm} of bacterial culture at induction time, IPTG concentration, temperature and time (after induction)), medium composition [glucose, K_2HPO_4/KH_2PO_4 ratio, $MgSO_4 \cdot 7H_2O$, $(NH_4)_2SO_4$ and trace-elements solution] and also their significant interactions were optimized by using the Taguchi statistical method. For the screening of the above factors and interaction between four factors: OD_{600nm} of induction, glucose and IPTG concentration and time (after induction) at two levels, The L16 orthogonal array was designed (Tables 2). After the primary process, more effective factors and their interactions were optimized using L9 (Tables 3) and L4 (Tables 4) orthogonal arrays. Qualitek-4 software was used for automatic design and analysis of variance. At the end of the experiments, analysis of variance determined the effect of each factor and suggested the ideal conditions.

Media and culture conditions

Optimized and modified M9 minimum medium was used for *E. coli* BL21(DE3) pLysS culture. After autoclaving the medium at 121°C for 20 min, filter sterilized $MgSO_4 \cdot 7H_2O$ and Glucose were added separately. Kanamycin was also steriled by filtration and then added to the sterile M9 medium. A 250 ml Erlenmeyer flask containing 50 ml of sterile M9 was inoculated with *E. coli* previously grown on Luria–Bertani agar. The inoculated medium was incubated at 37°C for about 3 h with shaking at150 rpm/min till the OD_{600nm} of bacteria reached 0.8.

Expression and identification of the N-ter IpaD peptide

E. coli BL21 (DE3) pLysS which contains pET-28a(+)-*ipad* expression system, was cultured in the optimized and modified M9 minimum medium at 37°C. When the OD_{600nm} of bacteria reached to 0.8, it was induced by 1 mmol/L isopropylthio-β-D-galactoside (IPTG) and incubated for 5h. The bacteria were collected by centrifugation at 5000g for 5 min at 4 °C and the cell pellet was broken by lysis Buffer (NaH_2PO_4:13/8gr, Tris.HCl:1/2gr ,urea 480/5gr, add DDW to 1liter, pH= 8). An un-induced bacterial culture was used as a negative control blank. Protein expression and molecular weight of N-terminal IpaD truncated protein was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Immunoblot analysis

Immuno-reactivity of N-terminal-IpaD peptide was determined by Western blot. For Western blotting, proteins were transferred to 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 incubated in the blocking buffer [3% (w/v) bovine serum albumin (BSA)/ phosphate-buffered saline (PBS)] with gentle 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 1:1000 dilution of Mouse monoclonal Anti-IpaD antibody with gentle shaking for 1 h at room temperature (25°C). Subsequently, the membrane was incubated in a goat polyclonal anti-mouse Horseradish peroxidase (HRP) conjugated antibody (1:5000). After washing three times with washing buffer, nitrocellulose membrane was incubated in 2 ml of substrate of HRP staining solution (DAB). Once the protein band was visualized, chromogenic reaction was stopped by rinsing the membrane twice with water.

Assessment of N-terminal IpaD peptide

Cytoplasmic proteins from recombinant *E. coli* were extracted following cell lysis. The induced cells were harvested by centrifugation at 5000 rpm at 4°C for 10 min. The pellet was re-suspended in 200 µl of 8 M urea buffer and was followed by an hour of incubation at room

temperature (25°C). The cell suspension was then sonicated for four short bursts of ultrasonic pulses (on ice). Thereafter the lysate was centrifuged, 10000g for 10 min, to remove cell debris. The total protein in the resulting supernatant was quantified using the Bradford dye method and BSA as a standard[10]. The supernatant culture samples obtained, were tested for the protein expression through sodium dodecyl sulfate poly acryl amide gel electrophoresis (SDS–PAGE). The gel was stained with a colloidal Coomassie Blue solution and destained with deionized water. The relative abundance of IpaD peptide was determined by densitometry of gel bands, using a Gel Doc densitometer (Ettan DIGE Imager GE Health Care).

Purification of IpaD peptide

The supernatant obtained, was loaded onto an Ni-NTA (Ni2+-nitrilotriacetate) resin, the column was washed with phosphate buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl), and the soluble fraction was collected. Thereafter, the column was washed by 10ml of phosphate buffer containing 50 mM immidazole. Tightly bound protein was eluted with 2 ml of 100 mM, 3 ml of 150 mM and 2 ml of 200 mM immidazole. All the fractions were analyzed by 12 % SDS–PAGE and stained with Coomassieblue.

RESULTS

Taguchi statistical design for optimization of the Significant Variables in electrotransformation

The recombinant plasmids pET-28a(+)-*ipad* were extracted from transformed *E. coli* and digested by *EcoR*I and *Hind*III and analyzed on

Table 1. Ingredients of basic M9 medium

Regents	Volume
Glucose (g/l)	10
$MgSO_4 \cdot 7\ H_2O$ (g/l)	4
$(NH_4)2SO_4$(g/l)	3.5
K_2HPO_4 (g/l)	30
KH_2PO_4 (g/l)	15
Citric acid (g/l)	2
NaCl (g/l)	0.5
Trace-elements solution (ml per liter)1	1

1(g/l in 1M HCl): $MnCl_2 \cdot 4H_2O$, 2; $CoSO_4 \cdot 7H_2O$, 2.8; $FeSO_4 \cdot 7H_2O$, 2.8; $CuCl_2 \cdot 2H_2O$, 0.2; $ZnSO_4 \cdot 7H_2O$, 0.3; $CaCl_2 \cdot 2H_2O$, 1.5

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

Factor L16	(A)		(B)		(C)		(D)		(E)		(F)		(G)			(H)	(I)	
& levels screening array	0.5	0.8	0.2	1	1	2	5	15	32	37	0.5	1.5	1.5	3.5	10	15	2	4
1	•		•		•		•		•		•		•		•		•	
2	•		•		•		•			•		•		•		•		•
3	•		•			•		•	•		•			•		•		•
4	•		•			•		•		•		•	•		•		•	
5	•			•	•			•	•			•	•			•		•
6	•			•	•			•		•	•			•	•		•	
7	•			•		•	•		•			•		•	•		•	
8	•			•		•	•			•	•		•			•		•
9		•	•		•			•	•			•		•	•			•
10		•	•		•			•		•	•		•			•	•	
11		•	•			•	•		•			•	•			•	•	
12		•	•			•	•			•	•			•	•			•
13		•		•	•		•		•		•			•		•	•	
14		•		•	•		•			•		•	•		•			•
15		•		•		•		•	•		•		•		•			•
16		•		•		•		•		•		•		•		•	•	

A) OD_{600nm} Of induction; B) IPTG Concentration(mM); C) K_2HPO_4/KH_2PO_4 ration; D) Time after induction (h); E) Temperature after induction (°C); F) Trace Elements (ml /liter); G) $(NH_4)_2SO_4$ (g/l); H) Glucose concentration (g/l); I) $MgSO_4 \cdot 7\ H_2O$ (g/l)

agarose gel electrophoresis (Fig. 1). As shown in this figure Presence of 333 base pair bond substantiate the accuracy of the cloning process.

Western blot analysis using a specific antibody against N-terminal region of IpaD

The expression of the recombinant N-terminal region of IpaD in the cells, were evaluated by SDS-PAGE analysis, before and after induction. A 16.7 kDa band corresponding to IpaD peptide was obtained after gel staining (Fig. 2). The identity of the IpaD peptide was then verified using Western blotting (Fig. 3).

IpaD peptide purification

After expression of the IpaD peptide in optimized conditions for the bacterial growth, the purification of recombinant IpaD peptide was performed by Ni-NTA affinity chromatography. After loading the supernatant onto a Ni-NTA column, N-terminal region peptide of IpaD was allowed to bind selectively to the Ni-NTA resin through the His6-tag. The target peptide was eluted in presence of imidazole (150 mM). Thereafter the

Table 3. L9 orthogonal array of the Taguchi design for optimization of significant factors

Factors & levels L9screenin garray	OD_{600nm} Of induction			K_2HPO_4/KH_2PO_4 ration			Time after induction (h)			Temperature after induction (°C)		
	0.6	0.8	1	1.5	2	2.5	3	5	7	35	36	37
1	•			•			•			•		
2	•				•			•			•	
3	•					•			•			•
4		•		•				•				•
5		•			•				•	•		
6		•				•	•				•	
7			•	•					•		•	
8			•		•		•					•
9			•			•		•		•		

Table 4. L4 orthogonal array for studying the interaction between: IPTG concentration and Time after induction (Part:1) Time after induction and Glucose Concentration (Part:2) Glucose and IPTG concentration (Part: 3)

Part: 1		
Trial No:	IPTG concentration	Time after induction
1	0.2	5
2	0.2	10
3	1	5
4	1	10
Part:2		
Trial No:	Time after induction	Glucose Concentration
1	5	5
2	5	10
3	15	5
4	15	10
Part: 3		
Trial No:	Glucose Concentration	IPTG concentration
1	5	0.2
2	5	1 15 0.2
4	15	1

expression of the IpaD peptide was analyzed as mentioned before (Fig. 4).

Taguchi experimental design

L16 orthogonal Array for Evaluation of significant selected factors and Interactions

The standard L16 orthogonal array screened nine variables including four operational variables (OD_{600nm} of induction, IPTG concentration, temperature and time (after induction)) and five medium-composition factors including glucose, K_2HPO_4/KH_2PO_4 ratio, $MgSO_4 \cdot 7H_2O$, $(NH_4)2SO_4$ and trace-elements solution.

Table 5. ANOVA of the effects of assigned variables and interaction on N-terminus IpaD expression

Column	Factor	DOF	F-ratio	Percent	Optimum levels
1	OD_{600nm} of Induction (A)	1	1260.75	19.111	2
2	IPTG Concentration (B)	1	12	0.515	1
3	A×B	1	108	1.606	N. a.
4	K_2HPO_4/KH_2PO_4 ration (C)	1	3072	46.102	2
5	A×D	1	3	0.04	N. a.
6	B×D	1	396.75	5.941	2
7	Time after induction (D)	1	720.75	10.805	1
8	Temperature after induction (E)	1	330.75	4.95	2
9	A×H	1	90.75	1.347	N. a.
10	B×H	1	192	2.867	N. a.
11	Trace Elements (F)	1	27	0.55	2
12	D×H	1	147	2.192	N. a.
13	$(NH_4)_2SO4$ (G)	1	147	2.192	2
14	Glucose concentration (H)	1	60.75	0.896	2
15	$MgSO_4. 7 H_2O$ (I)	1	60.75	0.896	2

Table 6. ANOVA of the effects of assigned variables and interaction Between: IPTG concentration and Time after induction (Part:1) Time after induction and Glucose Concentration (Part: 2) Glucose and IPTG concentration (Part: 3)

Part:1 Factors	DOF	Variance	F-ratio	Percent	Optimized level
IPTG concentration (mM)	1	1225	12250000	85	1
Time after induction (h)	1	225	2250000	15.517	10
Part: 2					
Factors	DOF	Variance	F-ratio	Percent	Optimized level
Time after induction (h)	1	1482.250	658.777	95.1	2
Glucose Concentration (g/l)	1	72.250	32.11	4.9	15
Part: 3					
Factors	DOF	Variance	F-ratio	Percent	Optimized level
Glucose Concentration (g/l)	1	1406.250	5625.000	66.3	15
IPTG concentration (mM)	1	756.250	3025.000	33.7	1

Table 7. ANOVA of results of the significant factors obtained from L8 orthogonal array

Factors	DOF	F-ratio	percent	Optimized levels
OD_{600nm} of Induction	2	103.58	35.63	2
K_2HPO_4/KH_2PO_4 ration	2	108.951	41.60	2
Time after induction	2	22.05	10.933	2
Temperature after induction	2	24.493	11.83	3

Fig. 1. Agarose gel electrophoresis analysis of recombinant pET-28a(+)-*ipad,* Lane1: Double digests of recombinant pET-28a(+)-*ipad* with *EcoR*I and *Hind*III, Lane2: Recombinant pET-28a(+)-*ipad* without digestion, Lane3: 100bp DNA size marker (#SM0623 purchased from fermentas co.)

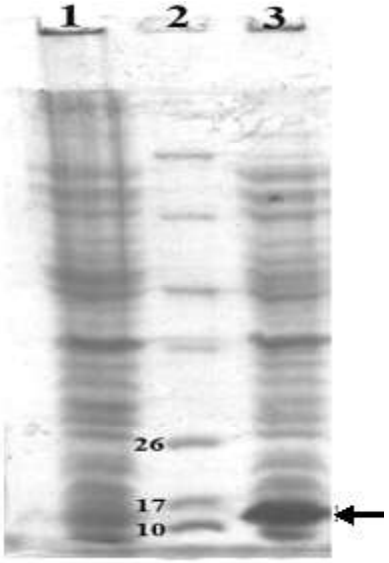

Fig. 2. Expression of IpaD protein in pET-28a(+)-*ipad*-BL21DE3 plysS, Lane 1: Negative control (Non-induced recombinant bacterial cells), Lane 2: Protein size marker (cat No:#SM0671 from fermentas co.), Lane 3: 16.7 kDa protein resulted from crud extraction of bacterial cells

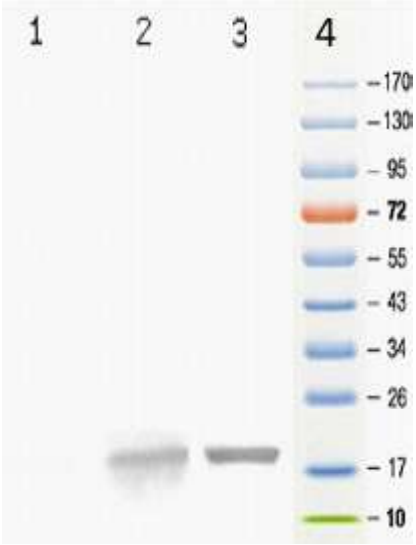

Figure 3. Western blotting analysis of expressed pET-28a-*ipad* products. Lane 1: Non-Induced bacterial cells as a negative control, Lane 2: Non-Optimized recombinant purified protein by nickel column, Lane 3: Optimized recombinant purified protein by nickel column, Lane 4: Protein size marker (cat No:#SM0671 purchased from fermentas co.)

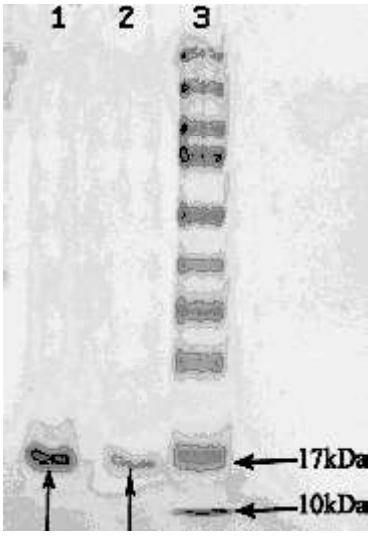

Fig. 4. IpaD peptide purification, Lane 1: Optimized recombinant purified protein by nickel column, Lane 2: Non-Optimized recombinant purified protein by nickel column, Lane 3: Protein size marker (cat No:#sm0671 purchased from Fermentas co.)

Results were analyzed by standard ANOVA (table 5). These data showed that the expression of the recombinant IpaD peptide in *E. coli* cells were affected by K_2HPO_4/KH_2PO_4 ratio, OD_{600nm} of induction, time and temperature (after induction) and trace elements variable factors. Some other factors such as, $(NH_4)_2SO_4$, glucose, $MgSO_4 \cdot 7H_2O$ and IPTG concentration, were not affecting yield of recombinant IpaD peptide. The results showed that the interactions between IPTG concentration and time(after induction), IPTG concentration and glucose concentration, and time(after induction) and glucose concentration had a significant contribution.

L4 orthogonal array for evaluating effects of Interaction

The interactions between IPTG concentration and time(after induction), IPTG concentration and glucose concentration, and time(after induction) and glucose concentration were studied by the L4 orthogonal array with the primary optimized conditions of OD_{600nm} of induction, temperature (after induction), K_2HPO_4/KH_2PO_4 ratio, $MgSO_4 \cdot 7H_2O$, $(NH_4)_2SO_4$ and trace-elements solution. The contribution rate of each variable and the optimum levels were obtained by standard ANOVA (Tables 6). Thereafter, the ideal levels for IPTG concentration, time (after induction) and glucose concentration were obtained.

Final optimization of significant factors by L9 orthogonal array

After screening the significant variables (OD_{600nm} of induction, K_2HPO_4/KH_2PO_4 ratio, time (after induction) and trace elements) they were applied. The results obtained by ANOVA, indicated the optimal conditions for IpaD expression (Table 7). After optimization, the concentration of the purified production was about 2.64 mg/ml which was increased by 2.2 fold compared to control (expression in modified M9 medium). Final results depicted that in order to induce high expression of N-terminal region of IpaD peptide in *Escherichia coli* BL21(DE3) pLysS the following factors needs to be taken into serious consideration such as OD_{600nm} of induction (0.8), IPTG concentration (1mM), K_2HPO_4/KH_2PO_4 ratio(2), time (after induction)(5h), trace elements (1.5 ml/l), $(NH_4)_2SO_4$ concentration (3.5 g/l), glucose concentration (15 g/l), $MgSO_4 \cdot 7H_2O$ concentration (4 g/l) and post-induction temperature (37°C).

DISCUSSION

In third world countries The main cause of shigellosis is *S. dysentriae*, which is a gram negative pathogenic bacterium characterized by its invasion capabilities to the epithelial cells of large intestine. Attachment of the microorganism to the host cells may induce an explosive secretion of proteins via the type three secretion system (TTSS) of the bacterium. The TTSS is an essential determinant for *Shigella*.. It was recently reported by Olive et al that in presence of bile salts as environmental signals IpaD recruits IpaB onto the *Shigella* needle tip complex[11]. Espina et al showed that the essential virulence protein IpaD works primarily as a TTSA needle tip protein. As described above they also reported that the antibodies that recognize the N-terminal region of IpaD can block the ability of the microorganism to induce pores on the eukaryotic cells[4]. Allaoui et all reported that antibodies raised against IpaD have an inhibitory effect on entry of *S. dysentriae* into its host cells[5]. Therefore IpaD and its functional derivatives are potentially able to be an effective vaccine. Purification of IpaD from the wild type of *S. dysentriae* or producing it in recombinant manner is required in order to have certain amount of IpaD peptide. It is difficult to Purify IpaD from the wild type of *S. dysentriae,* because the microorganism produces the IpaD protein in a low level and expensive and sophisticated biochemical techniques are needed to purify this protein among plenty of other proteins. furthermore, the risks of working with the wild type of *S. dysentriae* and also the high costs of these experiments are making them impractical[12].

In this study, IpaD was expressed in order to obtain high amounts of antigenic protein. we Insertion of a His-Tag to the N-terminal of the regarding peptide can facilitate the downstream protein purification steps. Since electroporation is an efficient method, it was used for plasmid transformation. By the results of the previous research[8], the yield of pET-28-*ipad* plasmid transformation reaches to 5×10^8 CFU/µg.

Optimization of transformation and expression were done by the Taguchi method in order to obtain suitable results. In other studies, this method was successfully used to produce specific biomolecules in microorganisms[13-14]. Since

M9 medium has simple and is capable of optimizing, it was selected as the medium for the IpaD expression.

Independent factors that affected the rate of expression as well as medium components were evaluated by this method. As shown in table 5, recombinant IpaD expression by *E.coli* Bl21 DE3 was mostly effected by K_2HPO_4/KH_2PO_4 ratio, while OD_{600nm}, time and temperature (after induction) had a slight effect on it. Using L4 orthogonal array and based on the results which were obtained from ANOVA, interactions between IPTG concentration and time(after induction), IPTG concentration and glucose concentration, and time(after induction) and glucose concentration had a significant influence on the IpaD production. L9 orthogonal array showed that the optimum level for K_2HPO_4/KH_2PO_4 ratio, Glucose concentration, Time (after induction), and Trace Elements are 2, 15g/lit, 5h and 1.5 ml/lit respectively. Similar to most mesophil bacteria, Optimum temperature for *ipad* expression in *E.coli* Bl21 DE3 as a host, was 37°C and reducing the temperature to 32°C declined the yield of protein expression.In this study it is shown that the Optimum concentration of IPTG for Ipad expression in *E.coli* BL21 DE3 is 1mM, Whereas Yari et al reported that over expression of *Clostridium botulinum* neurotoxin heavy chain recombinant protein was induced with the IPTG concentration of 0.7 mM [6]. Similarly, Yari used Taguchi statistical method for optimization. They reported that the yield of *Clostridium botulinum* neurotoxin heavy chain expression with the statistically optimized condition yielded 4.1 fold higher than the basic condition. Our results showed that after optimization, the rate of recombinant N-terminal IpaD expressed was 2.64 mg/ml which was 2.2 fold more than the control (expression in modified M9 medium). This study is the first report that has used taguchi statistical method with the aim of optimizing the transformation of the plasmid and the expression of the recombinant IpaD in *E.coli,* simultaneously.

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