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Design and Expression Optimization of a Chimeric Derivative of NetB, Alpha-Toxin and Metallopeptidase Proteins as a Subunit Vaccine Against *Clostridium perfringens*

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ABSTRACT

Avian necrotic enteritis (NE) is a multi-virulence disease caused by the bacterium *Clostridium perfringens*. Several toxins of this bacteria are components of different candidate vaccines, and have been considered as important factors in pathogenesis of NE. A fusion subunit protein composed of immunodominant segments of NetB, Alpha-toxin and metallopeptidase proteins (NAM) was designed. The high level production of NE subunit vaccine candidate is important for the *in vitro* and *in vivo* evaluation and later to decrease the production costs. Therefore, the Response Surface Methodology (RSM) was used for optimizing *E. coli* culture condition. To this end, induction conditions including cell optical density prior induction (OD600nm), IPTG concentration, post-induction temperature and time were modified. The statistical analysis revealed that all variables except IPTG had significant effects on production and solubility of rNAM. A 7.39 fold increase in production of soluble rNAM was achieved when the post induction temperature, IPTG concentration, the pre-induction OD and time were 19 ºC, 0.55 mM, 0.8 respectively in 8 h after induction. Our study indicated that the RSM method is a simple and superior strategy for protein expression improvement which is considered as a major limitation in production of vaccine candidate and other recombinant proteins using different hosts.

INTRODUCTION

Necrotic enteritis (NE) is caused by clostridium perfringens type A, an anaerobic Gram-positive bacteria. Several virulence factors are involved in disease induction in chickens. In recent years protein based subunit vaccines have provided a safe and most attractive technology for protection against multifactorial disease like NE [10, 34]. Previous studies demonstrated that immunizing ability for protection against NE was associated with several virulence factors. The most important of these virulence factors are phospholipase C known as Alpha-toxin, β-pore forming toxin, NetB, and a zinc metallopeptidase protein [7,8, 28]. Utilizing a fusion of non toxic variants of these proteins as a recombinant subunit vaccine candidate could enhance protective immunity [31, 6].

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To develop an effective subunit vaccine candidate against NE, it is important to optimize in a suitable heterologous expression system.

To this end, *E. coli*, and Salmonella, were used to express recombinant toxin of NE [12]. *E. coli* expression system is one of the most attractive and well characterized expression system because of the fast growth, high yield and low cost of protein expression. In spite of these advantages, sometimes, high level production of soluble recombinant protein may be quiet challenging [29, 22]. Generally, the aggregated proteins, known as inclusion bodies, are misfolded and therefore are biologically inactive. In many cases, solubilization and refolding of insoluble protein is a cumbersome and time consuming process and increase the complexity of downstream processing [24, 32, 33]. To address these concerns, multiple strategies designed to improve solubility rates of the recombinant protein. One of the easy, inexpensive and efficient methods is to modify the culture conditions [23]. Starting time of induction, post-induction tezmperature, time and inducer concentration are the most parameters that affect the solubility [3, 26].

The conventional optimization method of 'one factor at a time' requires numerous experimental data set to describe the interaction between parameters and time consuming. To overcome these obstacles, an efficient statistical model is needed. Therefore, evaluating the independent variables and assessing the interaction among them and simultaneously utilizing randomization and replication are applied. Statistical approaches like response surface methodology (RSM) that is based on full factorial central composite design (CCD) is ideal in industrial biotechnology processes. Numerous biochemical processes, including recombinant protein expression utilizing RSM to estimate optimum operating conditions results in identifying minimum or maximum responses. The statistical design allows quick identification of the important parameters for screening and optimization culture conditions [20, 23].

In this study, we designed a fusion trivalent protein including the immunogenic epitopes of Alfa-toxin, NetB and a metaloprotease proteins as a candidate vaccine against NE and optimized the expression of this fusion protein using statistical design.

MATERIALS and METHODS

Materials, Plasmid and Expression System

Bacterial cultures and kanamycin antibiotic were purchased from Sigma. Restriction enzymes were obtained from Thermo (US). PCR reagents were obtained from SinaClone (IRAN).

Construction of Expression Plasmid

The amino acid sequences and structure characteristics of three selected proteins for construction the fusion protein was retrieved from uniProtKB with accession number 4h56, 2wxu, 5kdj for NetB, Alpha toxin and metalopeptidase protein, respectively. To make a fusion protein, the carboxy terminal of NetB and Alpha-toxin and a strongly immunogenic epitopes of Zinc metallopeptidase were joined with A(EAAAk)4A as a flexible helical linker. The fusion protein was back translated to nucleic acid and synthesized by Biomatic (Canada), the flanking restriction sites EcoRI and HindIII were inserted using p1 (ATAGAgaattcATGGTTTCTAATTCAATCGGA) and p2 (CATAAaagcttTTACTCTTCACCCAAAGCAA) primers (table 1).

Table 1. Rare codon analysis at after and before codon optimization based on codon usage of tobacco as host organism

Codon optimization parameters	Before optimization		After optimization	
	Tobacco	*E. coli*	Tobacco	*E. coli*
CAI	0.85	0.63	0.93	0.63
GC%	31.76	31.76	35.69	35.69
CFD%	0	11	0	9
Negative CIS elements	35	0	0	1
Negative repeat elements	1	1	0	1

CAI: codon adaptation index, **CFD**: low frequency codon

The NAM gene was then cloned into expression vector pET28a (+) (Novagene) under control of the strong T7 RNA polymerase promoter. This prokaryotic expression system was named as pET28-NAM. The resulting pET28a-NAM plasmid was introduced into *E.coli* BL21 (DE3) (Stratagene) cells according to heat shock method and grown in LB agar plate supplemented with 50mg/l kanamycin. The optimization runs were carried out in 100 ml Erlenmeyer flask containing 20 ml of LB medium, under different conditions of temperature and 150 rpm rotational speed.

Protein Expression and Characterization

A single colony of BL21 (DE3) cells harboring the expression recombinant vector inoculated in 5 ml LB medium supplemented with 50 mg/l kanamycin at 37 ºC overnight. The pre-culture was inoculated into 20 ml LB medium at 1% (v/v). Expression of rNAM was induced by adding 1mM IPTG when OD 600 reached to 1. After 6 h, the cells were harvested by centrifugation at 5000x g for 10 min. The harvested cells were resuspended in 0.5 ml lysis buffer containing 100mM sodium phosphate buffer(pH:8), 10 mM Tris, 0.05% Tween 20 and lysozyme (0.1 mg/ml) and incubated at 37 ºC for 20 min . Then mixture was sonicated on ice (5×20 s with 20 s pause at amplitude 60) and centrifuged at 10000x g for 15 min. Total extract containing whole soluble proteins and other fractions of protein in lysate (same as insoluble proteins) separated by SDS-PAGE on 10 % gel using a Mini-protean Tetra Cell System (BioRad). Proteins were visualized with Coomassie Blue R-250 (BioRad).

Western blotting was performed using antibody against 6his-tagged NAM recombinant protein. The chimeric protein was transferred to a nitrocellulose membrane (sigma, USA). The membrane was blocked with 3% BSA for 1 hour and then incubated with a monoclonal anti-6xHis tag antibody (Abcam,US) at 1:3000 dilution in PBST/BSA (PBS containing 1% tween 20 and 2% BSA) by gentle shaking at 4 ºC overnight. Following membrane was incubated with HRP-conjugated goat anti mouse IgG (sigma) as secondary antibody diluted 1:5000 in PBST/BSA for 1 hour and then the membrane stained using deaminobenzidine (Sigma Aldrich, USA) solution.

Experimental Design and Optimization of Expression

Attempt to optimize overexpression of soluble rNAM protein was conducted based on RSM [2]. Four independent variable factors including post-induction temperature (factor 1), IPTG concentration (factor 2), OD600nm prior induction(factor 3) and post induction time (factor 4) were screened on the expression yield of recombinant chimeric protein rNAM in *E.*

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coli BL21 (DE3) using rotatable central composite design (Design Expert v. 8.0.6, Stat–Ease, Inc., Minneapolis, MN, USA). A total of 30 experimental trails were performed involving 8 star points and 6 replicates at the central points. Details of cultivation condition layout were shown in Table 2.

Analytical methods for protein concentration determination

The expression level of rNAM in each run was measured by SDS-PAGE and quantified by image J densitometry quantification software (NIH) and the protein concentration was determined by the Bradford method [5] by using bovine albumin as a standard. Responses of variables were calculated using design Expert software package. Linear and quadratic coefficients were analyzed by ANOVA test. The F-test was performed for estimation of the significance of the model terms and model equation while the quality of fitted model equation was analyzed by the coefficient of determination (R2), adjusted R2 and ‘‘adequate precision’’. The significance of the model terms was confirmed by p-value less than 0.05. ‘‘Point optimization’’ process tool of Design Expert software was performed to optimize the level of each factor for maximum solubility of final product.

RESULTS

Expression of Recombinant Construct and Western Blotting

We designed a chimeric NAM protein based on the nontoxic c-terminal domains of phospholipase c, a truncated form of NetB that is impaired in binding and pore forming on the target cell surface and ZMP, a partial form of a zinc metallopeptidase protein. The three functional domains were separated by two hydrophobic linkers. The chimeric construct was successfully cloned into pET28a vector between the EcoRI and HidIII sites and named pET28-NAM. The recombinant construct was further confirmed by digestion and sequencing (Fig. 1). The expression of recombinant NAM was induced by addition IPTG.

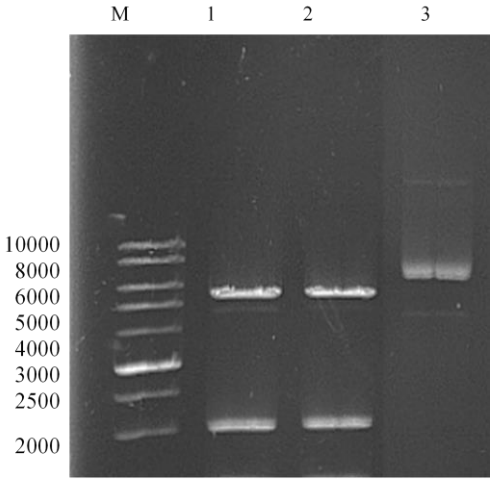


Fig. 1. Cloning validation. Lane M: DNA ladder. Lane 1 and 2: double digestion of recombinant vector with EcoRI and HindIII resulted a 2000 bp and 5400 bp bands corresponding to NAM and pET28a, respectively. Lane 3: single digestion pET28a/NAM resulted a 7400bp band.

Whole bacterial cell lysates was prepared and the total extracted protein was subjected to SDS-PAGE and a band corresponding to a 78 kD rNAM was detected on the gel (Fig. 2). The majority of the rNAM protein were found as an aggregate within the inclusion body while only a small amount was detected in the soluble fraction. For confirmation anti his-tag antibody specially targeted chimeric protein of corresponding molecular weight to rNAM from crude bacterial cell extract (Fig. 3).

Table 2. Culture conditions and the assigned levels for each factor.

Factors	unit	Low star points	Low level	Center points	High level	High Star points
		-α	-1	0	+1	+α
Post-induction temperature(A)	ºC	19	25	31	37	45
Concentration of IPTG(B)	mM	0.05	0.3	0.55	0.8	1.05
Starting induction time(C)	OD	0.4	0.6	0.8	1	1.2
Post induction time(D)	H	0.5	3	5.5	8	10.5

*The central point (0) corresponds to intermediate values between the -1 and +1 interval of each variable.

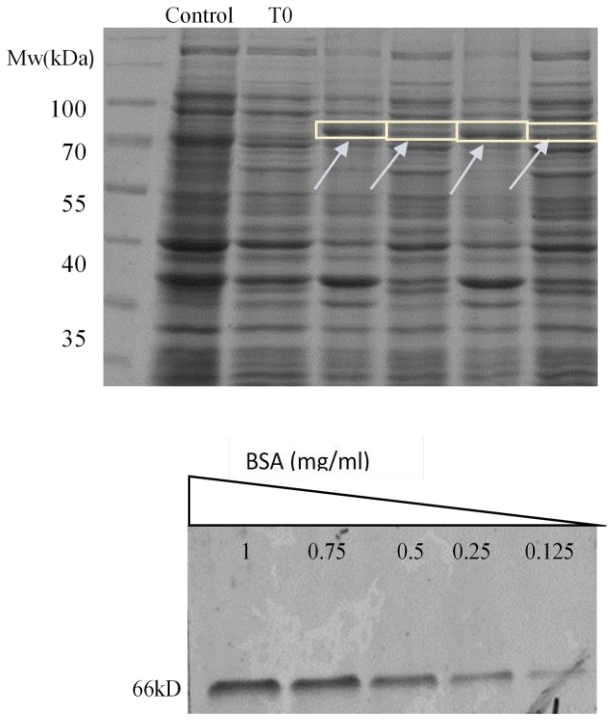


Fig.2. protein quantification using Image J software. BSA standard curve was used to estimate yields of the rNAM in SDS-PAGE 10 %. Soluble protein fraction of induced BL21 (DE3)-pET28a/NAM cells at different runs were illustrated. Lane control: induced BL21 (DE3)-pET28a without insert. Lane T0: uninduced BL21 (DE3)-pET28a/NAM cells.

Central Composite Design and Condition of Optimized Expression

The rotatable central composite design was applied to determine optimal level of factors to maximize the expression

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rate of rNAM (Table 2). The protein expression was analyzed at 30 different expression condition and the soluble protein produced were measured and the corresponding results are represented in Table 4.

The model F-value of 37.12 indicates that the model was significant. P-value prob>F less than 0.05, imply that the model terms was significant.. In this model terms, temperature, Time, OD and the interaction between temperature and IPTG concentration (AB), post induction time and cell density (OD600) (CD) were significant factors illustrated in figure 6 as contour and 3D surface plots (Table 3 and Fig. 4). The "lack of fit F-value" of 4.32 was insignificant indicated that the model was fit with observed value and was acceptable for the following experiment. Another criteria for checking the model quality is coefficient R2. The value R-squared (0.97) indicates that 97% of the total variations in system can be explained by model. In addition, the coefficient determination (R2-adjusted) of 0.94 suggested a good compatibility between actual and predicted value of expression of soluble rNAM protein.

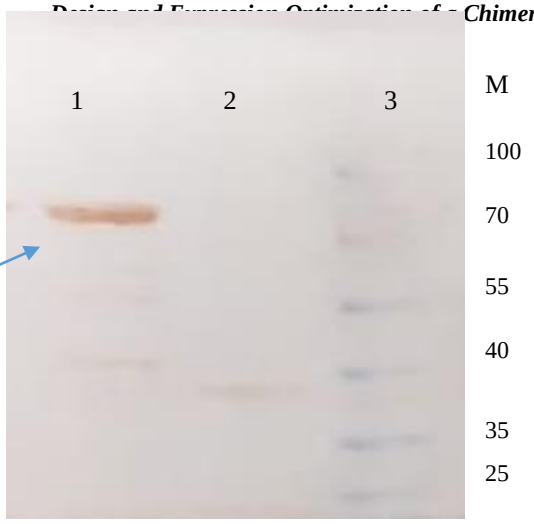


Fig 3. Western blot of expressed rNAM using anti His-tag antibody. 1: Extract of IPTG induced *E. coli* containing NAM construct. ~78kD. 2: uninduced *E. coli* cells (T0). 3: MW marker

In this model, Adeq precision was 24.48 indicating that there was an adequate signal.

Table 3. Analysis of ANOVA for response surface quadratic model.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	4.04	14	0.28	36.00	0.0001	significant
A-temperature	3.37	1	3.25	416.73	0.0001	significant
B-IPTG	0.027	1	0.026	3.32	0.0883	
C-OD	0.067	1	0.071	9.15	0.0085	significant
D-Time	0.31	1	0.31	39.18	0.0001	significant
AB	0.025	1	0.037	4.76	0.0454	
AC	3.875E-003	1	1.785E-003	0.23	0.6394	
AD	2.678E-003	1	8.418E-003	1.08	0.3155	
BC	4.523E-003	1	3.875E-003	0.50	0.4918	
BD	0.010	1	0.012	1.47	0.2435	
CD	0.035	1	0.039	5.01	0.0408	
A^2	0.055	1	0.056	7.15	0.0174	
B^2	0.079	1	0.078	10.02	0.0064	
C^2	0.017	1	0.018	2.26	0.1537	
D^2	3.709E-004	1	4.597E-004	0.059	0.8115	
Residual	0.12	15	7.804E-003			
Lack of Fit	0.10	10	0.010	4.34	0.0593	not significant
Pure Error	0.012	5	2.417E-003			
Cor Total	4.15	29				

Std. Dev.	Mean	C.V.%	R-Squared	Adj R-Squared	Pred R-Squared	Adeq Precision
0.088	0.65	13.48	0.97	0.94	0.85	24.84

*the p-value indicates the significance level of the variables and their interactions, Variables with $p < 0.05$ were considered statistically significant

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Based on multiple regression analysis, the full quadratic equation for prediction the optimal expression of rNAM soluble protein using coded factors was expressed as follows:

$$Y= 0.64 – 0.37A + 0.034B + 0.053C + 0.11D – 0.039AB + 0.016AC – 0.013AD + 0.017BC + 0.026BD – 0.047CD + 0.045A2 – 0.054B2 + 0.025C2 + 3.68E\text{-}0.03D2 \quad (1)$$

Where Y as a response is the soluble rNAM production, A, B, C and D are the coded factors for four variable factors. Positive and negative effects of each factors and interaction between them are represented as plus (+) and minus (–) symbols.

Different combinations of post induction temperature (19-43 ºC), IPTG concentration (0.05-1.05 mM), OD 600nm prior induction (0.4-1.2) and post induction time (0.5-10.5 h) resulted in soluble rNAM production from 0.058 to 1.48 mg/ml (Table 4). The lowest amount of soluble recombinant (0.058 mg/ml) was obtained at the center point of IPTG concentration, OD 600nm and post induction time (0.55 mM, 0.8 and 5.5 h, respectively) while post induction temperature was 43 ºC. This result revealed that a high temperature is not favorable for

Table 4. The observed and predicted values for the expression of rNAM in *E.coli* under various culture condition based on CCD.

Run	A:Temprature (ºC)	B: IPTG concentration (mM)	C: cell density (OD_{600})	D: Induction time (h)	Actual response (mg/ml)	Predicted response
1	37	0.8	0.6	3	0.11	0.018
2	19	0.55	0.8	8	1.48	1.57
3	31	0.55	0.8	0.5	0.25	0.424
4	25	0.8	0.6	8	1.25	1.25
5	37	0.3	0.6	3	0.21	0.11
6	37	0.8	0.6	8	0.32	0.37
7	43	0.55	0.8	5.5	0.058	0.067
8	25	0.3	1	8	0.97	1.03
9	25	0.3	0.6	3	0.81	0.79
10	37	0.8	1	8	0.39	0.44
11	25	0.8	0.6	3	0.87	0.85
12	31	0.05	0.8	5.5	0.27	0.35
13	31	0.55	0.8	10.5	0.96	0.88
14	31	0.55	0.8	5.5	0.6	0.63
15	25	0.8	1	8	1.299	1.266
16	31	0.55	0.8	5.5	0.64	0.63
17	31	1.05	0.8	5.5	0.48	0.49
18	37	0.3	0.6	8	0.34	0.36
19	25	0.8	1	3	1.101	1.05
20	37	0.3	1	3	0.37	0.311
21	31	0.55	0.8	5.5	0.68	0.638
22	37	0.3	1	8	0.42	0.369
23	25	0.3	1	3	1.04	0.92
24	25	0.3	0.6	8	1.13	1.087
25	31	0.55	0.8	5.5	0.71	0.638
26	37	0.8	1	3	0.319	0.28
27	31	0.55	0.4	5.5	0.58	0.63
28	31	0.55	0.8	5.5	0.58	0.638
29	31	0.55	1.2	5.5	0.8	0.84
30	31	0.55	0.8	5.5	0.62	0.63

* Recombinant NAM concentration yield (mg/L) obtained from densitometry analysis with different expression conditions of IPTG concentration, OD_{600}, temperature and induction time using a central composite design for four variables.

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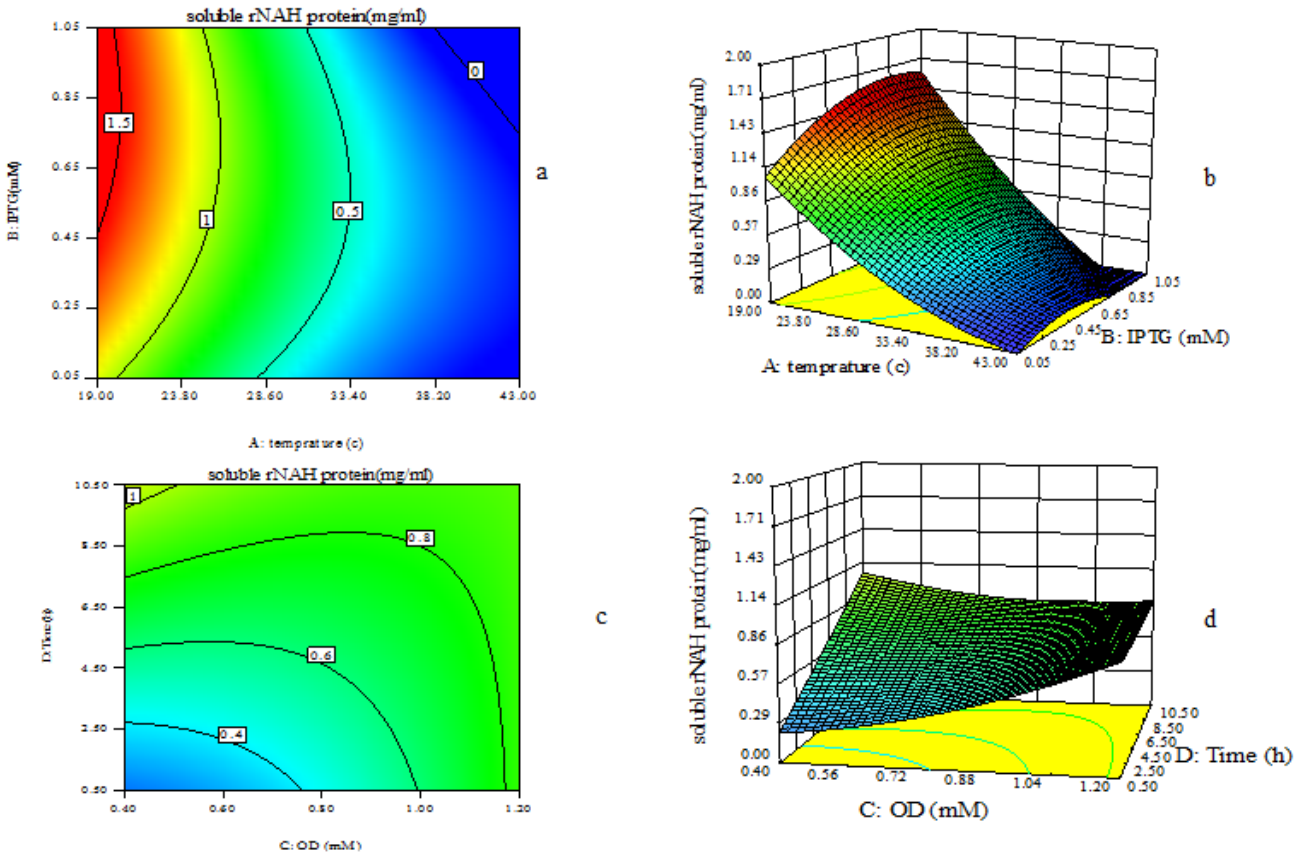


Fig. 4. 3D surface and contour curves of soluble rNAM protein showing (a, b) interaction between temperature and IPTG concentration at cell density 0.8 and post induction time 5.5h, (c, d) interaction between cell density and time at the post induction temperature 31 ˚C and IPTG concentration 0.55mM.

rNAM production. Moreover, at the low temperature (19 ºC) and the central point value of IPTG concentration and OD 600nm (o.55 mM and 0.8) at the 8 h induction time we achieved the highest value of rNAM production. Therefore, by increasing the induction temperature above 20 ºC the higher amounts of rNAM is accumulated as inclusion body. The model's authenticity test explained that the experimentally measured values are located around the statistically predicted ones, approving the model's sufficiency (Fig. 5)

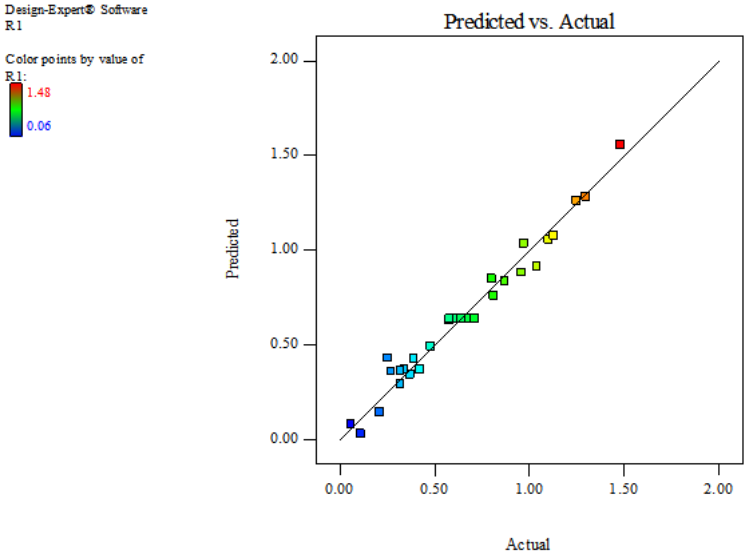


Fig 5. Linear plot of Predicted vs. actual values of soluble rNAM protein. X axis: experimental value of produced rNAM under optimization process, Y axis: a prediction of produced rNAM under optimization process

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DISCUSSION

There is a growing need to develop a vaccine for NE. The designation of fusion subunit vaccine encompassing the immunogenic segments from individual toxin against NE is a promising recent approach [8]. Mot et al. and Uzal et al, have shown for protection against NE, administration of multiple antigens like Alpha-toxin and NetB toxin could elicit better immunogenicity and protection than each antigen alone [16, 32]. Rostami et al, designed a fusion protein consisting of immunogenic parts of three virulence factors of NetB-Alpha toxin-Tpel(NAT) to be used as vaccine against necrotic enteritis[27]. Most of NAT was expressed as an inclusion body. The protein was denatured, purified and refolded but the refolding efficiency of the protein was low as detected by circular dichroism analysis which could be due to the hydrophobic nature of Tpel fragment in the chimeric protein.

There are some evidences that the Tpel have little effect on pathogenesis of NE and induces partial protection against NE [7,8]. However, there is no consensus in this regard and some researchers believe that it increases virulence of *C. perfringens* while others found no evidence of involving Tpel in the NE pathogenesis. Moreover, it is demonstrated that metallopeptidase protein, with cell surface anchoring feature, has important role in *C. perfringence* pathogenesis [18]. This result was confirmed by Kulkarni et al., [11] which showed that immunization with metallopeptidase and PFOR as a part of multicomponent vaccine resulted in the protection against a sever challenge.

Hence, a recombinant fusion protein, rNAM, was designed using bioinformatics tools (data not shown) to preserve critical immunogenic segments of Alpha-toxin, NetB, and metallopeptidase that are involved in *C. perfringens* pathogenesis.

The bioactivity of the therapeutic proteins like subunit recombinant vaccines depends on their correct folding to induce a complete immune response. Aggregation of recombinant protein as inclusion body is one of the main problems in preparation of functional protein [32,33].

In many cases the yield of production of heterologous proteins in *E. coli*, encounters with two important challenges including low expression and misfolding into insoluble aggregates. Several studies demonstrated that codon optimization has a critical role in high expression of heterologous protein. Production of plant codon optimized protein, as soluble form in *E. coli* in acceptable amount is considered to be major obstacles in using one gene sequence for both systems [4,13]. Moreover, only 30-50 % of recombinant proteins produced in *E. coli* are sufficiently soluble [13,17].
Firstly, due to higher percentage of rare codons, and codon adaptation index of 63% for *E.coli* the rNAM is expressed poorly (Table 1). This is in accordance with Menzella et al. study. They have shown that the CAI of 66% resulted in reduced production of chymosin in *E. coli* [15].

The solubility of rNAM produced at standard growth condition (run 1, Table 4) in *E. coli* was less than refolded form. This could be as a result of changes in secondary structure of refolded rNAM compared to the native form that was shown by data obtained from circular dichroism analysis (manuscript under review). Different approaches are designed to eradicate the inclusion bodies including codon optimization strategy, fusion tags utilization [22], changing the expression host [9], and culture condition [26, 14, 29] such as lower growth temperature [24].

Rigi et al. [29] reported that 5 fold productivity of Staphylococcal protein A was attained using culture condition optimization by RSM. Using the same method Papaneophytou et al. [22] has reported increasing up to 11% of the soluble TNF-α after determination of optimum induction conditions in *E. coli*.

Our results is in accordance with the above studies. In this study, application of RSM software has resulted in Table 3 presenting modifications of parameters for increasing production of soluble protein. The results of optimum response by regression equation using coded factors fitted to a second-order poly-nominal mode (Eq.(1)) defined that the linear coefficients A (temperature), C (starting time induction) and D (induction time), unlike the linear coefficient B (IPTG concentration) have a positive effect on production of soluble rNAM Although, the interactions between temperature and inducer concentrations were statistically significant where p-value was 0.045 Such as modification of the growth temperature had the most significant effect.

The maximum enhanced soluble rNAM protein production was found in run 2 that revealed that lower post-induction temperature at 20ºC. This is in accordance with Volonte et al. [35] that have reported the highest productivity of soluble recombinant glutaryl-7-aminocephalosporanic acid acylase (GLA) was achieved by lowering temperature from 37 to 25. An increase in the amount of Tumor Necrosis Factor-α at lower temperature has also been reported [23]. Furthermore, several studies have demonstrated that culturing at low temperatures, due to a decrease in many metabolic processes can lead to a higher yield of the soluble fraction of many proteins [21, 19, 29, 1].

Based on results of this study, post-induction time is also an important factor, the longer post induction time resulted in a higher production of soluble rNAM, and thus maximum 8 h was selected as the optimized induction time.

In this study, the IPTG concentration showed insignificant effect on production of soluble form (Table 3). Therefore, based on optimum level of other three factors for obtaining the highest yield, the RSM software has proposed 0.55mM for IPTG concentration. This is in accordance with Shafiee et al., and Ramirez et al.,[25, 30]. who demonstrate that the inducer concentration lower than 1 mM does not affect the expression level of the recombinant protein.

The represented experimental data showed that the production of the soluble rNAM was maximized when post induction temperature, IPTG concentration, the starting time of induction and duration of induction were 19 ºC, 0.55mM, 0.8 and 8 h respectively. Based on the prediction of Design Expert, the optimum condition for highly production of soluble rNAM were achieved at 20.36 ºC with, 0.49 mM of IPTG, an OD600 of 0.8 for start of the induction, and 7.85h of induction time for the predicted rNAM production of 1.57 mg/ml. Therefore, the optimization process led to 7.39 fold increase in the soluble protein fraction (i.e. 1.48 mg/ml versus 0.2mg/ml) (Table 4).

In summary, this research is the first report presenting the data on optimization the expression of subunit candidate vaccine rNAM against avian necrotic enteritis in *E. coli*, using RSM methodology. We demonstrated that with limited number of experiments will facilitate 7 fold increase in production of soluble protein. The data would increase the knowledge of expression of heterologous proteins for research and industrial applications.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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