Original Article

Medium Optimization for Synaptobrevin Production Using Statistical Methods

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Abstract

Background: Botulinum toxin, the most potent biological toxin, has become a powerful therapeutic tool for a growing number of clinical applications. Molecular studies have identified a family of synaptic vesicle-associated membrane proteins (VAMPs, also known as synaptobrevins) which have been implicated in synaptic vesicle docking and fusion with plasma membrane proteins.

Materials and Methods: Using the synaptobrevin as a substrate for in vitro assay is the method to detect BoNT activity. We have been working on optimizations of bacterial expression conditions and media for high-level production of synaptobrevin peptide. Statistics-based experimental design was used to investigate the effect of medium components (E. coli strain, peptone, IPTG, yeast extract, ampicillin, and temperature) on synaptobrevin production by E. coli.

Results: A 24 fractional factorial design with center points revealed that IPTG and temperature were the most significant factors, whereas the other factors were not important within the levels tested. This purpose was followed by a central composite design to develop a response surface for medium optimization. The optimum medium composition for synaptobrevin production was found to be: IPTG 29 mM, peptone 10 g/L, yeast extract 5 g/L, temperature 23°C and ampicillin 100 mg/L. This medium was projected to produce, theoretically, 115 mg/L synaptobrevin.

Conclusion: The optimum medium composition synaptobrevin production was found to be: BL21 (E.coli strain), LB medium (peptone 10 g/L, Yeast 5 g/L), Ampicillin (100 mg/L), IPTG (0.29 mg/L) and temperature (23°C).

Keywords: synaptobrevin; E. coli; experimental designs; central composite design; medium optimization

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Introduction

Tetanus toxin and botulinum neurotoxins are neurotoxin belonging to the same family, which cause tetani and botulism respectively (1). These have been

successfully used as a therapy to treat disorders caused by abnormal muscular contractions such as strabismus, cerebral palsy, anal contractions and torticollis. Recently these toxins have been used even in cosmetics against wrinkles (2, 3).

Several laboratory assays for the detection of BoNTs and tetanus toxin have been developed. The currently used in vivo mouse bioassay is the standard method to detect BoNT activity, and the only assay approved by the FDA(4). In this assay, mice are injected intraperitoneally or intravenously with toxin or and observed for signs of toxicity and death (5).

An alternative in vitro assays include endopeptidase assays(6) can be used to quantitate BoNT's and tetanus toxin in vitro and in foods and clinical samples (6). Reliably detection of BoNT and tetanus toxin can be achieved by Western blot assay of the cleaved synaptobrevin as a target protein(6-8). The synaptobrevin peptide is intended for development of cell-free assays to test inhibitors of BoNT catalytic activity by monitoring reductions in the cleavage of the synaptobrevin fragment by BoNT/B(9).

The production of high-yield recombinant proteins from Escherichia coli is highly desirable for biotechnological process and industrial settings. In order to gain maximum efficiency, a number of central elements are essential in the design of recombinant expression systems (10) such as strain development, medium optimization, bioprocess optimization, and mathematical modeling have been widely used. Concentrations of produced protein and growth of cells are strongly influenced by medium composition such as the carbon, nitrogen source and IPTG concentration (11-15). The traditional method of optimization involves varying one factor at a time, while keeping the others constant. This method is simple and easy to implement with no need for statistical analysis; although, it may require a relatively large number of experiments and frequently fails to anticipate the optimal condition(16). This important shortcoming is due to the inability of the approach to consider the effect of possible interactions between factors. The deficiency can be overcome by applying more efficient, statistically based experimental design (16, 17). For this purpose, factorial design and response surface analysis are important tools to determine the optimal process conditions. Experimental designs for optimization have been used in many areas of biotechnology such as optimization of a culture medium (18-21), enzyme production (22-24), ethanol production and biomass production (18, 25-27).

Previous studies has indicated that the most important aspects of the process parameter is medium composition, where the concentration of carbon and nitrogen sources play a major role(28, 29). Therefore an investigation was performed to statistically optimize the medium components for the production of the recombinant synaptobrevin from E. coli strains. Response surface methodology (RSM) is the most widely used statistical technique for medium optimization. Response surface experiments identify the response of a system as a function of explanatory variables. RSM is most often used to determine the optimum response for the specific range of variable conditions. The interaction among the possible influencing parameters can be evaluated with limited number of experiments(30, 31).

The aim of this work was to define of five factors that will be affected a basal culture medium. The selected proportions of these factors are expected to maximize the quantity of active secreting cells, the productivity and the quality of the produced peptide.

The optimization of IPTG, yeast, peptone, ampicillin concentration and temperature of induction and also selection of E.coli strain for high synaptobrevin production by recombinant E. coli were carried out using the statistical optimization method. Experimental design using response surface methodology (30, 32, 33) was used in the present study to enhance synaptobrevin production by optimizing the induction and culture medium conditions.

Methods

Plasmid and expression system. E. coli strains (BL21 and Rosetta) were used as the hosts. The synaptobrevin gene was cloned into the expression vector pET-15b under the control of Lac promoter. Synaptobrevin was expressed as intracellular protein from plasmid PET-15b by IPTG induction under the control of the strong promoter.

Medium Composition. Two types of medium have been used throughout this study, a minimal medium (LB) and the enriched medium (SB) for recombinant protein production.

Overexpression. E. coli cells were transformed with appropriate plasmids and grown overnight at 37°C on Luria-Bertani (LB) agar plates containing 100 mg ampicillin/ml. The overnight colonies were transferred to a shake flask containing LB broth with 100 mg ampicillin/ml and were grown at 37°C for the required time interval. Fermentation was carried out in a 1L flask with 100 ml working volume of LB medium with 100 mg/ml ampicillin. The pH of the media was maintained at 7.0. The culture was induced with different concentrations of IPTG when the concentration of exponentially growing cells was equivalent to an optical density (O.D) at 550 nm between 0.4 and 0.70.

Experimental Design and Data Analysis. A two-step experimental design was used in developing a model for optimal synaptobrevin production in this study. The predictor variables were coded according to the following equation:

$$Xi = (Xi-Xi,0)/\Delta Xi \quad (1)$$

Where Xi is the coded value of an independent variable, Xi is the independent variable's real value, Xi,0 is the independent variable's real value at the center point, and ΔXi is the step change value. The synaptobrevin concentration was taken as the dependent variable or response.

Fractional Factorial Designs. At first, Fractional factorial design (FFD) carried out to identify medium ingredients that had a significant effect on the synaptobrevin production. The major advantage of using a factorial design is the obtaining of maximum information with reduced number of experiments that need be performed. At the end, the factorial design allows the effect of a given factor to be determined at several levels of the other factors, so, the conclusions are valid over a range of experimental conditions (34). The most important class of the factorial design is to investigate ''k'' factors, each at only two levels and requires 2k experiments.

Because, the main effects and lower-order interactions are usually the most significant terms(35), determining these effects by conducting a fractional factorial design without loss of any important information is possible. In this study, a fractional factorial design with 24 experimental runs was performed. In this case, the first-order model was fitted to the data from the FFD experiments.

Central Composite Design. A Box-Wilson experimental design (36) with five coded levels was performed to describe the behavior of the response in the optimum region,. To provision a central composite design (CCD) for the two remaining factors, a full 22 factorial design was combined with 6 replications of the center points and 5 axial points the two factors are at their center point levels. In order to prediction of optima, quadratic model was fitted to experimental results.

Data Analysis. The statistical analysis of the results was performed with the accessory of Design Expert version 7.0.1 statistical software (Stat- Ease Inc., Minneapolis, MN). The qualities of the fitted polynomial models were examined by the coefficient of determination R2. The synaptobrevin concentration was analyzed using the analysis of variance (ANOVA) combined with the F-test to evaluate if a given term had a significant effect ($p \leq 0.05$). The location of the optimum was determined by solving the set of equations derived by the differentiation of the final quadratic model.

Results

Fractional Factorial Design. The aim of the first optimization step was to identify the factors and the components of the medium that have a significant effect on synaptobrevin production. Five factor were assessed. The range and the levels of the variables are given in Table I.

A full factorial design would need 32 experiments, which is a high number. Consequently, a 24 fractional factorial design consisting of 18 factorial runs along with 6 other experiments at the center of the design for analysis of variance was performed. The experimental design and the results of the FFD are summarized in Table II. The synaptobrevin concentrations shown are maximum values observed during each growth period. Based on these experimental values, statistical testing was carried out using Fisher's statistical test. The model F-value of 6.06 implies that the model is significant. Also, P-

values less than 0.05 indicate model terms that are significant at the probability level of 95%. The P-value of 0.0001 for IPTG shows that it has a very significant positive effect on synaptobrevin production. The low temperature allowed the strain to produce a higher level of synaptobrevin. According to the analysis of variance procedure, the combination of low temperature and IPTG effects accounted for nearly 91% of the variability in the synaptobrevin production, whereas ampicillin, E.coli strain and medium (yeast extract and peptone) did not significantly influence synaptobrevin production within the levels tested.

A first-order model was fitted to the data acquired from the fractional factorial design experiments in order to obtain optima. The values of the regression coefficients were calculated and the following equation was derived using the coefficients of the coded variables:

$$Y=14554+2122_{x1}-3892_{x2}-8133_{x3}-7859_{x1x3}+2718_{x2x3}-2513_{x1x4} \quad (2)$$

The Analysis of variance for FFD refined model is summarized in Table III. The curvature F-value of 12.51 indicates that model curvature is not significant throughout the design space, and there is 0.01% chance that a curvature F-value this could occur due to noise. Furthermore, the lack of fit value of 6.06 shows this source of variation is not significant relative to pure error. The assumption of normal error distribution was confirmed by a normal probability plot of the studentized residuals for the model.

Central Composite Design. To explain the nature of the response surface in the optimum region, a central composite design was performed, and the levels of the two significant variables, IPTG (X2) and temperature (X3) were optimized. For the two factors, this design comprised a full 22 factorial design with its 4 cubic points, augmented with 5 replications of the center points, and the 4 axial points. The level of the three non-significant factors (Medium, E.coli strain and ampicillin) was kept at the central point of the FFD (LB medium and BL21 strain selected). The range of the variables were investigated in this step are given in Table IV; the experimental design and the results are presented in Table V.

The experimental results of the CCD were fitted with a second-order polynomial expression. The values of regression coefficients were calculated and the fitted equation (in the terms of coded values) for predicting synaptobrevin production was:

$$Y=3.67-0.98_{x2}-2.05_{x3}-1.98_{x1x3}+0.69_{x2x3} \quad (3)$$

The experimental results of the CCD were fitted with a second-order polynomial expression. The model P-value of 0.0001 implies that the model is significantly fit. The goodness of fit was expressed by the coefficient of determination R2, which was calculated to be 0.90, indicating nearly 90% of the variability in the response could be explained by the model. Furthermore, the final quadratic model had R2Adj equal to 0.90 in comparison to 0.88 calculated for the full quadratic model. This supports the hypothesis that the model is sufficient to describe the

Table 1: Applied levels of independents variables in the FFD.

Variable	component	Applied levels	
		(Low) g/L, °C, Type	(High) g/L, °C, Type
X1	E.coli strain	Rosetta	BL21
X2	IPTG	0.05 mM	0.1 mM
X3	Temperature	15 °C	37 °C
X4	Medium	Yeast: 5 g/L Peptone: 10 g/L	Yeast: 20 g/L Peptone: 32g/L
X5	Antibiotic	10 mg/L	250 mg/L

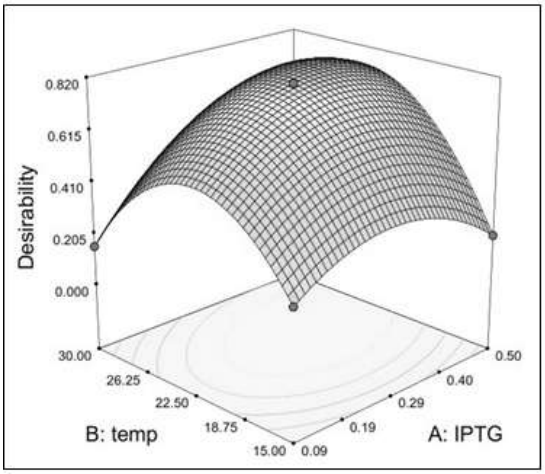

Figure 1. Response surface of synaptobrevin concentration.

response of the experimental observations pertaining to synaptobrevin production. The analysis of variance showed that the model F-value of 12.36 is significant and there is only 0.01% likelihood that this large model F-value could occur by chance. Also, the lack of fit value of 0.16 implies that the lack of fit is not significant relative to the pure error.

The response surfaces shown in Figure 1 were based on the final model, varying the two factors within their experimental range. Figure 1a illustrates the response surface for the optimum level of IPTG and temperature. The minimum response (40 mg/L) occurs when IPTG were 0.09 and 0.5 mg/L, temperature were 15 and 30 ° C. At the 23°C of (88 mg/L) the response indicates a maximum nearly at the middle of peptone level. Also, at different levels of IPTG the response varies noticeably along the axis representing temperature axis. These suggest considerable interaction between the two factors.

At the optimum level of IPTG (Fig.1), maximum response (115 mg/L) was seen with a high level of temperature. Analysis of response at the different levels of the factors revealed that there is a significant interaction between interaction IPTG and temperature. Also, the minimum value of the response (40 mg/L) is located at the lowest and highest level of IPTG and temperature.

Table 2: Experimental design and the result of FFD.

Run	Applied levels					Expression mg/L
	X1	X2	X3	X4	X5	
1	BL21	0.53	26	LB	125	51.39
2	Rosetta	0.53	26	LB	125	7.93
3	Rosetta	1	15	SB	10	55.97
4	Rosetta	0.53	26	LB	125	1.12
5	Rosetta	0.53	26	SB	125	23.13
6	BL21	1	37	SB	10	25.15
7	Rosetta	0.05	15	LB	10	109.9
8	Rosetta	1	37	SB	250	1.78
9	BL21	1	15	SB	250	13.33
10	Rosetta	0.53	26	SB	125	14.57
11	BL21	0.53	26	SB	125	80.09
12	BL21	0.05	15	LB	250	30.05
13	BL21	0.05	15	SB	10	63.24
14	Rosetta	0.05	15	SB	250	92.7
15	Rosetta	0.05	37	SB	10	1.001
16	BL21	1	15	LB	10	21.7
17	Rosetta	0.05	37	LB	250	1.38
18	BL21	0.53	26	LB	125	62.37
19	BL21	0.05	37	LB	10	16.69
20	BL21	0.53	26	SB	125	81.11
21	Rosetta	1	37	LB	10	2.75
22	BL21	1	37	LB	125	23.31
23	BL21	0.05	37	SB	250	57.62
24	Rosetta	1	15	LB	250	71.37

Discussion

The classical method of medium optimization based on changing one parameter at a time while keeping the others at fixed levels is laborious and time consuming. This method requires a complete series of experiments for every factor of interest. Moreover, such a method does not provide means of observing possible factor interactions. In contrast,

Table 3: Analysis of variance for FFD refined model.

Source of variation	*SSi*	*df*	*MSi*	*F*	*P>F*
Model	161.91	5	32.38	23.29	< 0.0001
X1	4.60	1	4.60	3.31	0.0905
X2	15.45	1	15.45	11.11	0.0049
X3	67.47	1	67.47	48.52	< 0.0001
X1X3	62.99	1	62.99	45.30	< 0.0001
X2X3	7.54	1	7.54	5.42	0.0354
Curvature	69.56	4	17.39	12.51	0.0002
Residuals	19.47	14	1.39		
Lack of Fit	18.26	10	1.83	6.06	0.0487
Pure error	1.21	4	1.30		
Total	250.94	23			

Table 4: Frequency of Dermatomycoses among students in a school dormitory.

	Applied levels				
Independent variables	Star-low	Low	Center	High	Star-high
X2	0.01	0.09	0.29	5.0	0.58
X3	11.89	15	22.5	30	33.1

factorial experimental designs offer a number of important advantages.

For instance, the researcher could easily determine factor effects with considerably less experimental effort, identify factors, find optima, offer greater precision(37) and facilitate system modeling (38).

In this report, a fractional experimental design proved to be a valuable tool in optimizing the medium for recombinant synaptobrevin production. Fractional factorial design used as the first step was efficient to screen which medium components among the selected factors were significant. More specifically, yeast extract, peptone, E.coli strain and ampicillin concentration were proven to be not very important parameters with regard to synaptobrevin production, while IPTG and temperature were very significant.

Finally, central composite design and response surface analysis were useful to determine the optimum levels of the components that significantly influence the protein production. The optimum medium composition for the production of synaptobrevin by E.coli was established as follows: BL21 E.coli strain), LB medium (peptone 10 g/L, Yeast 5 g/L), Ampicillin (100 mg/L), IPTG (0.29 mg/L) and temperature (23°C).

Conflicts of Interest

There is no conflict of interest

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