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Research Article

EXPRESSION AND OPTIMIZATION OF CAPSULAR POLYSACCHARIDE PRODUCTION BY *NEISSERIA MENINGITIDIS* SEROGROUP-A USING STATISTICAL DESIGNS AND SURFACE PLOTS

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ABSTRACT

Statistical designs were used to optimize the fermentation variables for the enhanced production of polysaccharide by Neisseria meningitides serotype A. Among the seven independent variables studied by using Plackett-Burman design, three variables Yeast extract, Glucose and NaCl were found to be significantly affecting for polysaccharide production. The optimum concentrations of these three variables were predicted by a second order polynomial model fitted to the results obtained by Box-Behnken design. The predicted optimal levels of these variables were as follows, Yeast extract 3g/L, Glucose 7.36g/L and NaCl 3.26g/L. There was thirty percent increase in polysaccharide production as compared with polysaccharide obtained at original levels of these variables.

Keywords: Plackett-Burman design, Response Surface Methodology, Neisseria meningitides, Polysaccharide (PS).

INTRODUCTION

Meningitis[1] is an inflammation of the meninges, the lining that protects the brain and spinal cord. It is almost always caused by an infection, usually by a bacterium (bacterial meningitis) or a virus (viral meningitis[2]). In rare cases it can be triggered by a fungus or parasite. A number of bacterial agents can cause a severe and often fatal form of meningitis[3]. The most common etiologies present today are *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae*. College-aged individuals are at increased risk for bacterial meningitis due to living conditions and social practices that facilitate pathogen.

Meningococcal Polysaccharide vaccine (A, C, Y & W135) is to protect from Meningococcal meningitis serogroups A, C, Y & W135. This vaccine is made from bacterial capsular polysaccharides through fermentation of each individual serogroup of *Neisseria meningitides* in bioreactors. Then the polysaccharides are purified, formulated and lyophilized using preservatives and stabilizers to make a vaccine product.

In this present study for developing maximum polysaccharide expression, two experimental designs were sequentially applied as a tool for optimizing polysaccharide expression. In the first optimization step, Plackett-Burman experimental design[4] was applied to test the relative importance of various medium components on polysaccharide expression. In the following, Box- Behnkn design[5] was applied for further optimization to the most significant variables. This design is a response surface methodology[6] employed to find factor settings that produce the best response, find factor settings that satisfy operating or process specifications and model a relationship between the quantitative factors and the response.

MATERIALS AND METHODS

Microorganism and media

The microorganism used in this study was *Neisseria meningitides type* A. The culture media used was basal salt medium[7] containing gL^{-1}; L-Glutamic acid, 1.6; L-Cystein.HCl, 0.02; NaCl, 6.00; NH_4Cl, 1.25; $MgSO_4.7H_2O$, 1.23; Glucose, 5.00; Yeast extract, 2.00; $Na_2HPO_4.2H_2O$, 6.24 and KCl, 0.09.

Seed preparation

The meningococcal stock cultures are maintained in 1ml basal medium at -70°C with 2.5 OD_{600} and were streaked on Muller-Hinton[8] agar slant tubes. The tubes were incubated at 35°C for 15hours in an incubator for adequate growth. The bacterial lawn was scraped, resuspended in 20ml of basal medium and transferred to 1liter conical flask containing 250 ml of basal medium and incubated in a shaker incubator at 30°C, 200 rpm for adequate growth of 2.0-3.0 OD_{600}.

Design of experiment (DOE)

Experiments were designed and analyzed by Placket-Burman[9], response surface methodology[10,11] using STATISTICA software. Plackett-Burman (PB)[12] is a technique designed to screen the important variables affecting the product formation. Response surface methodology is used to optimize the major variables which are screened through PB design.

Fermentation process

A 6L fermentor equipped with automatic control of temp, DO and pH was used. The fermentation process[13] was carried out at the following parameters, temperature 35°C; air flow rate 5 L/min (0.125 vvm); agitation frequency 300-500 rpm; vessel pressure 6psi; pH 7.0; DO 30-40 %; and inoculum size 5 % for 16 hours. The DO level was maintained by pure oxygen enriched air.

ANALYTICAL METHODS

Cell concentration

Expressed as OD (Optical density) and Dry biomass[14], Optical density (OD) was estimated at OD_{600} and dry biomass determinated by a centrifugation of a sample at 10,000 g, followed by the pellet drying at 60°C for 48 hours.

Polysaccharide analysis

Polysaccharide was precipitated[15,16] by the addition of Cetavlon to the sample; after centrifugation the supernatant was eliminated and the precipitated pellet was resuspended in 1M $CaCl_2.2H_2O$ solution. The suspension was centrifuged again and the supernatant was collected for the polysaccharide determination using phosphorus and O-acetyl groups estimation. One gram of purified polysaccharide group A will give greater than 2mmol of O-acetyl groups[17] or 80mg of phosphorus[18].

EXPERIMENTAL DESIGNS

Plackett and Burman design

The Plackett and Burman experimental design, a fractional factorial design, was used to reflect the relative importance of various media components on polysaccharide expression in fermentation cultures. Seven independent variables were screened in eight combinations organized according to the Plackett and Burman design matrix (Table 1) for each variable; a high (+) as well as low (-) level was tested. All trials were performed and the polysaccharide expression

results were treated as responses. The main effect of each variable (Table 2) was determined with the following equation.

$E_{xi} = (\Sigma M_{i+} - \Sigma M_{i-}) N^{-1}$

Where E_{xi} is the variable main effect, M_{i+} and - ΣM_{i-} are the polysaccharide in trials where the independent variable (xi) was present in high and low concentrations, respectively and N is the number of trials divided by 2. A main effect with a positive sign indicates that the high concentration of this variable is near to the optimum and a negative sign indicates that the low concentration of this variable is near to the optimum. Significance of variables was determined using Statistica software.

Table 1: Plackett -Burman experimental design for 7 factors

Trial	Independent variables							Polysaccharide (g/L)
	GA	CH	NC	NH	MG	GL	YE	
1	-1	-1	-1	1	1	1	-1	118
2	1	-1	-1	-1	-1	1	1	130
3	-1	1	-1	-1	1	-1	1	120
4	1	1	-1	1	-1	-1	-1	110
5	-1	-1	1	1	-1	-1	1	112
6	1	-1	1	-1	1	-1	-1	109
7	-1	1	1	-1	-1	1	-1	110
8	1	1	1	1	1	1	1	120
9 (C)	0	0	0	0	0	0	0	100

Table 2: Factors examined as independent variable affecting polysaccharide and their levels in the Plackett-Burman experiment

Variable	Symbol	Level (g/L)			Main effect	t-value
		-1	0	+1		
L-Glutamic Acid	GA	0.80	1.60	2.40	2.25	0.20930
L-Cystein.HCl	CH	0.01	0.02	0.03	-2.25	-0.02093
NaCl	NC	3.00	6.00	9.00	-6.75	-0.62791
NH4Cl	NH	0.625	1.25	1.875	-2.25	-0.20930
MgSO4.7H2O	MG	0.615	1.23	1.845	1.25	0.11628
Glucose	GL	2.50	5.00	7.50	6.75	0.62791
Yeast Extract	YE	1.00	2.00	3.00	8.75	0.81395

Box-Behnken design

Experimental design, which is a central composite design, was applied[19,20]. In this model, the most significant independent variables, designated (X1), (X2), and (X3) were included and each of them was examined at three different levels(Table 3), low (-), high (+) and central or basal (0). According to the applied design described in the result section, fifteen combinations were tried. For predicting the optimal point, second order polynomial model was fitted to correlate relationship between independent variables and response variable. The values of the coefficients were calculated using STATISTICA software and the optimum concentrations were predicted.

Table 3: Variables and their levels for Box-Behnken experiment

Variables	Symbols	Levels (g L-1)		
		−1	0	+1
Yeast Extract	X1	2	3	4
Glucose	X2	5	7.5	9.5
NaCl	X3	1	3	6

Table 4: Examined concentrations of the key variables and results of the Box-Behnken experiment

Trial	Yeast Extract (X1)	Glucose (X2)	NaCl (X3)	Polysaccharide (g L^{-1})
1	2	5	3	110
2	4	5	3	115
3	2	9.5	3	100
4	4	9.5	3	105
5	2	7.5	1	119
6	4	7.5	1	132
7	2	7.5	6	109
8	4	7.5	6	112
9	3	5	1	115
10	3	9.5	1	101
11	3	5	6	107
12	3	9.5	6	105
13	3	7.5	3	120
14	3	7.5	3	125
15	3	7.5	3	119

RESULTS AND DISCUSSION

Factors affecting polysaccharide production (Plackett-Burman design)

For the explanation of medium components affecting polysaccharide production, the independent variables examined in the Plackett-Burman experiment and their settings are shown in Table1. The main effect of each variable was calculated according to polysaccharide expression gL^{-1} (Table 2). The data indicated that, the presence of high levels of GA, MG, GL and YE in the growth medium affects polysaccharide production positively. On the other hand, the presence of CH, NC and NH at their lowest levels would

result in high production. According to this results it can be predicted that the near optimum medium for polysaccharide production by *N.meningitides* serotypeA is (gL^{-1}): L-Glutamic acid, 2.4; L-Cystein.HCl, 0.01; NaCl, 3.00; NH_4Cl, 0.625; $MgSO_4.7H_2O$, 1.845; Glucose, 7.50; Yeast extract, 3.00; $Na_2HPO_4.2H_2O$, 6.24; and KCl, 0.09.

In order to evaluate the accuracy of the applied Plackett-Burman screening test, a verification experiment was carried out in triplicate. The predicted near optimum levels of independent variables were examined and compared to the basal settings. The average of polysaccharide production was recorded. Polysaccharide was achieved approximately 1.5times higher than obtained from basal medium. On the basis of calculated t-values (Table 2) YE, GL and NC were chosen for further optimization, since these factors had the most significant effects on the polysaccharide production.

Optimization of nutrient levels by Box-Behnken design

In this second optimization step the levels of the three significant independent variables YE, GL and NC were further investigated each at three different levels. Near optimum levels of the other factors, suggested by the Plackett - Burman experimental results were used in all trials. Experiments were performed and the observations (polysaccharide expression) were recorded (Table 4) and the results were presented in the form of surface plots, showed that High Yeast extract concentration together with basal level of Glucose (Figure1) greatly supported polysaccharide production, while high Yeast extract concentration together with low NaCl appeared to be positive with respect to polysaccharide expression (Figure2). On the other hand the interaction between Glucose and NaCl concentration (Figure3) illustrated that high NaCl concentration together with high glucose level inhibits polysaccharide production.

The predicted optimal concentrations of the tested three variables were obtained from desirability charts (Figure4), constructed using Response Surface Regression in STATISTICA software. These levels were as follows: Yeast Extract concentration: 3 g/L, Glucose concentration: 7.36 g/L and NaCl concentration: 3.26 g/L.

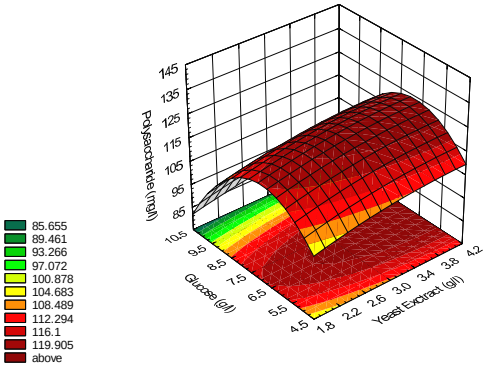

Fig. 1: Effect of glucose and yeast extract on polysaccharide (PS) production

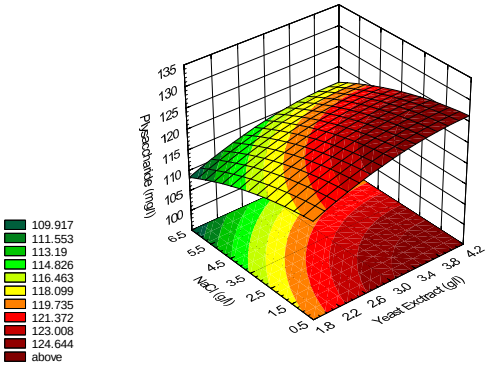

Fig. 2: Effect of NaCl and yeast extract on polysaccharide (PS) production

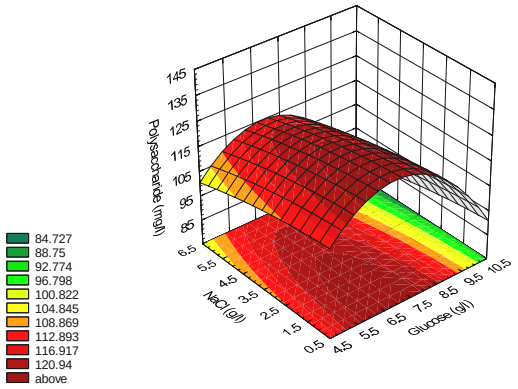

Fig. 3: Effect of NaCl and glucose on polysaccharide (PS) production

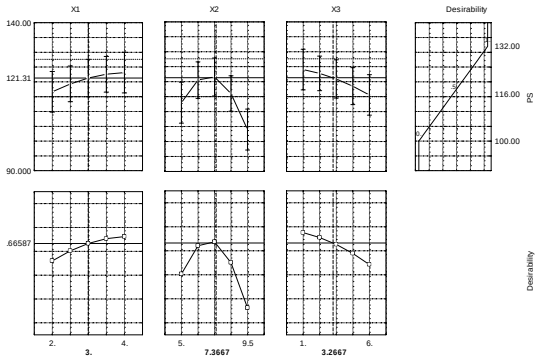

Fig. 4: Desirability charts for optimum concentration of yeast Extract(X1), glucose(X2) and NaCl(X3)

Model verification

In order to determine the accuracy of the model and to verify the optimization results, an experiment was performed under basal and predicted optimal conditions. Under the optimized condition, the polysaccharide was increased 1.4times as compared to the original medium.

CONCLUSION

Response surface methodology was used to optimize medium composition for *Neisseria meningitides* serotype-A polysaccharide production. It was found to be a very efficient method for optimal media setting. We screened three significantly effecting variables from seven in the first step and the optimal levels of these three variables were sorted out in the second step. Using that optimal levels polysaccharide yield was increased up to 1.4 folds than the yield obtained at their original levels.

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