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# Optimization of Capsular Polysaccharide Production by *Streptococcus pneumoniae* Type 3

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**Response surface methodology (RSM) examining the effects of five-level-three-factors and their mutual interactions was utilized to optimize the fermentation conditions to enhance capsular polysaccharide (CPS) production of *Streptococcus pneumoniae* type 3. Twenty experiments conducted in an 8-l lab-scale fermentor were designed to assess fermentation pH, supplemented glucose concentration, and stirring rate. The predicted highest CPS production by the obtained optimization model equation was 256.14 mg/l at optimal conditions [pH 7.5, stirring rate 180 rpm, and supplemented glucose concentration 1% (w/v)]. The validity of the response model was confirmed by the good agreement between the predicted and experimental results. The maximum amount of CPS obtained was 255.03±2.23 mg/l.**

**Keywords:** Capsular polysaccharide, optimization, response surface methodology, *Streptococcus pneumoniae*, vaccine

*Streptococcus pneumoniae* is a human pathogen that is a frequent cause of pneumonia, septicemia, meningitis, and otitis media [14]. Pneumococcal infections carry a high risk of mortality and morbidity, particularly in infants [16] and the elderly [5, 18]. The capsule, which protects encapsulated bacteria against phagocytosis by polymorphonuclear leukocytes, has long been recognized as a major virulence factor. Additionally, capsular polysaccharide (CPS) has been considered a major target for the development of a human vaccine [4]. Presently, the vaccines available for pneumococcal disease are the 23-valent pneumococcal polysaccharide vaccine (PPV23) and the 7-valent pneumococcal conjugated vaccine (PCV7). More recently, PCV9 (PCV7+serotypes 1 and 5; Wyeth, Madison, NJ, U.S.A.) and PCV11 (PCV9+serotypes 3 and 7F; Glaxo SmithKline, Middlesex, U.K.) have been developed to improve these vaccines. Moreover, PCV 13 is anticipated to become available before 2010 [18].

Type 3 CPS is composed of 16 repeat β-1→3 and β-1→4 glycosidic linkages of D-glucopyranose and D-glucopyranose uronic acid [16]. Optimizing and maximizing type 3 CPS production is desirable because it is an important component of PCV 11, and so could enhance the vaccination effect. Although some studies have reported the production of type 3 CPS using heterologous expression systems such as *Escherichia coli* (240.00 mg/l) [1] and *Lactococcus lactis* (120.00 mg/l) [12], CPS production did not attain levels sufficient for industrial purposes, because of the instability of pneumococcal DNA in *E. coli* (the produced pneumococcal proteins are detrimental to *E. coli* or to the presence of large numbers of random sequences with strong promoter activity that results in instability in *E. coli*) [8] and low productivity in *L. lactis*.

Response surface methodology (RSM) is a popularly used optimization procedure that is based mainly on full factorial central composite design (CCD) [2]. In this study, *S. pneumoniae* type 3 CPS production was optimized in 8-l lab-scale fermentor experiments using CCD, examining the simultaneous effects of three independent variables.

## Materials and Methods

### Cultivation Conditions

*S. pneumoniae* type 3 strain (ATCC 6303; American Type Culture Collection, Manassas, VA, U.S.A.) was initially grown in tryptic soy agar (TSA) containing 5% (w/v) defibrinated sheep blood (Difco, Detroit, MI, U.S.A.) at 37°C for 24 h. The seed culture was prepared by inoculating a recovered colony into a 250-ml Erlenmeyer flask

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**Table 1.** RSM experimental codes, ranges, and levels of the independent variables.

| Variables | Units | Symbol code | Levels[a] | | | | |
|---|---|---|---|---|---|---|---|
| | | | -1.682 | -1 | 0 | +1 | +1.682 |
| pH | | $X_1$ | 4.97 | 6 | 7.5 | 9 | 10.02 |
| Glucose concentration | % | $X_2$ | 0.33 | 0.6 | 1 | 1.4 | 1.67 |
| Stirring rate | rpm | $X_3$ | 79.09 | 120 | 180 | 240 | 280.91 |

[a]Based on program design value.

containing 50 ml of brain heart infusion (BHI) medium (BD Biosciences, Franklin Lakes, NJ, U.S.A.) at 37°C for 24 h. To determine the optimal medium for *S. pneumoniae* type 3 strain growth, Todd–Hewitt broth (THB, Difco), tryptic soy broth (TSB, Difco), and casitone-based broth (CAT, Difco) were investigated instead of BHI medium. To optimize fermentation conditions, 40 ml of the seed culture was inoculated in an 8-l lab-scale fermentor (BioG-Micom, Korea) containing 4 l of medium at 37°C for 24 h with aeration (1 gas volume flow per unit liquid volume per min; vvm).

The used lab-scale fermentor was performed by a top drive motor (mechanical drive, 50–1,200 rpm) with a 2-Rushton turbine impeller (15×15 mm). The agitation speed was detected by a magnetic hall sensor. The usable temperature range was from 3°C above the cooling water temperature to 80°C (accuracy±0.1°C) and controlled (digital temperature controller) by a RTD (Pt-100) sensor. The supplied air was pass through the 0.2-μm air filter cartridge and sparged by a ring sparger made of multiple holes to help uniform foam distribution. The Mettler Toledo electrode (pH 2.0–12.0) was used for pH control.

### Dry Cell Weight and CPS Determinations

After centrifugation (13,000 ×*g*, 4°C, 5 min) of 4 ml of culture broth, the recovered cells were washed with distilled water two times using the same centrifugation conditions. The final pellet was resuspended in 4 ml of distilled water and the optical density was measured at 600 nm. The suspension was completely dried in an 80°C oven for 24 h, and dry cell weight was calculated from the relationship with optical density. CPS was quantified, determined using phenol-sulfuric acid as described previously [9] with minor modifications. The sample (25 μl) and 5% (v/v) phenol (25 μl) were mixed well in a strip cap tube at 4°C prior to the addition of 125 μl of sulfuric acid. The reaction aliquots were heated at 100°C for 10 min and then the absorbance was recorded at 490 nm using a microplate reader (Bio-Rad, Hercules, CA, U.S.A.).

### Purification of Type 3 CPS

Type 3 CPS was purified as described previously [15] with minor modifications. After centrifugation (13,000 ×*g*, 4°C, 5 min) to remove cells, the supernatant was treated with 0.5% (v/v) sodium deoxycholate (Sigma-Aldrich, St. Louis, MO, U.S.A.) for 30 min at 37°C. The mixture was then heated for 10 min after adjusting the pH to 4.5 with acetic acid. Precipitated proteins and cell debris were removed by centrifugation (1,000 ×*g*, 4°C, 30 min). CPS was recovered by fractionation with 30%–60% ethanol precipitation and was dissolved in 400 ml of distilled water. The solution was treated twice with 80 ml of chloroform:butanol [5:1 (v/v)] and the CPS concentrate was obtained by centrifugation at 13,000 ×*g* (4°C, 5 min). The concentrated CPS was applied to a Sephadex G-100 column (Pharmacia, New York, NY, U.S.A.). The resulting pure CPS was lyophilized. During all the procedures, total CPS was quantified as described above.

### Experimental Design

To optimize the conditions for CPS production, Design-Expert 6.0.11 CCD RSM software (State-Ease, Minneapolis, MN, U.S.A.) was used. This design centered the values of the highest CPS productivity, which were preliminarily obtained using the "single-variable-at-a-time" technique [6]. The values were coded according to the chosen design and assessed at five coded levels (-1.682, -1, 0, +1, +1.682), as shown in Table 1. Twenty experiments were conducted; the full experimental plans with regard to their values in actual and coded forms are provided in Table 2. The CPS yield (Y, mg/l) in each trial was the average of triplicate determinations.

### Statistical Analysis and Modeling

The experimental RSM data was fitted *via* the response surface regression procedure using the following second-order polynomial equation [3]:

**Table 2.** CCD matrix for the experimental design and predicted responses for CPS production.

| Run no. | Coded levels | | | CPS production (mg/l) | |
|---|---|---|---|---|---|
| | $X_1$ | $X_2$ | $X_3$ | Actual[a] | Predicted |
| 1 | -1 | -1 | -1 | 30.43 | 42.37 |
| 2 | 1 | -1 | -1 | 33.21 | 56.48 |
| 3 | -1 | 1 | -1 | 47.90 | 52.24 |
| 4 | 1 | 1 | -1 | 47.90 | 53.99 |
| 5 | -1 | -1 | 1 | 50.48 | 71.95 |
| 6 | 1 | -1 | 1 | 23.41 | 46.63 |
| 7 | -1 | 1 | 1 | 94.55 | 98.84 |
| 8 | 1 | 1 | 1 | 45.56 | 61.18 |
| 9 | -1.682 | 0 | 0 | 14.46 | 2.75 |
| 10 | 1.682 | 0 | 0 | 10.20 | 0 |
| 11 | 0 | -1.682 | 0 | 133.10 | 98.88 |
| 12 | 0 | 1.682 | 0 | 124.15 | 119.40 |
| 13 | 0 | 0 | -1.68 | 111.37 | 97.52 |
| 14 | 0 | 0 | 1.682 | 153.55 | 128.43 |
| 15 | 0 | 0 | 0 | 253.61 | 256.14 |
| 16 | 0 | 0 | 0 | 258.75 | 256.14 |
| 17 | 0 | 0 | 0 | 256.46 | 256.14 |
| 18 | 0 | 0 | 0 | 253.01 | 256.14 |
| 19 | 0 | 0 | 0 | 255.52 | 256.14 |
| 20 | 0 | 0 | 0 | 252.80 | 256.14 |

[a]The observed values of CPS production were the mean values of triplicates.

$Y=-3019.34+641.53X_1+671.58X_2+5.86X_3-41.37X_1^2-324.83X_2^2-0.01X_3^2-5.15\,X_1X_2-0.11X_1X_3+0.18X_2X_3$

$$Y_i = \beta_0 + \sum_i \beta_i x_i + \sum_{ii} \beta_{ii} x_i^2 + \sum_{ij} \beta_{ij} x_i x_j$$

in which $Y_i$ is the predicted response, $x_i x_j$ the independent variables, $\beta_0$ is the offset term, $\beta_i$ is the *i*th linear coefficient, $\beta_{ii}$ is the *i*th quadratic coefficient, and $\beta_{ij}$ is the *ij*th interaction coefficient. In this study, however, the independent variables were coded as $X_1$, $X_2$, and $X_3$. Thus, the second-order polynomial equation can be presented as follows:

$$Y=\beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_{11}X_1^2+\beta_{22}X_2^2+\beta_3X_3^2+\beta_{12}X_1X_2+\beta_{13}X_1X_3+\beta_{23}X_2X_3$$

Design-Expert software was also utilized for the regression analysis and graphical analysis of the data obtained during the whole experiments. Analysis of variance (ANOVA) was used to estimate the statistical parameters. The second-order polynomial equation was employed to fit the experimental data. The significance of the model equation and model terms was evaluated by Fisher's test [3]. The quality of fit for the polynomial model equation was expressed by the coefficient of determination ($R^2$) and the adjusted $R^2$. The fitted polynomial equation was expressed as three-dimensional surface plots to demonstrate the relationship between the responses and the experimental levels of each of the variables utilized in the design. The combination of different optimized parameters required to yield the maximum response was determined in an attempt to confirm the validity of the model.

## Results and Discussion

### Culture Medium Selection

CPS production was investigated in the different medium compositions. The final concentration of CPS was 150.38 mg/l for BHI broth, 106.26 mg/l for THB, 147.58 mg/l for TSB, and 98.17 mg/l for CAT. The maximum production obtained with BHI broth was much higher than that previously reported (120 mg/l) [12]. BHI was selected as the basal medium for further experiments.

### CCD-Mediated Optimization of Fermentation Conditions for CPS Production

The selected fermentation conditions including pH ($X_1$), supplemented glucose concentration ($X_2$), and stirring rate ($X_3$) were investigated. CCD was utilized to assess the interaction of these variables within a range of −1.682 to +1.682 in relation to CPS production in the 8-l lab-scale fermentor (Table 1). A total of 20 experiments was conducted utilizing different combinations of these factors (Table 2). The response surface plots and their contour plots described by the second-order polynomial equation are shown in Fig. 1. CPS production varied with changes in $X_1$, $X_2$, and $X_3$. As ascertained from the central points of the corresponding contour plots, the three variables were $X_1$=7.5, $X_2$=1% (w/v), and $X_3$=180 rpm. At these conditions, the maximum type 3 CPS production was 255.03 mg/l, which was 15.03 mg/l higher than previously achieved in *Escherichia coli* (240.00 mg/l) [1] and 135.03 mg/l higher than previously achieved in *Lactococcus lactis* (120.00 mg/l) [12]. The previously determined optimal pH (8.0) [10] of the essential enzyme, Cps 3S [1], in the CPS biosynthetic pathway [11] is in good agreement with our determined optimal pH. It is apparent that control of pH is important for type 3 CPS production in a fermentor system.

*S. pneumoniae* is an aerotolerant microorganism. Consistent with this, the CPS productivity was 98.40 mg/l in unstirred cultures and increased up to 255.03 mg/l in stirred cultures. However, at stirring rates exceeding 180 rpm, CPS production gradually decreased, perhaps due to elevated shear stress. Previous observations are that *S. pneumonia* type 1 CPS production is highest at stirring rates of 50 rpm or lower, indicating that CPS production by different serotypes may vary with fermentation conditions such as the stirring rate [13].

Glucose is an important component in the CPS biosynthetic pathway. Preliminary experiments established that the glucose component of BHI was completely consumed by

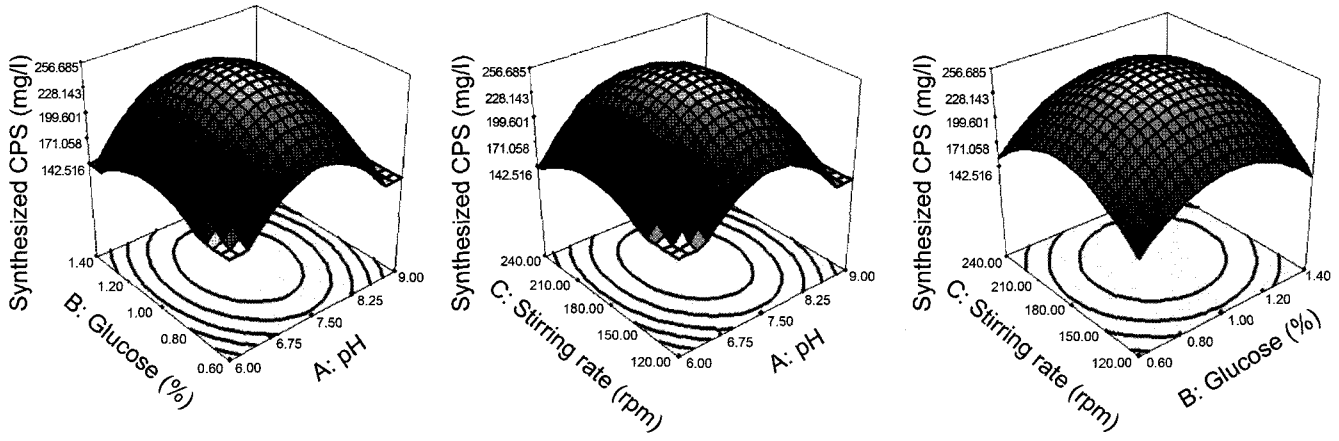


**Fig. 1.** Three-dimensional response surface plot and its contour plot for CPS production.
**A**. Interactive effects of initial pH and supplemented glucose concentrations at a stirring rate of 180 rpm. **B**. Interactive effects of fermentation pH and stirring rate at a supplemented glucose concentration of 1% (w/v). **C**. Interactive effects of supplemented glucose concentration and stirring rate at a pH of 7.5.

**Table 3.** ANOVA for the parameters of RSM fitted to the second-order polynomial equation.

| Source | Sum of squares | Degree of freedom | Mean square | F-value | *P*-value>F |
|---|---|---|---|---|---|
| Model | 175,200 | 9 | 19,471.51 | 39.46 | <0.0001 |
| Residual | 4,934.40 | 10 | 493.44 | | |
| Lack of fit | 4,907.21 | 5 | 981.44 | 180.46 | <0.0001 |
| Pure error | 27.19 | 5 | 5.44 | | |
| Cor. total | 180,200 | 19 | | | |

Standard Deviation=22.21, $R^2$=0.9726, Adj-$R^2$=0.9480, C.V.=18.13.
Cor. total: corrected total.

8 h incubation (data not shown). Supplementation of the medium with glucose increased CPS productivity up to 1% (w/v) (Fig. 1A and 1C). This may have been due to the accumulation of UDP-glucuronic acid (UDP-GlcUA), which is an important cytosolic intermediate for CPS synthesis. Consistent with this notion, the presence of high concentrations of UDP-GlcUA favors a steady state with a high proportion of the synthase in the synthesized polymer [11], with UDP-GlcUA being the final glucose transferred product in the type 3 pneumococcal CPS biosynthetic pathway [17]. The observed decline of CPS concentration and cell growth that occurred in the presence of over 1% (w/v) glucose may reflect limitation in the fermentation capability of this sugar [7]. Supplementation of BHI with 1% glucose was judged to be optimal for maximum CPS production.

### Determination of Polynomial Equation Coefficients for CPS Production

The design matrix and the corresponding results of the RSM experiments are provided in Table 2, along with the mean predicted values. From ANOVA, values of "Prob>F" <0.05 indicated that the model terms were significant. In this case, $X_1^2$, $X_2^2$, and $X_3^2$ were significant model terms. The model F-value was 39.46 and the F-value for lack of fit was 180.46 (Table 3).

Regression equation coefficients were calculated and the data were fitted to a second-order polynomial equation. The response, CPS production by *S. pneumoniae* type 3, was expressed in terms of the following regression equation:

$$Y=-3019.34+641.53X_1+671.58X_2+5.86X_3-41.37X_1^2-324.83X_2^2-0.01X_3^2-5.15X_1X_2-0.11X_1X_3+0.18X_2X_3$$

in which $X_1$ is pH, $X_2$ is the supplemented glucose concentration, and $X_3$ is the stirring rate.

The regression equation obtained from ANOVA indicated that a $R^2$ (multiple correlation coefficient) value of 0.9726 (a value>0.75 indicates fitness of the model) was reasonably consistent with the adjusted $R^2$ of 0.9480. This was an estimate of the fraction of overall variation in the data accounted for by the model and, thus, the model was capable of explaining 97.26% of the variation in response. The "adequate precision value" of the present model was 17.39, which indicates that the model can be used to navigate the design space. The "adequate precision value" is an index of the signal-to-noise ratio, and values exceeding 4 are desirable prerequisites for a model to be a good fit.

To the best of our knowledge, the present use of a CCD design for production of type 3 CPS is novel. The experimental CPS production (255.03 mg/l) was entirely consistent with predicted CPS production (256.14 mg/l). This confirms the validity of the model, suggesting that RSM might be effectively applied to the process optimization of the production of a variety of CPSs from different serotypes. Further study for the quality of purified type 3 CPS is in progress.

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