Original Article

Strain Selection and Statistical Optimization of Culture Conditions for 19F Polysaccharide Production from *Pneumococcus*

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Abstract

Introduction: Capsular polysaccharides of pneumococci are principle antigenic constituents of vaccines against pneumococci. Enhancing the yield of capsule production decreases costs of these vaccines and increases the vaccine coverage in developing countries. This study aims to optimize the capsule production from serotype 19F pneumococcus in terms of the applied pneumococcal strain and environmental culture conditions.

Materials and Methods: Thirteen serotype 19F *Streptococcus pneumoniae* strains were screened for the capsule production in modified Hoeprich culture medium using the stains all assay. The optimal ranges of environmental culture conditions for the selected strain were determined using single factor at a time (SFAT) strategy and utilized for the design of experiments based on the response surface methodology (RSM).

Results: *S. pneumoniae* 82218 showed the highest capsule production, and thus used for further studies. The maximum capsule production (1.364 mg/ml) was attained under optimal conditions (pH 7.26, 35.5 ºC, 30 rpm) predicted by the RSM derived quadratic model. The capsule production under the optimal conditions increased to 1.9 mg/ml using the buffered culture medium.

Conclusion: These results are much higher than those reported for pneumococcal capsule production in published studies [1, 2] and thus can be used to design suitable systems for the serotype 19F capsule production in the vaccine manufacturing process.

Keywords: Models, Pneumococcal vaccines, Polysaccharides, Serogroup, *Streptococcus pneumoniae*

1. Introduction

Streptococcus pneumoniae (the pneumococcus) is a noteworthy pathogen worldwide and provokes infectious maladies such as pneumonia, septicemia, and meningitis [3,4]. World Health Organization (WHO) has evaluated that 476000 yearly passing among youngsters under 5 years old are caused by pneumococcal diseases [5]. Antibiotic treatment of pneumococcal disease is being compromised by emerging multidrug resistant strains [6,9]. The pneumococcal capsule has been perceived as its primary factor of virulence, comprising of a biopolymer of rehashed oligosaccharides [10,12] . Each pneumococcal serotype corresponds to a different chemical structure of the capsule. S. pneumoniae serotypes containing 1, 5, 6A, 6B, 14, 19F, and 23F is the main reason of most invasive

pneumococcal infections in kids under 5 years old globally [13].

Pneumococcal capsules are main antigenic components of pneumococcal vaccines, which have been utilized for prevention of pneumococcal diseases. However, the capsular immunizations are ineffectively immunogenic in youthful kids and cannot prompt anamnestic immune responses upon revaccination. The capsule is therefore, changed from a T-cell free antigen to a T-cell activating antigen via conjugation to an immunogenic carrier protein. As a result, the antibody response is enhanced and the immunological memory is elicited. The manufacturing of pneumococcal conjugate vaccines (PCVs) requires bioprocesses containing several steps and is costly. WHO advises to incorporate PCVs in national vaccination programs for kids, whereas high production expenses of these vaccines restricts their usage in developing nations [3,5,13]. Production costs of microbial compounds are diminished through enhancing the production yield through optimizing conditions of microbial cultivation [14,15]. Nonetheless, few investigations have been reported on improving cultivation conditions of *S. pneumoniae* for increasing capsule production. The importance of selecting an appropriate *S. pneumoniae* strain for enhancing the serotype 14 pneumococcal capsule production was demonstrated in the present study [16]. The production of capsular polysaccharide by the serotype 4 pneumococcus was observed to be dependent on the culture medium pH [1]. Moreover, the serotype 3 pneumococcal capsule productivity increased in agitated cultures. However, the capsule production decreased at agitation rates exceeding 180 rpm which was attributed to the damaging effect of elevated shear stress on pneumococcal cells [17]. Cimini et al. investigated the influence of temperature on the capsular polysaccharide production by *Escherichia coli* K4. Their results demonstrated that the capsule production got enhanced at higher temperatures [18].

Response surface methodology (RSM) has been a popular and efficient statistical technique that has been widely used for optimization studies in biotechnological processes. It can be used for designing experiments, constructing accurate mathematical models to describe the process, assessing the effects of factors alone or in combination on the response, and specifying optimum conditions of factors to obtain desirable responses [17, 19]. Based on the authors' current knowledge, there are no reports assessing and optimizing impacts of bacterial strains and environmental culture conditions for the capsule production by serotype 19F *S. pneumoniae* as one of the main serotypes leading to most invasive pneumococcal infections in kids under 5 years of age. Therefore, in the current study, a suitable pneumococcal strain was determined as well as appropriate ranges of environmental culture conditions including temperature, pH and agitation rate for the serotype 19F capsule production using single factor at a time (SFAT) method. Then, RSM based on the SFAT results was used to obtain an optimized culture method for the capsular polysaccharide preparation.

2. Materials and Methods

2.1 Microorganisms and Stock Cultures

This study was a basic research in the field of biotechnology and was approved by the research ethics committee of Shahid Beheshti University of Medical Sciences under the code: SBMU.REC.1392.494. Six clinically isolated strains of *S. pneumoniae* including 62P, 64P, 65P, 70P, 87P, and BbP were supplied by Pediatric Infections Research Center (PIRC) (Tehran, Iran). Four other clinical strains of *S. pneumoniae* including 67H, 101H, 80H and 110H were provided by Health Reference Laboratories Research Center (Tehran, Iran). *S. pneumoniae* ATCC 6319 (American Type

Culture Collection, Rockville, MD, USA), *S. pneumoniae* ATCC 49619, and *S. pneumoniae* 82218 (Statens Serum Institute, Copenhagen, Denmark) were also used in this study. Pneumococcal strains were propagated in tryptic soy broth (TSB) including 5% defibrinated sheep blood under 5% CO_2 at 37 °C for 18 h and kept up at -70 °C in a similar medium containing 20% (v/v) glycerol [20].

2.2 Cultivation Conditions

S. pneumoniae strains were cultivated using Hoeprich's modified medium (HMM). This medium composed of glucose (20g/l), hydrolyzed soybean meal (soytone) (20 g/l), yeast extract (20g/l), sodium bicarbonate (1 g/l), Dipotassium hydrogen phosphate (5 g/l), asparagine (100 mg/l), L-glutamine (624 mg/l), manganese (II) sulfate (0.36 mg/l), Iron (II) sulfate (5 mg/l), magnesium sulfate (500 mg/l), zinc sulfate (0.8 mg/l), choline (10 mg/l), and 10% (v/v) thioglycolic acid (0.1% v/v). The culture medium pH was set to 7.5 and sterile filters (0.22 µm) were applied to sterilize the broth. Glucose, soytone and yeast extract were products of BD-Difco (Sparks, USA). All other compounds were from Sigma-Aldrich (Saint Louis, USA). Potassium phosphate buffer (0.1M) at appropriate pH was used to dissolve HMM constituents rather than distilled water when buffering was required. Tryptic soy agar (TSA) plates supplemented with defibrinated sheep blood (5%) were inoculated by frozen stock cultures of *S. pneumoniae* strains. The incubation of the plates was done under CO_2 (5%). After incubating the plates for 18h at 37 ºC, The optical density at 600 nm (OD_{600}) of HMM was adjusted to 0.15 by transferring plate colonies. The obtained broth was utilized for inoculating HMM (10% (v/v)) in 50 ml bottles, which were kept 18 h at 37 °C with tight lids at desired agitation speed [2, 20].

2.3 Capsule Measurement

Stains all assay was used to quantify the capsule production. Briefly, *S.pneumoniae* cells were harvested by centrifugation and suspended in a buffer containing magnesium sulfate (1mM) and tris-hydrochloric acid (150 mM, pH 7.0) to obtain a final OD_{600} of 5. One ml of the suspension was centrifuged to separate the cells from the supernatant. The cells were then suspended in the buffer (0.5 ml). The autolysis of bacteria was prompted by adding deoxycholate to the cell suspension to a final concentration of 0.1% wt/vol followed by incubating for 15 min at 37 °C. Ribonuclease A (50 µg), deoxyribonuclease I (50 µg), and mutanolysin (100 U) were added to the pneumococcal cells and the incubation was further continued for 18 h. The supernatant was treated similar to the cells without using mutanolysin. Proteinase K (50 µg) was added to the samples, which were kept for 4h at 56 °C and then stored at -20 °C. Two hundred microliters of water and 1 ml of stains-all solution were added to 50 µl of the cellular samples and OD_{640} was measured. The supernatant samples (250 µl) were also mixed with stains-all solution (1 ml) prior to reading OD_{640}. A standard calibration graph correlating values of OD_{640} to the concentration of *S. pneumoniae* serotype 19F capsule (Statens Serum Institute, Copenhagen, Denmark) was used for the capsule quantification. The sum of the cellular capsule and the capsule in the supernatant was used to report capsule production values in the culture [21].

2.4 Experimental Design, Statistical Analysis and Modeling by RSM

The appropriate bacterial strain for serotype 19F pneumococcal capsule production was selected via measuring the capsule production following cultivation in HMM. The statistical significance of results

was analyzed using Tukey test of SPSS software (IBM Corp, NewYork, USA). Specifying suitable ranges of environmental culture conditions including pH, temperature, and agitation speed for the selected bacterial strain was carried out using SFAT method. Experiments were performed in duplicate and the average values for the capsule production and standard deviations were reported. RSM was then designed to optimize the environmental culture conditions for producing capsule at maximum level. Design Expert Software (Statease, Minneapolis, USA) was used to provide the RSM face centered central composite design resulting in 20 experiments. The variables were evaluated at three levels (-1, 0, +1) as indicated in Table 1. The tests were carried out in duplicate and the average values for the capsule production and standard deviations were reported. A polynomial equation was used to fit the obtained results using the response surface regression procedure. Statistical parameters were determined using Analysis of variance (ANOVA). ANOVA is based on decomposition of the total variability of the response to two sources including the experimental error and the model to demonstrate whether the variation from the model is significant compared to the variation due to the experimental error. This comparison is done using Fisher's F-test value, which is the ratio between the model and the error mean squares. Fisher's test was therefore applied to assess the equation of the model and its components in terms of statistical significance. The determination coefficient (R^2) was used to examine the model fit quality. Three dimensional surface plots of the model equation were used to demonstrate the correlation between the response and variables. The verification experiment was performed under optimal conditions predicted by the model for capsule production. P-values less than 0.05 are considered as statistically significant [17, 19].

2.5 Protein Profile Analysis of Pneumococcal Strains

Pneumococcal cells cultivated in HMM (2.5 OD_{600}) were collected by centrifugation and washed once with phosphate buffered saline. The bacterial pellet was then suspended in the same buffer containing 0.1% sodium deoxycholate and incubated for 1h at 37 ºC. For analysis of the samples, SDS-PAGE (10%) was used and the gel was stained using Coomassie Brilliant Blue R250 [22].

3. RESULTS

3.1 Screening of Pneumococcal Strains for Serotype 19F Capsule Production

The pneumococcal capsule is a cell surface exposed structure of polysaccharide nature, which is firmly connected with the capacity of pneumococci to induce invasive diseases [10]. The serotype 19F *S. pneumoniae* strains were examined in terms of capsule production following cultivation in HMM, which is an animal component free medium and is acceptable for the vaccine production. The maximum capsule production was observed for *S. pneumoniae* 67H among the clinical strains (Table 1). However, the capsule production by *S. pneumoniae* 82218 (1.053 mg/ml) was 1.6 fold higher than that of the obtained one, using *S. pneumoniae* 67H and the observed difference was statistically significant. Therefore, *S. pneumoniae* 82218 was selected as the appropriate strain for the serotype 19F capsule production and used in further studies to evaluate and optimize environmental culture conditions for the capsule production. The analysis of the bacterial protein profile using SDS-PAGE indicated the difference between the intensity of two protein bands in the range of 99-100 kDa in *S. pneumoniae* 82218 and *S. pneumoniae* 67H (Figure. 1). Therefore, these proteins were expressed differentially in the two studied pneumococcal strains. Therefore, these proteins were expressed differentially in the two studied pneumococcal strains. The results indicated

that expression levels of these proteins may affect the capsule production level. The pneumococcal genetic locus encoding the genes involved in the capsule formation (*cps* locus) constitutes an operon transcribing from a promoter in its upstream region. The regulatory and processing genes of the *cps* locus including *cpsA*, *cpsB*, *cpsC*, and *cpsD* are highly conserved among different pneumococcal serotypes. Other genes of the *cps* locus are required to encode necessary enzymes for production and export of the capsule of each pneumococcal serotype [10, 23]. The fifth gene of the *cps* locus (*cpsE*) encodes a glucose phosphate transferase enzyme, which catalyzes the transfer of the activated glucose phosphate to the lipid carrier. The capsule expression in *S. pneumoniae* was lost as a result of mutation of the *cpsE* gene [24]. In addition, Carvalho et al. demonstrated that inactivation of the pyruvate oxidase encoding gene in *S. pneumoniae* resulted in enhanced capsule production [25]. These studies together with the present study's results demonstrated that the identification of proteins affecting the pneumococcal capsule production paves the way for constructing appropriate pneumococcal strains for the vaccine manufacture.

Table 1. Serotype 19F capsule production by pneumococcal strains

Pneumococcal strain	Capsule production (mg/ml)
S. pneumoniae ATCC 6319	0,813 ± 0,043
S. pneumoniae ATCC 49619	0,343 ± 0,048
S. pneumoniae 82218	1,053 ± 0,010
S. pneumoniae 62P	0,370 ± 0,054
S. pneumoniae 64P	0,458 ± 0,037
S. pneumoniae 65P	0,514 ± 0,010
S. pneumoniae 70P	0,435 ± 0,030
S. pneumoniae 87P	0,540 ± 0,011
S. pneumoniae BbP	0,336 ± 0,026
S. pneumoniae 67H	0,656 ± 0,010
S. pneumoniae 101H	0,610 ± 0,032
S. pneumoniae 80H	0,387 ± 0,044
S. pneumoniae 110H	0,448 ± 0,039

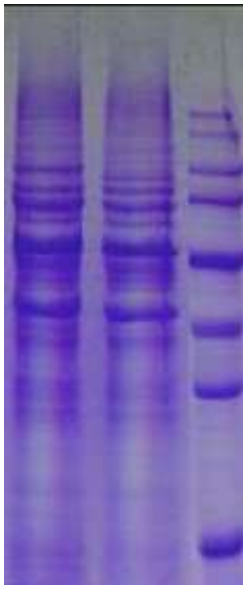

Figure 1. Analysis of protein profiles of pneumococcal strains. Lane 1, *S. pneumoniae* 67H; lane 2, *S. pneumoniae* 82218; lane M, molecular weight marker

3.2 Determining Optimal Ranges of Environmental Culture Conditions for Capsule Production Using SFAT Method

Several single factor experiments were carried out to determine appropriate ranges of environmental culture conditions for the capsule production using *S. pneumoniae* 82218. The tested variables included pH, temperature, and agitation speed. To determine the effect of pH on the serotype 19F capsule production, HMM was used in 5 different initial pH levels (from pH 6 to pH 8) for the cultivation of *S. pneumoniae* 82218. The bacterial growth enhanced with increasing pH (from pH 6 to pH 7.5) as measured by OD_{600}. OD_{600} in pH 8 was similar to that in pH 7.5 (Figure. 2a). The capsule production was best achieved in pH 6.5 to pH 7.5 and it was notably decreased in pH 6 and pH 8. The capsule production in pH 7.5 was 1.8 fold higher than that in pH 8.0 in spite of the similar OD_{600} for the bacterial growth.

To examine the influence of cultivation temperature, *S. pneumoniae* 82218 cells were cultivated in HMM and incubated at 34 ºC – 39 ºC. The capsule production at 37ºC was 2.35 fold higher than that at 34 ºC

(Figure. 2b). Moreover, the suitable temperature range for the capsule production was around 36ºC to 37ºC. It was further studied at different agitation speeds and was highest at agitation rates of 0-30 rpm. However, the capsule production was notably decreased at agitation rates of 50 rpm or higher. No bacterial growth was observed at 90 rpm or higher agitation rates (Figure. 2c). It was shown that the serotype 4 pneumococcal capsule production was inhibited by vigorous agitation (higher than 100 rpm) but was enhanced at the agitation rate of 50 rpm [1]. In addition, the serotype 3 pneumococcal capsule production was decreased at agitation rates exceeding 180 rpm [17]. These results were attributed to the elevated shear stress at higher agitation rates and its destructive effects on the cells [27].

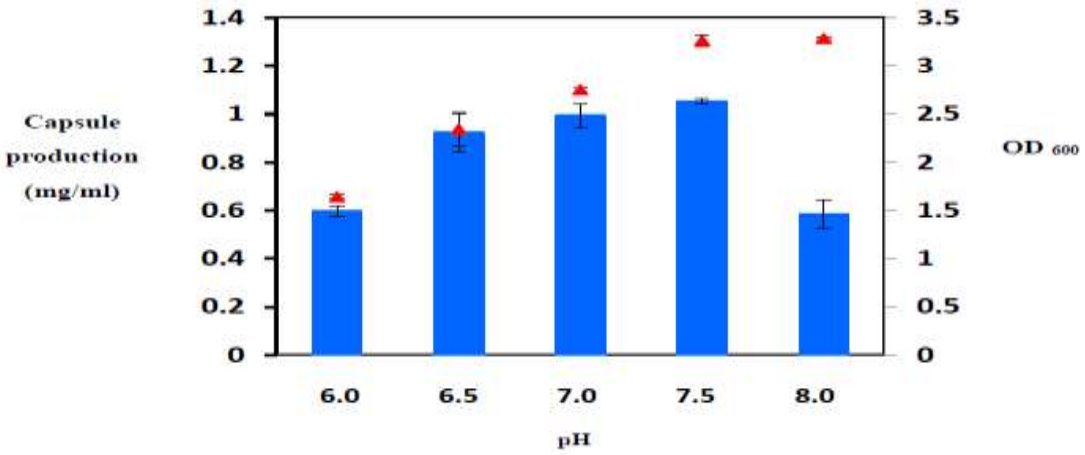

Figure 2a. The effect of pH on capsule production by *S. pneumoniae* 82218 and the bacterial growth obtained using SFAT. Bar lines and triangles indicate the capsule production and bacterial growth respectively

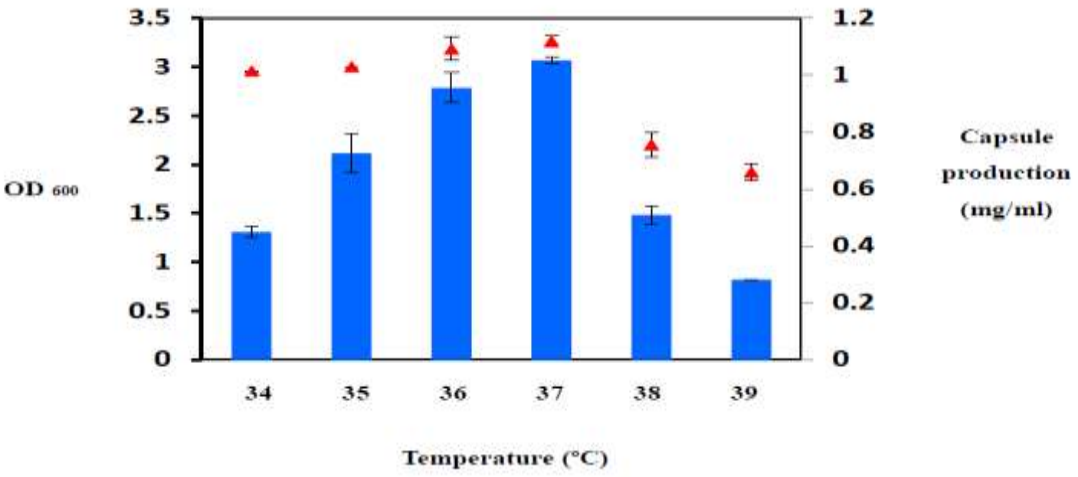

Figure 2b. The effect of temperature on capsule production by *S. pneumoniae* 82218 and the bacterial growth obtained using SFAT. Bar lines and triangles indicate the capsule production and bacterial growth respectively

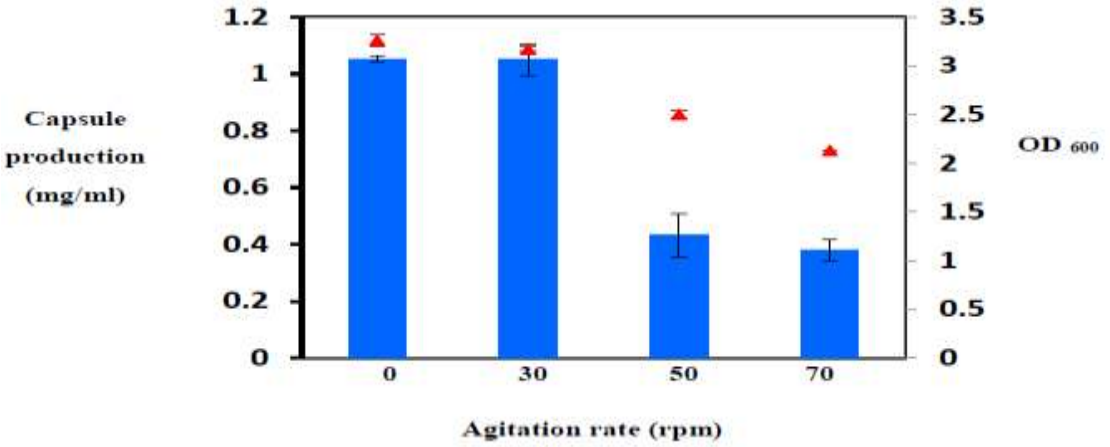

Figure 2c. The effect of agitation rate on capsule production by *S. pneumoniae* 82218 and the bacterial growth obtained using SFAT. Bar lines and triangles indicate the capsule production and bacterial growth respectively

3.3 Optimization of Capsule Production Using RSM

Using RSM for experimental design offers a number of advantages including evaluation of factor effects, development of a system model, as well as specifying optimal conditions with less experimental requirements [17,19]. Considering the suitable ranges of environmental factors for the capsule production obtained using SFAT experiments, the RSM experimental design was applied to determine the optimum conditions of environmental factors (Table 2). This study's results displayed that the highest capsule production (1.349 mg/ml) was obtained in run 6, while the lowest value (0.294 mg/ml) was attained in run 8. A quadratic polynomial equation demonstrated the correlation between capsule production and environmental conditions of the pneumococcal culture. The equation parameters were specified by multiple regression analysis of results of the experiments. The obtained quadratic polynomial model describing the empirical relationship between the response and variables is as follows:

$Y = 0.8 + 0.11A - 0.21\ B - 0.14\ C - 0.12\ AB - 0.031\ AC - 0.16\ BC - 0.18\ A^2 + 9.091E\text{-}005B^2 + 0.28\ C^2$ where the equation response (Y) is the capsule production (mg/ml), A is pH, B is temperature (ºC), and C is agitation speed (rpm). ANOVA of the response surface quadratic model is demonstrated in Table 3. The adequacy of the model equation was examined using Fisher's statistical test. The F-value of the model (37.03) and its p-value (< 0.0001) indicated the significance of the model and that the chance of the model F-value to happen by noise was just 0.01% [23]. A, B, C, AB, BC, A^2, and C^2 were significant model terms (p values < 0.05). The lack of fit was not significant and therefore, the model fitted the experimental data properly. A high R^2 value (0.9709) also ensured the model adequate fitness with the data and showed that 97.09% of the variation in the capsule production could be described by the model. The value of adequate precision specifies the signal to noise ratio, which is more than 4 for suitable models. The adequate precision value for producing the capsule was 22.276. This result implied a proper signal for the model [23]. The suitability of size of RSM design was evaluated using "Fraction of Design Space (FDS)" generated by Design expert software. An FDS value above 80% suffices for generating a precise fitted surface model. As for the case of the present study, an FDS value of 94% (standard deviation of 0.06 and half wide of the confidence interval of 0.1 for the optimal response) far exceeds 80% and indicated that 94% of the design space was precise enough to predict the optimal response ± 0.1[24,25].

Three dimensional response surface plots were graphical representations of the model equation and showed the correlation between the capsule production and levels of pH, temperature, and agitation rate (Figure. 3a, b). The maximum capsule production (1.352 mg/ml) predicted by the model was expected to attain at pH 7.26, 35.5 ºC and 30 rpm. The verification experiment was then carried out using the predicted optimum environmental culture conditions for the cultivation of *S. pneumoniae*. The capsule production under these conditions was 1.364 mg/ml, which was not significantly different from the predicted value (Table 4). This result therefore indicated the validity and effectiveness of the model.

Table 2. RSM based experimental design for capsule production and responses

Run	pH (A)*	Temperature (B)	Agitation rate (C)	capsule production (mg/ml)
1	6.5 (-1)	35.5 (-1)	0 (-1)	0.859 ± 0.00141
2	7.5 (+1)	35.5 (-1)	0 (-1)	1.289±0.002051
3	6.5 (-1)	37.5 (+1)	0 (-1)	0.923±0.003323
4	7.5 (+1)	37.5 (+1)	0 (-1)	1.039±0.01202
5	6.5 (-1)	35.5 (-1)	30 (+1)	0.89±0.03041
6	7.5 (+1)	35.5 (-1)	30 (+1)	1.349±0.01003
7	6.5 (-1)	37.5 (+1)	30 (+1)	0.457±0.00071
8	7.5 (+1)	37.5 (+1)	30 (+1)	0.294±0.02263
9	6.5 (-1)	36.5 (0)	15 (0)	0.524±0.01061
10	7.5 (+1)	36.5 (0)	15 (0)	0.754±0.05162
11	7 (0)	35.5 (-1)	15 (0)	1.013±0.01732
12	7 (0)	37.5 (+1)	15 (0)	0.632±0.03182
13	7 (0)	36.5 (0)	0 (-1)	1.254±0.00566
14	7 (0)	36.5 (0)	30 (+1)	0.952±0.09051
15	7 (0)	36.5 (0)	15 (0)	0.765±0.05940
16	7 (0)	36.5 (0)	15 (0)	0.795±0.07495
17	7 (0)	36.5 (0)	15 (0)	0.842±0.02899
18	7 (0)	36.5 (0)	15 (0)	0.806±0.04667
19	7 (0)	36.5 (0)	15 (0)	0.811±0.01131
20	7 (0)	36.5 (0)	15 (0)	0.658±0.01556

* Coded values of variables are indicated in parentheses.

Table 3. ANOVA for RSM model of capsule production

Source	Sum of Squares	F - Value	p - Value
Model	1.32	37.03	< 0.0001
A, pH	0.11	28.98	0.0003
B, Temperature	0.42	106.49	< 0.0001
C, Agitation rate	0.2	50.99	< 0.0001
AB	0.11	27.62	0.0004
AC	7.812E-003	1.97	0.1907
BC	0.21	53.44	< 0.0001
A^2	0.093	23.33	0.0007
B^2	2.273E-008	5.731E-006	0.9981
C^2	0.22	54.60	< 0.0001
Lack of Fit	0.019	0.91	0.5421

pH, temperature and agitation rate were independent variables. The capsule production was dependent variable. R^2 = 0.9709 and adequate precision 22.276. Adequate precision denotes signal to noise ratio and it should be more than 4 for appropriate models.

Table 4. The capsule production under RSM predicted optimal conditions and using buffered HMM

Cultivation conditions	Capsule production (mg/ml)	OD600	Final pH
*pH 7.26, 35.5 ºC and 30 rpm	1.364 ± 0.048	3.285 ± 0.12	4.93 ± 0.01
pH 7.26, 35.5 ºC and 30 rpm and using buffered HMM	1.9 ± 0.019	5.3 ± 0.1	6.1± 0.05

*RSM predicted optimal conditions for 19F capsule production.

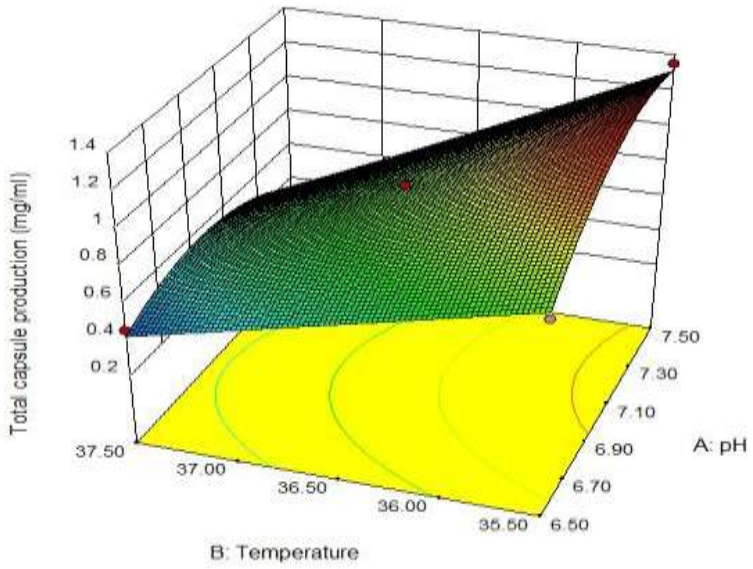

Figure 3a. The response surface plot for capsule production indicating effects of pH and temperature

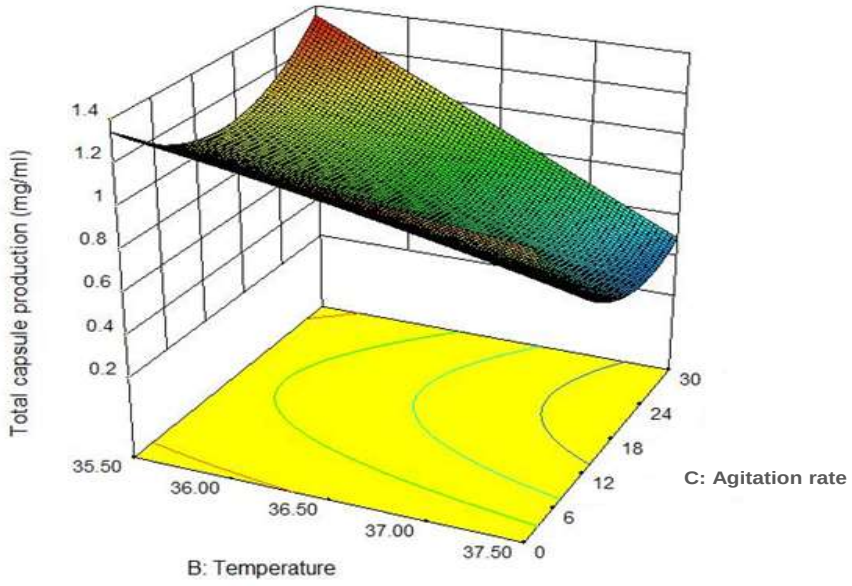

Figure 3b. The response surface plot for capsule production indicating effects of temperature and agitation rate

3.4 Capsule Production in Buffered Medium

S. pneumoniae is among lactic acid bacteria (LAB). The primary product of LAB is lactic acid, which can decrease the culture pH [26]. The primary product of LAB is lactic acid, which can decrease the culture pH [31] . *S. pneumoniae* 82218 was cultivated in buffered HMM under optimal environmental conditions obtained from RSM. The decrease in pH of buffered HMM was lower than that of HMM following cultivation of *S. pneumoniae* 82218 (Table 4). The bacterial growth was notably increased in the buffered HMM as indicated by OD_{600}. Moreover, the capsule production increased 1.39 fold through

buffering HMM. However, preparation of HMM is easier than that of the buffered HMM.

4. Discussion

In this research, *S. pneumoniae* 82218 was identified as the most suitable strain for 19F capsule production because it showed the highest level of the 19F polysaccharide production among the studied pneumococcal strains. The observed difference in the protein electrophoretic profile of *S. pneumoniae* 82218 and that of *S. pneumoniae* 67H indicated that expression levels of the identified proteins may affect the capsule production level. The pneumococcal genetic locus encoding the genes involved in the capsule formation (*cps* locus) constitutes an operon transcribing from a promoter in its upstream region. The regulatory and processing genes of the *cps* locus including *cpsA*, *cpsB*, *cpsC*, and *cpsD* are highly conserved among different pneumococcal serotypes. Other genes of the *cps* locus are required to encode necessary enzymes for production and export of the capsule of each pneumococcal serotype [10, 27]. The fifth gene of the *cps* locus (*cpsE*) encodes a glucose phosphate transferase enzyme, which catalyzes the transfer of the activated glucose phosphate to the lipid carrier. The capsule expression in *S. pneumoniae* was lost as a result of mutation of the *cpsE* gene [28]. In addition, Carvalho et al. demonstrated that inactivation of the pyruvate oxidase encoding gene in *S. pneumoniae* resulted in enhanced capsule production [29]. These studies together with the agitation rate of 50 rpm [1]. In addition, the serotype 3 pneumococcal capsule production was decreased at agitation rates exceeding 180 rpm [17]. These results were attributed to the elevated shear stress at higher agitation rates and its destructive effects on the cells [31].

Using RSM for experimental designs offers a number of advantages including evaluation of factor effects, development of the present study results demonstrated that the identification of proteins affecting the pneumococcal capsule production paves the way to construct appropriate pneumococcal strains for the vaccine manufacture.

Rault et al. showed that the intracellular pH of *Lactobacillus delbrueckii* decreased concomitantly with the extracellular pH decrease. The intracellular pH is a key factor influencing several cellular processes including proton motive force, transport kinetics, ATP generation, and enzyme activities [30]. Therefore, the present results can be attributed to the pH effect on activities of enzymes involved in the capsule biosynthesis in pneumococcal cells. These results also demonstrated that the 19F capsule production as well as the bacterial growth was best achieved in pH 6.5-7.5. Moreover, Kim et al. examined the effect of culture medium pH on the serotype 4 pneumococcal capsule production and showed that the capsule production in pH 5 to pH 8 did not enhance alongside the increase of bacterial growth [1]. In their study, the bacterial growth in pH 5 to pH 6 was higher than that in pH 7 to 8. However, the capsule production in pH 5 to pH 6 was less than one third of that in pH 7 to pH 8 [1]. It is then apparent that selecting an appropriate pH is necessary to achieve high yields of the capsule production.The 19F capsule production by *S. pneumoniae* 82218 and its growth were decreased by increasing the agitation rate to 50 rpm or higher. The serotype 4 pneumococcal capsule production was inhibited by vigorous agitation (higher than 100 rpm) but it was enhanced at a system model as well as specifying optimal conditions with less experimental requirements [17,19]. We designed RSM experiments based on SFAT results. ANOVA of the obtained response surface model indicated that the capsule production was dependent on pH, temperature, and agitation rate. Moreover, the interaction between pH and temperature as well as the interaction between temperature and

agitation rate significantly affected the capsule production. The pneumococcal capsule production reported by other studies was in the range of 0.17-0.21 mg/ml [1, 2], whereas the optimization procedures used in the present study including the pneumococcal strain selection, optimization of environmental culture conditions, and buffering the culture medium resulted in a notably high level of pneumococcal capsule production (1.9 mg/ml). Therefore, the results of this study are expected to be applicable for the pneumococcal capsule production in the vaccine manufacturing process.

Although buffering the culture medium compensates for the pH decrease, improving the buffering capacity through changing the buffer composition needs to be addressed in future studies. Moreover, designing an appropriate scale up procedure is an essential step towards capsule production for the vaccine manufacture.

5. Conclusion

The suitable yield of capsule production is necessary to ensure efficient pneumococcal vaccine preparation. The present study demonstrated the importance of bacterial strain as well as environmental culture conditions for achieving the appropriate yield of the 19F pneumococcal capsule production. The capsule production was expressed as a quadratic model using environmental factors of pneumococcal culture. Moreover, regarding the observed differences in the protein electrophoretic profile of pneumococcal strains possessing different levels of capsule production, identification of the designated proteins can pave the way to construct pneumococcal strains with high yields of the capsule production through manipulations of the relevant genes.

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Conflict of Interest

The authors declare no conflict of interest.

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