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Investigation of Appropriate Cultivation Approach for Capsular Polysaccharide Production by *Streptococcus pneumoniae* Serotype 19F

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Abstract With the aim of determining an appropriate cultivation approach for the capsular polysaccharide production by *Streptococcus pneumoniae* serotype 19F, the influence of environmental and culture medium conditions on the pneumococcal culture was investigated. Using 5% CO_2 atmosphere instead of using an aeration limited environment enhanced the capsule production 3.5 fold. Buffering the cultivation medium prevented the culture pH drop to the acidic condition and increased the capsule production almost 5 fold. Utilizing casamino acid as the nitrogen source of the culture medium instead of soytone provided 1.3 fold increase in the capsule production. Glucose, sorbitol, lactose and sucrose were investigated as carbon sources of the culture medium. Regarding costs of these sugars and their effects on the capsule production, lactose was the best carbon source. Our results demonstrated that buffering the cultivation medium had the most profound effect on the serotype 19F capsule production. The capsule was produced at 1.706 mg/ml in the buffered medium. Applying this culture method allows the cost effective production of the serotype 19F pneumococcal capsule for inclusion in pneumococcal vaccines.

Keywords: *capsular polysaccharide, cultivation, pneumococcal conjugate vaccines, serotype 19F, streptococcus pneumoniae*

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1. Introduction

Streptococcus pneumoniae is a major pathogen that causes diseases such as meningitis, pneumonia, and sepsis [1]. World Health Organization (WHO) estimated that 476000 annual deaths among children less than 5 years of age were caused by pneumococcal infections [2].

Pneumococcal vaccines have been used for protection against pneumococcal infections. The pneumococcal capsule is the main antigenic component of these vaccines. The capsule is poorly immunogenic in children and is not able to induce anamnestic antibody responses upon revaccination. In order to enhance the antibody responses and induce immune memory, the capsule is converted from a T-cell independent antigen to a T-cell dependent antigen through its chemical conjugation to an immunogenic carrier protein. Therefore, the PCVs manufacturing requires multistep processes and is expensive. WHO recommends inclusion of pneumococcal conjugate vaccines (PCVs) in national immunization programs for children [2]. However, high manufacturing costs of PCVs limit their implementation in developing countries [1,2]. Optimizing cultivation conditions for microbial products has been used to improve the product yield and thus to reduce the manufacturing costs [3,4]. *S. pneumoniae* is a member of lactic acid bacteria (LAB) and as is characteristic of LAB, it is a nutritionally fastidious facultative anaerobe. The bacterium obtains sufficient energy from carbohydrates via glycolysis to support the cell growth. The major catabolite of the carbohydrate metabolism is lactic acid, which decreases the culture pH and inhibits the cell growth [5]. Furthermore, pneumococci are not able to synthesis de novo all of 20 amino acids. To overcome this deficiency, pneumococci produce cell wall located proteases to digest proteins and transporters for uptake of amino acids [5,6,7,8]. There are few studies on *S. pneumoniae* cultivation and improvement of the capsule production. Gongalves et al. [9] enhanced serotype 23F pneumococcal capsule production by selecting an appropriate nitrogen source for the cultivation medium and using N_2 atmosphere for the bacterial growth. In addition, Leal et al. [10] demonstrated the importance of the pH control during the cultivation for increasing the pneumococcal capsule production by *S. pneumoniae* serotype 14. To our knowledge, there is no report evaluating the effects of cultivation methods on the capsule production by *S. pneumoniae* serotype 19F, one of the major pneumococcal serotypes causing most invasive pneumococcal disease in children less than 5 years [11]. In this study, therefore, we aim to investigate the influence of

environmental and culture medium conditions including use of CO_2 atmosphere, buffering the culture medium, using different nitrogen and carbon sources on the serotype 19F capsule production and a suitable culture method for the capsular polysaccharide preparation was then determined.

2. Materials and Methods

2.1. Bacterial Strain and Stock Cultures

S. pneumoniae serotype 19F strain ATCC 49619 (American type culture collection) was grown in tryptic soy broth (TSB) containing 5% defibrinated sheep blood at 37 °C with 5% CO_2 for 18 h and maintained in a deep freezer (-70 °C) in the same medium containing 20% (v/v) glycerol.

2.2. Culture Medium and bacterial growth Conditions

The Hoeprich's modified medium (HMM) was used for the cultivation. Each liter of this medium contains 20 g enzymatically hydrolyzed soybean meal (EHS), 20 g glucose, 20 g yeast extract, 5 g K_2HPO_4, 1 g $NaHCO_3$, 624 mg L-glutamine, 100 mg asparagines, 10 mg choline, 500 mg $MgSO_4$, 5 mg $FeSO_4$, 0.8 mg $ZnSO_4$, 0.36 mg $MnSO_4$, and 1 ml thioglycolic acid (10% v/v). The pH of the medium was adjusted to 7.5 by 5 N HCl and the broth was then filter sterilized using 0.22 µm sterile filters.

Frozen stock cultures of *S. pneumoniae* ATCC 49619 were used to inoculate TSB containing 5% defibrinated sheep blood. The tubes were incubated at 37 °C with 5% CO_2 for 18 h. The broth was used to inoculate tryptic soy agar plates containing 5% defibrinated sheep blood. The plates were incubated at 37 °C with 5% CO_2 for 18 h. The colonies were transferred to 260 µl of HMM to obtain an OD_{600} of 0.1. This broth was then used to inoculate 15 ml HMM in 50 ml bottles. The bottles were incubated with tightened lids at 37 °C for 18 h statically.

For buffering HMM, the medium components were dissolved in 0.1 M sodium phosphate buffer (pH 7.5) instead of distilled water. For investigating the effect of nitrogen source on the capsule production, soytone in HMM was replaced with casamino acid at the same concentration. For studying the effect of the carbon source on the capsule production, glucose in HMM was replaced with other carbon sources as indicated in the text at the same concentration.

2.3 Quellung Test

A loopful of the pneumococcal culture provided using HMM broth as mentioned above was spread on a glass slide and allowed to air dry. A loopful of the typing 19F antiserum applied directly to the dried spot on the slide. A few microliter of 1% aqueous methylene blue solution was then mixed with the antiserum on the slide. The slide was examined under immersion lense of an optical microscope. A negative control lacking the antiserum was prepared.

2.4. Capsule Measurement

The pneumococcal cells were collected by centrifugation and suspended in the suspension buffer (150 mM Tris-HCl (pH 7.0) - 1 mM $MgSO_4$) to an OD_{600} of 5. A 1 ml aliquot was pelleted using centrifugation and the cell pellet was resuspended in 0.5 ml of the suspension buffer. The bacterial autolysis induced by the addition of 0.1% (wt/vol) deoxycholate and incubation at 37 °C for 30 min. The samples were then incubated with 100 U mutanolysin (Sigma), 50 µg DNase I (sigma), and 50 µg RNase A (Sigma) at 37 °C for 18 h. The samples were incubated with 50 µg proteinase K (Sigma) at 56 °C for 4 h before storage at -20 °C. The capsule amount in the samples was determined by mixing the enzyme treated cells (50 µl of the cells plus 200 µl water) with 1 ml of Stains all solution and measuring the absorbance at 640 nm. The absorbance values were compared with a standard curve generated with known concentrations of purified pneumococcal serotype 19F capsular polysaccharide (Statens Serum Institute) to quantify the capsular polysaccharide in the samples [12,13].

3. Results and Discussion

3.1. Effect of Using CO_2 on Capsule Production

S. pneumoniae ATCC 49619 is a serotype 19F pneumococcal strain. The bacterial serotype was confirmed using Quellung test. The capsule swelling was observed in the bacterial sample. However, no capsule swelling was observed in the negative control lacking the antiserum (Figure 1).

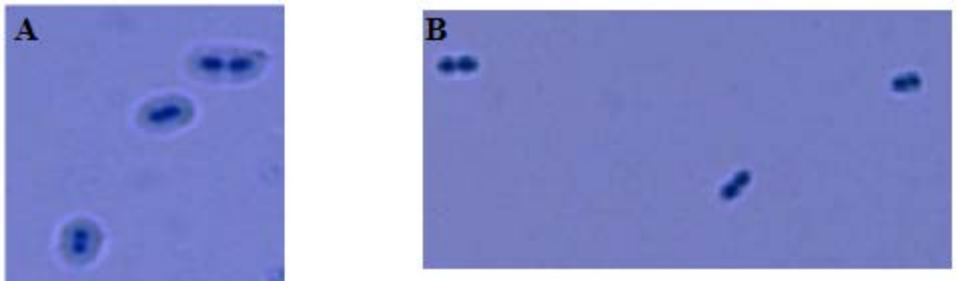

Figure 1. Quellung test with *S. pneumoniae* ATCC 49619. (A) Bacterial sample. (B) Negative control

S. pneumoniae ATCC 49619 was cultivated in the HMM broth using bottles with tightened lids. The broth was then centrifuged and the pneumococcal cells were separated from the culture supernatant. Our results showed that the capsule was in the cell associated form and we did not detect the capsule in the culture supernatant. The capsule was produced at 0.343 mg/ml in the HMM broth (Table 1). However, cultivation of the bacterium in bottles with loosen lids under 5% CO_2 atmosphere improved the

bacterial growth as measured by OD_{600} and increased the capsule production 3.5 fold.

Table 1. Serotype 19F capsular polysaccharide production using CO_2

Cultivation method	Capsule production (mg/ml)[1]	OD_{600}[2]
Without CO2	0.343 ± 0.048	2.48 ± 0.071
Using CO2	1.205 ± 0.043	3.38 ± 0.18

[1] The amount of the cell associated capsule (mg) per milliliter of the bacterial culture indicated.
[2] OD_{600} of the cells was measured after 18 h of cultivation in the HMM broth.
Data are mean values of two experiments ± standard deviations.

S. pneumoniae has been described as a facultative anaerobic microorganism, and highly aerated environments can inhibit the pneumococcal growth and the capsule production [9,14]. Since 5% CO_2 atmosphere is routinely used for the clinical isolation of pneumococci and also for the capsule production, we performed the experiments under 5% CO_2 atmosphere. Our results demonstrated that the bacterium was able to grow and produce the capsular polysaccharide in the aeration limited environment (static bottles with tightened lids). However, the bacterial growth and the capsule production in 5% CO_2 atmosphere were higher than those obtained in the aeration limited environment. Therefore, using 5% CO_2 atmosphere is advantageous over using the aeration limited environment for the serotype 19F pneumococcal capsule production.

3.2. Effect of Buffering Cultivation Medium on Capsule Production

Streptococcus pneumoniae is among LAB, so named for their primary metabolic byproduct (lactic acid), which decreases the culture pH [5]. The initial pH of the HMM broth was set to 7.5. Following the cultivation of *S. pneumoniae* ATCC 49619, the HMM broth pH dropped to 4.86, whereas the pH of the buffered HMM broth showed a slight decrease to 7.27 (Table 2). The capsule production in the buffered HMM broth was 4.97 fold more than that obtained in the HMM broth. In addition, the bacterial growth in the buffered HMM broth was much higher than that in the HMM broth as indicated by OD_{600}. These results indicated that the culture pH drop to the acidic condition due to the pneumococcal metabolism impaired the capsule production as well as the bacterial growth. However, buffering the medium showed a profound effect on the culture, enhancing both the bacterial growth and the capsule production. The pneumococcal capsule concentration obtained in this study using the buffered medium is much higher than those obtained in other reports for the pneumococcal capsule production (0.2-0.3 mg/ml) [9,10]. Moreover, this culture method is simple and cost effective. Therefore, buffering the culture medium is an appropriate approach for the capsule production by *S. pneumoniae* serotype 19F.

Table 2. Serotype 19F capsular polysaccharide production using buffered HMM

Cultivation medium	Capsule production (mg/ml)	OD_{600}	Final pH
HMM	0.343 ± 0.048	2.48 ± 0.071	4.86 ± 0.02
Buffered HMM	1.706 ± 0.030	5.62 ± 0.042	7.27 ± 0.014

Data are mean values of two experiments ± standard deviations.

3.3. Effect of Nitrogen Source on Capsule Production

To compare the effects of nitrogen sources on the pneumococcal culture, soytone in the HMM broth was replaced with casamino acid at the same concentration. Our results showed that using casamino acid as the nitrogen source of the culture medium instead of soytone increased the capsule production 1.3 fold by *S. pneumoniae* ATCC 49619 (Table 3). However, the bacterial growth in the culture medium containing soytone as the nitrogen source was better than that containing casamino acid as the nitrogen source. Therefore, casamino acid seemed to favor the capsule production, whereas soytone increased the bacterial growth. These results are in agreement with the results of Gongalves et al. [9] for the capsular polysaccharide production by *S. pneumoniae* serotype 23F. Soytone is enzymatically hydrolyzed soybean meal and casamino acid is acid hydrolyzaed caseine. The observed effects of these nitrogen sources on the pneumococcal culture were speculated to result from their different composition [15].

Table 3. Serotype 19F capsular polysaccharide production using casamino acid

Nitrogen source	Capsule production (mg/ml)	OD_{600}
Soytone	0.343 ± 0.048	2.48 ± 0.071
Casamino acid	0.449 ± 0.005	1.77 ± 0.042

Data are mean values of two experiments ± standard deviations.

3.4. Effect of Carbon Source on Capsule Production

Carbohydrates are the most common sources of energy required to produce essential compounds for the bacterial growth. *S. pneumoniae* possesses a high capacity for the uptake of carbohydrates, and the bacterium is able to metabolize up to 32 different carbohydrates [16]. For comparing effects of using different carbon sources on the pneumococcal culture, we replaced glucose in the HMM broth with other carbon sources including sorbitol, lactose and sucrose. Our results showed that sucrose was the best carbon source for the production of the capsular polysaccharide by *S. pneumoniae* ATCC 49619 and the bacterial growth (Figure 2). The capsule production using sucrose as the carbon source of the culture medium was 3.41 fold more than that obtained using glucose as the carbon source. In addition, the cell growth in the presence of sucrose was better than that in the presence of glucose. The capsule production and the cellular growth in the presence of sucrose as the carbon source of the culture medium were comparable with those obtained in the presence of lactose as the carbon source. On the other hand, the price of lactose is lower than that of sucrose. Therefore, lactose is an appropriate carbon source to include in the HMM broth instead of glucose for the serotype 19F capsule production.

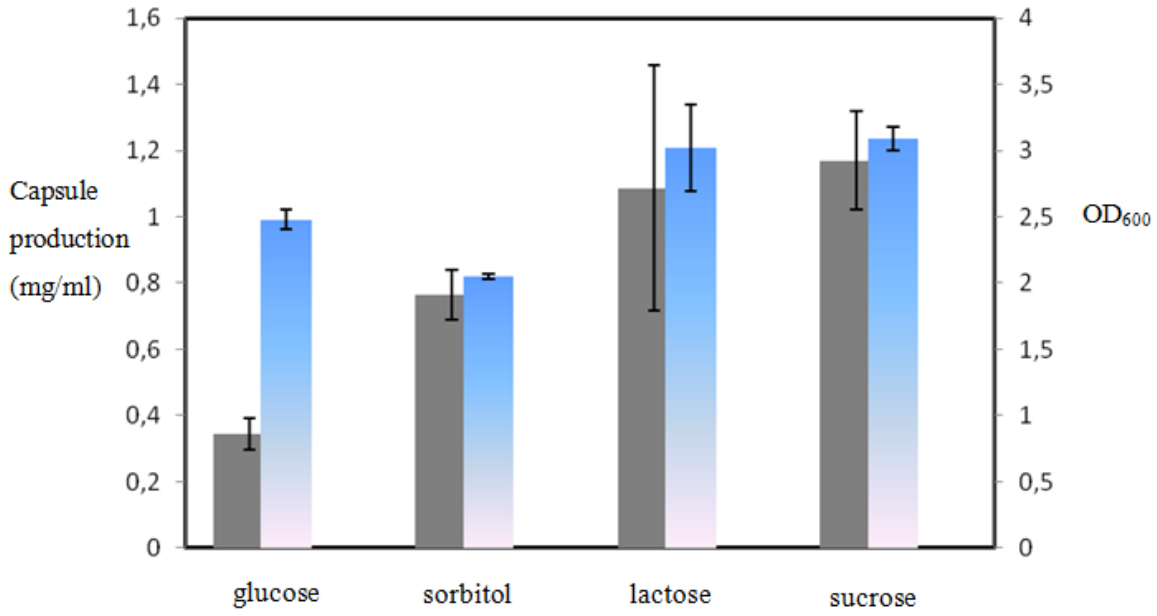

Figure 2. Using different carbon sources for capsular polysaccharide production. The grey bars indicate the capsule production and the gradient filled bars represent OD_{600} at the end of the cultivation. Data are mean values of two experiments and standard deviations are indicated

4. Conclusions

Our results showed that buffering the culture medium is the best approach for the production of the capsular polysaccharide by *S. pneumoniae* serotype 19F. In future, we will perform the pneumococcal cultivation in the buffered medium at higher scales.

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