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Optimization of Culture Conditions for Production of Pneumococcal Capsular Polysaccharide Type I

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Streptococcus pneumoniae **(pneumococcus), the most common cause of bacterial pneumonia, has an ample polysaccharide(PS) capsule that is highly antigenic and is the source of PS vaccine. This investigation was undertaken to optimize the culture conditions for the production of capsular PS by type 1 pneumococcus. Among several culture media, brain heart infusion (BHI) and Casitone based media were found to support luxuriant growth of pneumococcus type 1 at the same level. Because BHI medium is rather expensive and more complex than the Casitone based media, the Casitone based media was used to study optimization of the culture condition. The phase of growth which accomodated maximum PS production was logarithmic phase. Concentrations of glucose greater than 0.2% did not enhance growth or PS production. Substitution of nitrogen sources with other resources or supplemention of various concentrations of metal ion (with the exception of calcium ion) had adverse effects on growth and PS production. On the other hand, low level aeration was beneficial for increased PS production. Addition of 3 mg/l concentration of methionine, phenylalanine, and threonine were found to enhance growth and PS production. The synergistic effect of all the favorable conditions observed in pneumococcal growth assays provided a two-fold cumulative increase in capsular PS production.**

Key words: ***Streptococcus pneumoniae*** **type 1, polysaccharide, culture optimization**

Streptococcus pneumoniae (pneumococcus), is the most common cause of bacterial pneumonia, and an important cause of otitis media, meningitis, and septicemia. In spite of modern antimicrobial agents, it remains a leading cause of morbidity and mortality in persons of all ages (5, 10). *S. pneumoniae* is an encapsulated facultative anaerobe that can use a wide variety of fermentative carbohydrates. Its energy metabolism is primarily of the lactic acid type, but the amount of acid accumulating is small unless the culture is periodically neutralized (5, 10).

Pneumococcal capsules consist of complex polysaccharides that form hydrophilic gels on the surface of the organism. These polysaccharides (PSs) are antigenic and form the basis for the separation of pneumococci into 84 different serotypes (5, 10). Of the 84 types, PS of pneumococcus type 1, which contains D-glucose, 2-amino-2-deoxy-D-glucose, a 2-amino-2-deoxy- D-galactose, and D-galacturonic acid residues, and O-acetyl groups (7), is a component of the 23 valent PS vaccine (2) and is present in relatively large amounts than other pneumococcus types.

Several media including brain heart infusion broth, tryptic soy broth, defined media (1, 9), and casein-hydrolysate based culture medium (8) have been used for the culture of pneumococcus. But so far, no study has been reported on the optimization of pneumococcus culture. Therefore, the present study was undertaken to optimize culture conditions for the production of the pneumococcus type 1 PS.

Materials and Methods

Microorganism and culture

Streptococcus pneumoniae type 1 was from the American Type Culture Collection (Rockville, Maryland, USA) and initially grown in a brain heart infusion (BHI) agar that was supplemented with 5 % sheep blood for pneumococcal inoculum medium. Seed culture was prepared by inoculating a clone from the agar plate on BHI

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broth and incubating at 37°C until optical density at 550 nm reached 0.3. Ten %(v/v) glycerol was added to reach a final concentration of 10 %(v/v), and the culture was preserved at -65°C. One % of the seed culture was inoculated as an inoculum and incubated at 37°C without aeration unless otherwise mentioned. Growth was determined by reading optical density(O.D.) at 550 nm. Casitone based broth (CAT broth: 8) is composed of Casitone 1%, Tryptone 0.5%, NaCl 0.5%, Yeast Extract 0.1%, 0.175 M K_2HPO_4, and glucose 0.2%.

Isolation of PS

Pneumococcus was cultured in the CAT or BHI broth until optical density reached 0.6, and then Campbell and Pappenheimer's method (3) was used for PS preparation. To the culture, phenol was added to reach a final concentration of 0.1%. The clear centrifuged medium was brought up to a 50% ethanol concentration and the collected precipitate was resuspended in 1/20 volume of distilled water and extracted several times with 1/5 volume of chlololform to butanol (5 : 1) mixture. To the clear supernatant, hexadecyltrimethyl ammonium bromide was added to reach a final concentration of 0.2% (w/v). The PS was collected by precipitation with 2 volumes of 95% ethanol, and then washed with ethanol and acetone, and finally dried in vacuo. The resulting powder was dissolved in distilled water.

Analysis

Total sugar was determined by the Orcinol-Sulphuric acid method assay (11). When carbohydrates were used as carbon sources, capsular PS content was measured by deducting the amount of carbohydrate coprecipitated with the capsular PS.

Immunodiffusion

Double immunodiffusion studies (4) were made at 4°C in 1% agarose gel by using 1 mg/ml of PS and type 1 pneumococcal antisera from Statens Serum Institute (Copenhagen, Denmark).

Results and Discussion

Effect of culture media

Defined media (1), new synthetic medium (9), tryptic soy broth, the CAT broth (8), and the BHI broth were used to determine the medium best suited for supporting for pneumococcus cultures. Of these media tested, the CAT and BHI media were good for cultures of type 1 pneumococcus, and the maximum optical density attained in these media was 0.73. Defined media supported moderate growth of type 1 pneumococcus (the maximum optical density attained was 0.54), but the new synthetic medium and tryptic soy broth did not(data not shown). Because the BHI broth is rather expensive and more complex than the CAT broth, whereas the maximum growth attained by both broths are similar, only the CAT broth was used for the present medium optimization study.

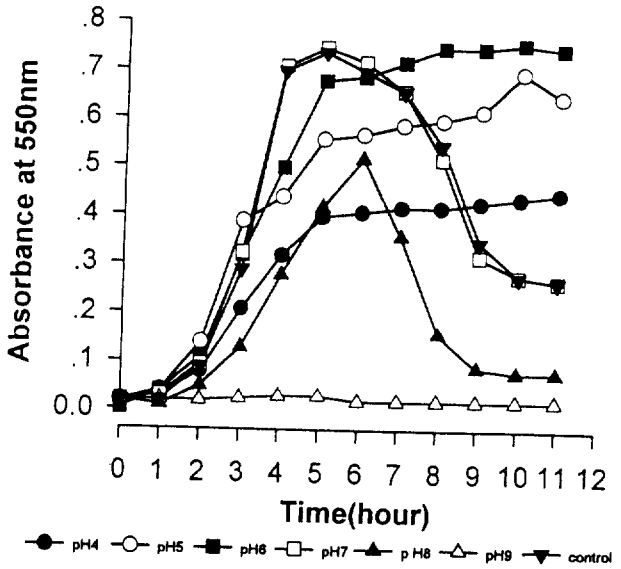


Fig. 1. Effects of initial pH on growth.

Effect of initial pH

To determine effect of initial pH on growth and PS synthesis, pH in the CAT broth was adjusted from 3~10 by adding HCl or NaOH to the medium. Low initial pH supported higher growth but it did not support good PS production. When initial pH of the CAT broth was adjusted below pH 4 or above pH 9, growth was limited to optical density of 0.1. Between pH 6 and 8, PS production did not increase in parallel with increase of growth, i.e., although the highest O.D. was obtained at pH 6, the largest PS production was obtained at the natural pH (Fig. 1). Therefore, the optimal pH for PS production seemed to be best at the natural pH.

Effect of incubation time

The optical density of type 1 culture increased rapidly and steadily during 1 to 3 hours of incubation in the CAT broth, but rapidly decreased after the exponential phase (Fig. 1). To determine the optimal time for harvesting the culture to gain maximum PS yield, cultures were harvested 1 hour before the highest O.D point, or just at the highest O.D point, or 1 hour after the highest O.D point. The maximum quantity of PS was produced when the culture was harvested 1 hour before the maximum O.D (Fig. 2).

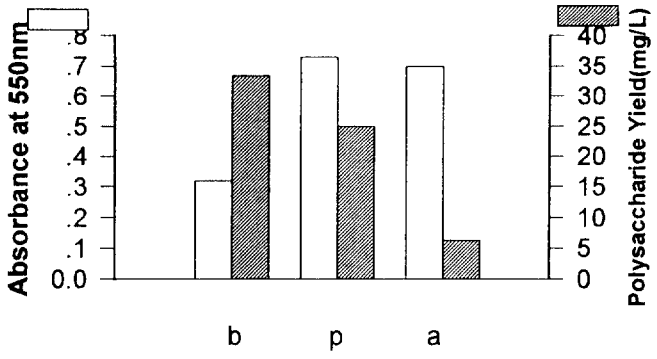


Fig. 2. Effect of incubation time on growth and PS production. Legend at the bottom describe harvest time of the culture : b, culture harvested 1 hr before the peak point; p, culture harvested at the peak point; a, culture harvested 1 hr after the peak point.

Effect of carbon and nitrogen sources

The Casitone based medium employed for the study of the effect of carbon sources on PS synthesis contained (w/v): Casitone 1%, Tryptone 0.5%, NaCl 0.5%, Yeast Extract 0.1%, and 0.175 M K_2HPO_4. Glucose, maltose, sucrose and soluble starch were added from 0.05 to 5% (w/v) concentrations as the carbon source. The greatest growth of pneumococcus was obtained on the media with 0.2% glucose or 2% sucrose. Concentrations of glucose and sucrose greater than 0.2% and 2%, respectively, did not enhance growth or PS production. There is no measurable growth when starch was used as a carbon source (data not shown).

The Casitone medium containing 0.2% glucose as the carbon source was used to investigate the effect of nitrogen sources on PS production. When nirtrogen sources in the CAT, i.e., yeast extract, Casitone, and Tryptone, were replaced by various concentrations of $(NH_4)_2SO_4$, peptone, Casitone, or yeast extract, growth and PS production did not change (data not shown).

Effect of metal ions

Metal salts, i.e., $MnSO_4$, $MgSO_4$, $FeSO_4$, $CuSO_4$, $CoCl_2$, $CaCl_2$, or EDTA, were supplemented to the CAT broth in the range of 0.5 mM~50 mM. The growth of type 1 pneumococcus with the CAT broth supplemented with metal ions other than Fe ion was not higher than growth without any supplementation (maximum O.D=0.66). When 0.5 mM and 1 mM concentrations of Fe ion was supplemented, O.D increased up to 0.8 and 0.75, respectively, but PS production severely decreased to less than one fourth of the PS yield in the CAT broth without any supplementation. Although 0.5 and 1 mM concentrations of Ca ion supplementation to the CAT broth did not stimulate pneumococcus growth, it did increase the PS production by 25% and 13%, respectively,

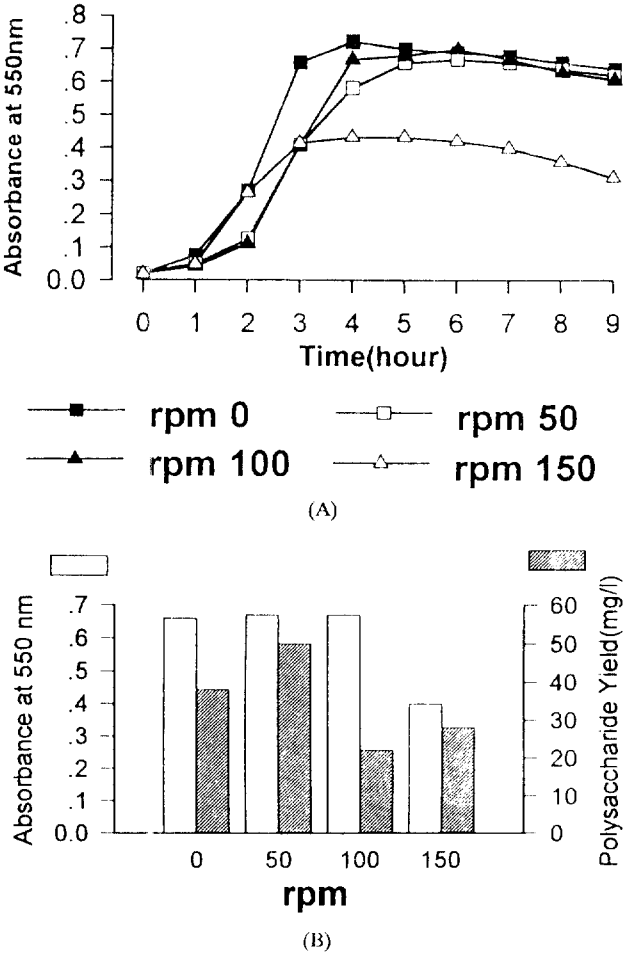


Fig. 3. Effect of aeration on growth [A] and PS production [B].

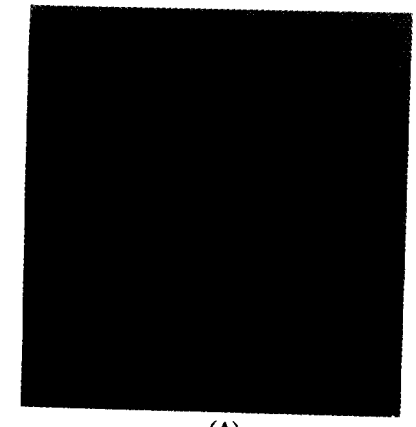
compared to broth without any supplementation (data not shown).

Effect of amino acid

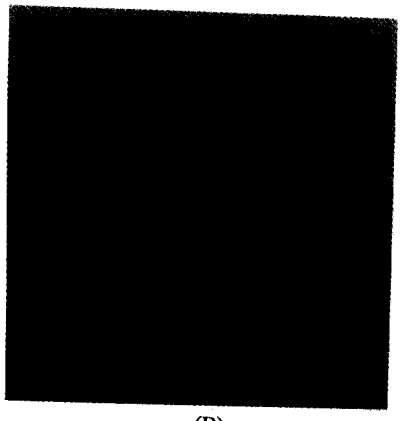
To determine supplementary effect of various amino acids to the CAT broth, 3 mg/ml concentration of cysteine, asparginе, phenylalanine, isoleucine, threonine, or methionine was added to the CAT broth. Pneumococcal growth on the CAT media supplemented with 3 mg/l of various amino acids did not change significantly. However, when methionine, phenylalanine, or threonine was supplemented, PS yield increased, respectively, to 1.68, 1.82, and 1.68 times greater than PS without any supplementation (data not shown).

Effect of aeration

Fifty ml of the Casitone based medium in 250 ml Erlenmeyer flasks were inoculated with 0.5 ml of the seed culture and incubated on rotary shakers (New Brunswick Scientific Co. Incubator Shaker) at various



(A)



(B)

Fig. 4. Immunodiffusion of pneumococcal type 1 PS with type 1 antisera. PS prepared from BHI broth [A] and the optimum CAT broth [B] The Concentration of PS was 0 mg/ml (well 1), 0.1 mg/ml(well 20, 0.25 mg/ml(Well 3), 0.5 mg/ml(well 4), and 1 mg/ml (well 5).

rpm. Vigorous aeration (higher than 100 rpm) inhibited both growth and PS production but low level aeration(50 rpm) gave rise to a 40% increase of PS production compared to incubation without aeration although there was no variation at growth (Fig. 3).

Optimum CAT broth and antigenicity

The synergistic effect of all the favorable conditions mentioned above for pneumococcal growth provided a cumulative increase in capsular PS production. When pneumococcal growth and PS yield were compared in the original CAT broth, and modified CAT broth (CAT broth supplemented with 0.5 mM concentration of $CaCl_2$, and 3 mg/ml concentration of methionine, phenylalanine, and threonine), the modified CAT broth did not stimulate maximum growth, but did increase PS production upto 2 fold.

In order to determine whether the PS produced on the modified CAT media had the same antigenic reactivity as the PS produced on the BHI broth, purified PS were crossreacted with specific antiserum using double immuno-diffusion method. On immunodifusion, the materials gave a strong precipitin line against type 1 pneumococcal antiserum (Statens Seruminstute). No difference was observed between the PSs produced on the modified CAT and BHI broth (data not shown), suggesting that PS prepared from the optimum CAT broth had same antigenic reactivity as the PS prepared from the BHI broth.

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