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

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The Pneumococcus, *Streptococcus pneumoniae*, has an ample polysaccharide (PS) capsule that is highly antigenic and is the main virulence factor of the organism. The capsular PS is the source of PS vaccine. This investigation was undertaken to optimize the culture conditions for the production of capsular PS by type 4 pneumococcus. Among several culture media, brain heart infusion (BHI) and Casitone based medium were found to support luxuriant growth of pneumococcus type 4 at the same level. Therefore in this study, the Casitone based medium was used to study optimization of the culture condition because of BHI broth's high cost and complex nature. The phase of growth which accomodated maximum PS production was exponential phase. Concentrations of glucose greater than 0.8% did not enhance growth or PS production. Substitution of nitrogen sources with other resources or supplementation of various concentrations of metal ion (with the exception of calcium, copper, and magnesium ions) had adverse effects on growth and PS production. On the other hand, low level aeration and supplementation of 3 mg/l concentration of asparagine, phenylalanine, or threonine were beneficial for increased 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 : *S. pneumoniae* type 4, Casitone medium, Polysaccharide, Culture optimization

INTRODUCTION

The pneumococcus is one of the most frequent causative agent of acute bacterial pneumonia, meningitis, and otitis media. The pneumococcus, *Streptococcus pneumoniae*, is a Gram positive nonspore forming coccus, and is an encapsulated facultative anaerobe that can use a wide variety of fermentative carbohydrates. Pneumococcal capsules consist of complex polysaccharides (PSs) that form hydrophilic gels on the surface of the organism. The capsule is the main virulence factor of the organism, which does not produce important exotoxins but causes disease by eliciting a powerful inflammatory reaction. The PS is antigenic and forms the basis for the separation of pneumococci into 84 different serotypes (Joklik *et al.*, 1988; Storch, 1989). Of the 84 types, chemical composition of capsular PS type 4 has been characterized very well. The type 4 PS is a component of the 23 valent PS vaccine and is composed of D-galactose, 2-acetamido-2-deoxy-D-galactose, 2-acetamido -2-deoxy-D-mannose, a 2-acetamido-2,6- dideoxygalactose (N-acetylfucosamine) and pyruvic acid in the molar portions 3 : 3 : 2 : 3 : 3 (Kenne and Lindberg, 1983).

Several media including brain heart infusion broth, tryptic soy broth, defined media (Adams and Roe, 1945: Sicard, 1964), and casein-hydrolysate based culture medium (Porter and Guild, 1976: Rhee, 1995: Kim and Rhee, 1995) 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 condition for production of the pneumococcus type 4 PS.

MATERIALS AND METHODS

Reagents

Chemical reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise mentioned. Type 4 pneumococcal antiserum was from Statens Seruminstitute (Copenhagen, Denmark) and culture media were from Difco Laboratories (Detroit, MI, USA).

Microorganism and culture

Streptococcus pneumoniae type 4 was purchased from American Type Culture Collection (Rockville,

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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 broth and incubating at 37°C until optical density (OD) at 550 nm reached 0.3. Glycerol was added to reach a final concentration of 10% (v/v), and the culture was preserved at -65°C until used. To obtain pneumococcus culture, 1% of the seed culture was inoculated as an inoculum and incubated at 37°C without aeration unless otherwise mentioned. Growth was determined by reading OD at 550 nm using 10×15 mm culture tube as a cuvette directly.

Casitone based broth (CAT broth: Porter and Guild, 1976: Rhee, 1995: Kim and Rhee, 1995) 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%. CAT broth was prepared as described previously (Kim and Rhee, 1995).

Isolation of PS (Campbell and Pappenheimer, 1966)

Pneumococcus was cultured in the CAT or BHI broth until OD reached 0.6, and then 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 chloloform 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 (White and Kennedy, 1986). 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 (Coligan *et. al.*, 1991) were made at 4°C in 1% agarose gel by using 1 mg/ml of PS and type 4 pneumococcal antisera from Statens Seruminstitute (Copenhagen, Denmark). Precipitin lines were allowed to develop over 3 days.

RESULTS AND DISCUSSION

Effect of culture media

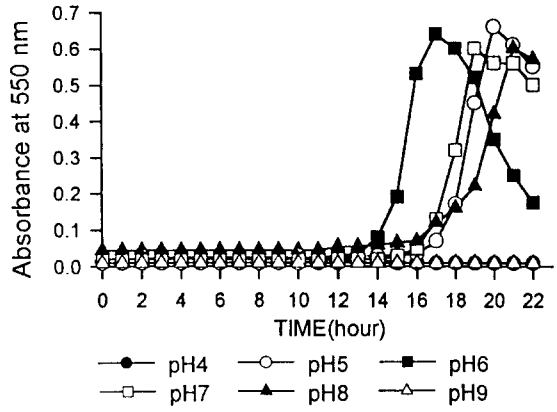

Fig. 1. Effect of initial pH on growth

Defined media (Adams and Roe, 1945), new synthetic medium (Sicard, 1964), tryptic soy broth, the CAT broth (Porter and Guild, 1976: Kim and Rhee, 1995), and the BHI broth were used to determine the medium best suited for pneumococcus cultures. The growth in the defined media, new synthetic medium, and the tryptic soy broth was very poor. Therefore in the subsequent studies, only the CAT and the BHI broth were employed. In the BHI broth, the maximum OD of type 4 culture was 0.56, and the value of maximum OD in the CAT broth was quite similar to that attained in the BHI broth although the maximum OD of type 4 in the CAT broth reached a few hours later than in the BHI broth. Because the BHI broth is rather expensive and is 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. 2).

Effect of initial pH

To determine effect of the initial pH on growth and PS synthesis, 7 initial pH levels (from pH 3 to pH 10) of the CAT broth were tested by addition of 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 OD of 0.1 (Fig. 1). Between pH 5 to 8, PS production did not increase in parallel with increase of growth, i.e., the CAT broth in pH 5 and pH 6 showed higher growth than in pH 7 and pH 8 but PS yield in pH 5 and 6 was less than 1/3 of PS yield in pH 7 and pH 8 (Table I). Therefore, the pH 8 CAT broth seemed to be the best for pneumococcus growth and PS production.

Effect of carbon sources

The Casitone based medium employed for the study of the effect of carbon sources on PS synthesis

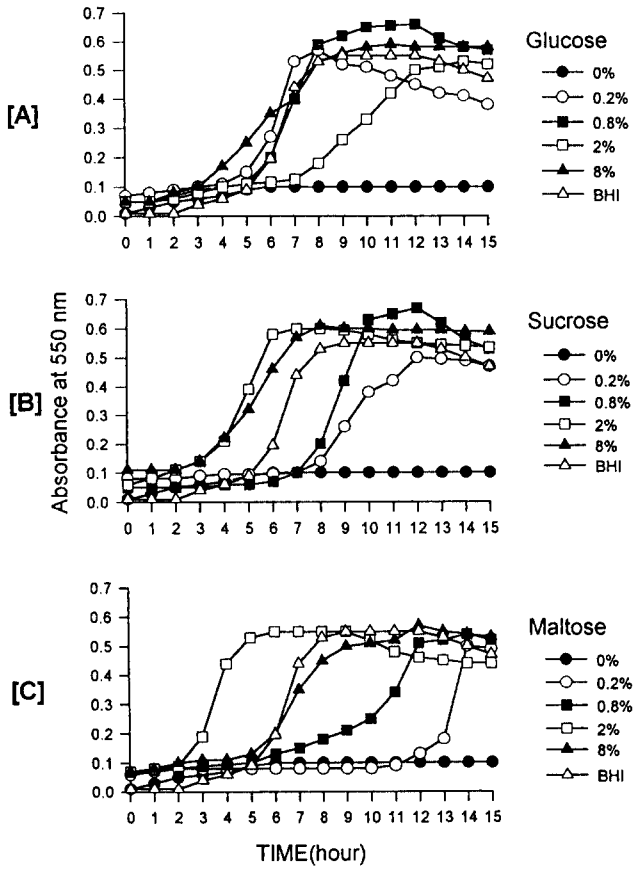

Fig. 2. Effect of carbon source on growth

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 or soluble starch were added to 0.05 to 5% concentrations as the carbon source. Fig. 2 shows growth of pneumococcus in the CAT broth with various carbon sources in the course of 15 hours. The greatest growth of pneumococcus was obtained in all experimental conditions on the media with 0.8% glucose or sucrose. Concentrations of glucose or sucrose greater than 0.8% did not enhance growth or PS production. There is no measurable growth when starch was used as a carbon source (data not shown). In the presence of maltose as a carbon source, 6 to 8 hours of a lag period was observed to get to the maximum OD and the overall PS synthesis was relatively poor than with glucose and sucrose as carbon sources.

Effect of nitrogen sources

The Casitone medium containing 0.2% glucose as the carbon source was used to investigate the effect of nitrogen sources on PS production. 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 (Fig. 3).

Table I. Effect of initial pH on PS production

Initial pH	Harvest OD[@]	PS yield (mg/L)
5	0.58	13.7
6	0.62	25.5
7	0.56	72.6
8	0.54	84.3

[@]Culture was harvested at the indicated OD.

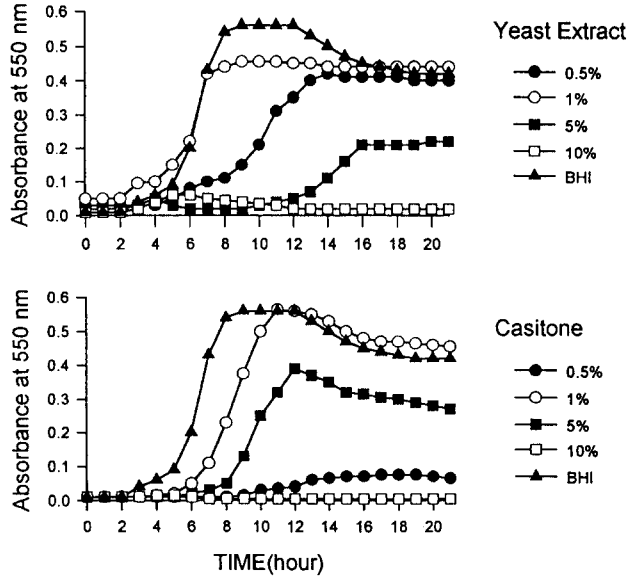

Fig. 3. Effect of nitrogen source on growth

There is no measurable growth when ammonium sulfate or Peptone was used as a nitrogen source. Employment of yeast extract as a nitrogen source seems to stabilize the culture not to lyse the cells although employment of the higher concentration of yeast extract as a nitrogen source decreased both the growth and PS production of type 4 pneumococcus. Also employment of Casitone as a nitrogen source did not result in good growth as high as the original CAT broth. Therefore, substitution of nitrogen sources with either one of them did not have positive effects on growth nor PS production.

Effect of metal ions

Fig. 6 shows supplementary effect of various concentrations of metal ions to CAT broth. Metal salts, i.e., $MnSO_4$, $MgSO_4$, $FeSO_4$, $CuSO_4$, $CoCl_2$, $CaCl_2$ (or EDTA as a control), were supplemented to the CAT broth in the range of 0.5 mM-50 mM. Supplementation of Co or Mn ion to the CAT broth arrested growth of the cell completely but supplementation of the other metal ions (Mg, Fe, Cu, Ca) in the range of 0.5-1.0 mM stimulated growth although metal ions of Mg, Fe, Cu, and Ca higher than 1 mM concentration decreased growth. Supplementation of 1 mM Mg to the CAT broth decreased PS

Table II. Effect of metal ion on growth and PS production

Metal Ion (mM)	Harvest OD[@]	PS yield (mg/L)
Ca 0.5	0.42	138.3
1.0	0.40	67.9
Cu 0.5	0.62	68.0
1.0	0.54	26.0
Fe 0.5	0.39	77.2
1.0	0.26	40.0
Mg 0.5	0.46	26.1
1.0	0.60	16.8
Mn 0.5	0.30	63.3
1.0	0.16	51.6

[@]Culture was harvested at the indicated OD.

production to less than half of the CAT broth without any supplementation. However, supplementation of 0.5 mM concentration of Ca ion increased PS production to 1.9 times than the control without any change in growth (Table II).

Effect of amino acid

To determine supplementary effect of various amino acids to the CAT broth, 3 mg/ml concentration of cysteine, aspargine, phenylalanine, isoleucine, threonine, or methionine was added to the CAT broth. Complete arrest of growth was found when cysteine or methionine was supplemented. When isoleucine was supplemented to the CAT broth, lag phase was almost abolished and exponential phase was extended. Therefore supplemtation of aminoacid at 3 mg/ml concentration did not increase growth or PS production significantly (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. Edison, New Jersey, U.S.A.) at various rpm at 37°C for 12 hours. Vigorous aeration (higher than 100 rpm) inhibited both growth and PS production but low level aeration (50 rpm) gave rise to increase of growth and PS production despite of unaerobic nature of pneumococcus culture (Fig. 4).

Synergistic effect of all the favorable conditions in CAT broth

The synergistic effect of all the favorable conditions observed in pneumococcal growth provided a cumulative increase in capsular PS production. The modified CAT broth (CAT broth [initial pH 8] supplemented with 0.8% of glucose, and 0.5 mM concentration of $CaCl_2$) was shown to increase growth and PS production than the original CAT broth. The

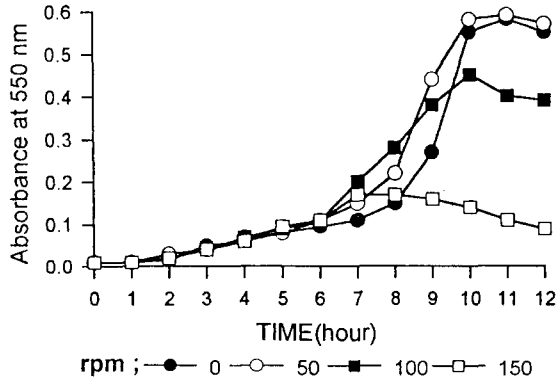

Fig. 4. Effect of aeration on growth

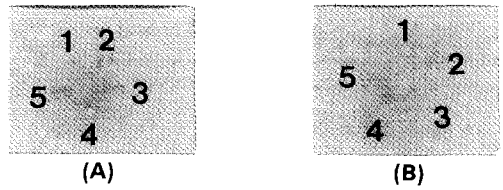

Fig. 5. Immunodiffusion of pneumococcal type 4 polysaccharide with type 4 antisera. Polysaccharide prepared from BHI broth[A] and the optimum CAT broth[B]. Antibody was placed on the center well and the PS was placed on the outer wells. The concentration of polysaccharide was 0 mg/ml (well 1), 0.1 mg/ml (well 2), 0.25 mg/ml (well 3), 0.5 mg/ml (well 4) and 1 mg/ml (well 5).

modified CAT broth was beneficial for both reducing the time required to get to the highest OD up to 2 hours and increasing the PS yield to twice than the original CAT broth. Above all, it increased PS production upto 2 times than the BHI broth.

Immunodiffusion

In order to determine whether the PS produced in the modified CAT media had the same antigenic reactivity as the PS produced in the BHI broth, purified PS was cross-reacted with type specific antiserum using double immuno-diffusion method. On immunodifusion, the materials gave a strong precipitin line against type 4 pneumococcal antiserum (Statens Seruminstute). No difference was observed between the PSs produced on the modified CAT and BHI broth (Fig. 5) 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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