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Research Article

EFFECT OF COMPLEX NITROGEN SOURCE ON PRODUCTION OF CAPSULAR POLYSACCHARIDE BY *STREPTOCOCCUS PNEUMONIAE* – 6B

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ABSTRACT

Production of capsular polysaccharide from *Streptococcus pneumonia-6B* in single step fermentation using cost effective production medium was studied. Eight types of complex organic nitrogen supplements Beef extract, Casein hydrolysate, Casamino acids, Corn steep liquor, NZ case, Peptone Soya peptone, Tryptone and Yeast extract were evaluated for their potential to yield more CPS. Soya peptone and yeast extract were found to be the best organic nitrogen supplements for CPS production in this study.

Keywords: Capsular polysaccharide (CPS), Nitrogen supplement, Fermentation, Streptococcus pneumoniae-6B (S.pneumoniae- 6B)

INTRODUCTION

Streptococcus pneumonia is an important human pathogen causing invasive diseases such as pneumonia, bacteremia, and meningitis. Control of pneumococcal diseases is being complicated by the increasing prevalence of antibiotic-resistant strains[1] and the suboptimal clinical efficacy of existing vaccines. The capsular polysaccharide (CPS) of *S. pneumoniae* is essential for virulence because it protects S. pneumoniae from the nonspecific defence system of the host organism[2]. All fresh isolates from patients with pneumococcal infection are encapsulated, and spontaneous nonencapsulated derivatives of such strains are almost completely avirulent[3]. 84 different CPS have been identified so far based on immunological and chemical techniques [4, 5]. Of the 84 serotypes, 6B is one of the predominant serotype because of its prevalence and multi drug resistantance[6, 7]. Pneumococcal vaccines available in market are not affordable to common man due to high cost of vaccine. With an aim to reduce the vaccine cost by increasing the yields using a cost effective medium was attempted. Semi defined medium with various complex nitrogen sources with varying concentration was attempted. Concentration of glucose and nitrogen greater than 2.0% & 3.0% respectively did not enhance CPS production. Substitution of various complex nitrogen sources with varying concentrations along with synergistic effect of all favorable conditions observed in pneumococcal, growth assays provided a two-fold cumulative increase in CPS production.

MATERIALS AND METHODS

Bacteria strain

S.pneumoniae Type-6B strain (ATTC 6303), was obtained from ATTC (American Type Culture Collection, Manassas, VA, U.SA.).Lyophilized vial was revived in Tryptic soy agar (TSA) containing 5% (w/v) defibrinated sheep blood (Difco, Detroit. MI, U.S.A.) At 37^{0}C for 24h. 5ml of Holt's medium[8] was inoculated by a single clone and incubated in shaker incubator for 12hrs at 37^{0}C/220 rpm.100ml of liquid medium was inoculated with 5ml of above mentioned culture. Equal volume of 20% glycerol solution was added to young culture grown for 6 -8hrs at 37^{0}C/220 rpm. 5ml aliquots were made into a 10ml cryo vials and stored at -70^{0}C.

Inoculum

A volume of 5 ml of stock culture was used to inoculate 100ml of Holt's culture medium grown in a 500ml shake flask for 8-12hrs, 250rpm at 37^{0}C which is further used for inoculating the fermentor

Medium composition and preparation medium

Composition of Holt's medium includes (g/L): 10g of glucose, 20.0 g of proteose peptone, 5.0 g of yeast extract, 2.5 g of sodium chloride, 0.5 g of monobasic potassium phosphate, 0.4 g of $MgSO_4$ 7H20, 0.01 g of calcium chloride, 0.15 g of L-cysteine hydrochloride in a final volume of one liter distilled water. The pH was adjusted to 7.2 using 20% (w/v) Na_2CO_3 solution. The medium was sterilized at 121^{0}C for 30 minutes except glucose, $MgSO_4$ and cystine stocks which were added aseptically after the sterilization using the 0.22µm filter. The bioreactor BioFlo 3000 (New Brunswick Scientific Co.) has temperature control, pH electrode, oxygen probe for measurement of DO (Dissolved Oxygen), Three peristaltic pumps to acid , alkali for pH control and anti foam. The bioreactor keeps the temperature, DOT and pH at set values automatically. The DO probe keeps the desired value by changing the stirring speed and by amount of air uptake; pH electrode feeds information to acid/alkali pumps and maintains the pH at set point.

Cultivations: Initial experiments were performed in 250ml shake flasks with 100ml of Holt's medium. Composition of the medium was unaltered except for varying complex nitrogen sources. After screening the effective nitrogen source, similar experiments were performed to determine the optimal concentration. Confirmatory experiments were conducted in a Biostat C (B.Braun) with a 2L working volume, under the following conditions: stirring speed of 200 - 600 rpm, temperature of 37°C, pH 7.0 and 0.1-0.2VVM (volume of air per volume of medium per minute) of air.

The experiments conducted were as follows:

(1) Eight flask level experiments with different nitrogen sources (Table1)

(2) Five fermentation batches with variable concentrations of Hisoya

(3) Five fermentation batches with variable concentrations of Tryptone

Kinetic parameters: The kinetic parameters were estimated based on the results obtained from biomass formation and CPS production in shake flask experiments and batch cultivations. Specific productivity is the amount of CPS per gram of cell mass (Y_{CPS}). All parameters like optical density (OD), Cell Mass (CM) and CPS concentration were estimated at the end of fermentation. Maximum CPS concentration was attained in between 10- 12 hrs of fermentation.

ANALYTICAL METHODS

Biomass concentration: Optical density of the culture was measured at 590nm using spectrophotometer (Thermo). Dry cell weight (DCW) was determined in 10-mL samples collected in preweighed tubes. After centrifugation at 3220*g* and 4°C for 30 min, the pellet was resuspended in 10 mL 0.15 M NaCl and centrifuged

again. The centrifuge tube containing the cells was dried at 60°C to achieve constant weight. True cell mass was defined as cell mass minus CPS concentration. CPS content was defined as the percentage of the ratio of CPS to dry cell weight. The yield of CPS was defined as grams of CPS per liter broth and grams of CPS per gram cell mass (Y_{CPS}).

Table 1: Shake flask experiment design

Expt.No	Initial medium	Nitrogen source	Code	Quantity (g/L)
1	Holts	Bacto peptone	BP	20
2	Holts	Beef extract	BE	20
3	Holts	Caseinhydrolysate	CH	20
4	Holts	Casaminoacids	CA	20
5	Holts	Corn steep liquor	CL	20
6	Holts	Hisoya	HS	20
7	Holts	NZ case	NZ	20
8	Holts	Tryptone	TP	20

Glucose concentration

A new method[9] using polarographic oxygen sensor with a circuit modified to record the rate of oxygen consumption was used to determine the glucose concentration in culture broth. The maximum apparent rate of oxygen consumption relative to the rate obtained with a glucose standard provides a direct measure of the glucose.

CPS concentration

Samples of 10mL were withdrawn from bioreactor and centrifuged at 3220xg at 4°C for 60min. CPS concentration in the supernatant was determined by high performance size-exclusion chromatography (HPSEC) having ultraviolet (UV), multi angle laser light scattering (MALLS) and refractive index (RI) detectors. Size exclusion (gel filtration) columns (Shodex columns SB-803 HQ and SB-806 HQ, Munich, Germany) were used in the assay. Concentrations of polysaccharide samples were calculated from the RI using the dn/dc factor which is the change in a solution's refractive index with a change in the solute concentration. Standard peak area of known concentration was compared with the peak area of unknown concentration found at the same resolution time.

Note: dn/dc is the Specific Refractive Index Increment which describes the change of the refractive index of a polymer solution with change of the polymer concentration.

Alternately CPS was quantified, determined using phenol – sulfuric acid[10] with minor modifications. 25µl of sample was mixed well with equal volume of 5%phenol (v/v) in a screw cap vial at 4^{0}C. 125 µl of sulfuric acid was added and the reaction aliquots were heated in a water bath at 100^{0}C for 10minutes followed by absorbance at 490nm.

RESULTS AND DISCUSSION

When streptococcus pneumonia- 6B was grown in a complex Holt's medium, Cell mass and CPS content obtained in 10-12h were high as 3.5g and 0.23g per liter of culture broth respectively. Results and findings from various batch cultures with different media suggested for streptococcus pneumonia- 6B required complex nitrogen sources for the efficient synthesis and accumulation of CPS. Since our aim in this study was to find a complex nitrogen sources that could promote better CPS synthesis during batch culture, we decided to use Holt's medium both in flask and fermentor since the enhancing effect of complex nitrogen sources on CPS synthesis might be different in different media.

Eight complex nitrogen sources at fixed concentrations were examined for their ability to enhance CPS synthesis. The results are summarized in Table 2. As a comparison, Optical density, Cell mass, CPS concentration and Y_{CPS} were analyzed. Out of eight types of nitrogen sources tested, Hisoya and Tryptone given high CPS production compared to other nitrogen sources (Fig.1).

Table 2: Shake flask results

Nitrogen source	Conc	OD	CM	CPS	Y_{CPS}
Bacto peptone	20	4.2	3.5	0.23	0.075
Beef extract	20	2.4	2.4	0.15	0.062
Caseinhydrolysate	20	3.6	3.1	0.18	0.058
Casaminoacids	20	3.9	3.4	0.2	0.058
Corn steep liquor	20	2.7	2.3	0.15	0.065
Hisoya	20	5.6	4.9	0.52	0.106
NZ case	20	3.2	2.9	0.16	0.055
Tryptone	20	4.9	3.6	0.32	0.088

OD - Optical density at 590nm, CM - Cell Mass (g/l), CPS - Capsular polysaccharide (g/l), Y_{CPS} - CPS/CM (g/g)

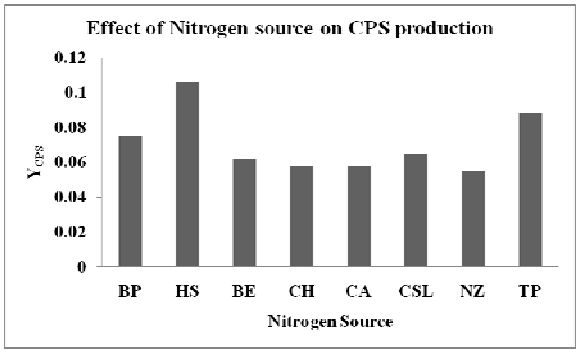

Fig. 1: *Streptococcus pneumonia-6B* in shake flask: Nitrogen source Vs Y_{CPS}

CPS production in Holt's medium with variable concentration of tryptone

The experimental results show significant production of CPS with high concentrations of bacterial cells substituting Tryptone in Holts medium (Fig.2). Five batch fermentations were run with different concentrations of Tryptone, 20, 25, 30, 35 and 40g per liter medium to verify the concentration effect. Lower CPS production was observed at 2% when compared to other concentrations. Maximum CPS of 0.36 g per liter of culture broth was observed at 3 %.(Fig.2). The present study explains that tryptone can be used as alternated nitrogen source for bacto-peptone.

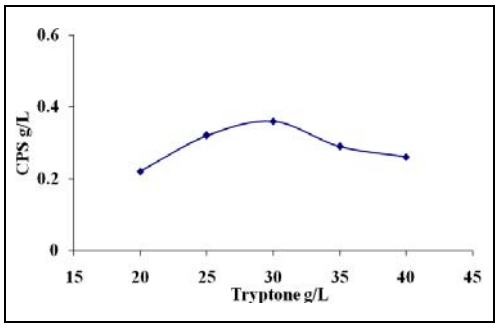

Fig. 2: CPS production by *streptococcus pneumonia-6B from* different concentration of Tryptone

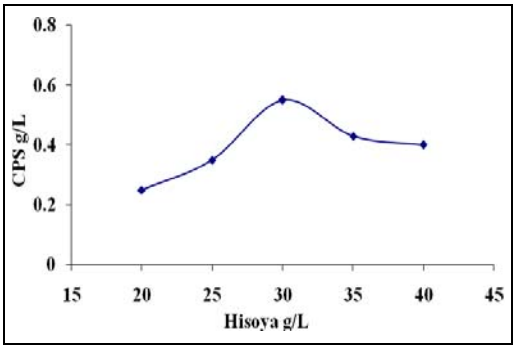

Fig. 3: CPS production by *streptococcus pneumonia-6B from* different concentration of Hisoya

CPS production in Holt's medium with variable concentrations of Hisoya

High CPS was observed with Hisoya in place of Bacto-peptone in Holt's medium was compared to other types of nitrogen sources. Five batch fermentations were performed with different concentrations of Hisoya, 20, 25, 30, 35 and 40g per liter medium to verify the concentration effect. 0.55g of CPS per liter of culture medium was obtained at a concentration 3% (Fig.3). CPS production was not steep with increased soya concentration however better than 2%.

CONCLUSION

CPS could be produced with low cost nitrogen substrates in fermentation medium with high productivity. CPS is the starting material of polysaccharide vaccines and conjugate vaccines. 60% of manufacturing cost is attributed to CPS production. Current research targeted effective vaccine production with minimum manufacturing cost. High CPS producing nitrogen sources in combination with Holt's basal medium were screened for fermentation of *streptococcus pneumonia-6B.* Of all the nitrogen sources tested, Hisoya and Tryptone could replace bacto peptone in Holt's medium. We observed two fold increase in CPS with usage of Hisoya in fermentation medium which will indirectly reduce the vaccine making cost.

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