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Investigation of cultivation conditions for capsular polysaccharide production by *Streptococcus pneumoniae* serotype 14

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Abstract *Streptococcus pneumoniae* (pneumococcus) is among the most significant causes of bacterial disease in humans. Capsular polysaccharide (CPS) production is essential for pneumococcal virulence. Pneumococcal CPS has been widely used as vaccine antigen. This study is focused on the influence of culture conditions of *Streptococcus pneumoniae* serotype 14 as for developing an industrial method for polysaccharide production. The pH proved to be a highly important variable in batchwise culture. Using the pH control all glucose added was consumed resulting in a four-fold increase in polysaccharide productivity relative to cultivation without pH control. *S. pneumoniae* is a lactic acid bacterium, so named for its primary metabolic byproduct (lactate), which has an inhibitory effect on cell growth in concentrations ranging from 4 to 5 g/L. An increase of 30% in polysaccharide productivity was observed using glucose pulses with 5.5 hrs of growth, resulting in a maximum polysaccharide concentration of 185.2 mg/L. Our data suggest the possibility of using a medium of non-animal origin and employing pH control for the cultivation of pneumococcus to produce a polysaccharide vaccine.

Keywords: polysaccharide capsular, vaccine, *Streptococcus pneumoniae*

INTRODUCTION

Streptococcus pneumoniae is an important pathogen in young children and older adults and causes invasive disease, meningitis, and otitis media worldwide. More than 90 pneumococcal serotypes have been identified, and each possesses an immunologically distinct capsular polysaccharide which is the main bacterial virulence factor (Bogaert et al. 2004; Garau and Calbo, 2007; Weinberger et al. 2009).

The impact of pneumococcal disease on young children is a matter of great concern in developing countries, where the disease causes estimated 1.2 million deaths of young children annually (Hausdorff et al. 2000).

In South America the pneumococcal disease incidence and the mortality rates in children below 5 years of age vary a lot among different countries. Brazil, Peru, Bolivia and Colombia presented disease incidence from 750 to 1,000 cases per 100,000 inhabitants in 2000. On the other hand Guyana, Paraguay and Suriname presented higher incidence (2,000-3,000 cases/100,000). However in the same period Brazil and Bolivia displayed higher mortality rates (80-160 deaths/100,000). Moreover Argentina and Uruguay showed low mortality rates (ca of 10 deaths/100,000) and an incidence

average of 1,000 cases/100,000 inhabitants. Chile was the only country with low rates of mortality (5 deaths/100,000) and disease incidence (250 cases/100,000) (www.preventpneumo.org) in that year. Nowadays Brazil shows 15% of bacterial meningitis caused by *S. pneumoniae*. In this country, pneumococcal meningitis has presented an incidence coefficient of 7.5 per 100,000 inhabitants in children under five years, in the last 10 years (www.saude.gov.br/svs). Data from Vaccine Regional System II in 2009 for pneumococcus disease in Latin America, show the serotype 14 as the most prevalent in 58% of the countries. In Brazil it has been accounted almost 40% of pneumoccocal infections in children below 5 years of age caused by serotype 14. Penicillin resistance is detected in 29% of meningitis isolates (Organización Panamericana de la Salud, 2010).

The bacterial capsule has been used as an antigen in pneumococcal vaccines since the 1980s which led to the development of a 23-valent polysaccharide vaccine that have proven to be efficacious against pneumococcal disease in adults (Institut Merieux, 1980). However, pneumococcus polysaccharide vaccines are not effective in young children and have a low efficacy in older adults. Because polysaccharide antigens are T-independent molecules, they do not induce immunological memory and long-lasting immune response (Weintraub, 2003). Therefore, these antigens will likely be replaced by new, conjugated vaccines whose efficacy depends on the inclusion of prevalent regional serotypes to be successful in protection. Pneumococcal conjugated vaccines have been licensed around the world, beginning with a seven-valent from Pfizer (formerly Wyeth) against serotypes 4, 6B, 9V, 14, 18C, 19F and 23F (Jefferies et al. 2010). More recently, a 10- valent from GSK (Sinflorix) and 13 valent from Pfizer (Prevnar 13) vaccines were licensed in Brazil.

Although *S. pneumoniae* disease and new vaccineshas been extensively studied, few publications have focused on different fermentation processes and bioreactor up-scale cultivation currently used in vaccine antigens production. Considering that the serotype 14 is one of the most prevalent in Brazil and in other South American countries, this study estimated the kinetic parameters and evaluated the PSC production yield of serotype 14 cultivation in 2.5 liter-bioreactor.

MATERIALS AND METHODS

Microorganism and stock cultures

S. pneumoniae serotype 14 strain St 113/95 was clinically isolated and is deposited in the Instituto Adolfo Lutz, Seção de Bacteriologia, SP, Brazil. Stock cultures of *S. pneumoniae* 14 were grown in non-animal Trypict soy broth (TSB) for 5 hrs at 37ºC with 5% CO_2 and maintained in a deep freezer (-70ºC) in the same medium, supplied with 20% (v/v) glycerol.

Medium composition and preparation

A modified Catlin medium (Catlin Bio) was specially developed for this study. The original Catlin 6 (Fu et al. 1995) was added by $Fe_2(SO4)_3$ (2.31 mg/L) instead of iron citrate. The other components added were: 10 mg of choline, 625 mg of L-glutamine, 100 mg of asparagine, 360 mg of $MnSO_4.7H_2O$, 1 mL of thioglycolic acid (10% v/v) and 1 g $NaHCO_3$ in 1 L of the final medium. The other components added were: 10 mg of choline, 625 mg of L-glutamine, 100 mg of asparagine, 360 mg of $MnSO_4.7H_2O$ and 50 mL of dialyzed yeast extract solution (90 g/L) in 1 L of the final medium. The lower molecular weight fraction obtained from yeast extract dialysis was prepared using membrane cut-off 12-14 kDa in distilled water. All medium components were sterilized by passage through a 0.22 µm polyetersulfone membrane.

Bioreactor batch cultivation

To prepare the inoculum for the experiment in bioreactor, a frozen stock (1 mL) of *S. pneumoniae* culture was used to inoculate a TSA (trypticase soy agar) Roux bottle, supplemented with 5% (v/v) sheep blood and incubate at 37ºC with 5% CO_2 for 16 hrs. After incubation, the cells were transferred to two 125 mL flasks containing 62,5 mL of Catlin Bio medium at an optical density (OD_{550nm}) of 0.4 as the first pre-culture. After 4 hrs of growth, the flasks were used to prepare the second pre-culture in 250 mL flasks containing 125 mL of Catlin Bio medium. The second pre-culture, after 4 hrs of incubation, was used to inoculate a 2.5 L bioreactor. A 10% v/v of inoculum was used in the bioreactor step. Batch

cultures were carried out in 2.5-L bioreactor (Bioflo 110, New Brunswick, USA) with 1.5 L of medium, at 37ºC and 100 rpm. Comparative studies without and with pH control (pH 7.2) were performed by addition of 5 M NaOH (Institut Merieux 1980). Glucose was added (20 mL of a 50% w/v solution) after 5 hrs and 30 min of growth.

Analytical methods

The culture broth samples were centrifuged at 9,500 g for 30 min at 4ºC. Then, the cell-free supernatants were filtered through 0.20-µm membrane filter and used for chemical analyses of glucose, lactic acid and PSC concentrations. The glucose and lactic acid concentrations were measured using the enzymatic-colorimetric assay oxidase enzyme kit (Laborlab, Brazil) and lactate dehydrogenase enzyme kit (Katal Biotecnológica, Brazil), respectively. The CPS production, in each one hour of growth, was determined by the method of Dubois (Dubois et al. 1956), after extensive dialysis against distilled water. A model mixture of the hexoses in the same molar ratio as in the polysaccharide structure was used as the standard for the calibration curve (Cuesta et al. 2003). A correlation between dry cell concentration and optical density (550 nm) was previously done to be used to evaluate the bacteria growth in the bioreactor.

RESULTS AND DISCUSSION

Accordingly to kinetic profiles of bioreactor cultures showed in Figure 1, the polysaccharide production was associated to cell growth without pH control. There was no lag phase, because the cells immediately entered the exponential phase growth, indicating that they were adapted to the culture conditions. The cell growth profiles were similar in the shaker flask and bioreactor scales, displaying the same specific growth rate (μ_x = 0.75 h^{-1}) (data not shown).

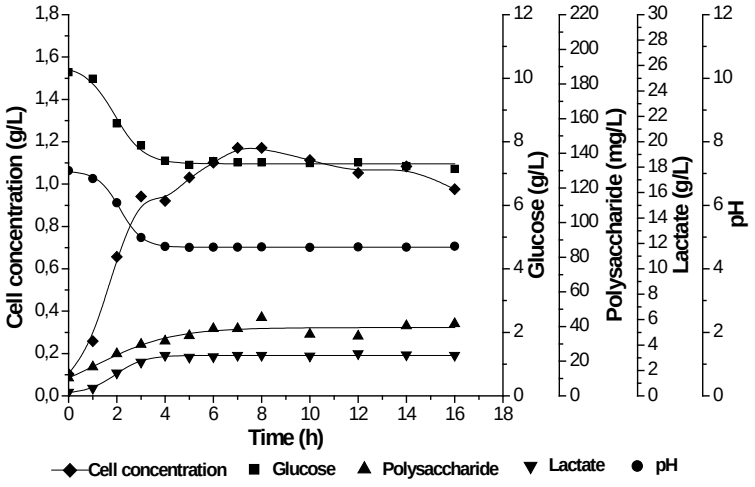

Fig. 1 Kinetic profile of growth, polysaccharide production, glucose consumption and lactic acid formation in Catlin Bio medium. Bioreactor without pH control. Strain 113/95. Number of repetitions: 02.

The glucose consumption and the lactic acid production, the main byproduct of pneumococci glucose metabolism (Hoskins et al. 2001), ceased after 4 hrs of fermentation. The production of lactic acid caused a decrease in pH (pH = 4.7), which may have led to a halt in glucose uptake. The amount of glucose reached a residual level of 70% of the initial concentration, indicating the importance of pH control during bacterial growth.

The results of the experiment with pH control are depicted in Figure 2, which shows that the rate of polysaccharide production is clearly associated with cell growth. The value of specific growth rate (μ_x = 0.853 h^{-1}) was greater than the one obtained without pH control, reinforcing the importance of this variable. In contrast to the results of culture condition without pH control, in 10 hrs of pH-controlled fermentation, the glucose was entirely consumed, even after cell growth had ceased. The secretion of lactic acid was only partially associated with cell growth, as indicated by its continued production even after cell growth cessation. This type of kinetic behaviour of lactic acid fermentation has been previously reported (Gonçalves et al. 2002).

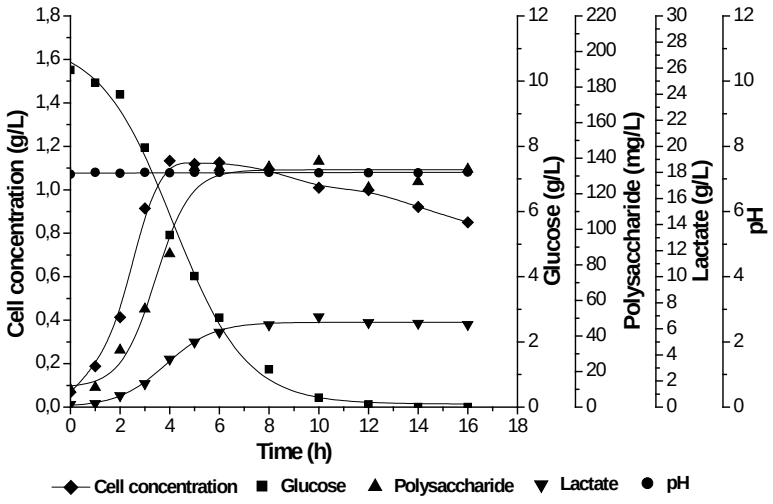

Fig. 2 Kinetic profile of growth, polysaccharide production, glucose consumption and lactate formation in modified Catlin Bio medium. Bioreactor with pH control. Strain 113/95. Number of repetitions: 02.

The polysaccharide volumetric productivity (Q_P) at 16 hrs of fermentation with pH control was approximately four times higher than the fermentation without pH control.

With the goal of improving the production of PSC, a pulse of glucose was fed into the bioreactor at 5.5 hrs of batch fermentation. Usually, glucose is added glucose in fed batch fermentation when the substrate is completely consumed; however, we fed the bioreactor at the end of the exponential growth phase because at the stage, the cell metabolic activity had not yet been reduced. To avoid a change in the working volume in the bioreactor, the feeding medium contained a 50% w/v concentration of glucose. Thus, the variation in volume resulting from the addition of glucose was negligible (≤20 mL).

Using pH control, the glucose was completely consumed (Figure 3) in 24 hrs, at which time the lactate concentration was 12.1 g/L.

Cell growth has ceased when the concentration of lactic acid reached approximately 4 to 5 g/L, as was observed in batchwise experiment, which suggests that this metabolite may inhibit bacterial growth. By employing acid casein hydrolysate and dialyzed yeast extract in the growth of *S. pneumoniae* serotype 23F, Gonçalves et al. (2002) observed that the lactic acid-inhibiting concentration of bacterial growth was close to 10 g/L. In contrast, with a glucose pulse and pH control, we found that polysaccharide production continued even after cell growth has ceased, as is typical of a process whose production kinetic is partially associated with cell growth. Under certain conditions of growth, several factors that are not well understood in growing population display a particularly effective quorum sensing system that activates several potent autolysins (Restrepo et al. 2005). Among various autolysins LytA seems to be the major pneumococcal lysin. This enzyme is an amidase that cleaves the N-acetyl-muramoyl-L-alanine bound of pneumococcal peptidoglycan (Balachandran et al. 2001; Kadioglu et al. 2008). The

cell lysis, which has been described as a phenomenon that occurs around the last part of the exponential phase of growth, accelerated the CPS liberation. Gonçalves et al. (2006) found that a portion of the polysaccharide by serotype 23F was trapped in the cell wall, and its release was also linked to cell lyses. The patent of the Merieux Institut (1980) also describes cell lyses as a way to increase the amount of polysaccharides recovered. In the downstream process of CPS recover the rapid lysis upon exposure to sodium deoxycholate is based on its capacity of triggering the LytA activity (Garcia et al. 1999).

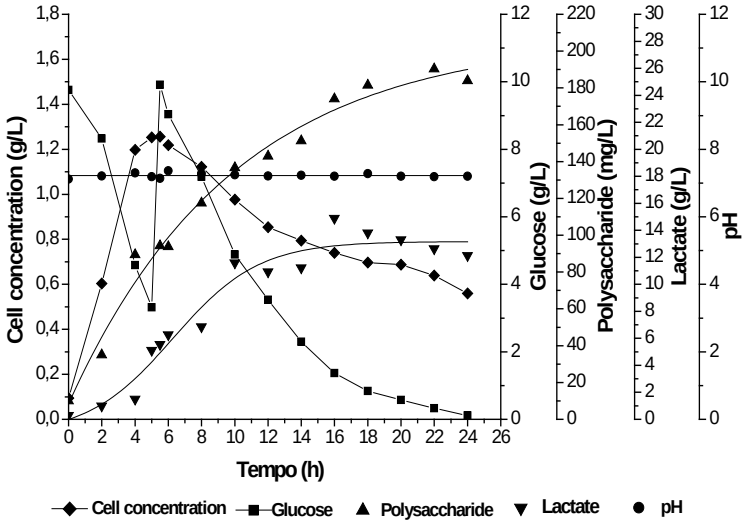

Fig. 3 Kinetic profile of growth, polysaccharide production, glucose consumption and lactate formation in Catlin Bio medium. Bioreactor with pH control and glucose addition time 5 hrs and 30 min. Strain 113/95.

It is shown in Figure 3 that cell growth declined even after the addition of glucose, probably due to the complete consumption of an essential nutrient. The results suggest that other nutrients could be essential to prevent bacterial lysis. Choline chloride added to the growth medium has been shown to inhibit LytA pneumococcal autolysin. This amino alcohol prevents the LytA attachment to wall teicoic acids avoiding cell lysis. Culture media added of 2% of choline induces the loss of autolitytic properties of autolysin that is detected normally in the last part of exponential phase of pneumococcus growth. The lack of choline in *S. pneumoniae* culture media modifies the peptidoglycana synthesis (Garcia et al. 1999). In our study the choline concentration in Catlin Bio medium was 0,1% which seems to be lower than the amount described in the literature to bacterial lysis inhibition. Nonetheless, the growth limitation by essential nutrients in Catlin Bio medium and also the avoidance of bacterial lysis should be better studied in future experiments.

The increasing polysaccharide concentration after cell growth cessation may be ascribed to cell lyses, which led to a greater release of polysaccharide into the culture medium. The total amount of polysaccharide after cell growth is partially related to the release by cell lyses and also the production of viable cells, still consuming the glucose as nutrient. In the present work, the final polysaccharide concentration after a glucose pulse and pH control was 185.2 mg polysaccharide/L. Other groups have reported a polysaccharide concentration produced by *S. pneumoniae* serotype 23F in the same order of magnitude as that obtained in this work. Gonçalves et al. (2002) and Cruz-Leal et al. (2006) worked with the same polysaccharide and reported concentrations of 240 mg/L and 250 mg/L, respectively. In addition Sheng-De et al. (2009) found 255 mg/L of serotype 3. The variables related to serotype 14 CPS production of the study are in accordance to the literature and are in Table 1.

The volumetric productivity of polysaccharide was 10.25 mg/L.h at 16 hrs of fermentation with pH control and the addition of glucose. This productivity corresponded to increases of 1.3 and 5.2 fold when compared to the experiment operated batchwise without and with pH control, respectively.

CONCLUDING REMARKS

The pH proved to be an important variable in batch culture for the production of polysaccharide by *S. pneumoniae*. An approximately four-fold increase in volumetric productivity was achieved by using pH control during fermentation. The addition of glucose to the fermentation broth had a significant effect on polysaccharide productivity, corresponding to an increase of 30% over the productivity of simple batchwise fermentation. It is noteworthy that cell lyses was related to an increase in polysaccharide release, which may also be ascribed to CPS production by the remaining viable cells because glucose continued to be consumed after cell growth has ceased. The results are of interests because strategies for increasing polysaccharide recuperation in downstream step are vital for lowering the process costs of obtaining a capsular polysaccharide for use as a vaccine against pneumococcal disease.

Table 1. **Results of response variables for cultivation of *S. pneumoniae* Catlin Bio medium.** Strain: 113/95. Results obtained between the initial time and 16 hrs of cultivation.

Experiment	$Y_{P/S}$[a] (mg P/g S)	Q_P[a] (mg P/L.h)	X_{max} (g/L)	L_{max} (g/L)	P_{max} (mg/L)
without pH control	10.02	1.96	1,17	3.21	39.19
with pH control	12.85	7.73	1.13	6.50	132.34
with pulse of glucose 5,5h[b]	10.29	10.25	1.25	13.36	185.22

X_{max}: maximum cell concentration; L_{max}: maximum lactate concentration; P_{max}: maximum polysaccharide concentration; $Y_{P/S}$: cell yield; Q_P: volumetric productivity. a: Calculation of coefficients $Y_{P/S}$ and Q_P with 16 hrs of culture. b: Experiment with pH = 7,2 control.

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