Abstract The influence of medium composition and culture conditions on *Streptococcus pneumoniae* serotype 23F cultivation was investigated in order to develop an industrial method for polysaccharide (PS) production. Acid-hydrolyzed casein (AHC) and dialyzed enzymatically hydrolyzed soybean meal (EHS) were investigated as nitrogen sources, and the vitamin solution of Hoeprich’s medium and dialyzed yeast extract as vitamin sources. The influence of initial glucose concentration was also evaluated. In flask experiments, the best nitrogen source for PS production was AHC; EHS yielded small amounts of PS without interfering with bacterial growth. Dialyzed yeast extract provided an approximately 2-fold increase in PS production when compared to Hoeprich’s vitamin solution. In a 5-L bioreactor, it was observed that the pneumococcus did not grow under aerobic conditions, CO2 did not increase PS yield, glucose was inhibitory above 30 g L−1, and the main glucose catabolism product was lactate, which had an inhibitory effect on cell growth. When anaerobic cultivation was performed under N2 flow using the optimized medium, 240 mg L−1 of soluble PS was obtained, which represents a 3-fold increase in yield as compared to that described in the published patent [Yavordios and Cousin (1983) European Patent 0 071515 A1]. Application of these results would considerably simplify upstream and downstream processes for PS production.

Introduction

*Streptococcus pneumoniae* is a pathogenic capsulated lancet-shaped Gram-positive bacterium, which grows in pairs or short chains and which has been classified as facultative anaerobe. *S. pneumoniae* lacks catalase and cytochrome oxidase enzymes and CO2-atmosphere is routinely used for clinical isolation (Holt et al. 1994). The capsule of *S. pneumoniae* has long been recognized as the major virulence factor (Austrian 1981) and consists of a high molecular weight polymer of repeated oligosaccharides. Ninety different pneumococci serotypes have been identified and each serotype corresponds to a different chemical composition of the capsule (Henrichsen 1995).

The capsule is currently used as an antigen in pneumococcal vaccines, either as free polysaccharide (PS) or as PS conjugated to proteins (Hollingshead and Briles 2001). The conjugated vaccines enhance PS immunogenic character, inducing T-dependent immune response and immunological memory (Klein and Ellis 1997). There are 90 distinct pneumococcus serotypes; however, most human infections are caused by only 20. Since PS antigens do not induce cross reactivity, vaccine efficacy will depend on inclusion of PS antigens from prevalent serotypes. Although a 23-valent PS vaccine has been used worldwide, it will probably be replaced by the new conjugated vaccines, which contain from 7 to 11 PS-protein conjugates and have been in clinical trials (Hollingshead and Briles 2001). A 7-valent vaccine, containing serotypes 4, 6B, 9V, 14, 18C, 19F and 23F, has recently been licensed for clinical use (Rubin 2000). Several different PS-protein conjugates in a single vaccine make the development and production of a pneumococcal conjugate vaccine a laborious and expensive task (Lieu et al. 2000).

Although *S. pneumoniae* has been extensively studied, few publications focus on large-scale cultivation and PS purification. To our knowledge, there are no data on process parameters such as specific growth rates and yield coefficients. The two main studies are presented as
Materials and methods

Microorganism and stock cultures

*S. pneumoniae* serotype 23F strain St 99/95 is a clinical isolate and is deposited in the Instituto Adolfo Lutz, Seção de Bacteriologia, SP, Brazil. Stock cultures of *S. pneumoniae* 23F were grown in acid-hydrolyzed casein (AHC)/yeast extract medium for 16 h at 36°C in a candle jar and maintained in a deep freezer (−70°C) in the same medium supplied with 30% (v/v) glycerol.

Medium composition and preparation

The first set of flask experiments was carried out using Hoeprich’s medium (Hoeprich 1955) with different concentrations of glucose and AHC. Hoeprich’s medium contains (per liter): 20 g AHC; 12.5 g glucose; 5 g K₂HPO₄; 1 g NaHCO₃; 150 mg L-cystine; 20 mg tryptophan; 200 mg tyrosine; 625 mg l-glutamine; 100 mg asparagine; 10 mg choline; 500 mg MgSO₄; 5 mg FeSO₄; 0.8 mg ZnSO₄; 0.36 mg MnSO₄; 1 ml thioglycolic acid (10%); 0.02 ml HCl and distilled water to 1 l. The lower molecular weight fraction obtained from yeast extract dialysis (membrane cut off, 12–15 kDa in discontinuous procedure: samples were treated with 1% sodium deoxycholate to achieve cellular lysis and centrifuged at 17,696 g for 1 h to remove cellular debris. The supernatant. PS was determined as rhamnose (Dische and Shettles 1948) after extensive dialysis against distilled water. According to the PS formula (Richards and Perry 1988), the rhamnose concentration was multiplied by 2.3 to obtain the PS concentration.

In shake flask experiments, total PS was determined from the whole culture (cell-bound plus soluble) by the following procedure: samples were treated with 1% sodium deoxycholate to achieve cellular lysis and centrifuged at 17,696 g for 1 h to remove cellular debris. The supernatant was then dialyzed against distilled water. The recovery of PS from the dialysate per gram of cells, glucose consumed, and biomass yield on glucose (Yₓₛ) after 12 h culture are shown in Table 1. An increase in the PS yield was observed for higher initial glucose concentration. The effect of glucose concentration on PS production was more evident when a low concentration of AHC (10 g l⁻¹) was used. In this case, PS production was positively influenced by high glucose concentration (18.75 g l⁻¹) and a C/N ratio of 1.9 (Table 1, experiment 1.4). The highest average value for biomass yield on glucose (Yₓₛ =0.23 g g⁻¹) was achieved using higher initial concentrations of glucose (18.75 g l⁻¹) and AHC (30 g l⁻¹) (Table 1, experiment 1.2). The C/N ratio was kept the same as in Hoeprich’s medium (0.6).

Bioreactor batch cultivation

Frozen stock culture (1 ml) was used to inoculate a 250-ml flask containing 25 ml of the medium selected in the shaker flask experiments, with the following composition: 30 g AHC; 18.75–30 g glucose; 20 g dialyzed yeast extract; 5 g K₂HPO₄; 1 g NaHCO₃; 625 mg l-glutamine; 100 mg asparagine; 10 mg choline; 500 mg MgSO₄; 5 mg FeSO₄; 0.8 mg ZnSO₄; 0.36 mg MnSO₄; 1 ml thioglycolic acid (10%); 0.02 ml HCl and distilled water to 1 l. The flasks were incubated at 36°C and 50 rpm for 12 h. In the first set of experiments, five cultivation media of different compositions were investigated: standard Hoeprich’s medium (triplicate) and test media containing 6.25–18.75 g glucose and 10–30 g AHC (duplicate). For the second set of experiments (duplicate), eight combinations of cultivation media containing 100 ml of medium to obtain an initial optical density (OD) of 0.1 at 625 nm. The flasks were incubated at 36°C for 16 h and two precultures were static-cultivated in a candle jar at 36°C for 16 h and two precultures were static-cultivated in 5 l reactors. This study was performed using *S. pneumoniae* serotype 23F, one of the most prevalent serotypes worldwide, comprising many strains with antibiotic multiresistance (Barnes et al. 1995; Coffey et al. 1998).

Analytical methods

Culture broth samples were centrifuged at 3,220 g and 10°C for 60 min. The supernatant was used for chemical analyses of glucose, lactic acid and PS concentrations. As vitamin sources, either EHS or AHC as nitrogen source and Hoeprich’s vitamin solution or 20 g l⁻¹ dialyzed yeast extract was employed. The lower molecular weight fraction obtained from yeast extract dialysis (membrane cut off, 12–15 kDa in discontinuous procedure: samples were treated with 1% sodium deoxycholate to achieve cellular lysis and centrifuged at 17,696 g for 1 h to remove cellular debris. The supernatant was then dialyzed against distilled water. According to the PS formula (Richards and Perry 1988), the rhamnose concentration was multiplied by 2.3 to obtain the PS concentration.

In shake flask experiments, total PS was determined from the whole culture (cell-bound plus soluble) by the following procedure: samples were treated with 1% sodium deoxycholate to achieve cellular lysis and centrifuged at 17,696 g for 1 h to remove cellular debris. The supernatant was then dialyzed against distilled water. According to the PS formula (Richards and Perry 1988), the rhamnose concentration was multiplied by 2.3 to obtain the PS concentration.

In shake flask experiments, total PS was determined from the whole culture (cell-bound plus soluble) by the following procedure: samples were treated with 1% sodium deoxycholate to achieve cellular lysis and centrifuged at 17,696 g for 1 h to remove cellular debris. The supernatant was then dialyzed against distilled water. According to the PS formula (Richards and Perry 1988), the rhamnose concentration was multiplied by 2.3 to obtain the PS concentration.

Results

Influence of carbon and nitrogen sources

Flask cultures were used to study the influence of glucose and AHC concentration on capsular PS production. Total PS, biomass, specific PS production per gram of cells, glucose consumed, and biomass yield on glucose (Yₓₛ) after 12 h culture are shown in Table 1. An increase in the PS yield was observed for higher initial concentration of nitrogen source (30 g l⁻¹ of AHC); thus, PS production seemed to be more dependent on the nitrogen than on the carbon source (Table 1, experiments 1.2 and 1.5). The effect of glucose concentration on PS production was more evident when a low concentration of AHC (10 g l⁻¹) was used. In this case, PS production was positively influenced by high glucose concentration (18.75 g l⁻¹) and a C/N ratio of 1.9 (Table 1, experiment 1.4). The highest average value for biomass yield on glucose (Yₓₛ =0.23 g g⁻¹) was achieved using higher initial concentrations of glucose (18.75 g l⁻¹) and AHC (30 g l⁻¹) (Table 1, experiment 1.2). The C/N ratio was kept the same as in Hoeprich’s medium (0.6).