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ORIGINAL PAPER

Pneumococcal whole-cell vaccine: optimization of cell growth of unencapsulated *Streptococcus pneumoniae* in bioreactor using animal-free medium

C. Liberman · M. Takagi · J. Cabrera-Crespo · M. E. Sbrogio-Almeida · W. O. Dias · L. C. C. Leite · V. M. Gonçalves

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Abstract The high cost of the available pneumococcal conjugated vaccines has been an obstacle in implementing vaccination programs for children in developing countries. As an alternative, Malley et al. proposed a vaccine consisting of inactivated whole-cells of unencapsulated *S. pneumoniae*, which provides serotype-independent protection and involves lower production costs. Although the pneumococcus has been extensively studied, little research has focused on its large-scale culture, thus implying a lack of knowledge of process parameters, which in turn are essential for its successful industrial production. The strain Rx1Al$^-$ ery^R was originally cultured in Todd–Hewitt medium (THY), which is normally used for pneumococcus isolation, but is unsuitable for human vaccine preparations. The purposes of this study were to compare the strains Rx1Al$^-$ ery^R and kan^R, develop a new medium, and generate new data parameters for scaling-up the process. In static flasks, cell densities were higher for ery^R than kan^R. In contrast, the optical density (OD) of the former decreased immediately after reaching the stationary phase, and the OD of the latter remained stable. The strain Rx1Al$^-$ kan^R was cultivated in bioreactors with medium based on either acid-hydrolyzed casein (AHC) or enzymatically hydrolyzed soybean meal (EHS). Biomass production in EHS was 2.5 times higher than in AHC, and about ten times higher than in THY. The process developed for growing the strain Rx1Al$^-$ kan^R in pH-controlled bioreactors was shown to be satisfactory to this fastidious bacterium. The new culture conditions using this animal-free medium may allow the production of the pneumococcal whole-cell vaccine.

Keywords Pneumococcal whole-cell vaccine · *Streptococcus pneumoniae* culture conditions · Culture medium development

C. Liberman (✉) · M. Takagi · J. Cabrera-Crespo · M. E. Sbrogio-Almeida · W. O. Dias · L. C. C. Leite · V. M. Gonçalves
Centro de Biotecnologia, Instituto Butantan, Av. Vital Brasil 1500, São Paulo, SP 05503-900, Brazil
e-mail: libercel@butantan.gov.br

Introduction

According to the World Health Organization, 1.6 million deaths worldwide caused by *S. pneumoniae* are reported each year [9]. This pathogen affects the human respiratory tract, causing otitis media, sinusitis, pneumonia, meningitis and sepsis. Although the 90 different serotypes of *S. pneumoniae* prevail according to the world's geographic regions, some vaccines have been developed and licensed solely on the basis of those capsular polysaccharide serotypes which are currently prevalent in the USA and Europe. One of them aggregates 23 purified polysaccharides, but has reduced or no immunogenicity in high-risk groups such as children and elderly, respectively. Another one, composed of seven polysaccharides individually conjugated to a carrier protein, is effective in protecting high-risk groups, but expensive and laborious to be produced in developing countries. Thus, to meet the demand for higher efficiency and cost reduction, Malley et al. [7] proposed an intranasal vaccine which would be technologically simpler to be manufactured at lower costs for being composed of inactivated whole-cells of unencapsulated *S. pneumoniae*. Later, these authors demonstrated that such a vaccine prevents nasopharyngeal colonization in mice through a CD4+T cell-mediated immune response [8].

The *S. pneumoniae* is one of the most studied microorganisms on account of its great importance for public health

and its unique ability to develop natural competence. However, little research focuses on its large-scale culture, thus implying a lack of knowledge of process parameters such as specific growth rate and coefficient yields. *S. pneumoniae* is a fastidious Gram-positive bacterium which occurs in diplococcal forms or in short chains [4]. It is classified as facultative anaerobe, anaerobe [2] or anaerobe aerotolerant [1] and obtains energy mainly producing lactate from glycolysis. Indeed, the impossibility to knockout the gene of lactate dehydrogenase is indicative of the essential role of this enzyme [5]. Hence, the pneumococcal metabolism is restricted to substrate level phosphorylation which imposes constraints upon the productivity of the microorganism. Moreover, the growth of *S. pneumoniae* cells is inhibited by end-products of fermentation such as lactic and acetic acids.

The inactivated whole-cell vaccine was originally prepared with the strain Rx1Al$^-$ ery^R, an autolysin negative mutant resistant to erythromycin, which would be inadequate for human vaccine preparation, since the erythromycin is an antibiotic widely employed in the treatment of infection human disease [7]. Furthermore, Malley et al. [7] employed Todd–Hewitt medium supplemented with yeast extract (THY), which is normally used for *S. pneumoniae* isolation, but it is unsuitable for human vaccine preparations due to the presence of bovine heart extract [10]. Thus, in order to produce pneumoccocal polysaccharide vaccine, new media based on either acid-hydrolyzed casein (AHC) or enzymatically hydrolyzed soybean meal (EHS) was developed in our laboratory [6]. In that study, EHS-based medium was shown to be favorable to biomass formation in flask experiments, but, due to pH decrease, the differences between the two media were subtle for biomass and the study followed using only the AHC in bioreactors, since it presented much better results for polysaccharide production. In addition to replacing THY with AHC or EHS, the current study aimed to characterize culture conditions for growing the strains Rx1Al$^-$ ery^R and kan^R (resistant to erythromycin and kanamycin, respectively), scale-up the culture process to bioreactors, compare the AHC and EHS media in bioreactors, and enhance biomass yield.

Materials and Methods

Microorganisms

The strain Rx1 is a rough derivative from D39 serotype 2 strain with loss of encapsulation. The strains Rx1Al$^-$ ery^R and kan^R were obtained by insertion-duplication mutagenesis of the *Streptococcus pneumoniae* chromosomal autolysin gene (*lyt*A) [3], where the erythromycin resistance gene (ery^R) or the kanamycin resistance gene (kan^R) was used to select the mutants with the disrupted *lyt*A gene. The strains were kindly supplied by Dr. Malley (Division of Infectious Diseases and Emergency Medicine, Harvard School of Public Health, Harvard University, USA).

Culture media

Three culture media were employed: (1) Todd–Hewitt supplemented with yeast extract 5 g L^{-1} (THY); and Hoeprich's modified media [6] based on (2) acid-hydrolyzed casein (AHC) (20 g L^{-1}) or (3) enzymatically hydrolyzed soybean meal (EHS) (20 g L^{-1}). Each liter of Hoeprich's modified medium contained 20 g AHC or EHS, 20 g glucose, 20 g yeast extract, 5 g K_2HPO_4, 1 g $NaHCO_3$, 624 mg L-glutamine, 100 mg asparagine, 10 mg choline, 500 mg $MgSO_4$, 5 mg $FeSO_4$, 0.8 mg $ZnSO_4$, 0.36 mg $MnSO_4$, 1 ml thioglycolic acid (10% v/v), and 0.02 ml HCl. The antibiotic for selection of each strain was added to all media at the following concentration: 0.3 mg L^{-1} erythromycin and 200 mg L^{-1} kanamycin. All media were adjusted to pH 7.5 and sterilized by filtration in 0.22 µm. Todd–Hewitt, yeast extract, AHC (Casamino acids), and EHS (Soytone) were supplied by BD-Difco, USA. All other chemicals were of analytical grade.

Comparison of strains and media in flasks

The strains were compared by culturing them in static flasks at 36 °C and 3% CO_2. For the pre-culture, 50 mL AHC, EHS or THY medium was inoculated with 100 µl frozen stock cultures and incubated at 36 °C and 3% CO_2 for 11 h. The culture was transferred to tubes containing 50 ml of AHC, EHS or THY medium in order to obtain an initial OD of 0.1. The tubes were incubated at 36 °C and 3% CO_2. Samples were collected in 1-h interval up to the beginning of the exponential phase, and after this phase in intervals of 30 min, in order to monitor OD, CFU and pH. At least three independent experiments were performed with each medium.

Bioreactor cultivation

The strain Rx1Al$^-$ kan^R was further cultured in 5-L bioreactors with AHC- or EHS-based medium. Frozen stock culture (100 µl) was used to inoculate 500 mL of the medium and the cell suspension was incubated at 36 °C and 3% CO_2 for 11 h. This pre-culture was inoculated into a bioreactor so as to obtain an initial OD of 0.1. Batch cultures were carried out in 5-L bioreactors (BioFlo 2000, New Brunswick, USA) with 4.5 L of medium, at 36 °C, 150 rpm, 0.5 L min^{-1} N_2, and 0.1 bar, with or without pH control. The pH was controlled at 7.0 by addition of 5 M NaOH. Polypropylene glycol was used as an antifoam agent. Samples were harvested for analyses.

Analytical methods

Culture broth samples were used to measure both the OD at 600 nm and the colony-forming units (CFU ml^{-1}). One unit of OD corresponds to 0.4 g L^{-1} of dry cell weight (DCW), according to the calibration curve prepared as follows: cells were inactivated with 0.2% formalin for 18 h, centrifuged at 4,000*g* for 30 min, washed with 0.9% NaCl, and filtered in pre-weighted 0.22 μm membrane. The cells were dried at 60 °C until reaching constant weight.

After centrifugation of culture broth samples at 20,000*g* and 4 °C for 10 min, glucose, lactate and acetate were determined in the supernatant using high-performance liquid chromatography (HPLC, Shimazdu) with an Aminex HPX 87H column (300 × 7.8 mm, BioRad) at 60 °C, and 5 mM H_2SO_4 was used as solvent with a flow rate of 0.6 mL min^{-1}.

Results

Comparison between the strains Rx1Al$^-$ ery^R and kan^R

In flasks, cell densities were higher for ery^R than for kan^R in both tested media: THY and AHC. In contrast, the OD of the former decreased immediately after reaching the stationary phase, and the OD of the latter remained stable (Fig. 1). Both strains showed higher cell densities and slightly higher CFU in AHC medium, the pH markedly decreased in all conditions and the cell growth ceased when the pH of the culture falls below 6.0 (Fig. 1).

Effect of pH control on cultivation of the strain Rx1Al$^-$ kan^R

In order to verify whether the pH control could be beneficial to the cell growth, bioreactors were used to cultivate the strain Rx1Al$^-$ kan^R with or without pH control. The culture of the strain Rx1Al$^-$ kan^R using AHC medium in bioreactor without pH control resulted in an OD of 2.5; a dry cell weight of ~1.0 g L^{-1}; a maximum specific growth of 1.03 h^{-1}; a yield coefficient on biomass of 0.15 g dry cell weight per gram consumed glucose; a lactate concentration of 6.0 g L^{-1}. The culture using AHC medium with pH control resulted in an OD of 3.6; a dry cell weight of ~1.4 g L^{-1} (40% higher than without pH control); a maximum specific growth of 1.18 h^{-1}; a yield coefficient on biomass of 0.11 g dry cell weight per gram consumed glucose; a lactate concentration of 14 g L^{-1}. In both cases, no acetate formation was observed (Fig. 2).

Effect of EHS on cultivation of the strain Rx1Al$^-$ kan^R

In flasks, when AHC was replaced to EHS in the medium composition, the OD increased from 2.5 to 3.5 (Fig. 3). When the EHS medium was used for bioreactor culture with pH control, the biomass production was 2.5 times higher than that using AHC also in pH-controlled bioreactor; the OD reached 8.9 (3.5 g L^{-1} dry cell weight); the yield coefficient on biomass was 0.14 g dry cell weight per gram consumed glucose; the maximum specific growth rate was 0.82 h^{-1}; and the lactate and acetate concentration, respectively, reached 32 and 9.0 g L^{-1}, which means 1.28 g lactate and 0.3 g acetate produced per gram consumed glucose (Fig. 4).

Discussion

The current work dealt with the problem of bringing a process from the laboratory initial research steps to the bench scale with the aim of developing an industrial production process. First, the original strain, which carries the erythromycin

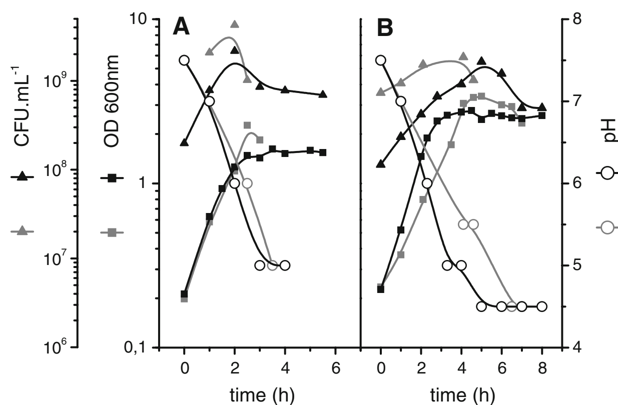

Fig. 1 Comparison between *S. pneumoniae* strains in flasks. Typical curves are shown. **a** In THY medium. **b** In AHC medium. In *grey* strain Rx1Al$^-$ ery^R and in *black* strain kan^R. *Squares* OD; *triangles* CFU; *open circles* pH

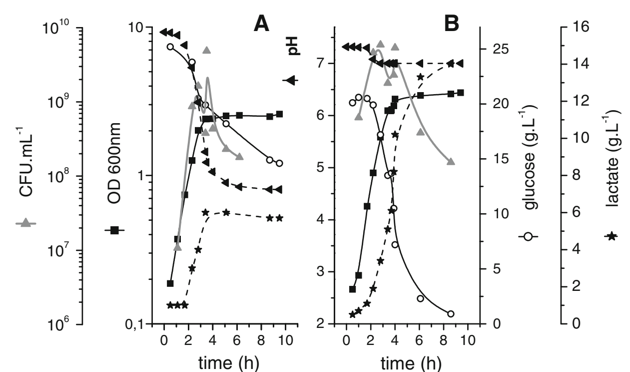

Fig. 2 Typical growth profile of *S. pneumoniae* RxAl$^-$ kan^R in bioreactor with AHC medium. **a** without pH control, **b** with pH control. *Black squares* OD; *light grey triangles* and *solid line* CFU; *dark grey triangles* and *dashed line* pH; *open circles* glucose; *stars* and *dashed line*lactate

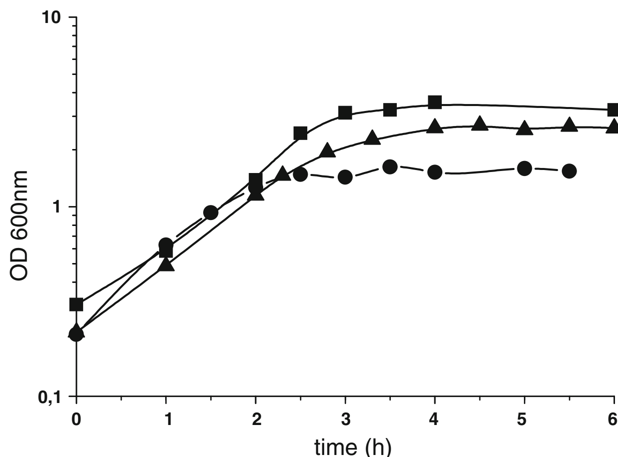

Fig. 3 Comparison of *S. pneumoniae* RxAl$^-$ kan^R cell growth in flasks with different media. Typical results are presented. *Circles* THY medium; *triangles* AHC medium; *squares* EHS medium

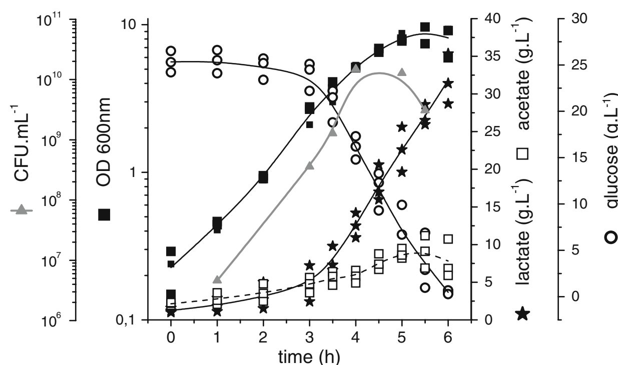

Fig. 4 Growth profile of *S. pneumoniae* RxAl$^-$ kan^R in bioreactor with EHS medium and pH control. *Symbols* represent the results of three independent experiments and *lines* are the average curves. *Dark squares* OD 600 nm; *grey triangles* CFU mL^{-1} (results of one experiment is presented); *open circles* residual glucose (g L^{-1}); *stars* lactate (g L^{-1}); *open squares* acetate (g L^{-1})

resistance gene, was compared with a strain carrying the kanamycin resistance gene, inasmuch as the former is unsuitable for human vaccine production. Second, the medium containing animal tissue extract, Todd–Hewitt, was replaced for a casein-based medium, which is also from animal origin, but is industrially used for tetanus vaccine production. Moreover, there are no reported cases of diseases associated with casein. Although allowing higher cell densities than with Todd–Hewitt medium, the results obtained with casein-based medium were still below the desired for industrial processes, even when the pH-controlled bioreactor was employed for cultivation.

The EHS was previously shown to be favorable to cell growth in flask experiments only, while AHC led to higher polysaccharide production [6]. Therefore, a new process was developed for growing unencapsulated *S. pneumoniae* in pH-controlled bioreactor using enzymatically hydrolyzed soybean meal, which resulted in 2.5 times higher cell density than using casein-based medium, confirming the tendency observed for polysaccharide production in flasks [6]. Interestingly, the growth profile of the encapsulated and unencapsulated strains in pH-controlled bioreactor using AHC medium differed significantly: the biomass reached 3.5 g L^{-1} for the serotype 23F encapsulated strain [6], but only 1.4 g L^{-1} for unencapsulated strain Rx1Al$^-$ kan^R (Fig. 2b).

In comparing the composition of acid-hydrolyzed casein and enzymatically hydrolyzed soybean meal, some compounds were observed in higher amount in the latter. In this sense, several assays were performed adding those compounds to the casein-based medium, but none of them was able to produce the same results as the soybean-based medium (results not shown). Since the percentage of free amino acids is substantially lower in enzymatically hydrolyzed soybean meal than in acid-hydrolyzed casein (BD/Difco catalog), further studies are necessary to verify whether some peptides could be responsible for the observed effect.

The process developed in the current study was shown to be satisfactory for this fastidious bacterium. The new culture conditions using this animal-free medium may allow the production of the pneumococcal whole-cell vaccine.

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