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Characterization of Polysaccharide Production of *Haemophilus influenzae* Type b and Its Relationship to Bacterial Cell Growth

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Abstract

Haemophilus influenzae type b (Hib) causes invasive infections in infants and young children. Vaccines consisting of Hib capsular polysaccharide (polymer of ribosylribitol phosphate [PRP]) conjugated to a protein are effective in the prevention of such infections. The production of capsular polysaccharide type b was studied in three cultivation conditions: single, glucose pulse, and repeated batch. Specific polysaccharide production ($Y_{p/x}$) was calculated for all experiments, showing the following values: 67 (single-batch cultivation), 71 (glucose pulse), 75 (repeated-batch cultivation, first batch), and 87 mg of PRP/g of dry cell weight (DCW) (repeated-batch cultivation, second batch). Biomass concentration reached ~1.8 g of DCW/L, while polysaccharide concentration was about ~132 mg/L in the three fermentation runs. Polysaccharide synthesis is associated with cell growth in all studied conditions as established by Kono's analysis and Luedeking-Piret's model.

Index Entries: *Haemophilus influenzae*; capsular polysaccharide; polymer of ribosylribitol phosphate; batch cultivation.

Introduction

Haemophilus influenzae type b (Hib) is a small Gram-negative coccobacillus that in clinical specimens presents a filamentous or pleomorphic

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shape. Meningitis is the most common and serious form of invasive Hib disease. Invasive Hib disease occurs mainly in children younger than age 5 yr and in adults with predisposing conditions such as an immunodeficient state. The incidence rate of this invasive disease in children younger than age 5 is variable, depending on geographic and social factors *(1)*. In developing countries, 40–50% of the cases occur between 6 and 9 mo of age and the mortality rate owing to meningitis can reach 40% even after antibiotic treatment, in contrast to 5% for treated cases in developed countries *(2)*.

In Brazil, in children younger than age 1 yr, the incidence of meningitis owing to invasive Hib corresponded to 17.7 cases per 100,000 children in 1987–1991. By contrast, because of widespread vaccination in developed countries, the rate of *H. influenzae* meningitis decreased by >90% *(3,4)*.

Several surface structures of Hib seem to be important determinants of the pathogenesis. The polysaccharide capsule, a repeating polymer of ribosylribitol phosphate (PRP) found in the outermost structure of capsulated bacteria, is considered the main cause of its virulence *(5)*. The six Hib conjugate vaccines currently licensed for use are all based on Hib capsular polysaccharide conjugated to a carrier protein *(6)*.

The *Haemophilus* vaccination will be included in the World Health Organization Expanded Program on Immunization as a tetravalent vaccine (tetanus-diphtheria-pertussis-haemophilus b). For this reason, a detailed study on the kinetic of capsular polysaccharide production during cultivation must be conducted in order to improve the cultivation process so that PRP can be obtained in high yield and at a minimal cost.

A number of studies describe aspects related to Hib cultivation *(7–9)*. However, there is a lack of studies on kinetic models, an important tool to design and control a microbial process. The building of such kinetic models consists of comparing assumed models to experimental data in order to obtain more relevant information *(10)*. To contribute to the knowledge and improvement of PRP production, three types of fermentation—a single, pulse, and repeated-batch process—were established in the present study. The experimental data were used to investigate the relationship between polysaccharide production and cell growth.

Materials and Methods

Strain

A pathogenic strain of Hib, GB 3291, was obtained from the Centers for Disease Control and Prevention (Atlanta, GA), and the manufacturing master seed was prepared in the Department of Bacteriology at the Adolph Lutz Institute (São Paulo, SP, Brazil).

Preparation of Working Seed

Hib GB 3291 from one freeze-dried culture vial was suspended in 0.15 *M* NaCl, spread on four chocolate agar slant tubes, and incubated in a

candle jar at 37°C for 16–18 h. The chocolate agar medium contained 3.7% broth heart infusion, 2% agar, and 10% sterile defibrinated horse blood. The grown cells were resuspended in Greaves medium *(11)* containing 10% glycerol as a cryoprotector agent, distributed into 1-mL vials, and kept in a deep freezer at –70°C. Each vial contained approx 10^9 viable cells/mL.

Composition and Preparation Medium

The cultivation medium, MP, was made according to Carty et al. *(12)*: 10.0 g of soy peptone (Difco, Detroit, MI), 5.0 g of dialized yeast extract (Merck), 2.5 g of K_2HPO_4 (Merck), 13.1 g of Na_2HPO_4 (Sigma, St. Louis, MO), 3.3 g of NaH_2PO_4 (Sigma), 5.0 g of glucose (Merck), 10.0 mg of hemin chloride (Sigma), and 10.0 mg of nicotinamide adenine dinucleotide (NAD) (Sigma) in a final volume of 1 L of distilled water. The pH was adjusted to 7.5 with 5.0 *N* NaOH. The MP medium was sterilized by filtration in a Millipore system with a 0.22-µm membrane previously autoclaved at 120°C for 15 min and aseptically transferred to the fermentation vessel or Erlenmeyer flasks.

Preparation of Inocolum

The frozen working seed culture was thawed, transferred to four chocolate agar slant tubes, and incubated for 18 h at 37°C in a candle jar. The surface-grown cells were harvested and resuspended in 2 mL of cultivation medium. The resulting cell suspension was then transferred to 2-L Erlenmeyer flasks containing 400 mL of fresh culture medium and incubated at 37°C in a shaker incubator at 150 rpm for 5 to 6 h.

Cultivation Conditions

The experiments were conducted in a Bioflo 2000 (New Brunswick Scientific) with a 13-L nominal volume, under the following conditions: stirring speed of 100–600 rpm, initial medium volume of 7.4 L, temperature of 37°C. Overlay aeration was employed with a flow rate of 2 L/min. The experiments were carried out without pH control. The bioreactor containing fresh MP medium was inoculated with 400 mL of midexponential growth–phase culture prepared as described in Preparation of Inocolum. Identity and purity of inoculum were confirmed by agglutination with specific antiserum and by Gram staining. Three groups of experiments were performed: (1) batch cultivation four 24 h, (2) batch cultivation with glucose pulse at h 9, and (3) repeated-batch cultivation. The repeated-batch cultivation proceeded as a simple batch during the first 12 h after inoculation. At that time, approx 90% of cultivated medium was withdrawn and the fermentation vessel was supplied with fresh medium up to the initial volume.

Analytical Procedures

Cell Concentration

Dry cell weight (DCW) was determined in 10-mL samples collected in preweighed tubes. After centrifuging at 3220*g* and 4°C for 60 min, the

pellet was resuspended in 10 mL 0.15 *M* NaCl and submitted to centrifugation as in the previously described conditions. The supernatant was discarded, and the centrifuge tube containing the cells was dried at 60°C for 24 h and weighed.

Glucose Concentration

Glucose concentration was determined in cell-free samples by the glucose oxidase method *(13)*.

PRP Concentration

PRP concentration was determined using the modified Bial reaction *(14)* with D-ribose (Sigma) and purified PRP as standards. To exclude the interference of glucose, the method described by Drury *(15)* was used. The samples were pretreated to minimize interference of culture medium and cell component; an approx 100-mL sample of cultivated medium was precipitated using 0.5 *M* hexadecyltrimethylammonium bromide (Cetavlon®; Sigma) at pH 6.5. After centrifuging at 17,000*g* and 4°C for 1 h, the supernatant was discarded; the polysaccharide present in the precipitate was dissolved in 0.4 *M* NaCl and centrifuged again at 17,000*g* and 4°C for 30 min to remove cell debris. Thereafter, PRP concentration was obtained by measuring pentose concentration in the supernatant. The PRP concentration was estimated using a conversion factor in which 1 mg of ribose corresponded to 2.55 mg of PRP. The value for the conversion factor was based on the PRP structural formula reported by Crisel et al. *(16)*.

Model for Growth and Product Formation

Kono and Asai *(17)* studied product formation behavior and proposed a classification for fermentation processes based on a graphic method, in which the rate of product formation and the growth rate are plotted against biomass concentration. Through the analysis of the curves representing the growth and product formation rates, the fermentation processes are classified as: (1) processes in which product formation is growth associated, (2) processes in which product formation is not growth associated, and (3) processes in which product formation is combined.

Luedeking and Piret *(18)* proposed a model to correlate product formation and growth, in which the specific rate of product formation (r_p) is given by Eq. 1. The first term is the growth-related product (μ) formation, and the second term (m_p) is the non-growth-related product formation. The second term can also be interpreted as the formation of product owing to the maintenance process in the cell:

$$r_p = \alpha\mu + m_p \tag{1}$$

in which r_p is the specific rate of product formation, μ is the specific growth rate, α is the stoichiometric coefficient of product formation per biomass, and m_p is the maintenance-associated specific metabolic product formation.

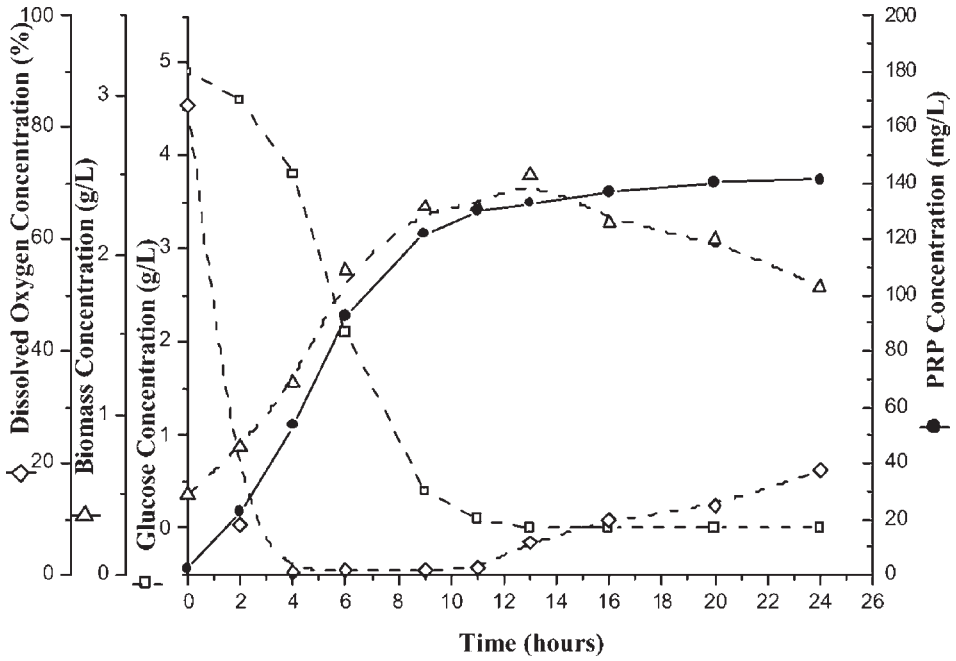

Fig. 1. Single-batch cultivation of Hib. (◇) DO concentration (%); (□) glucose concentration (g/L); (△) biomass concentration (g/L); (●) polysaccharide concentration (mg/L).

Results and Discussion

Single-Batch, Batch With Glucose Pulse, and Repeated-Batch Cultivations

Hib was studied in three kinds of fermentation: single-batch, batch with glucose pulse, and repeated-batch cultivation. Figure 1 shows some parameters measured in single-batch cultivation: cell growth, oxygen consumption, glucose consumption, and PRP production. The following characteristics can be observed: First, instead of the usual exponential growth phase, there was a linear behavior in the first 6 h. Second, fermentation reached the stationary growth phase at h 9, and after 14 h of cultivation, cell death occurred owing to a decrease in glucose concentration. Third, dissolved oxygen (DO) concentration remained below 10% saturation during the first 13 h of fermentation and increased slowly afterward as a consequence of cell death. Fourth, during cultivation, pH decreased from 7.5 to 5.9 (data not shown). Finally, the PRP production curve showed a profile parallel to the cell growth curve. At the end of fermentation, PRP concentration reached 140 mg/L.

In the next experiment, a glucose pulse (5.0 g/L) was applied at h 9 of fermentation (Fig. 2) to restore the initial glucose concentration. However, only a small amount of the additional glucose supply was consumed, prob-

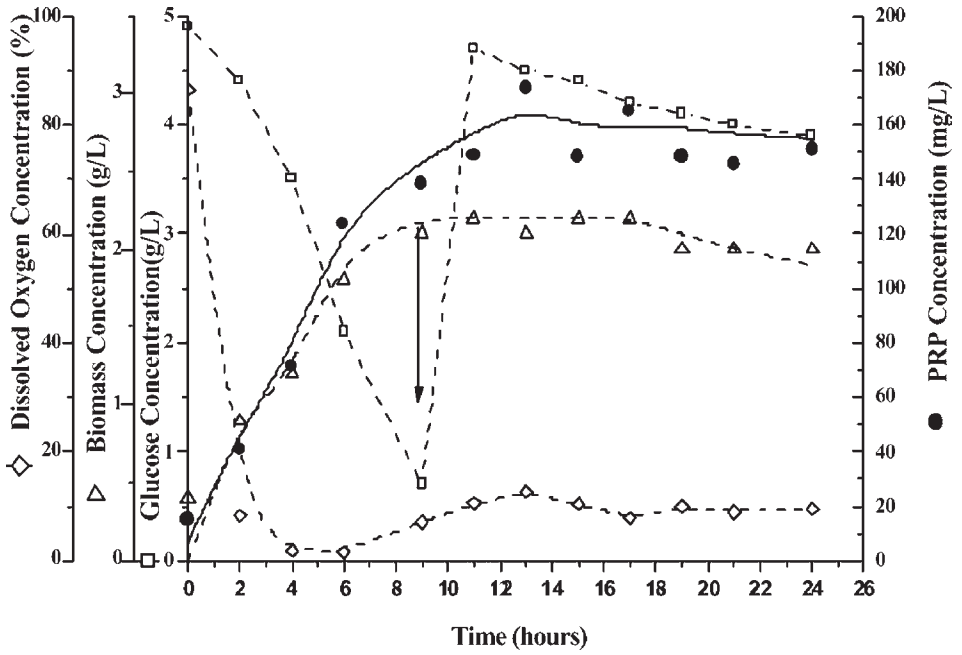

Fig. 2. Batch with glucose pulse cultivation of Hib. (◇) DO concentration (%); (□) glucose concentration (g/L); (△) biomass concentration (g/L); (●) polysaccharide concentration (mg/L). The arrow shows time of glucose addition.

ably being used for cell maintenance since neither PRP production nor biomass concentration increased. This inability of the bacteria to metabolize the additional glucose was probably owing to the limiting growth factor concentration and/or accumulation of cell waste inhibitors in the culture medium.

To improve PRP production, a repeated-batch process was investigated. The results of a typical experiment are shown in Fig. 3. All the parameters measured, such as glucose consumption, biomass formation, and PRP production, followed similar patterns in both cultivation conditions. The values of PRP and biomass concentrations as well as the specific polysaccharide production were calculated for the three independent experiments (Table 1) and indicated that PRP and cell production in both stages were similar and reproducible. Furthermore, the final PRP concentration at the end of the repeated-batch cultivation was more or less the same as that of single-batch cultivation.

Linear growth phases were observed in all experiments as a consequence of some limitation in the culture condition; this behavior was modeled by Moser *(19)*. Reuss and Wagner *(20)* reported that lack of an exponential growth phase is owing to insufficient oxygen supply in the cultivation. In the present work, oxygen supply could also be insufficient owing to overlay aeration, and, in addition, hemin and NAD, essential growth factors, are perhaps at a limited concentration.

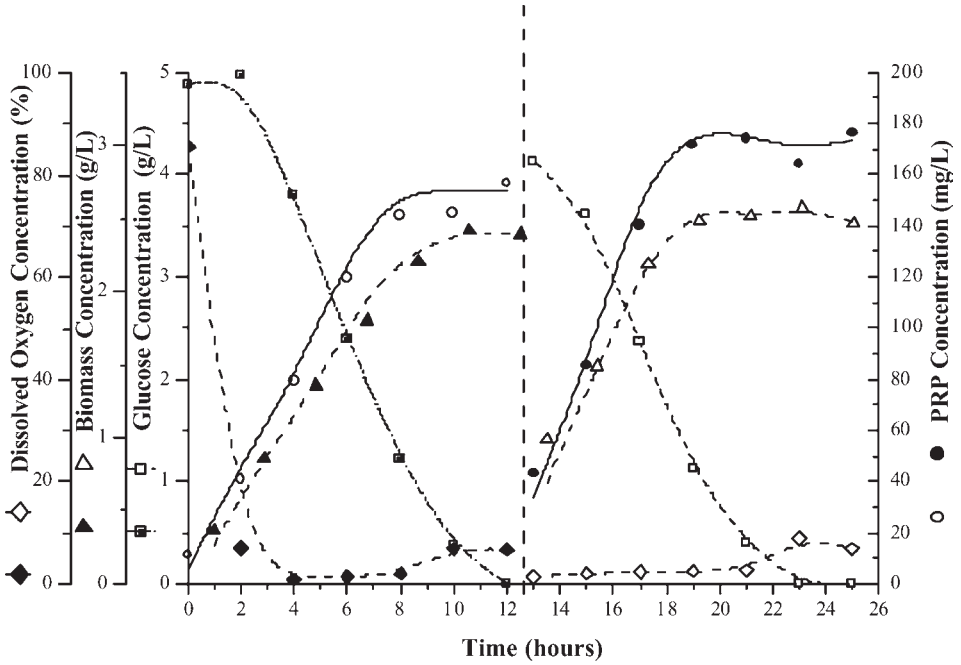

Fig. 3. Repeated-batch cultivation of Hib. (◇, ◆) DO concentration (%); (■, □) glucose concentration (g/L); (▲, △) biomass concentration (g/L); (○, ●) polysaccharide concentration (mg/L).

Association Between Growth and Polysaccharide Synthesis

To define efficient strategies for design and operation of bioprocesses, it is important to elucidate the interdependence between growth and product formation. Anderson et al. *(21)* observed that the major part of polysaccharide was released to the supernatant during the exponential growth phase. In addition, Inzana *(8)* showed that the higher the number of surviving cells the higher the polysaccharide concentration. These facts suggest that there is a relationship between growth cell and polysaccharide formation. The nature of this relationship is the aim of our study. For this purpose, the experimental data were analyzed according to the Kono-Asai *(17)* classification and Luedeking-Piret's model *(18)*.

Kono-Asai's *(17)* approach analyzes *dp/dt* and *dx/dt* to describe the association between growth and product formation. The values of the differentials *dp/dt* and *dx/dt* were obtained by derivation of the third-order polynomial fitted to the experimental data represented in Figs. 1–3. Figure 4 shows the profile for cell growth rate (*dx/dt*) and product formation rate (*dp/dt*) as a function of biomass concentration. Both curves confirm that Hib polysaccharide synthesis is totally associated with the cell growth.

The Luedeking-Piret's model *(18)* was applied using the experimental results presented in Figs. 1–3. The specific polysaccharide formation rates (r_p) and specific growth rates (μ) were correlated by a linear regression (Eq. 1),

Table 1
Values for Maximum Polysaccharide and Biomass Concentrations at 12-h Time Intervals

Experiment	PRP (mg /L)	Biomass (g /L)	$Y_{(p/x)}$ (mg PRP/g biomass)[a]	μ_{max} (h^{-1})	$Y_{x/s}$ (g biomass/g glucose)[b]	$Y_{p/s}$ (mg PRP/g glucose)[c]
Single batch	134 ± 14	2.12 ± 0.03	67 ± 7	0.22 ± 0.02	0.43 ± 0.00	28 ± 3
Batch with glucose pulse	155 ± 23	2.00 ± 0. 06	71 ± 14	0.17 ± 0.05	0.39 ± 0.00	34 ± 6
Repeated batch (I)	144 ± 22	2.10 ± 0.00	75 ± 11	0.17 ± 0.05	0.40 ± 0.03	28 ± 3
Repeated batch (II)	132 ± 5	1.55 ± 0.10	87 ± 2	0.19 ± 0.01	0.37 ± 0.01	32 ± 1

Note: All results are expressed as average and confidence interval (at 95%).
[a]$Y_{(p/x)}$, specific polysaccharide production.
[b]$Y_{(x/s)}$, overall yield for cell growth on glucose.
[c]$Y_{(p/s)}$, overall yield for polysaccharide production on glucose.

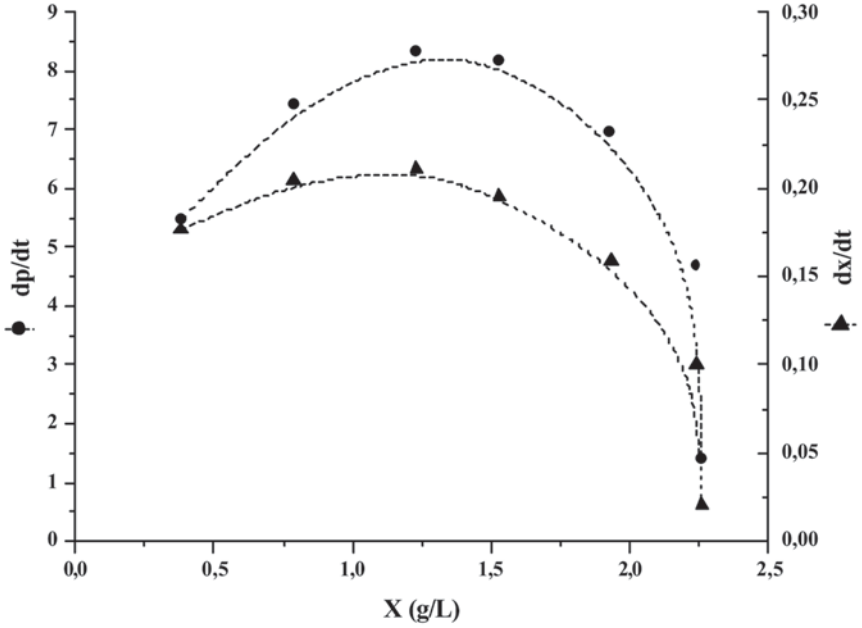

Fig. 4. Association between growth and product formation according to Kono-Asai classification: (▲) *dx/dt*; (●) *dp/dt*. X, biomass concentration (g/L); p, product concentration (mg/L).

Table 2
Values of Parameter α Estimated
for All Experiments According to Luedeking-Piret's Model

Experiment	No. of points	α	r^2
Single batch	5	60.8	0.7955
Single batch	5	66.7	0.9906
Batch with pulse	6	78.5	0.9649
Batch with pulse	6	82.4	0.8512
Repeated batch (1a)	6	62.1	0.9912
Repeated batch (1b)	5	98.9	0.8843
Repeated batch (2a)	6	98.7	0.8852
Repeated batch (2b)	4	83.2	0.9738
Global[a]	43	74.1	0.8852

[a] $F_{cal} = 1.88 < F_{tab} = 2.36$ (at 95%).

and the values for the stoichiometric coefficients α, as well as r^2, are given in Table 2. The values obtained for the correlation coefficient r^2 show that the data can be described by the linear model with a high level of confidence. Furthermore, product formation is associated with bacterial growth, since the high values estimated for the stoichiometric coefficient α are higher than the values of the maintenance coefficient m_p ($m_p = 0$).

A statistical comparative *F* test with 95% confidence was applied to verify parallelism between the linear correlations. Thus, all data could be represented by only one correlation, whose coefficients are indicated in Table 2.

Therefore, applying the classification of Kono-Asai as well as the model of Luedeking-Piret proved that polysaccharide formation is associated with cell growth. With this result any kind of modification of culture media or fermentation process that provides an increase in the biomass will be advantageous for the production of polysaccharide.

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