Neisseria meningitidis serogroup C polysaccharide production by varying the bacteria inoculum and the initial glucose concentration

Produção de polissacarídeo de *Neisseria meningitidis* sorogrupo C variando o inóculo e a concentração inicial de glicose

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Luciana Juncioni de ARAUZ[1]*, Júlia BARUQUE-RAMOS[2], Haroldo HISS[3], Rose Leila MOTA[3], Maria Esther RICCI-SILVA[3], Isaías RAW[3]

* Endereço para correspondência: [1]Instituto Adolfo Lutz, Seção de Meios de Cultura, Av. Dr. Arnaldo, 355, CEP 01246-902, São Paulo, SP. E-mail: ljarauz@ial.sp.gov.br

[2] Universidade de São Paulo (ECHA – USP), Av. Arlindo Bettio, 1000 - CEP 03828-000, São Paulo, SP.

[3] Instituto Butantan, Centro de Biotecnologia, Av. Dr. Vital Brazil, 1500 - CEP 05503-900, São Paulo, SP.

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RESUMO

A produção de polissacarídeo para a produção de vacina específica contra *N. meningitidis* sorogrupo C é o principal produto obtido dos cultivos deste microorganismo. Para uma análise comparativa da produção de polissacarídeo variando as quantidades de inóculo e as concentrações iniciais de glicose, realizaram-se quatro ensaios, empregando planejamento fatorial 2^2, em duplicata. As amostras foram retiradas a cada duas horas para a determinação da concentração celular, pH, glicose e concentração de polissacarídeo. O critério analítico foi baseado na concentração final de polissacarídeo e no fator de conversão célula/polissacarídeo ($Y_{P/X}$). Os melhores resultados de concentração final de polissacarídeo (0,105 g/L) e $Y_{P/X}$ (0,078) ocorreram na condição de cultivo com maior inóculo (densidade óptica de cerca de 0,1 a 540 nm). O polissacarídeo obtido ao final desse cultivo foi eluído em gel-cromatografia para determinação de peso molecular, sendo um antígeno adequado para a produção da vacina.

Palavras-Chave. *Neisseria meningitidis*, concentração de glicose, inóculo, meio de cultura, vacina, polissacarídeo.

ABSTRACT

Polysaccharide for producing a specific vaccine against *N. meningitidis* (serogroup C) is the principal product obtained from this microorganism cultures. In order to assess the polysaccharide production varying the bacteria inoculum bulk **,** and the initial glucose concentrations, four assays were carried out in duplicate based on factorial 2^2 planning design. Samples were collected from the cultures every two hours for determining cell concentration (expressed as dry biomass), pH, glucose and polysaccharide concentration. The comparative criteria were based on the final polysaccharide concentrations**,** and the cell/polysaccharide ($Y_{P/X}$) yield coefficient. The bacteria culture employing the highest inoculum bulk (optical density ≅ 0.1 at 540nm) presented the best result of final polysaccharide concentration (0.105 g/L)**,** and $Y_{P/X}$ (0.078). The polysaccharide further purified on gel chromatography column following the molecular mass criterion, was the suitable antigen for vaccine production.

Key Words. *Neisseria meningitidis*, glucose concentration, inoculum, culture medium, vaccine, polysaccharide.

INTRODUCTION

Meningococcal disease occurs both as an endemic and epidemic disease in most parts of the world with significant morbidity and mortality. Despite all the advances in prevention, diagnosis and treatment, the disease continues to have high mortality (5 – 10%)[1]. The five major pathogenic serogroups of the Gram-negative encapsulated bacterium *Neisseria meningitidis* are A, B, C, Y, and W135[2].

Since 1971, two great epidemics have occurred in Brazil: the first epidemic was caused by *N. meningitidis* group C and the second by group A. The last outbreak began in April, 1974 with a higher incidence than the previous one[3].

Meningococcal vaccine development began in the 1930s with killed whole-cell and exotoxin vaccines, but widespread use of polysaccharide vaccines did not begin until the 1970's[4]. Currently available polysaccharide vaccines are effective in preventing disease caused by serogroups A, C, Y, and W-135 in older children and adults but do not promote good long-term protection in young children. Vaccines that protect against serogroup B disease are still in development[5].

For the vaccine production, a great amount of *N. meningitidis* (serogroup C) polysaccharide, which constitutes the antigen for the vaccine against meningitis is needed. But little information is available in the literature about production of this polysaccharide on an industrial scale[6].

The most employed culture medium for growth of *N. meningitidis* is Frantz medium, according the study of Paz et al.[6]. These authors compared two other culture media in assays carried out in a bioreactor, which presented a worse polysaccharide yield than the Frantz medium.

Aiming at the setting of operational parameters in order to increase polysaccharide yield, the present study investigated the effects of variation of inoculum (determined by optical density) and initial glucose concentration on cell and polysaccharide production. Molecular weight and gel chromatography partition coefficient (K_d) of the polysaccharide in the best condition were higher than 100 kDa and 0.3, respectively, as required to induce an adequate immunogenic response[7].

MATERIAL AND METHODS

Cultivation of the microorganism

The inoculum was prepared according to Gotschlich *et al*.[8]. The contents of two ampoules, containing the meningococcal strain IMC 2135 (supplied by Instituto Adolfo Lutz, SP, Brazil), maintained in 0.5 mL of Greaves medium[9] at 70ºC, having a mean value of 6.0×10^6 CFU.mL^{-1}, were streaked out onto four Müller-Hinton agar slant tubes[10]. The tubes were incubated at 35ºC during 12 h in a candle jar (5-10% CO_2). The microorganisms from each slant tube were resuspended in the culture medium and transferred to 500 mL conical flasks, containing 100 mL of this medium. The flasks were incubated at 35ºC for 12 h in a rotary shaker (New Brunswick Model G25, New Jersey, NY) at 140 rpm. Possible contaminations of cultures were monitored by Gram staining technique[11]. The medium for cultivation was sterilized by filtration through a 0.2 µ€m Millipore filter. Frantz medium[12] composition (per liter) was: L-glutamic acid, 1.6 g; NaCl, 6.0 g; $Na_2HPO_4.7H_2O$, 4.67 g; NH_4Cl, 1.25 g; KCl, 0.09 g; L-cystein.HCl.H_2O, 0.02 g; dialyzed yeast extract, 2.0 g; $MgSO_4.7H_2O$, 1.23 g; glucose, 5.0 g. The pH was adjusted to 7.4 with a 5 N NaOH solution. Four cultivation runs were carried out in duplicate according to the factorial 2^2 planning design[13]. Assays were conducted for 12 hours and the molecular weight for the best result was verified.

Analytical methods

Inoculum cell concentration was determined by optical density (O.D.) at 540 nm using 1 cm optical pathway glass chambers. Cell concentration was expressed as dry biomass weight per liter (g.L^{-1}) after centrifugation of a known-volume sample at 8,700x*g* during 30 minutes, followed by pellet drying at 60ºC for 48 h. Measurements of pH were performed with a bench pHmeter (Micronal, mod. 374, São Paulo, Brazil). Glucose concentration was measured using the glucose oxidase colorimetric method[14]. Polysaccharide concentration was determined after cell disruption and precipitation by addition of Cetavlon (Merck Lab., Darmstadt, Germany) to the sample, according to Gotschlich et al.[8]. After preliminary centrifugation of the sample (8,700x*g*, 30 minutes), the supernatant was discarded, while the biomass was resuspended in 1.0 M $CaCl_2.2H_2O$ and centrifuged again under the same conditions. The supernatant, containing the suspended polysaccharide, was used for polysaccharide determination by the resorcinol-HCl colorimetric method[8,15], which is sensitive regarding polysaccharide monomers (sialic acids) formed after acid hydrolysis of the supernatant. For molecular weight determinations, a column (1.5 x 85 cm) previously filled up with Sepharose 4B-CL (Pharmacia, Uppsala, Sweden) was frequently calibrated using a saturated mixture of riboflavin and Blue Dextran (to determine the void volume) and eluted with a 1.0 M ammonium acetate solution (pH = 7.0) containing 0.1% SDS[16,17]. Dextrans with known molecular weights were eluted through the column for calibration. Dextran concentrations were determined by the phenol-sulfuric method[14] and the correspondence between the logarithm of the molecular weights and the elution volume was established. The polysaccharides from samples purified with ethanol were injected (0.2 mL) and the content of collected fractions (3 mL each) was determined by the above resorcinol-HCl method. The partition coefficient (K_d) represents a way to normalize the peak of elution measured in different gel chromatography columns[17]. It is defined as:

$$K_d = \frac{V_e - V_o}{V_t - V_o} \qquad \text{(Equation 1)}$$

where: Ve = elution volume (located at sample peak); Vt = total volume (between Blue Dextran and riboflavin peaks) and Vo = void volume (located at Blue Dextran peak).

RESULTS AND DISCUSSION

Figure 1 shows the behavior kinetics of each group of assays (defined in Table 1). According this Table, assays 1 and 2 are performed with low initial O.D. values (respectively 0.023 and 0.026) whereas assays 3 and 4, with the greatest ones (respectively 0.105 and 0.085). On the other hand, initial values of glucose concentration of 3 and 5 $g.L^{-1}$ were alternated with low and high values of initial O.D. The highest concentrations of polysaccharide were obtained in assays 3 and 4 (respectively, 0.104 and 0.105 $g.L^{-1}$ at the end of the culture), where the initial O.D. values were the highest, even considering that these assays had different initial concentrations of glucose. In this way, contrary to the inexpressive effect of initial glucose concentration at the employed range in the present study, the effect of the initial value of O.D. in the studied range is actually significant on polysaccharide production (according to Student's *t* test at 5% significance level).

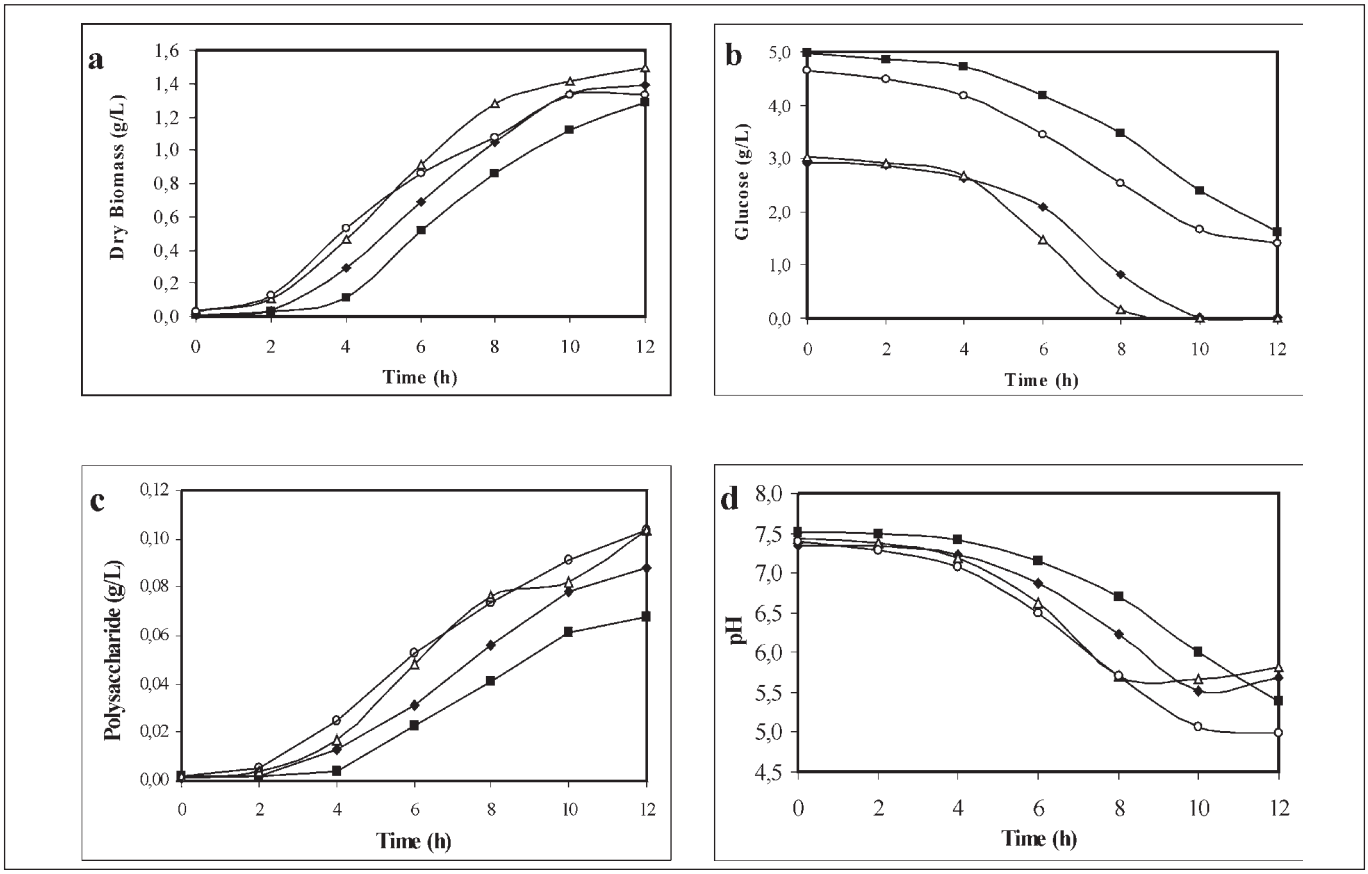

Figure 1. (a): Growth, **(b):** glucose consumption, **(c):** polysaccharide formation and **(d):** pH kinetics –**Assay 1** (♦): initial O.D.=0.023 and initial glucose concentration= 3$g.L^{-1}$; **Assay 2** (■) : initial O.D.=0.026 and initial glucose concentration=5 $g.L^{-1}$; **Assay 3** (Δ): initial O.D.=0.105 and initial glucose concentration=3 $g.L^{-1}$; **Assay 4** (o): initial O.D.=0.085 and initial glucose concentration=5 $g.L^{-1}$.

Table 1. Average values of initial O.D. (540 nm), initial glucose concentration, maximum specific growth rate (μ_{max}), final dry biomass concentration (X_f), final polysaccharide concentration (P_f) and cell/polysaccharide yield factor ($Y_{p/x}$) for assays 1 to 4.

Assay number	Initial O.D. (540 nm)	Glucose ($g.L^{-1}$)	μ_{max}	X_f($g.L^{-1}$)	P_f ($g.L^{-1}$)	$Y_{P/X}$($g.L^{-1}$)
1	0.023	3.0	0.70	1.39	0.088	0.063
2	0.026	5.0	0.59	1.28	0.068	0.053
3	0.105	3.0	0.47	1.50	0.104	0.069
4	0.085	5.0	0.54	1.33	0.105	0.078

All the assays began with pH values near 7.4 (Fig. 1d), which was decreasing along the cultivation time, due to formation of acid metabolites produced during cell growth. In assays 2 and 4, which had initial elevated glucose concentration (near 5 $g.L^{-1}$), the final pH values were near 5.0 at the end of the process, whereas in assays 1 and 3 (with initial glucose concentration near 3 $g.L^{-1}$), the pH values at the end of the process were near 6.0. All pH curves showed the same behavior until the 8th cultivation hour. However in assays 1 and 3, the glucose concentration became low from the 8th cultivation hour on and disappeared completely at the end of the process (Figure 1b). During this period (between the 8th and 12th cultivation hours), pH values increased slightly, probably due to the use of amino acids as alternative carbon source. On the other hand, in assays 2 and 4, which still had glucose for consumption, the pH values continued to fall and finished with values near 5 at the end of cultivation. However, the lack of glucose did not affect the polysaccharide production (Table 1 and Figure 1c). In addition, considering that in assays 2 and 4 the residual values of glucose were near 1.5 $g.L^{-1}$ (Figure 1b), its concentration can be adjusted in further formulations to avoid waste of this compound at the end of culture.

Figure 1a shows bacterial growth kinetics profile in the exponential phase until the 10th hour, when cultivations reach the stationary growth phase. Comparing these results with glucose consumption kinetics (Figure 1b), it seems that substrate consumption had little influence on cell growth and polysaccharide production kinetics (Figure 1c). However, even in the stationary growth phase, the production of polysaccharide continues. Thus, polysaccharide formation could or not have been associated with cell growth. This behavior had already been observed by the authors[6,18,19].

The data are comparable with the results described in the literature for bioreactor scale production. Paz et al.[6] obtained a concentration of 0.06 $g.L^{-1}$ for a 12-hour bioreactor cultivation. This value is compatible with the results obtained in the current study (Table 1 and Figure 1c). These authors also studied other

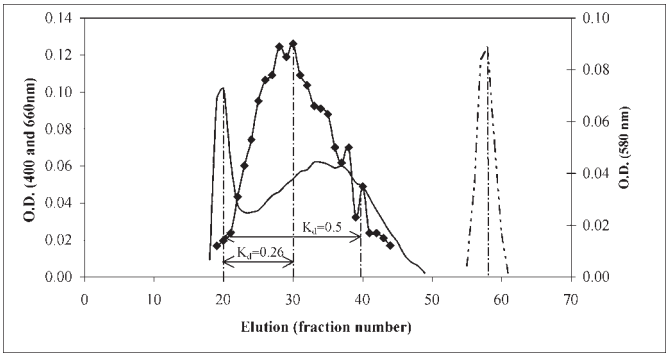

Figure 2. Gel chromatography profile: (—) Blue Dextran 2.10^6 Da (O.D. 660 nm); (—) Riboflavin 376.4 Da (O.D. 400 nm); (♦€) Polysaccharide from assay 4 (O.D. 580 nm measured after resorcinol-HCl reaction of the elution fractions). Partition coefficient K_d=0.26, recovery yield (percent amount of polysaccharide recovered before reaching K_d=0.5) close to 92% and estimated molecular weight of 480 kDa.

synthetic media and demonstrated that Frantz medium presented the best results for the obtention of polysaccharide from *Neisseria meningitidis* (serogroup C) culture.

The relationship between biomass and polysaccharide production is extremely important in large-scale production. Since, in order to lower the costs of capsular polysaccharide production (through the culture of serogroup C *N. meningitidis*) and the purification process of the obtained capsular polysaccharide, two criteria are essential: attaining the maximum polysaccharide concentration at the end of the culture process in a shaker (P_f) and simultaneously attaining the maximum cell/polysaccharide yield factor ($Y_{P/X}$), bearing in mind that, in the purification process, the rest of the cell structure is a contaminant[6].

In the present study, from a comparison of results presented in Table 1, it is evident that the greatest polysaccharide final concentration was obtained in assays 3 and 4 (namely, 0.104 and 0.105 $g.L^{-1}$ at the end of cultivation), but assay 4 shows the best process condition to present the best $Y_{P/X}$ (0.078 $g.g^{-1}$). The worst results were those obtained in assays 1 and 2 (respectively, 0.063 and 0.053 $g.L^{-1}$), where the greatest values of maximum specific growth rate (0.70 and 0.59 h^{-1}) occurred.

The polysaccharide obtained from assay 4, which presented the greatest final polysaccharide concentration and $Y_{P/X}$ was injected in the chromatography column to determinate molecular weight. The purified polysaccharide must be analyzed for purity (chemical determinations) and molecular size for potency prior to formulation of the final product. According to the World Health Organization, until 1980, molecular weight determination was carried out with Sepharose 4B resin and K_d value could not be greater than 0.4[20]. Since 1981 the determination must be accomplished with Sepharose CL-4B and the polysaccharide ought to be eluted before reaching $K_d = 0.5$, which represents at least 75% of recovery for total *N. meningitides* (serogroup C) polysaccharide[21,22].

The determination, as presented in Figure 2, performed on sample from assay 4, showed a mean molecular weight in the range of 400-500 kDa, with K_d lower than 0.3 and recovery rate (percent amount of polysaccharide recovery reaching $K_d = 0.5$) higher than 92%, being suitable as antigen for the vaccine elaboration.

CONCLUSION

The effect of initial glucose concentration is inexpressive in the employed range in the present study, whereas the effect of the inoculum in the studied range is actually significant as regards polysaccharide production. Polysaccharide was produced in all the assays, however the best results were obtained using the greatest inoculum values. The acquired data are comparable with the results described in the literature for bioreactor scale production. In addition, the analyzed polysaccharide sample followed the standard criteria for molecular weight, being suitable for vaccine elaboration.

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