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Capsular polysaccharide production by *Neisseria meningitidis* serogroup C: Optimization of process variables using response surface methodology

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Abstract

The production of capsular polysaccharide by *Neisseria meningitidis* serogroup C in Frantz medium cultivations was studied. Influence of process variables such as temperature, pH and agitation speed on the polysaccharide production were investigated by means of a three-factor face-centered central composite design. An optimal set of those process variables values were calculated using response surface methodology, and results showed that optimal values were: temperature equal to 37 °C, pH controlled at 7.0 and agitation speed maintained at 1300 rpm, which correspond to a oxygen global mass transfer coefficient (k_La) of 36 h^{-1}. The capsular polysaccharide concentration achieved under these conditions was equal to 155 ± 4 mg/L.

Keywords: *Neisseria meningitidis*; Capsular polysaccharide; Face-centered central composite design; Response surface methodology; Process variables optimization

1. Introduction

Neisseria meningitidis (or meningococcus) is an encapsulated pathogen that causes bacterial meningitis, one of the most feared infectious diseases in the world due to its rapid progression and tendency to cause outbreaks and epidemics. Meningococci are divided into groups on the basis of their chemically and serologically distinct capsular polysaccharide. Five serogroups (A, B, C, Y and W135) can cause meningococcal disease but approximately 90% of cases are associated to serogroups A–C [1]. Bivalent (A and C) and tetravalent (A, C, Y and W135) polysaccharide vaccines have been widely available since the early 1970s [2]. Nevertheless, the use of purified polysaccharide vaccines has been limited due to their poor immunogenicity in infants and young children as well as the relatively short duration of immunity induced [3]. Experience with the *Haemophilus influenzae* type B (Hib) and pneumococcal vaccines had shown that the immunogenicity of polysaccharides could be improved by chemical conjugation to protein carrier. The resulting polysaccharide–protein conjugate vaccines are safe; immunogenic in young infants, and induce long-term protection. Following the successful introduction of the Hib conjugate vaccines, considerable progress has been made in the development of similar conjugate vaccines based on meningococcal group C polysaccharide [4,5]. This way, the optimization of meningococcal polysaccharide production is an interesting goal to be achieved due to its importance either in purified polysaccharide or polysaccharide–protein conjugate vaccines manufactures.

Bacterial capsular polysaccharides are important virulence factors and there has been a growing body of evidence defining their role in the enhancement of pathogenesis. Capsules impart antiphagocytic and antibactericidal properties to the meningococcus and thus enhance meningococcal survive during invasion of the bloodstream or cerebrospinal fluid. Moreover, capsules provide protective (e.g., prevent desiccation, phagocytic killing) and antiadherent properties [6–8]. The major meningococcal capsular polysaccharides associated with invasive disease are

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composed, except for serogroup A, by sialic acid (*N*-acetyl neuraminic acid) derivatives. Serogroups B and C capsular polysaccharides are composed of homopolymers of α(2-8)-linked and α(2-9)-linked sialic acid, respectively, while Y and W135 are heteropolymers of sialic acid. Although polysialic acid is a relatively simple polymer, the biosynthetic pathway leading to the synthesis of this structure is a process that involves complex steps such as specific enzymatic reactions for sialic acid molecules synthesis, sialic acid polymerization and transport of the sialopolymer to the bacterial cell surface [9–12]. In last years, some studies have been addressed the importance of regulation by nutritional and environmental conditions (temperature, pH, oxygen level, etc.) of capsular polysialic acid biosynthesis by *Neisseria meningitidis* and other encapsulated bacteria such as *Escherichia coli* and *Pasteurella haemolytica* [13–16]. In most cases a relationship between capsule production and the synthesis or availability of enzymes and other intermediates directly involved in the formation of the sialopolymer were observed. Similar findings regarding regulation of capsule or other virulence factors production by other bacterial pathogens, including *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Staphylococcus aureus* and *Streptococcus pneumoniae*, had led to an appreciation that the amounts of capsular polysaccharide must be varied according to environment conditions [17–24].

Considering that capsule is a protective structure that interfaces bacterial cell surface and their surroundings during invasive infection of host cells, where the pathogen encounters a variety of environmental and nutritional conditions, many of which could exert effects on determining more or less pronounced expression of this important virulence factor in vivo, studies aiming to determine the influence of some variables such as temperature, pH, oxygen level, etc. on capsular polysaccharide production seem to be very important for implementing process conditions which drive to optimal polysaccharide production in vitro. Experimental designs based on statistical principles can be employed as an interesting strategy to achieve this objective. Nowadays, optimization through factorial design and response surface methodology (RSM) is a common practice in several biotechnological and industrial processes [25–30]. These are useful techniques for approaching the region of optimum response, performing a minimum number of experiments. At this work, the influence of some process variables (temperature, pH and agitation) on capsular polysaccharide production by *Neisseria meningitidis* serogroup C in bioreactors was investigated. Factorial design and RSM were used for optimal environmental conditions determination as an initial step in the optimization scheme of polysaccharide production for meningococcal vaccine manufacture.

2. Materials and methods

2.1. Microorganism

Neisseria meningitidis serogroup C2135 strain, obtained from Mérieux Institute, France, was used in this study.

2.2. Cultivation medium

The Frantz medium [31] composed of glutamic acid 1.6 g/L, NaCl 6.0 g/L, Na_2HPO_4 2.5 g/L, NH_4Cl 1.25 g/L, KCl 0.09 g/L, L-cysteine hydrochloridre 0.02 g/L, $MgSO_4.7H_2O$ 0.12 g/L, yeast extract 2.0 g/L and glucose 5.0 g/L was prepared according to the previously described procedure and had been used in flask and bioreactor submerged cultivations carried out in this study.

2.3. Inoculation procedure and cultivation conditions

The bacterial strain was made to grow in Agar Müller Hinton plates at 37 °C during 16 h in a candle jar (5–10% CO_2) and harvested by washing with 2 mL sterile Frantz medium. The bacterial suspension was used to inoculate 50 mL of Frantz medium dispensed in 250 mL Erlenmeyer flasks, which were incubated at 37 °C under 200 rpm in a shaker (New Brunswick Scientific Co., Edison, NJ, USA) for 4 h. The culture flasks content were then inoculated in 450 mL Frantz medium dispensed in 2 L Erlenmeyer flasks maintained at the same conditions previously described. After this propagation procedure, growth suspension was used as 10% (v/v) inoculum for experiments conducted in bioreactors.

2.4. Bioreactors experimental conditions

Experimental design runs were conducted in a set of four 1.0 L glass vases bioreactors (BIOSTAT Q, B. Braun Biotech International Diessel GmbH, Germany) with 0.5 L working volume. Bioreactors were equipped with in situ sterilizable pH and polarographic dissolved oxygen electrodes, pt-100-temperature sensors and magnetic agitation speed control. The fermentation conditions were controlled according to the experimental design schedule. Airflow rate was maintained at 0.8 L/min with an upper aeration system. Temperature ranged from 33 to 39 °C, pH was automatically controlled by NaOH 5N addition and ranged from 6 to 8, agitation speed ranged from 900 to 1500 rpm. These agitation speed limits resulted in an equivalent volumetric oxygen transfer coefficient (k_La) range of 22–42 h^{-1}.

2.5. Analytical assays

Samples were collected from bioreactor culture broth and analytical assays for biomass, glucose and polysaccharide concentration were performed.

Optical density of the culture was measured at 600 nm using a Beckman DU-530 spectrophotometer. Dry cell weight (DCW) was determined by centrifuging 10 mL of culture broth at 10,000 × *g* and 4 °C for 45 min. The supernatant was removed and the pellet was re-suspended in 10 mL of distillated water. The solution was centrifuged as above and the supernatant discarded. The pellet was dried until constant weight (80 °C for 72 h) in pre-weighed aluminum pans and then the mass of the cell pellet determined. DCW was demonstrated to be a linear function of optical density for $0.0 < OD_{600} < 0.8$.

Polysaccharide concentration was obtained through the quantification of sialic acid amount by HCl–resorcinol colorimetric method [32]. The monomer concentration was determined by centrifuging 10 mL of culture broth at 10,000 × *g* and 4 °C for 20 min to remove cells. The supernatant was filtered with 0.22 μm membranes (GSWP 04700-Milipore Co., USA) and dialysed against distilled water with 12–14 kDa dialysis tubing (VWR Scientific Co., USA) at 4 °C for 24 h for removing interfering substances.

Glucose concentration was determined using the GOD/POD enzymatic method (Bayer Co., USA).

2.6. Experimental designs

Aiming to evaluate the influence of temperature (*T*), agitation speed (*W*) and pH on bacterial growth (*X*) and capsular polysaccharide production (PS), statistical optimization experiments were initially carried out according to a three-factor face-centered central composite design (FCCCD). For a 3^3 FCCCD including three repetitions of central point, a set of 15 runs was carried out. The range and levels of these variables are given in Table 1. The central point conditions were selected aiming to be closed to the previous process conditions ($T = 37$ °C, initial pH 7.0 and $k_La = 36\ h^{-1}$ or $w = 1300$ rpm for 1.0 L glass vase bioreactors with 0.5 L working volume). STATISTICA 5.0 software from

Table 1
Coded factor levels and real values for FCCCD

	T (°C)	Ph	W (rpm)	k_La (h^{-1})
Level +1	39	8	1500	42
Central point (0)	36	7	1200	32
Level −1	33	6	900	22

StatSoft Inc. was used for regression and graphical analysis. The relationship between PS and the three studied factors (T, W, pH) was evaluated through a second-order polynomial model obtained by multiple regression analysis of experimental data. Optimal values for T, pH and W were estimated.

3. Results and discussion

The statistical design approach using response surface methodology was used to study the influence of some physical factors on polysaccharide production by *Neisseria meningitidis* serogroup C. Temperature, pH and agitation speed of the cultivation broth was chosen as the major variables to be optimized. Initially, the interactive effects of the studied variables were examined through RSM following the FCCCD. The experimental design and the results are shown in Table 2. The highest polysaccharide concentration (113 ± 7 mg/L) was observed at run number 15 where the variables were maintained at their intermediary values (central point). Moreover, data obtained at these conditions had also shown the highest values of specific capsular polysaccharide production (Y_{PX}), which is the ratio of polysaccharide and biomass concentrations, a parameter that express the average quantity of capsule produced by unit of microorganism. The profiles of biomass growth, glucose uptake, polysaccharide production and oxygen level of the central point run are displayed in Fig. 1.

Aiming to evaluate the effects of studied factors – temperature, pH and agitation speed (or k_La) – on the capsular polysaccharide formation, data were analyzed by non-linear multiple regression using the STATISTICA software (Statsoft, v.5.0). Table 3 depicts the regression analysis results, presenting the estimates and hypothesis tests to the coefficients of regression. As it can be seen, only the coefficients non-associated to agitation speed (w) can be considered statistically significant at 95% confidence level. The quadratic model obtained for capsular polysaccharide quantity as a function of the more significant variables is expressed by Eq. (1):

$$PS_f\,[mg/L] = -7183.6 + 301.56T + 503.91pH - 3.67T^2 - 25.15pH^2 - 4.38TpH \quad (1)$$

Table 4 shows the analysis of variance (ANOVA) for capsular polysaccharide production. The p-level value, which represents the probability of error that is involved in accepting the model prediction result as valid, was equal to 0.0007. The coefficient of determination (R^2) was 0.8274, explaining approximately 83% of the variability in the response, which ensured an acceptable adjustment of the quadratic model to the experimental data. Moreover, according to the F-test with 95%

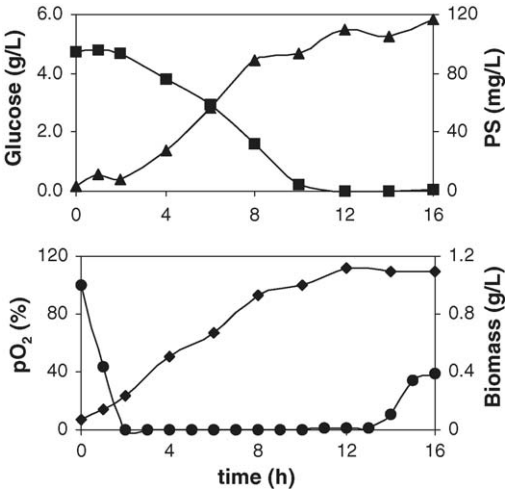

Fig. 1. Time profiles of biomass growth (◆), glucose uptake (■), capsular polysaccharide formation (▲) and oxygen level (●) for run number 15 (T = 36 °C; pH 7.0; W = 1200 rpm).

Table 2
Face-centered central composite design and experimental data for three-level three-factor response surface analysis

Run	Experimental values			[Biomass] (g/L)	[PS] (mg/L)	Y_{PX} (g/g)
	T (°C)	pH	W (rpm)			
1	−1	−1	−1	0.381	8.3	0.0218
2	−1	+1	+1	1.111	51.2	0.0461
3	+1	−1	+1	1.279	106.7	0.0834
4	+1	+1	−1	0.684	55.7	0.0814
5	+1	+1	+1	0.794	32.1	0.0405
6	+1	−1	−1	0.894	71.3	0.0797
7	−1	−1	+1	1.322	50.2	0.0380
8	−1	+1	−1	0.610	22.4	0.0367
9	0	0	−1	1.402	107.3	0.0766
10	0	0	+1	1.190	93.6	0.0787
11	−1	0	0	1.419	39.8	0.0281
12	+1	0	0	1.338	107.2	0.0801
13	0	−1	0	1.711	74.0	0.0432
14	0	+1	0	1.163	88.8	0.0763
15(a)	0	0	0	1.120	116.2	0.1038
15(b)	0	0	0	1.119	110.2	0.0985
15(c)	0	0	0	1.406	113.1	0.0804

Table 3
Statistical analysis of non-linear multiple regression

Factor	Coefficient	Standard error	t Stat	p-Value	Confidence level (%)
Intercept	7591.18				
T	299.06	80.11	3.73	0.007	99.2
pH	518.49	155.34	3.34	0.012	98.7
W	0.65	0.37	1.74	0.125	87.4
T^2	−3.49	1.09	−3.20	0.015	98.5
pH^2	−23.62	9.83	−2.40	0.047	95.2
W^2	-5×10^{-5}	1×10^{-4}	−0.46	0.658	34.4
TpH	−4.39	1.89	−2.31	0.054	94.6
TW	-8×10^{-3}	6×10^{-3}	−1.29	0.237	76.3
pHW	−0.03	0.02	−1.58	0.157	84.3

Table 4
Analysis of variance

Source of variation	Sum of squares	Mean square	Degrees of freedom	*p*-Level	*F*-value
Regression	16399.7	3279.95	5	0.00066	10.55
Residual	3420.6	310.96	11		
Total	19820.3				
Correlation coefficient (R^2)	0.8274				
$F_{\text{listed value}}$					$F_{0.95;5,11} = 3.20$

of confidence, the model can be considered statistically significant since the calculated F-value is about four times greater than the listed value [25].

The response surface plotted using Eq. (1) to estimate capsular polysaccharide concentration over independent variables temperature and pH is shown in Fig. 2. As it can be seen, the lowest values of final capsular polysaccharide concentration were attained at low values of temperature and pH (T = 33 °C and pH 6.0). An optimal response region for capsular polysaccharide concentration can be visualized through a broad plateau surrounding temperature 36–38 °C and pH 7.0–7.5.

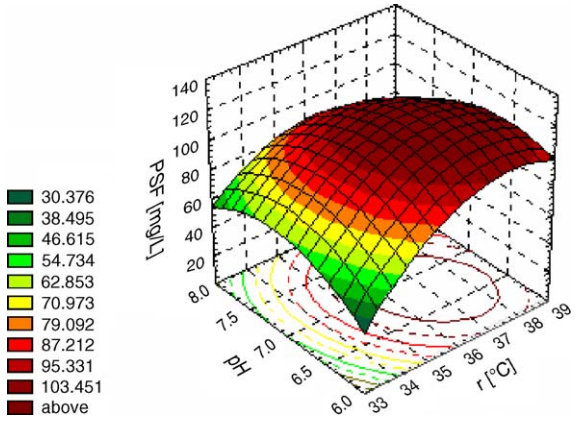

Fig. 2. Response surface plot of capsular polysaccharide production.

The effect of temperature can be better examined through the analysis of runs 15, 11 and 12 results — Figs. 1, 3 and 4, respectively. Data show that although biomass growth and glucose uptake were practically not affected by temperature at this range, the quantity of produced capsular polysaccharide varied significantly. The specific capsular polysaccharide production obtained at 36 °C is 3–4 folds higher than that obtained at 33 °C, but is not significantly different at 39 °C. This behavior – unfavorable production of capsule under low temperature values – can be confirmed by the results of the other runs conducted at 33 °C, which presented the lowest values of Y_{PX} (Table 2). Moreover, these results also indicated that conditions that favor bacterial growth are not always associated to high polysaccharide productivity.

The behavior observed for the effect of temperature on the polysaccharide production might be in part associated to the regulated activity of some enzymes involved in the capsular polysaccharide biosynthesis. There are much evidence that temperature regulates the activity of some enzymes that take part in polysialic acid capsule biosynthesis, such as NANA-synthetase, CMP-NANA-sythetase and sialyltransferase. According to latter studies, low activity of those enzymes was observed either at low or high temperature values and an optimal value had been achieved at 37 °C [15,16]. Otherwise, the improvement of capsular polysaccharide production attained when temperature was increased from 33 to 36–39 °C might be also explained by the protective capacity of the

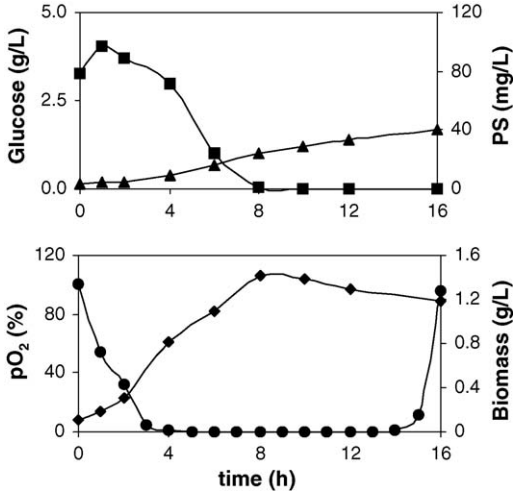

Fig. 3. Time profiles of biomass growth (◆), glucose uptake (■), capsular polysaccharide formation (▲) and oxygen level (●) for run number 11 (T = 33 °C; pH 7.0; W = 1200 rpm).

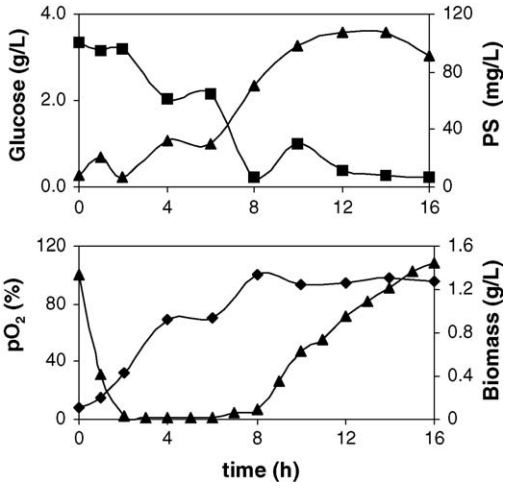

Fig. 4. Time profiles of biomass growth (◆), glucose uptake (■), capsular polysaccharide formation (▲) and oxygen level (●) for run number 12 (T = 39 °C; pH 7.0; W = 1200 rpm).

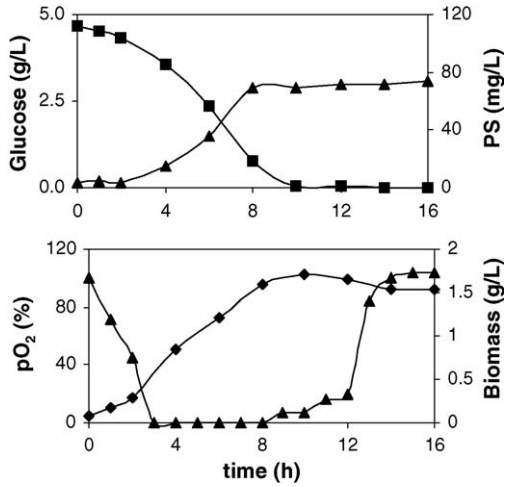

Fig. 5. Time profiles of biomass growth (◆), glucose uptake (■), capsular polysaccharide formation (▲) and oxygen level (●) for run number 13 (T = 36 °C; pH 6.0; W = 1200 rpm).

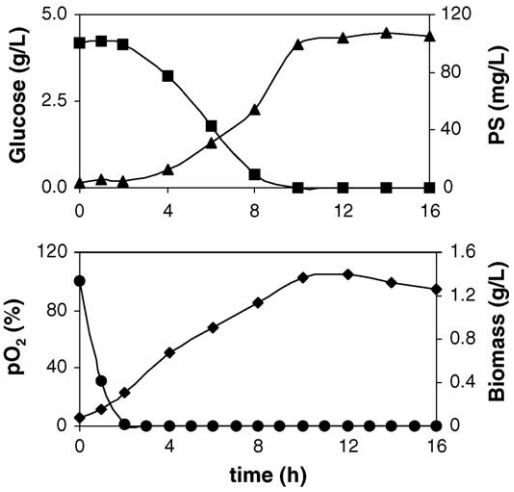

Fig. 7. Time profiles of biomass growth (◆), glucose uptake (■), capsular polysaccharide formation (▲) and oxygen level (●) for run number 9 (T = 36 °C; pH 7.0; W = 900 rpm).

bacterial capsule against cell desiccation effects caused by high temperature conditions, which trigger off the expression of some enzymes involved in the capsule formation [33].

The response surface (Fig. 2) shows that both low and high pH values are not favorable conditions for capsular polysaccharide production. The influence of pH on the surrounding of the central point run can be observed by the results of runs 15, 13 and 14 — Figs. 1, 5 and 6, respectively. Experimental run conducted at pH 7.0 presented the highest values of polysaccharide concentration and specific capsular polysaccharide production. In regard to the influence of pH on the microbial growth, it could be seen that the conditions that favored microbial growth were not necessarily the same conditions for optimal polysaccharide production. The highest value of final biomass quantity (X_f = 1.7 g/L) of the experimental design was achieved at run number 13, which was conducted at pH 6.0. However, the final polysaccharide concentration (PS_f = 74 mg/L) of this run was approximately 35% lower than that obtained at run number 15 ($\overline{X}_f = 1.21 \pm 0.41$ g/L and $\overline{PS}_f = 113 \pm 7$ mg/L). The results indicated that cells cultivated at pH 7.0 although in smaller quantity when compared to those cultivated at pH 6.0 presented on the average two-fold higher capsule quantity associated to them. The optimal value for polysaccharide production near pH 7.0 might be partially associated to competitive mechanisms of some enzymes during enzymatic capsule formation. The enzyme CMP-NANA hydrolase, which is a meningococcal enzyme that inhibits the sialyltransferase activity due to its high affinity for CMP-NANA, a common substrate for both enzymes reactions, was found to regulate the sialopolymer formation [34]. According to latter studies, the enzyme CMP-NANA hydrolase had shown an optimal activity near pH 8.0 and reached its minimal activity at pH 7.0. Since CMP-NANA hydrolase is a competitive enzyme that inhibits capsule

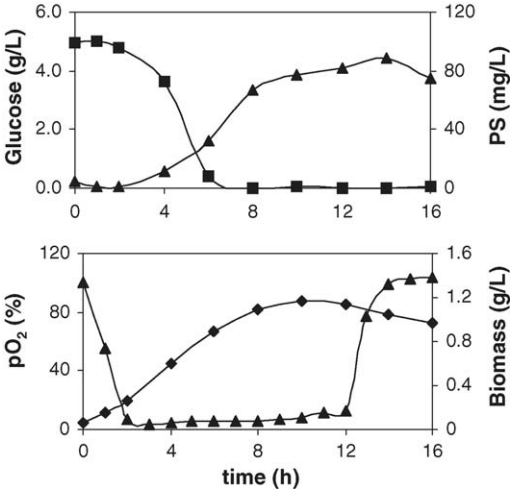

Fig. 6. Time profiles of biomass growth (◆), glucose uptake (■), capsular polysaccharide formation (▲) and oxygen level (●) for run number 14 (T = 36 °C; pH 8.0; W = 1200 rpm).

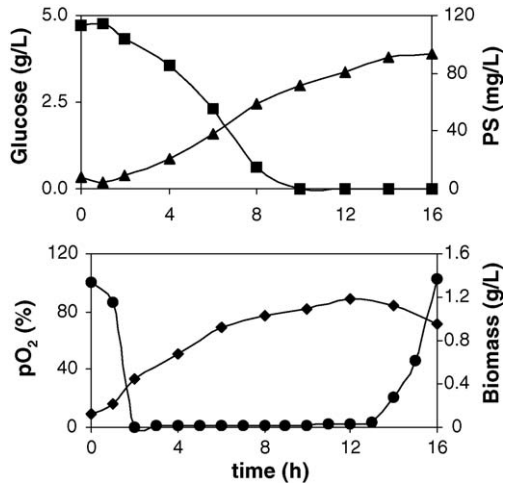

Fig. 8. Time profiles of biomass growth (◆), glucose uptake (■), capsular polysaccharide formation (▲) and oxygen level (●) for run number 10 (T = 36 °C; pH 7.0; W = 1500 rpm).

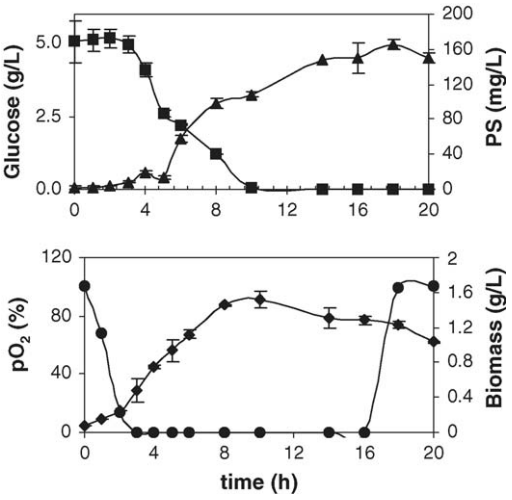

Fig. 9. Time profiles of biomass growth (◆), glucose uptake (■), capsular polysaccharide formation (▲) and oxygen level (●) for optimal run (T = 37 °C; pH 7.0; W = 1300 rpm).

biosynthesis, the formation of capsular polysaccharide is then favored under CMP-NANA hydrolase lower activity conditions. Furthermore, the decrease of capsular polysaccharide yield at pH 6.0 might be related to the maximum microbial growth attained at this condition. There has been evidence for the involvement of a common lipid intermediate – undecaprenyl phosphate (UP) – in the synthesis of sialic acid capsular polysaccharide and peptidoglycan [35]. This hypothesis supports the idea that under optimal growth conditions, the use of UP for the synthesis of essential peptidoglycan is favored and consequently this lipid intermediate would be less available for the biosynthesis of the relatively nonessential capsular polymer.

The effect of agitation speed or volumetric oxygen transfer coefficient (k_La) on capsular polysaccharide formation and microbial growth in the range of studied conditions were not statistically significant. Figs. 1, 7 and 8 display the results of runs 15, 9 and 10, respectively. The specific capsular polysaccharide production seem to be somewhat better at run number 15, but it is not possible to securely affirm that it is different from those attained at runs 9 and 10.

Based on the quadratic model (Eq. (1)), the optimal working conditions to attain high capsular polysaccharide productivity were defined. This optimal point corresponded to T = 37 °C and pH 7.0. Aiming to evaluate the significance of this prediction, a new set of experimental runs was conducted under the calculated optimal point. Since no significant effect of agitation speed was observed, this variable was set at 1300 rpm. This agitation speed is associated to a volumetric oxygen transfer coefficient (k_La) equal to 36 h^{-1}, the value that is usually used in capsular polysaccharide production. The capsular polysaccharide concentration achieved under these conditions was equal to 155 ± 4 mg/L. The profiles of biomass growth, glucose uptake, polysaccharide production and oxygen level obtained under the determined optimal conditions are shown in Fig. 9.

In conclusion, statistical optimization methods were used efficiently for the study of the influence of some important process variables on the production of capsular polysaccharide by *Neisseria meningitidis* serogroup C. The behavior observed for temperature and pH were in accordance with some hypothesis latter proposed for sialic acid capsular polysaccharide production. Once fermentation process variables are optimized, further optimization strategies for improving capsular polysaccharide production by *Neisseria meningitidis* serogroup C such as culture nutrients analysis, process control schemes, bioreactor operation mode modification, etc. can be addressed in future works.

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