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Polysaccharide Production in Pilot Scale Bioreactor Cultivations of *Neisseria meningitidis* Serogroup C

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Abstract: Serogroup C polysaccharide from *Neisseria meningitidis* (PS) constitutes the antigen for the respective vaccine production. In order to investigate the enhancement of the final PS concentration (P_f), as well as the overall yield factor (PS/biomass) ($Y_{P/X}$), 13 total cultivations distributed in 6 series (from A to F) were carried out in Frantz medium (40 L plus inoculum) in a 80L bioreactor at 35°C, 0.4 atm, 120 rpm, airflow rate of 5 L/min and $K_La = 4.2\ h^{-1}$. The series (A-F) correspond to different experimental conditions as follows: A) without pH and dissolved O_2 controls; B) pH control at 6.5; C) pH control at 6.5 and glucose pulse at the 10th hour; D) dissolved O_2 control at 10% saturation value; E) pH control at 7.4; F) dissolved O_2 limitation (set rotation at 55 rpm). Concentrations of dry biomass, PS, cellular nitrogen, residual glucose, organic and inorganic nitrogen in the medium were measured. The best results were represented by series A (averages of P_f = 0.15 g/L and $Y_{P/X}$ = 107 mg/g). The presented findings could be useful for a proper Frantz medium reformulation in order to obtain a greater amount of PS and improve the vaccine development in industrial scale-up production.

Keywords: *Neisseria meningitidis*, vaccine, polysaccharide, batch, fed-batch, kinetics.

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INTRODUCTION

Meningococcal disease is a major cause of death and morbidity worldwide. *Neisseria meningitidis* is responsible for one third of all bacterial meningitis cases on the planet, and, if this disease is not treated, mortality can reach 100%. Bacterial infections caused by meningococcus (*N. meningitidis*) remain a serious health problem, infecting 500,000 to 1.2 million people and killing between 50,000 and 135,000 per year worldwide. These infections may present a spectrum of illnesses, with the most common being meningitis and septicemia, but also including pneumonia, septic arthritis, pericarditis, conjunctivitis and urethritis [1, 2]. A major cause of childhood morbidity and mortality in developing countries, *N. meningitidis* is responsible for epidemics in Africa and Asia, most notably in the sub-Saharan Africa or the so-called "meningitis belt" [3]. *N. meningitidis* has been classified into 13 serogroups according to the chemical composition of the capsular polysaccharide (PS), of which serogroups A, B, C, W-135, X and Y are responsible for most meningococcal disease cases [4, 5]. During the past decades, large-scale epidemics were primarily caused by infections by serogroups A and C. With mass immunization campaigns, the incidence of serogroups A and C has been significantly reduced in the past few years [3, 6, 7].

Gotschlich *et al.* [8] developed an effective method, which has been adopted for serogroup A and C PS purification [9, 10]. The compound obtained was stable, suitable for purification and the final product presented a good immunological response.

The first successful capsular PS vaccines against A and C serogroups were developed in response to outbreaks of meningitidis [11, 12]. Thereby, due to the severity of the disease, bivalent (A and C) and tetravalent (A, C, Y and W135) PS vaccines have been widely available since the early 1970s [13]. All polysaccharide capsules of *N. meningitidis* are composed of sialic acid derivates, except serogroup A, and they provide the bacterium with an antiphagocytic coat that enhances survival in the blood stream and central nervous system during invasion [14]. So, polysaccharide constitutes the antigen for the respective vaccine production. Conventional vaccines based on meningococcal polysaccharide elicit an immune response against the capsular polysaccharide [15]. Nevertheless, the use of purified PS vaccines has been limited due to the poor immunogenicity in newborn and young children as well as the relative short duration of induced immunity [16]. These capsules are considered T-cell-independent antigens, so their use in vaccines has been optimal only with protein-carrier chemical conjugation to form PS-conjugate vaccines, which are polysaccharide-protein

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hybrids formed by covalent attachment of a protein to a bacterial PS [15].

In order to solve this problem, a new generation of conjugated capsular PS covalently linked to carrier proteins, such as tetanus toxoid, diphtheria toxoid or CRM197 (a mutant peptide related to diphtheria toxoid), has been used to improve vaccines against meningococcal disease [17]. Since 1999, meningococcal conjugate vaccines against serogroup C have been available and widely employed [4].

For the respective vaccine production, the industrial production of PS from *N. meningitidis* C is necessary. Additionally, a great amount of *N. meningitidis* (Serogroup C) polysaccharide is needed. The cost for cultivation and the production of polysaccharide is generally high and involves a series of production and purification steps [18]. However, little information is available in the literature about the production of this PS on pilot or industrial scale [19]. For polysaccharide production, searches in literature show studies in small laboratorial batch scale [20, 21]. The knowledge about bacterial metabolism is essential for the development of an efficient process [22]. For this purpose, the fed-batch operation could be better than the conventional batch operation, especially when concentration variations of one or more nutrients affect the yield or productivity of the desired metabolite [23, 24]. Therefore, to prevent the depletion of glucose in the medium during cultivations, a pulse of glucose could recover the cellular growth and, thus, an increase is expected in the PS production.

Therefore, this study investigated the enhancement of *N. meningitidis* C final PS concentration (P_f) as well as the overall yield factor (PS/biomass) ($Y_{P/X}$) in Frantz medium aiming to establish the operational parameters (varying pH, dissolved oxygen controls and glucose feeding) for the industrial production of this vaccinal antigen in standard cylindrical pilot bioreactor.

MATERIAL AND METHODS

Process Conditions

The inoculum of *N. meningitidis* IMC 2135 (supplied by Adolfo Lutz Institute, SP, Brazil – IAL WDCM 282) was prepared according to Gotschlich *et al.* [8]. The content of two ampoules containing $6.0x10^6$ viable cells of the meningococcal strain, maintained at -70°C in 1.0 mL of Greaves medium [25], was streaked out onto four Müller-Hinton agar [26] slant tubes (supplemented with 2% normal equine serum) and incubated at 35°C for 18-20 h in a candle jar (5-10% CO_2). The cells from each slant tube were resuspended in liquid Frantz medium [27] containing, per liter: 1.60 g L-glutamic acid, 6.00 g NaCl, 4.67 g $Na_2HPO_4.7H_2O$, 1.25 g NH_4Cl, 0.09 g KCl, 0.02 g L-cysteine.HCl, 2.00 g dialyzed yeast extract, 1.23 g $MgSO_4.7H_2O$, 5.00 g glucose, and whose pH was adjusted to 7.4 with 5 N NaOH solution. They were then transferred to four 250 mL conical flasks, each one containing 50 mL Frantz medium, and incubated at 35ºC for 5-6 h on a rotary shaker at 120 rpm. In order to verify possible contaminations, microscopic examinations were carried out using the Gram technique [28]. This culture was then transferred to four 2 L conical flasks, containing 400 mL of the same medium, and cultivated under the conditions previously described. The contents of four conical flasks, corresponding to an initial optical density around 0.1 at 540 nm, were employed to inoculate the 80 L (total capacity) bioreactor (New Brunswick, model MPP 80, New Jersey-NY, USA) containing 40 L Frantz medium. The medium for the inoculum and bioreactor was sterilized by filtration through a 0.22 μm pore size filter (GSWP, Merck Millipore, Darmstadt, Germany).

The cultivation conditions were as follows: temperature = 35.0ºC; airflow rate = 5 L/min (0.125 vvm); upper aeration; oxygen volumetric transfer coefficient K_La = 4.2 h^{-1} before the inoculation (t=0 h); rotational speed = 120 rpm in the absence of dissolved O_2 control, and in the range of 55-400 rpm when dissolved O_2 was controlled at 10% of saturation point (the 100% point was calibrated 1 hour before the inoculation at 400 rpm and submerged aeration). The vessel head pressure was maintained at 0.4 atm. During the experiments with pH control, this parameter was kept at 6.5 or 7.4 by the addition of 3 N NaOH solution. The height and the diameter of the vessel were 72 and 40 cm, respectively. Two Rushton six blade disc turbines with 16.5 cm diameter were used for mixing, one located at 10.0 cm from the vessel bottom and the other at 35 cm. Four baffles were installed, in order to enhance the mixture efficiency. No antifoam was employed. The pH was measured by an on-line sterilizable electrode, Ingold model 465-90 (Mettler-Toledo, Alphaville, Brazil) and conferred employing a bench pHmeter model 374 (Micronal, Sao Paulo, Brazil). The dissolved O_2 concentration was monitored by an on-line polarographic electrode, Ingold model 531 (Mettler-Toledo, Alphaville, Brazil). The feed volumetric flow for the glucose solution was estimated in order to establish the balance between the total glucose solution volume added and the summation of the sample volumes. In this way, the effects of dilution are minimized despite the fact that these dilution values were taken into account for the calculation of kinetic parameters.

Analytical Methods

Cell concentration was expressed as dry biomass weight per liter (g/L) after centrifugation of a known-volume sample at 8,700 *g* for 30 min, followed by pellet drying at 60°C for 48 h. The glucose concentration was measured using the glucose oxidase colorimetric method [29]. PS concentration was determined according to Gotschlich *et al.* [8], after cell disruption and debris precipitation by the addition of Cetavlon (Merck Lab., Darmstadt, Germany). After the preliminary centrifugation of the sample (8,700 *g*, 30 min), the supernatant was removed and the precipitate was resuspended in 1.0 M $CaCl_2.2H_2O$ solution and centrifuged again under the same conditions. The supernatant was employed for PS determination by the resorcinol-HCl colorimetric method [8, 30]. The concentrations of supernatant and cellular nitrogen were estimated employing semi-micro Kjeldahl method [31]. The yield coefficients were calculated as the ratios between cell biomass generated and glucose consumption ($Y_{X/G}$), cell biomass generated and organic nitrogen consumption ($Y_{X/Norg}$) and between PS production and cell biomass generated ($Y_{P/X}$) after the determined cultivation time.

RESULTS

Subdivisions in 6 series (from A to F) aimed at facilitating the comparison between the results obtained in different process conditions and also to show that the results of repeated experiments are similar and reproducible within the same series. The main results of pilot scale cultivations are summarized in (Fig. **1** and Tables **1** and **2**). These results are related to 13 total cultivations distributed in 6 series (from A to F). Typical curves presenting the growth profiles are shown in (Fig. **1a**). The behaviors of glucose consumption, PS production, organic nitrogen consumption, pH and dissolved oxygen are illustrated in Fig. **1b** to **1f**, respectively.

According to the results presented in (Fig. **1a**), the absence of a "lag" phase in all cultivation growth profiles suggests that the inoculum cells were well adapted to the medium. In addition, as shown in (Fig. **1d**), for all cultivations there was no organic nitrogen limitation.

A continuous oxygen supply is needed to cultivations. In this way, the gas-liquid mass transfer coefficient K_La was determined at beginning of cultivations. This coefficient is also often employed as a scale-up constant factor [32].

Cell Growth and PS Production

In order to reduce the cost of PS production and further purification, it is necessary to achieve maximum PS concentration at the end of the cultivation (P_f) and simultaneously attain the maximum yield factor of PS on biomass ($Y_{P/X}$), since the rest of the cell structure is a contaminant in purification process [33, 34].

When comparing the correspondent biomass and PS curve profiles (Fig. **1a** and **1c**), PS formation could have been associated or non-associated with cellular growth in cultivations without pH and dissolved O_2 controls (series A) and with pH control at 6.5 and glucose pulse at the 10^{th} process hour (series C) (Fig. **1a** and **1c**). This behavior [19, 31, 33, 34] is well denoted by the inflexion in the correspondent PS curves and the increase of PS formation even after the beginning of the stationary growth phase (Fig. **1c**).

The cultivations with pH control at 6.5 or 7.4 (respectively series B and E) and dissolved O_2 limitation (series D) presented a PS formation entirely associated with cellular growth (Fig. **1a** and **1c**). This behavior is due to the exhaustion of glucose between the 10^{th} and the 12^{th} cultivation hour (Fig. **1b**). However, experiments under the same process conditions with fed-batch glucose pulse at the 10^{th} cultivation hour (series C) indicated the resumption of PS production by the bacterium cultivation (Fig. **1a**, **1b** and **1c**). In this way, glucose consumption is related directly to the PS production kinetics. The experiment performed under severe oxygen limitation (with rotation set at 55 rpm) (series F) also presented a PS formation entirely associated with cellular growth.

The cultivations with dissolved oxygen control at 10% (series D) presented PS formation associated to the cellular growth (Fig. **1a** and **1c**). This probably occurred due to the impossibility of achieving the stationary phase in the presence of continuous oxygen availability and no substrate limitation (Fig. **1b**, **1d** and **1f**). The high oxygen content can develop killing effect in cells during the early phase of bacterial growth whereas low oxygen content may not satisfy the growth needs of the bacteria in the logarithmic growth phase [3].

The highest possible values of P_f and $Y_{P/X}$ are desirable in order to make the subsequent product purification easier and more effective. The results of final biomass concentration (X_{max}), final PS concentration (P_f) and yield of PS on biomass ($Y_{P/X}$), represented by averages and standard deviations (Table **2**), from respective series (from A to D), were compared among each other and the conclusions were confirmed employing a Student's "t" statistical test at 5% significance level. The respective individual values of series E and F were compared with the confidence interval determined for series A to D. The results for series C, with control of pH at 6.5 and glucose fed-batch pulse at the 10^{th} cultivation hour, presented the highest values for P_f and $Y_{P/X}$ (respectively, averages of 0.20 g/L and 150 mg/g) followed by series A, without pH or dissolved oxygen controls (respectively, averages of 0.15 g/L and 107 mg/g).

The results of series D, with dissolved O_2 control at 10%, despite presenting similar values for P_f (average of 0.15 g/L) when compared to results of series A and C, exhibited the lowest $Y_{P/X}$ values (average of 63 mg/g). The lowest $Y_{P/X}$ values for series E (with pH control at 7.4) (respectively, averages of 0.078 g/L and 46 mg/g) and series F (with limitation of dissolved oxygen and rotation set at 55 rpm) (respectively, averages of 0.058 g/L and 62 mg/g) were also remarkable. Therefore, the process conditions of these experiments (series D, E and F) are not recommended for industrial PS production. However, despite the observed variations for P_f and $Y_{P/X}$ values between the distinct series, a previous study reported no significant variation in *N. meningitis* C PS molar mass changing the bacterial growth process conditions [33].

As mentioned above, cultivations with dissolved O_2 control (series D) resulted in an inferior PS production in comparison to the other ones without the control of this process parameter.

pH Values and Glucose Consumption

In experiments without pH and dissolved O_2 controls (series A) and with dissolved oxygen limitation (series F) the pH started at 7.4 (Fig. **1e**) and decreased to 5.0 after 7-8 hours of cultivation. It is worthy of mention that the pH correction in the process (series B, C and E) has not increased the biomass values when compared to series without controls of pH and dissolved O_2. In these last series, the observed pH drop probably occurred due to the formation of acid metabolites during cellular growth [34]. On the other hand, in cultivations with pH control (series B, C and E) the glucose consumption has increased. There was no significant pH drop in bacterial cultivations with dissolved O_2 control (series D) and, consequently, probably no production of acid metabolites occurred.

In series A (without pH and dissolved O_2 controls) and F (with dissolved oxygen limitation), residual values of glucose near to 1.0 g/L and 2 g/L were verified at the end of the process respectively. On the other hand, in series B and E

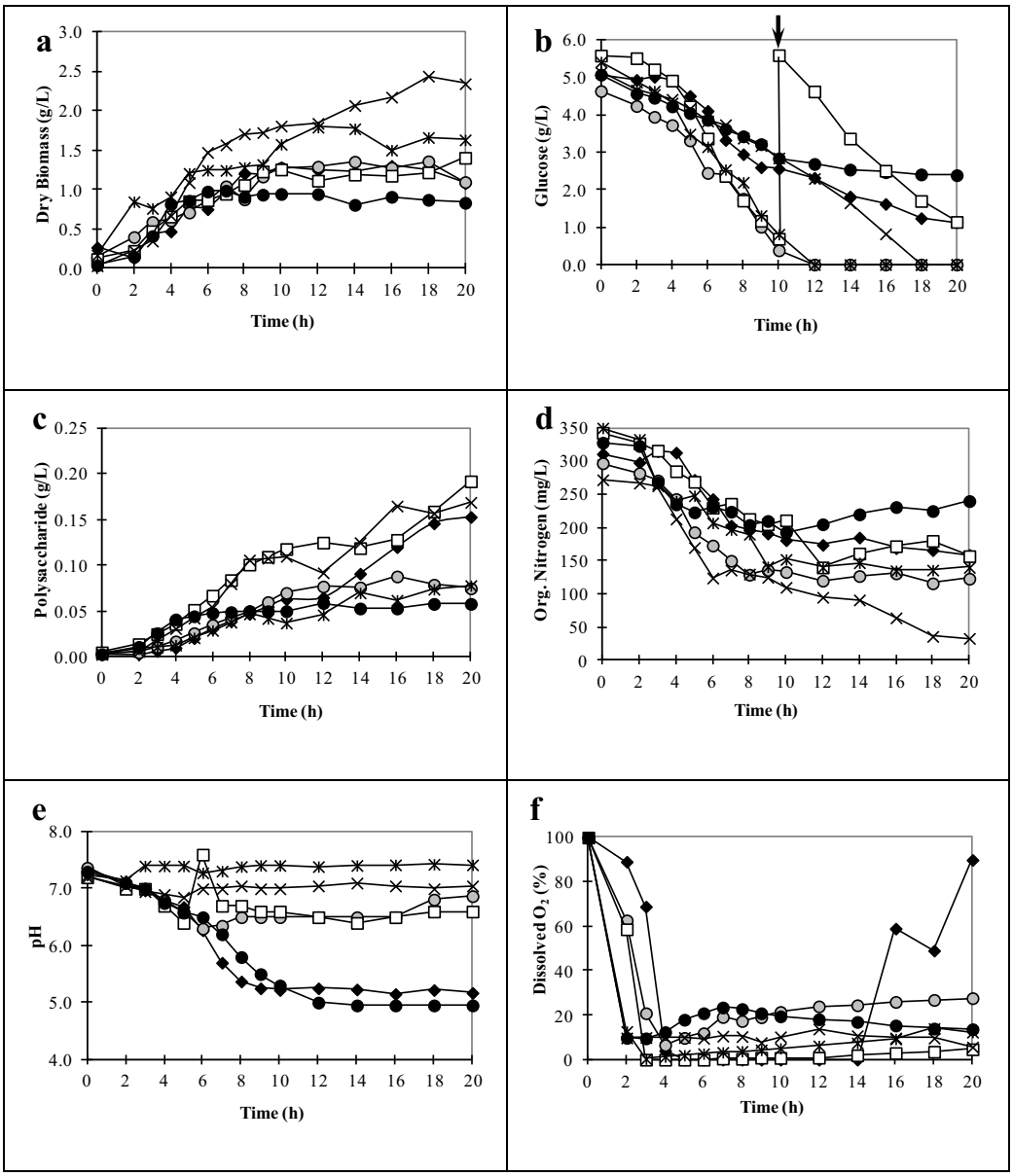

Fig. (1). Kinetics curves of bacterial growth (**a**), glucose consumption (**b**), PS formation (**c**), organic nitrogen consumption (**d**), pH (**e**) and dissolved oxygen (**f**). Pilot batch experiments: assay 1 (♦) without pH and dissolved O_2 controls, assay 6 (o) control of pH at 6.5, assay 8 (□) control of pH=6.5 and glucose pulse at the 10th process hour, assay 10 (x) control of dissolved O_2 at 10%, assay 12 (*) control of pH at 7.4, assay 13 (•) dissolved oxygen limitation (rotation set at 55 rpm).

(pH control at 6.5 and 7.4 respectively) the residual glucose concentration dropped to zero at the 12th cultivation hour (beginning of stationary phase) indicating a limitation of this substrate. For this reason, the assays of series C were designed with pH control at 6.5 and glucose fed-batch pulse (45 g/L feed solution) at the 10th hour of cultivation in order to verify the possibility of bacterial growth recovering. Instead, it was verified a continuous stationary growth phase with an increase in the PS production (Fig. **1a** and **1c**) and, consequently, an increase in the correspondent yield factor ($Y_{P/X}$) (Table **2**). As previously reported [33], this observation shows the influence of glucose concentration on the production of PS and indicates the existence of a relationship between glucose concentration and the specific rate of PS production.

In cultures with correction of dissolved O_2 at 10% saturation (series D), glucose exhausted at the 16-18th cultivation hour (Fig. **1b**). Nevertheless, as no pH change was observed in these cultivations (i.e. probably, there was no production of acid metabolites), it was assumed that carbon from glucose was employed in these cultivations for the growth of new bacteria, associated PS production, cell maintenance and carbon dioxide released from the system.

Organic and Inorganic Nitrogen Consumption and Cellular Nitrogen Concentration

In this study, the inorganic nitrogen in the medium (300 mg/L) and cellular nitrogen concentration in bacteria (13% w/w) did not change significantly along the cultivation time (data not shown). However, the organic nitrogen consumption

Table 1. Experimental conditions and consumption of substrates for *N. meningitidis* C pilot bioreactor cultivations.

Cultivation conditions	Run Number	Cultivation time (h)	pH control	Dissolved O_2 control at 10%	G_0 (g/L)[a]	N_{org0} (g/L)[b]	ΔG^e (g L^{-1})	ΔG^{e*} (g L^{-1})	$\Delta N_{org}{}^f$ (g L^{-1})	$\Delta N_{org}{}^{f*}$ (g L^{-1})	Series
Batch without controls	1	20	-----	-----	5.1	0.32	3.9		130		
of pH and dissolved O_2	2	20	-----	-----	5.0	0.29	3.5	3.8±0.2	136	139±22	**A**
	3	20	-----	-----	5.1	0.28	4.0		147		
	4	20	-----	-----	5.5	0.28	4.0		110		
	5	20	-----	-----	4.8	0.31	3.6		170		
pH control at 6.5	6	20	6.5	-----	4.8	0.30	4.7	4.8±0.1	174	177±4	**B**
	7	20	6.5	-----	4.9	0.31	4.9		180		
Pulse batch with control	8[c]	20	6.5	-----	5.5	0.34	9.4	9.3±0.1	186	167±28	**C**
of pH at 6.5	9[c]	20	6.5	-----	5.3	0.33	9.2		147		
Batch with dissolved O_2	10	20	-----	yes	5.1	0.28	5.1	5.3±0.3	239	225±20	**D**
control at 10%	11	20	-----	yes	5.1	0.30	5.5		211		
pH control at 7.4	12	20	7.4	-----	5.4	0.35	5.4	5.4	188	188	**E**
Dissolved O_2 limitation (55 rpm)	13[d]	20	-----	-----	5.1	0.33	2.7	2.7	88	88	**F**

[a]G_0 = initial glucose concentration in the bioreactor.

[b]N_{org0} = initial organic nitrogen concentration in the bioreactor.

[c]Glucose pulse (final concentration 5 g/L) at 10 h.

[d]Dissolved O_2 limitation (rotation frequency 55 rpm)

[e]ΔG= amount of glucose consumed during cultivations per unit reactor volume. In the runs 6, 7 and 12 the glucose was depleted at 12th cultivation hour. In runs 10 and 11, at 18th cultivation hour.

[f]ΔNorg = amount of organic nitrogen consumed during cultivations per unit reactor volume.

*Average and standard deviation values estimated for each group of runs.

was linearly related to cellular growth with constant yield factors (Table **2**) and no nitrogen absorption was observed after the beginning of the stationary growth phase (Fig. **1a** and **1d**). Despite Frantz medium being partially a non-defined medium due to the presence of dialyzed yeast extract (an organic nitrogen source), the other main organic nitrogen sources in this medium are L-glutamic acid and L-cysteine, which may be also used in the formation of new bacteria.

DISCUSSION

Generally it is expected that bioprocesses with controlled parameters provide better and more reproducible results than uncontrolled ones. Performing experiments in laboratory bench scale, [33, 34] reported this does not occur in the case of *N. meningitis* C cultivations aiming at PS production. In addition, pH and dissolved oxygen are very important factors related to PS production. Thus, changing the experimental process scale, it is necessary to demonstrate whether the same behavior could be maintained or not. Therefore, the different types of cultivations were chosen based on process conditions already reported in the scientific literature.

The formation of the polysaccharidic capsule in microaerophilic growth conditions is significantly greater than in aerobic conditions, i.e. the reduced availability of oxygen would lead to an upregulation in capsule expression. The bacterial cells *in vivo* conditions have the flexibility to alter their surface characteristics according to different requirements of colonization and infection [33, 35-37]. Clinical studies in patients, infected with *N. meningitidis* C, showed bacterial cells isolated from the nasopharynx have smaller capsular dimensions than those isolated from the cerebrospinal fluid (CSF), where there is less oxygen availability [33, 38]. According to Cole [39], pathogenic *Neisseria* are neither aerobes nor true anaerobes, but dedicated microaerophiles. These considerations suggest that oxygen control is not recommended for the whole duration of the process as it could negatively affect PS production.

In addition, the pH in the present research was controlled at 3 different values: 7.4, 7.0 (with dissolved O_2 control) and 6.5. *Neisseria meningitidis* is a human pathogenic bacterium. The CSF has pH near 7,3 and the human blood must be constantly maintained with the optimum pH between 7.36 and 7.42. Rapid changes of this blood pH will compromise not only the state of consciousness, but may also endanger life itself. If the blood pH falls to a value of 6.95 (slightly acidic), the person can go into coma [38]. The cultivation medium (Frantz medium) has its initial pH adjusted to near 7.4 in all experiments emulating the CSF and human blood conditions. So, all the assays began with this pH value, which decreased along the cultivation time in conditions of

Table 2. Results of *N. meningitidis* C pilot bioreactor cultivations carried out under different conditions.

Cultivation Conditions	Run Number	Cultivation time (h)	t_{st} [a] (g/ L)	X_{max} [b] (g/L)	P_f [c] (g/L)	P_f [c]* (g/L)	$Y_{P/X}$ [d] $\cdot 10^3$ (mg/g)	$Y_{P/X}$ [d]* $\cdot 10^3$ (mg/g)	$Y_{X/G}$ [e] (g/g)	$Y_{X/G}$ [e]* (g/g)	$Y_{X/Norg}$ [f] (g/g)	$Y_{X/Norg}$ [f]* (g/g)	Series
Batch without controls	1	20	12	1.4	0.15		107		0.28		8.38		
of pH and dissolved O_2	2	20	12	1.3	0.15	0.15±0.03	115	107±16	0.33	0.32±0.03	8.52	8.4±0.9	**A**
	3	20	12	1.4	0.12		86		0.35		9.66		
	4	20	12	1.3	0.13		100		0.29		8.40		
	5	20	12	1.4	0.18		129		0.34		7.24		
pH control at 6.5	6	20	12	1.3	0.09	0.11±0.02	69	81±16	0.25	0.26±0.01	6.63	7.8±1.7	**B**
	7	20	12	1.3	0.12		92		0.26		9.02		
Pulse batch with control	8	20	10	1.3	0.19	0.20±0.02	146	150±6	0.12	0.13±0.01	6.14	6.8±0.9	**C**
of pH at 6.5	9	20	12	1.3	0.20		154		0.13		7.40		
Batch with dissolved O_2	10	20	18	2.4	0.15	0.15±0.00	63	63±0	0.47	0.48±0.01	10.0	11±2	**D**
control at 10%	11	20	16	2.4	0.15		63		0.48		13.1		
pH control at 7.4	12	20	12	1.7	0.078	0.078	46	46	0.30	0.30	8.61	8.61	**E**
Dissolved O_2 limitation (55 rpm)	13	20	7	0.94	0.058	0.058	62	62	0.34	0.34	8.13	8.13	**F**

[a] t_{st} = stationary phase beginning.

[b] X_{max} = maximum biomass concentration obtained at the beginning of the stationary phase.

[c] P_f = final polysaccharide concentration.

[d] $Y_{P/X}$ = yield factor of polysaccharide on biomass calculated between the beginning and the end of cultivations.

[e] $Y_{X/G}$ = yield factor of biomass on glucose calculated between the beginning and the end of cultivations.

[f] $Y_{X/Norg}$ = yield factor of biomass on organic nitrogen calculated between the beginning and the end of cultivations.

*Average and standard deviation values estimated for each group of runs.

absence of pH or dissolved O_2 control, due to formation of acid metabolites produced during cell growth. The other pH values (7.0 and 6.5) were chosen because, in the case of pH=7.0, to be associated to a critical human meningitis illness condition and, in the case of 6.5, besides to be a value beyond this critical condition, pH=6.5 is employed for the beginning of PS purification process [19, 20, 33-35].

The PS concentration values reported in the literature are compatible or higher with those obtained in this study (Table **2**). They are higher than those related to batch cultivations carried out by Carty *et al.* [10], who reported a final PS concentration of 0.16 g/L after 22 h of cultivation. In addition, the results in this study are in accordance with an observation made by those authors, in which, under constant aeration and agitation (i.e. without dissolved O_2 control), a supplementary PS production took place related to oxygen-limited cell growth. In another study, conducted by Baruque-Ramos *et al.* [33], a set of experiments was carried out in Frantz medium in a 13 L bioreactor. The highest PS concentration (0.26 g/L) and the overall $Y_{P/X}$ yield (160 mg/g) were obtained in batch and partial fed-batch experiments in which the glucose concentration was maintained below 1.0 g/L, but these results were not significantly superior to the ones from batch experiments without pH or dissolved O_2 controls. Henriques *et al.* [16] reported a three-factor face-centered central composite design for PS production in Frantz medium, carried out in incubator shaker and using response surface methodology (RSM). The optimal values were temperature equal to 37°C, pH controlled at 7.0 and agitation speed maintained at 1,300 rpm, which correspond to K_La equal to 36 h^{-1} and PS concentration equal to 155.4 mg/L. However, this report have not taken in account all factors that could influence the PS production or the complexity of the metabolic pathways in *N. meningitidis* C, which can lead to totally different results if the process variables or/and range of experimental parameters were changed.

N. meningitidis adapts its metabolic pathways according to environmental conditions [34]. Therefore, upon employing RSM, it would be very difficult to identify how the factors would influence the effects, which could be a response to variations in the experimental parameters within the same metabolic pathway or/and could be a response to the changes in metabolic pathways due these variations in the experimental parameters. In order to correctly study this question, it is suggested, in further studies, an analytical analysis employing molecular labeling with radioactive isotopes.

N. meningitidis requires glucose (preferred substrate), pyruvate or lactate as sole carbon source and, during cultivation on any of these carbon sources, the secretion of acetate into the medium is expected. According to the genomic information for *N. meningitidis* Serogroup B, glucose can be completely catabolized through the Entner-Douderoff pathway (ED) and the pentose phosphate pathway (PP) [22]. *N. meningitidis* may employ different metabolic pathways for the carbon source uptake depending on the cultivation conditions. In the assays with pH control, productions of acetic and lactic acids occur and, consequently, a 2-3-fold increase in glucose consumption is observed in contrast to the other cultivation conditions [34].

The same behaviors, the increase in glucose consumption and the consequent reduction in the yield factor of biomass on glucose ($Y_{X/G}$), were also present in the assays carried out in this study (Fig. **1b**, Tables **1** and **2**). According to these findings, it is possible to conclude that, despite the highest values of P_f and $Y_{P/X}$ (respectively, averages of 0.20 g/L and 150 mg/g) presented in Series C assays (with control of pH at 6.5 and glucose fed-batch pulse at the 10^{th} cultivation hour) and due to the significant values related to glucose consumption observed at pH 6.5 (with consequent abundant release of acetic and lactic acids), pH control is not recommended for the industrial process set-up. Therefore, the best results are represented by series A, without pH or dissolved O_2 controls, despite slightly lower values for P_f and $Y_{P/X}$ (respectively, averages of 0.15 g/L and 107 mg/g).

Capsular PS from *N. meningitidis* C cultivations is the main antigen for manufacturing the vaccine against the disease caused by this bacterium. The final protein content allowed in this vaccine is 1% [9, 31]. Therefore, it is important to study the relationship between nitrogen consumption and cellular growth (related to PS production) and cell nitrogen content.

Santos *et al*. [40, 41] studied the consumption of amino-acids in Catlin defined medium during batch process of *N. meningitidis* B and observed that L-lactate and L-glutamic were the main limiting carbon and nitrogen source, respectively. The L-glutamic acid consumption contributed to the formation of ammonia and the increase of pH during in cultivations and L-cysteine.HCl had small consumption. They suggested that the original Catlin medium composition should be reformulated in order to enhance antigen production from the *N. meningitidis* serogroup B cultivations.

Thus, considering the low final protein content (< 1%) allowed in PS for vaccine formulation [9, 31] and the high protein percentage present in the bacteria, to employ the PS released in the medium instead of the capsular PS obtained after cell disruption could represent a cleaner process with easier and cheaper further purification procedure.

CONCLUSION

Serogroup C PS from *Neisseria* constitutes the antigen for the vaccine against the disease caused by this bacterium. This study investigated the possibility of increasing PS from *N. meningitidis* C cultivations setting operational parameters in standard cylindrical pilot bioreactor.

Despite of the highest values of P_f and $Y_{P/X,}$ respectively averages of 0.20 g/L and 150 mg/g, presented in assays with control of pH at 6.5 and glucose fed-batch pulse at 10^{th} cultivation hour (series C), because the significant glucose consumption (with consequent abundant release of acetic and lactic acids), it is concluded that these process conditions are not recommended for the industrial process set-up. In this way, the best results were represented by series A, without pH or dissolved O_2 controls, despite of values slightly lower of P_f and $Y_{P/X}$ (respectively averages of 0.15 g/L and 107 mg/g).

At the present study, inorganic nitrogen concentrations in the medium did not change significantly along the cultivation time with average values of 300 mg/L. The organic nitrogen consumption was linearly related to cell growth with constant yield factors and no nitrogen absorption was observed after the stationary growth phase beginning. The structural nitrogen concentration from bacteria showed an average value of 13% (w/w) with respect to dry biomass, regardless of cultivation conditions.

The employment of the PS released in the medium, instead of the capsular PS extracted from the bacterial capsids after the cell disruption process, could represent a cleaner process with facilitated and cheaper further process purification.

Finally, the presented findings could be useful to a proper Frantz medium reformulation in order to obtain a greater amount of PS and to improve the vaccine development in industrial scale-up production.'

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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