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ORIGINAL PAPER

Júlia Baruque-Ramos · Haroldo Hiss
Attilio Converti · Viviane Maimone Gonçalves
Isaías Raw

Accumulation of organic acids in cultivations of *Neisseria meningitidis* C

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Abstract Aiming at the industrial production of serogroup C meningococcal vaccine, different experimental protocols were tested to cultivate *Neisseria meningitidis* C and to investigate the related organic acid release. Correlations were established between specific rates of acetic acid and lactic acid accumulation and specific growth rate, during cultivations carried out on the Frantz medium in a 13 l bioreactor at 35°C, 0.5 atm, 400 rpm and air flowrate of 2 l min^{-1}. A first set of nine batch runs was carried out: (1) with control of dissolved oxygen (O_2) at 10% of its saturation point, (2) with control of pH at 6.5, and (3) without any control, respectively. Additional fed-batch or partial fed-batch cultivations were performed without dissolved O_2 control, varying glucose concentration from 1.0 to 3.0 g l^{-1}, nine of which without pH control and other two with pH control at 6.5. No significant organic acid level was detected with dissolved O_2 control, whereas acetic acid formation appeared to depend on biomass growth either in the absence of any pH and dissolved O_2 control or when the pH was kept at 6.5. Under these last conditions, lactic acid was released as well, but it did not seem to be associated to biomass growth. A survey of possible metabolic causes of this behavior suggested that *N. meningitidis* may employ different metabolic pathways for the carbon source uptake depending on the cultivation conditions.

J. Baruque-Ramos (✉)
Universidade de São Paulo (EACH-USP),
Av. Arlindo Bettio, 1000, 03828-000 São Paulo, Brazil
E-mail: jbaruque@usp.br
Tel.: +55-11-30911027
Fax: +55-11-30911027

H. Hiss · V. M. Gonçalves · I. Raw
Instituto Butantan, Centro de Biotecnologia,
Av. Dr. Vital Brazil, 1500, 05503-900 São Paulo, Brazil

A. Converti
Department of Chemical and Process Engineering "G. B. Bonino",
Genoa University, Via Opera Pia 15, 16145 Genova, Italy

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Introduction

Meningococcal disease is the 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% [29]. However, only a few work has been carried out in neisserial physiology [17–19, 21].

Gotschlich et al. [14] developed an effective method that has been adopted for serogroup A and C polysaccharide purification [1, 6]. To this purpose, the concentrated bacterial culture supernatant obtained from batch cultivations was employed. Owing to their anionic properties, these polysaccharides were precipitated by a cationic detergent (hexadecyl trimethyl ammonium bromide) and then submitted to various steps of organic solvent extraction and ultracentrifugation. The compound obtained by this protocol was stable and suitable for purification, and the final product showed a good immunological response.

Little information is available about the microorganism behavior on the production scale, for which only the batch process is presently applied. The fed-batch process has recently been tested with success [4]. However, alteration of the process conditions simultaneously influenced the consumption of substrate, the formation of polysaccharide and the release of undesired byproducts such as organic acids. There is no systematic report in the literature, to the best of our knowledge, on the formation of organic acids during *N. meningitidis* serogroup C cultures.

To select the optimum protocol for the industrial production of serogroup C meningococcal vaccine, we tested different modes of operation either in the presence or in the absence of pH and/or dissolved oxygen controls and investigated the effect of

organic acids release on cultivations in a cylindrical bioreactor.

Materials and methods

Inoculum preparation and medium composition

The inoculum of *N. meningitidis* IMC 2135 was prepared according to Gotschlich et al. [14]. The content of one ampoule containing 6.3×10^9 viable cells of the meningococcal strain, maintained at −70°C in 0.5 ml of the medium of Greaves [16], was streaked out onto two agar slant tubes [25] 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 the liquid medium of Frantz [11] that contained (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-cystein·HCl·H_2O, 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 two 500 ml conical flasks, each containing 100 ml of the same medium and incubated at 35°C for 4 h on a rotary shaker at 120 rpm. In order to verify possible contaminations, microscopic examinations were carried out using the Gram technique. The contents of both the conical flasks were used to inoculate a 13 l bioreactor, model Bioflo 2000 (New Brunswick, Edison, NJ, USA), containing 9 l of the above Frantz medium.

The medium used for both inoculum preparation and fermentations in the bioreactor was sterilized by filtration through Millipore filters with 0.2 μm mean pore diameter.

Fermentation conditions

The fermentation conditions were: temperature = 35°C; air flow rate = 2 l min^{-1} (0.22 vvm); upper aeration, $k_La \cong 14.4\ h^{-1}$; rotational speed = 400 rpm in the absence of dissolved O_2 control, and in the range 50–850 rpm when dissolved O_2 was controlled at 10% of its saturation point (as described subsequently). The vessel head pressure was maintained at 0.5 atm. During the test with pH control, this parameter was kept at 6.5 by addition of 3 N NaOH solution. Two Rushton six blade disc turbines with 9 cm diameter were used for mixing, one located at 10.0 cm from the vessel bottom and the other at 11.5 cm from the first turbine. The pH was measured by an on-line sterilizable electrode. Dissolved O_2 concentration was monitored by an on-line polarographic electrode, Ingold model 531 (Mettler-Toledo, Alphaville, Brazil). The 100% point was calibrated 1 h before the inoculation under the same cultivation conditions. During fed-batch runs, glucose solutions were fed by means of a peristaltic pump.

The experimental schedule is summarized in Table 1. The feeding volumetric flow of glucose solution was estimated in order to make a balance between the total volumes of added glucose solution and taken samples (every 2 h). In this way, the effect of dilution was minimized, and these values were taken into account for the calculation of the kinetic parameters.

Table 1 Experimental conditions for the experiments 1 to 20

Run number	Culture duration (h)	pH control at 6.5	Dissolved O_2 control at 10%	S_o (g/l)[a]	S_1 (g/l)[b]	S_2 (g/l)[c]	Series
1	24	–	Yes	4.9	–	–	
2	24	–	Yes	5.0	–	–	A
3	24	–	Yes	5.0	–	–	
4	20	Yes[d]	–	4.8	–	–	
5	20	Yes[d]	–	5.0	–	–	B
6	20	Yes[d]	–	5.2	–	–	
7	20	–	–	4.6	–	–	
8	20	–	–	4.9	–	–	C
9	20	–	–	5.3	–	–	
10	20	–	–	1.7	55[e]	7.5[e]	D
11	20	–	–	1.8	55[e]	7.5[e]	
12	20	–	–	0.16	55[e]	7.5[e]	E
13	20	–	–	0.12	55[e]	7.5[e]	
14	20	–	–	0.10	55[e]	7.5[e]	F
15	20	–	–	0.20	55[e]	7.5[e]	
16	20	–	–	3.4	–	7.5[e]	G
17	20	–	–	4.0	–	7.5[e]	
18	20	Yes	–	0.27	105[f]	80[f]	H
19	20	Yes	–	4.6	–	80[f]	I
20	30	–	–	3.8	–	7.5[f]	J

[a] S_o = initial glucose concentration in the bioreactor
[b] S_1 = feed glucose concentration from 0 to 10 h
[c] S_2 = feed glucose concentration from 10 h to the end
[d] Glucose pulse (final concentration 5 g/l) at 10 h
[e] Constant feed volumetric flow of 1.0 ml/min
[f] Feed volumetric flow of 0.5 ml/min within 0–4 h and 1.0 ml/min within 4–10 h

Analytical methods

Cell concentration was expressed as dry biomass weight per liter (g l^{-1}) after centrifugation of a known-volume sample at 8,700*g* for 30 min, followed by pellet drying at 60°C for 48 h. Glucose concentration was measured using the glucose oxidase colorimetric method [32]. Polysaccharide concentration was determined according to Gotschlich et al. [14], after cell disruption and debris precipitation by addition of Cetavlon (Merck Lab., Darmstadt, Germany). After preliminary centrifugation of the sample (8,700*g*, 30 min), the supernatant was removed, while the precipitate was resuspended in 1.0 M $CaCl_2 2H_2O$ solution and centrifuged again under the same conditions. The supernatant was used for the polysaccharide determination by the resorcinol–HCl colorimetric method [14, 33], which is sensitive to polysaccharide monomers (sialic acids) formed by acid hydrolysis.

For molar mass determinations, a column (1.5×85 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 1.0 M

ammonium acetate solution (pH = 7.0) containing 0.1% sodium dodecylsulfate [15, 28]. Standard dextrans with known molar masses were eluted through the column for calibration, and the corresponding elution volumes were related to the logarithm of the molar masses. Dextran concentrations were determined by the phenol–sulfuric acid method [32]. Samples purified with ethanol were then injected (0.2 ml), and the polysaccharide content of the collected fractions (3 ml each) was determined by the above resorcinol–HCl method.

Organic acids concentrations were determined by HPLC, model $10A_{VP}$ (Shimadzu, Kyoto, Japan) using a HPX-87H column (Bio Rad, Hercules, CA, USA) after dilution (1:5) of samples from cultivations with 5.0 mM sulfuric acid solution and filtration through 0.2 µm-pores. A standard mixture of organic acids, made up of malic, acetic, succinic, oxalic, formic and lactic acids, was injected in the column at different dilutions to prepare their respective calibration curves. A 5.0 mM sulfuric acid solution was used as a mobile phase at 0.6 ml min^{-1} flowrate and 35°C. Integration of the chromatographic peaks and determination of organic acid concentrations were done using the software Class VP-Version 6.2.

The yield of polysaccharide on biomass ($Y_{P/X}$) was calculated as the mass ratio of polysaccharide to biomass generated after a given cultivation time. The specific rates of organic acid formations (v_i) were calculated by polynomial fitting of their experimental concentrations with the computer program Logiciel du Lissage based on the Spline method [20]. To correlate product formation with growth, we used the model of Luedeking and Piret [22]:

$$v_i = \alpha\,\mu + \beta \qquad (1)$$

where μ is the specific growth rate (h^{-1}), α a growth associated coefficient (g g_X^{-1}) and β a non-growth associated coefficient (g g_X^{-1} h^{-1}).

Results

Growth curves

Typical growth curves of runs 1 to 20, exhibiting similar profiles, are shown in Fig. 1a, d, while Fig. 1b, e illustrate glucose consumption and Fig. 1c, f polysaccharide formation, respectively. These behaviors suggest that substrate consumption had little influence on cell growth kinetics, whereas polysaccharide formation was remarkably influenced. The absence of a lag growth phase also points out that cells of the inoculum were well adapted to the medium. In spite of a certain variability in the initial biomass concentration, analysis of the exponential growth phases (data not shown) and a statistical "*F*" test (Snedecor distribution) demonstrated that there was no significant difference between the slopes of the straight lines describing runs 1 to 20 (at 5% significance level) and that glucose concentration had no appreciable effect on the specific growth rate.

Polysaccharide formation and conversion factor

To make comparison easier, the results of final biomass concentration (X_{max}), final polysaccharide concentration (P_f) and yield of polysaccharide on biomass ($Y_{P/X}$) (Table 2), collected from repeated runs under the same cultivation conditions, were grouped in homogeneous series (from A to J), and a Student's *t* statistical test between the series was performed at 5% significance level. A comparison of the results of series A, B and C showed that batch tests performed with dissolved O_2 control at 10% exhibited the lowest $Y_{P/X}$ values; therefore, these operating conditions were not selected for subsequent fed-batch operation.

Comparison of results of series B, H, I and J with the respective confidence intervals of series C and G shows that the highest values of P_f and $Y_{P/X}$, useful to make the subsequent product purification easier and more effective, were obtained with no pH or dissolved O_2 control either in batch (series C) or fed-batch (series G) process. Besides, the former parameter did not appreciably increase when the cultivation time was prolonged from 20 to 30 h. It should also be noticed that the residual glucose concentration in the medium at the end of run 20 (series J) was lower than in the other fed-batch tests (Fig. 1e), but the final values of P_f and $Y_{P/X}$ increased by only a small quantity; therefore, the fed-batch process did not ensure a significant improvement of these parameters when compared to the batch one.

A previous study reported average polysaccharide molar mass in the range 400–500 kDa, gel chromatography partition coefficient (K_d) lower than 0.3 and recovery rate (percentage of polysaccharide recovered before reaching $K_d = 0.5$) higher than 84% [4], which means that the product satisfies all the criteria for an antigen for vaccine preparation [12, 36, 37].

Organic acids formation

Typical organic acids curves of runs 1 to 20, exhibiting similar profiles, are shown in Fig. 2, whereas the final concentrations of acetic and lactic acids are listed in Table 2. All comparisons were performed employing the *t* test, and the statistical *F* test was used to compare organic acids kinetics as groups of distinct correlations. The release of oxalic, citric, malic, succinic, lactic, formic and acetic acids was also monitored versus time throughout all experiments. No oxalic and formic acids were detected, whereas citric and malic acids formed only at very low levels (< 0.05 g l^{-1}; data not shown).

During the batch runs with control of dissolved oxygen at 10% (runs 1 to 3), neither pH fall nor acetic and lactic acids formation took place up to 16 h (Fig. 1a). After this time, the control of dissolved O_2 was switched off, but no enhancement of polysaccharide production was observed, and the pH decreased from 7.0 to as low as 5.1, as a consequence of significant production of acid acetic (Fig. 2a and Table 2). Meanwhile, succinic acid

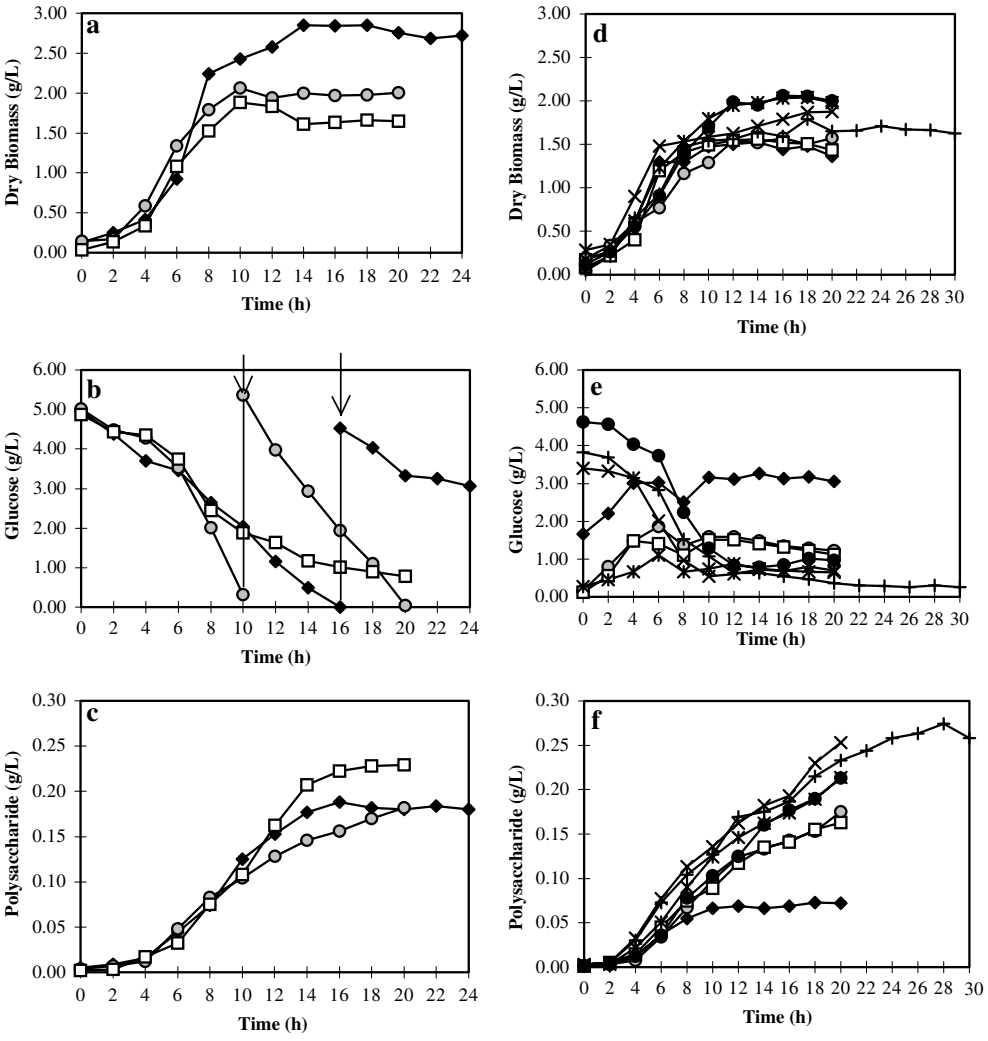

Fig. 1 Time behaviors of biomass growth, glucose consumption and polysaccharide formation. Batch experiments (**a**, **b**, **c**): run 2 (*filled diamond*) control of dissolved O_2 at 10%, run 5 (*open circle*) control of pH at 6.5, run 8 (*open square*) without pH and dissolved O_2 controls. Fed-batch experiments (**d**, **e**, **f**): runs 10 (*open diamond*), 12 (*open circle*), 14 (*open square*), 16 (*cross mark*) and 20 (*positive sign*) without pH and dissolved O_2 controls, 18 (*asterisk*) and 19 (*filled circle*) pH control at 6.5

concentration was appreciable even at the beginning of the run (~0.20 g l^{-1}), reached a maximum value after 8 h (~0.5 g l^{-1}), then decreased again to 0.20 g l^{-1}, and kept almost constant until the end of cultivation (Fig. 2a).

Batch and fed-batch cultivations performed without pH or dissolved oxygen control (runs 7 to 17 and 20) exhibited acetic acid formation partially associated to growth, according to the model of Luedeking and Piret [22], and similar values of both correlation coefficients (α and β) (Table 3); therefore, considering these runs as a whole, mean values of $\alpha = 0.128$ g_{Ac} g_X^{-1} and $\beta = 0.039$ g_{Ac} g_X^{-1} h^{-1} were estimated. On the contrary, no significant lactic acid formation was detected (Tables 2, 3 and Fig. 2c, d, f), whereas profiles of succinic acid were similar to those observed with dissolved O_2 control. In addition, acetic acid formation seemed to have no statistically significant effect upon polysaccharide production during these experiments.

zLikewise, considering batch and fed-batch cultivations with control of pH at 6.5 together (runs 4 to 6, 18 and 19), acetic acid formation turned out to be partially growth-associated (Table 3), showing higher mean values of both correlation coefficients ($\alpha = 0.254$ g_{Ac} g_X^{-1}; $\beta = 0.060$ g_{Ac} g_X^{-1} h^{-1}). On the other hand, lactic acid formation was shown not to be associated to the growth (Table 3) and exhibited only a constant value of the latter parameter ($\beta = 0.146$ g_{Lac} g_X^{-1} h^{-1}). These runs with pH control led to final concentrations of lactic acid almost twice that of acetic acid (Table 2, Fig. 2b, e) and, consequently, to a twofold to threefold increase in glucose consumption (ΔS) with respect to the other conditions (Table 2), while succinic acid production was very low (< 0.15 g l^{-1}) and progressively decreased throughout the runs.

Discussion

A kinetic correlation has been established, for the first time, between cell growth and partially associated acetic acid formation and/or non-associated lactic acid formation in cultivations of *N. meningitidis* serogroup C under different conditions. Other aspects, such as polysaccharide formation, either associated to growth or not,

Table 2 Results of *N. meningitidis* C cultivations performed under different conditions

Cultivation conditions	Run	Cultivation time (h)	S_{st}[a, b] (g l^{-1})	X_{max}[c] (g l^{-1})	P_f[d] (g l^{-1})	P_f[b, d] (g l^{-1})	$Y_{P/X}10^3$[e] (g g_X^{-1})	$Y_{P/X}10^3$[b, e] (g g_X^{-1})	Ac[f] (g l^{-1})	Ac[b, f] (g l^{-1})	Lac[g] (g l^{-1})	Lac[b, g] (g l^{-1})	ΔS[h] (g l^{-1})	ΔS[b, h] (g l^{-1})	Group
Batch with	1	24	–	3.0	0.17		62		–		–		6.9		
dissolved O_2	2	24	–	2.9	0.18	0.18	60	64	–	–	–	–	6.4	6.5	A
control at 10%	3	24	–	2.8	0.19		69		–		–		6.3		
Pulse batch	4	20	–	2.0	0.17		90		2.7		4.5		9.4		
with control	5	20	–	2.1	0.18	0.18	84	91	2.2	2.3	3.9	4.0	10	9.9	B
of pH at 6.5	6	20	–	2.0	0.19		98		2.1		3.5		10		
Batch without	7	20	–	1.6	0.20		139		1.1		–		3.7		
controls of pH	8	20	–	1.7	0.23	0.22	136	139	1.0	1.0	–	–	4.1	4.1	C
and dissolved O_2	9	20	–	1.7	0.23		141		0.91		–		4.6		
Fed-batch	10	20	3.0	1.5	0.072	0.078	48	52	1.2	1.2	–	–	3.0	2.8	D
	11	20	3.4	1.5	0.083		57		1.2		–		2.5		
Fed-batch	12	20	1.5	1.5	0.17	0.17	118	113	0.91	0.94	–	–	3.4	3.3	E
	13	20	1.3	1.5	0.16		107		0.97		–		3.3		
Fed-batch	14	20	0.65	1.6	0.20	0.21	126	131	1.0	1.1	–	–	4.0	4.0	F
	15	20	0.68	1.6	0.22		136		1.1		–		4.0		
Partial fed-batch[i]	16	20	0.68	1.8	0.25	0.25	145	143	0.88	0.95	–	–	3.3	3.5	G
	17	20	0.85	1.7	0.24		140		1.0		–		3.8		
Partial fed-batch with pH control at 6.5[i]	18	20	0.75	2.0	0.21	0.21	119	119	1.9	1.9	3.2	3.2	13	13	H
Fed-batch with pH control at 6.5	19	20	0.95	2.0	0.21	0.21	128	128	2.2	2.2	3.8	3.8	9.5	9.5	I
Partial fed-batch[j]	20	30	0.46	1.6	0.26	0.26	157	157	1.0	1.0	–	–	4.1	4.1	J

[a] S_{st} = glucose concentration at steady state. See Fig. 1e
[b] Average values estimated for each group of runs
[c] X_{max} = maximum biomass concentration obtained at the beginning of the stationary phase (14 h for runs 1–3 and 10 h for the others)
[d] P_f = final polysaccharide concentration
[e] $Y_{P/X}$ = yield of polysaccharide on biomass calculated between the beginning and the end of cultivations
[f] Ac = acetic acid concentration
[g] Lac = lactic acid concentration
[h] ΔS = quantity of glucose consumed during cultivations per unit reactor volume
[i] Fed-batch between 10 and 20 h
[j] Fed-batch between 10 and 30 h

nitrogen consumption and its assimilation by the cell, glucose consumption kinetics, as well as the relationship between polysaccharide and glucose concentration in batch and fed-batch processes had earlier been investigated by the authors [2–4, 27]. Most of the results of polysaccharide production by *N. meningitidis* serogroup C listed in Table 2 are better than those obtained batch-wise by Carty et al., who reported a maximum polysaccharide concentration of 0.16 g l^{-1} after 22 h of cultivation, under constant aeration and agitation conditions, as a consequence of oxygen-limited cell growth [6].

The organic acid formation detected in this study could be associated to metabolic features of this microorganism. Fu et al., who carried out cultivations of serogroup B *N. meningitidis* in a synthetic medium (MC6) with dissolved O_2 control, stressed that the main metabolic pathway for the carbon source (glucose) assimilation would be that of Entner-Doudoroff (EDP), which was responsible for about 80% of its consumption [13]. Through this pathway, pyruvic acid was likely to be stoichiometrically oxidized to acetic acid, being the TCA cycle, according to the same authors, only partially functional in pathogenic *Neisseria* species during glucose metabolism. Because they did not observe any activity of the Embden-Meyerhof-Parnas (EMP) pathway, the pentose–phosphate shunt could have accounted for the remaining 20% of glucose metabolization. In their experiments, carried out with dissolved O_2 control, acetic acid was the main byproduct of glucose neisserial metabolism. Fivefold acetic acid accumulation was observed when the cultivation was performed in a medium similar to the synthetic MC6 medium, but including yeast extract, whereas no extracellular formic, pyruvic, succinic and lactic acids were detected.

On the other hand, the use of yeast extract in our cultivation medium did not lead, in batch runs with dissolved O_2 control, to appreciable organic acid formation until 16 h. Under these conditions, the pH was kept near 7 without any control. From 16 h to the end of cultivation, dissolved O_2 control was switched off, the pH decreased to 5, and acetic acid formed at detectable levels (Fig. 2a). All other batch and fed-batch runs, with pH control at 6.5 or without pH and dissolved O_2 controls, exhibited abundant formation of acetic acid (Fig. 2b, f).

According to recent literature, *N. meningitidis* requires restricted carbon sources, only glucose, pyruvic and lactic acids being uptaken efficiently [21, 31]. Glucose was metabolized at pH 7.2 and 8.0 primarily via the Entner-Doudoroff pathway (about 80%), while the rest

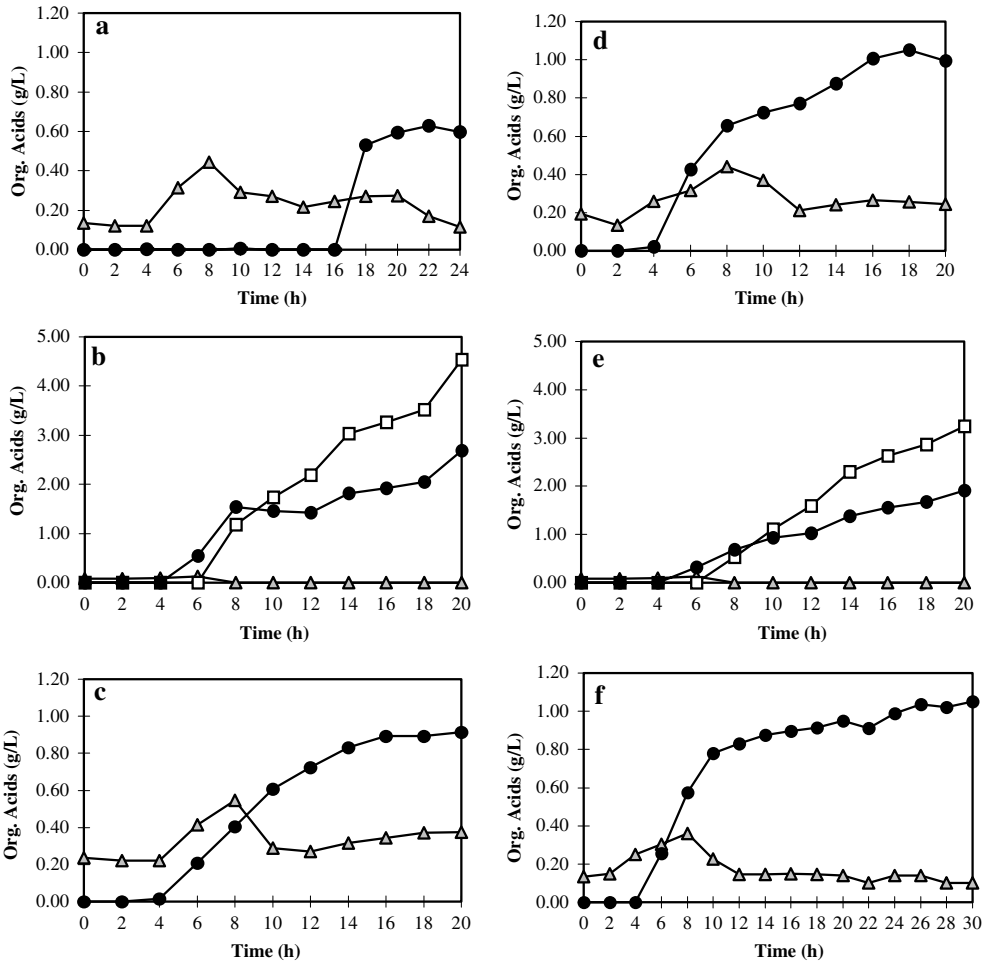

Fig. 2 Time behavior of organic acids. Acetic acid (*filled circle*), lactic acid (*open square*), succinic acid (*open triangle*). Batch experiments: **a** control of dissolved O_2 at 10% (run 1); **b** pH control at 6.5 (run 4); **c** without pH and dissolved O_2 controls (run 9); Fed-batch experiments: **d** without pH and dissolved O_2 controls (run 14); **e** pH control at 6.5 (run 18); **f** without pH and dissolved O_2 controls up to 30 h (run 20)

by the pentose–phosphate pathway (PPP). At pH above 7, most of the pyruvic acid and acetyl-CoA generated from glucose accumulated as acetic acid, only small quantities being oxidized by the tricarboxylic acid (TCA) cycle. However, at pH 6, the contribution of the PPP increased up to about 50% and additional acetyl-CoA was metabolized via the TCA cycle [24, 31]. In addition, a recent NMR and enzymatic study on the carbon metabolism in *N. meningitidis* has shown that growth on glucose, lactic acid and, especially, pyruvic acid, results in the excretion of significant quantities of acetic acid, via the phosphotransacetylase (PTA)-acetate kinase (ACK) pathway [21].

The small quantities of citric and malic acids detected in the present study can be associated to the release of these metabolites owing to the low or partial activity of the TCA cycle as well, whereas the appreciable levels of succinic acid detected since the beginning of cultivations performed without pH control could be due to its residual presence in the yeast extract-containing medium, as the result of metabolic activity during inoculum preparation and cultivation. On the contrary, only small quantities of succinic acid were detected with pH control (Fig. 2b, d). According to Pagliarulo et al., meningoccal gdhA, encoding the NADP-specific L-glutamate dehydrogenase (NADP-GDH), has two promoters: gdhA P1 and gdhA P2 [26]. Trans-activation of gdhA P2 was maximal in complex medium during the late log phase and was stimulated by glucose and inhibited by lactic acid, when the carbon source was present together with glutamic acid. Considering succinic acid a byproduct of aminoacid catabolism in the TCA cycle, this or a similar genomic system regulation could explain the increased production of this compound until the late exponential growth phase in cultivations performed without pH control and the small quantities of succinic acid detected in tests with pH control at 6.5.

Lactic acid release by *N. meningitidis* is not so well documented as that of acetic acid. Only one medical investigation was found that concerned the possibility of in vitro lactic acid formation [35], most of the remaining literature dealing with the consumption of lactic acid.

As far as the lactic acid consumption is concerned, there are three lactate-dehydrogenases (LDHs) responsible for the exclusive uptake of such a carbon source. Pyruvic acid produced by lactic acid oxidation is then used for gluconeogenesis, which is stimulated by lactic acid and inhibited by glucose [31].

Table 3 Values of α and β parameters of the model of Luedeking and Piret [22] relating the formation of acetic and lactic acids to cell growth

Cultivation conditions	Run	Cultivation time (h)	Points number	$\alpha^{a,b}$ Ac ($g_{Ac}\ g_X^{-1}$)	$\beta^{a,b}$ Ac ($g_{Ac}\ g_X^{-1}\ h^{-1}$)	r^2	Points number	β^{c} Lac ($g_{Lac}\ g_X^{-1}\ h^{-1}$)	Group
Batch with dissolved O_2 control at 10%	1	24	–	–	–	–	–	–	A
	2	24	–	–	–	–	–	–	
	3	24	–	–	–	–	–	–	
Pulse batch with control of pH at 6.5	4	20	4	0.277	0.059	0.973	9	0.158	B
	5	20	4	0.289	0.044	0.960	9	0.133	
	6	20	4	0.238	0.065	0.967	7	0.144	
Batch without controls of pH and dissolved O_2	7	20	6	0.105	0.042	0.920	–	–	C
	8	20	5	0.127	0.052	0.982	–	–	
	9	20	5	0.109	0.036	0.976	–	–	
Fed-batch	10	20	4	0.130	0.056	0.987	–	–	D
	11	20	5	0.172	0.049	0.979	–	–	
Fed-batch	12	20	5	0.140	0.026	0.969	–	–	E
	13	20	6	0.119	0.037	0.991	–	–	
Fed-batch	14	20	5	0.132	0.024	0.991	–	–	F
	15	20	4	0.139	0.038	0.976	–	–	
Partial fed-batch[d]	16	20	5	0.112	0.025	0.985	–	–	G
	17	20	4	0.112	0.047	0.951	–	–	
Partial fed-batch with pH control at 6.5[d]	18	20	6	0.219	0.062	0.964	7	0.133	H
Fed-batch with pH control at 6.5	19	20	5	0.274	0.079	0.983	9	0.159	I
Partial fed-batch[e]	20	30	4	0.133	0.034	0.972	–	–	J

[a]Runs 7–17, 20: $v_{Ac} = 0.128\mu + 0.039$, $r^2 = 0.824$; $F_{cal} = 2.12 < F_{tab} = 2.14$.
[b]Runs 4–6, 18, 19: $v_{Ac} = 0.254\mu + 0.060$; $r^2 = 0.880$; $F_{cal} = 3.02 < F_{tab} = 3.18$
[c]Runs 4–6, 18, 19: $v_{Lac} = 0.146$; $t_{cal} = 0.074 < t_{tab} = 2.353$
[d]Fed-batch between 10 and 20 h
[e]Fed-batch between 10 and 30 h

However, to the best of our knowledge, there is no clear evidence in the literature either of the existence of the glycolytic pathway or of lactic acid formation by it. Based on genomic data on *Neisseria gonorrhoeae* and *N. meningitidis* A and B, Smith et al. [31] stressed that these bacteria have all the enzymes for complete TCA cycle as well as for complete glycolysis/gluconeogenesis.

Tettlelin et al. [34] have recently suggested that a crucial factor in the commensal and pathogenic behavior of *N. meningitidis* B (MC58) could be its capacity to obtain and synthesize nutrients essential for its survival. These authors, who presented the genome of this strain and its functions, proposed that degradation of glucose, serine, proline, glycine, acetic, gluconic, glutamic, lactic, malic, oxaloacetic and pyruvic acids would be accomplished by an intact TCA cycle as well as by PPP and EDP. This bacterium would then be able to adapt glucose channeling through the three glycolytic routes depending on pH variations in the medium. Moreover, investigation on the metabolism of *N. gonorrhoeae* demonstrated that cells grown at pH 6.0 assimilated twice glucose than cells grown at pH 8.0 [23, 24].

Assuming for *N. meningitidis* a facilitated transport mechanism mediated by a glucose transporter located on the membrane and acting in the direction of the proton concentration gradient [7], the pH control at 6.5 in some of the runs performed in this work could have also favored the transport of glucose to the cell with respect to fermentations at higher pH, and the consequent carbon overflow upon the TCA cycle could have additionally stimulated the formation of organic acids.

Batch and fed-batch runs performed in this study with pH control showed, besides acetic acid, abundant formation of lactic acid as well (Fig. 2b, e). According to the model of Luedeking and Piret [22], the formation of lactic acid is non-growth associated, whereas the acetic acid formation is partially growth associated (Eq. 1). In case these metabolic features would depend on each other or be sequential, we could expect that equations describing them would have been linearly dependent, but they were not. Concluding, the present results suggest that acetic and lactic acid formations could have been the result of different pathways.

Analyzing the earlier information, it seems there be a contradiction: if lactic acid is one of the main carbon sources for *N. meningitidis*, why would this bacterium produce lactic acid as well?

Many of the studies dealing with the consumption of lactic acid by *N. meningitidis* are also worried about its relationship with virulence determinants. Interest is increasing in how bacteria behave and produce virulence determinants within the infected host. There are three main aspects related to this process: observations on the bacteria themselves, recognition of host factors that affect them and investigation of metabolic interactions between these. The first aspect is relatively easy to

investigate and attracts much interest. The second and third are difficult to handle and hence understudied [30]. In the case of lactic acid consumption by *N. meningitidis*, the three LDHs are also involved in bacteria virulence. In addition, lactic acid stimulates sialylation of lipopolysaccharide (LPS) by host-derived cytidine 5′-monophospho-*N*-acetyl neuraminic acid [31].

In *N. meningitidis* B, a major mediator of complement resistance is the polysaccharide capsule. This is composed by sialic acid, which is predicted to be synthesized from *N*-acetyl mannosamine and phosphoenol pyruvate (PEP), a reaction catalyzed by the enzyme SiaC, encoded on the horizontally acquired capsule biosyntesis locus. The PEP can be formed from glucose by a circuitous route via the EDP or directly from lactic acid after conversion to pyruvic acid, thus providing a potential connection between lactic acid availability and resistance against complement-mediated lysis. In addition, during meningococcal infection, acquisition of lactic acid may enhance meningococcal growth. In the cerebral-spinal fluid, where there are a few complement components, lactic acid contributes to bacterial growth, whereas available lactic acid contributes to resistance against complement during bacterial infection [10]. Investigating a *N. meningitidis* B mutant defective for the uptake of lactic acid, it was also demonstrated that the lactic acid is an important energy source for the bacterium during colonization and is necessary for its growth in nasopharyngeal tissue [10]. Besides, it was proposed, as the likeliest cause for the increased adhesion, the more extensive sialylation of the wild-type LPS with respect to that of the mutant, which may inhibit bacterial association with epithelial cells [9]. Moreover, meningococcal infection and disease show a pronounced seasonal variation, with the highest rates being observed during winter. There is also an association between influenza and invasive meningococcal infection. This could be explained with viral infection increasing the presence of activated phagocytic cells at the epithelial surface, thereby increasing local lactic acid concentrations. Lactic acid could then be used as a substrate for meningococcal growth and promote resistance to complement-mediated lysis, favoring survival of the bacterium in the blood-stream and increasing the likelihood that infection will lead to disease [9].

Despite the present work was performed with a different strain, the collected results and the related literature suggest that the production of lactic acid by *N. meningitidis* would constitute a strategy for colonization and resistance to complement-mediated lysis. Looking at a faraway hypothesis, lactic acid production by part of bacterial population could be acting as a kind of signal for the rest initiating the invasion and infection.

With regard to the polysaccharide production, because a pH decrease notoriously affects the dissociation equilibrium of weak acids, our cultivations performed without pH or dissolved O_2 controls (runs 7–9, 14–17, 20) exhibited the lowest formation of acetic acid. As a consequence, most values of P_f and $Y_{P/X}$ were higher than those obtained with pH control (runs 4–6, 18, 19), contrary to the generalized opinion that neutralization of inhibiting organic acids should anyhow enhance the formation of fermentation products. Contrary to the above interpretation, these results, although statistically insufficient, suggest the occurrence of some inhibiting or competitive effect exerted by acetic or lactic acid on polysaccharide formation, with particular concern to the abundant formation of lactic acid in experiments with pH control at 6.5.

Finally, even the formation of polysaccharide may be related to the way of glucose consumption and metabolization. Blacklow and Warren, investigating *N. meningitis* serogroup C, were able to concentrate up to 125 times, with respect to the original extract, an enzyme catalyzing the condensation of *N*-acetyl-mannosamine with PEP, leading to *N*-acetylneuraminic acid (polysaccharide monomer) and inorganic phosphorus [5], whereas the metabolic synthesis of *N*-acetyl-mannosamine from glucose was successively explained by Champe and Harvey [8]. According to these findings, a logarithmic relationship between glucose and polysaccharide concentrations along the cultivation time has recently been proposed [4], which is consistent with the abundant release of acetic and lactic acids as well as with the significant fraction of consumed glucose observed at pH 6.5; therefore, we can conclude that pH control is not recommended for the industrial set-up of the process.

Conclusions

The present work relates for the first time, to the best of our knowledge, the kinetics of organic acids formation to cell growth during cultivations of *N. meningitidis* serogroup C under different conditions. The coefficients α and β for the formation of acetic and lactic acids were estimated according to the Luedeking and Piret model. Acetic acid formation was partially growth-associated except when dissolved O_2 level was controlled at 10%, although its largest formation occurred when pH was controlled at 6.5. This last condition was the only one, among those investigated, that exhibited non-growth associated lactic acid formation. These results suggest that *N. meningitidis* could preferably employ distinct metabolic pathways according to pH. In addition, no improvements of final polysaccharide concentration and yield of polysaccharide on cell mass were observed with the fed-batch process when compared with the batch one, and the highest polysaccharide productions were obtained without any pH or dissolved oxygen controls.

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