Outer membrane vesicles (OMV) production of *Neisseria meningitidis* serogroup B in batch process

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A B S T R A C T

Serogroup B outer membrane vesicles (OMV) with iron regulated proteins (IRP) from *Neisseria meningitidis* constitute the antigen for the vaccine against the disease caused by this bacterium. Aiming to enhance final OMV concentration, seven batch experiments were carried out under four different conditions: (i) with original Catlin medium; (ii) with original Catlin medium and lactate and amino acids pulse at the 6th cultivation hour; (iii) with Catlin medium with double initial concentrations of lactate and amino acids and (iv) Catlin medium without glycerol and with double initial concentrations of lactate and amino acids. The cultivation experiments were carried out in a 7-L bioreactor under the following conditions: 36 °C, 0.5 atm, overlay air 1 L/min, agitation: 250–850 rpm, and O_2 control at 10%, 20 h. After lactate and amino acids exhaustion, cell growth reached stationary phase and a significant release increase of OMV was observed. According to the Luedeking & Piret model, OMV liberation is non-growth associated. Glycerol was not consumed during cultivation. The maximum OMV concentration value attained was 162 mg/L with correspondent productivity of 8.1 mg/(L h) employing Catlin medium with double initial concentrations of lactate and amino acids. The obtained OMV satisfied constitution and protein pattern criteria and were suitable for vaccine production.

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1. Introduction

Meningitidis and sepsis caused by serogroup B meningococcus are two severe diseases that continue to cause significant mortality [1,2].

Five major pathogenic serogroups have been identified on the basis of the chemical composition of the bacterial capsule (A, B, C, Y and W135) [3–5].

However, the capsular vaccine approach is not suitable for strains of serogroup B since that polysaccharide capsule has a structural homology to human embryonic neural tissue [6]. Thus, outer membrane proteins or outer membrane vesicles (OMV)-based vaccines were tested extensively in clinical trials [7].

An alternative approach to vaccine development is based on surface-exposed proteins contained in outer membrane vesicles [4,8,9]. OMV are released from the outer membrane of Gram negative bacteria. They consist of a phospholipid (PL) bilayer containing outer membrane proteins, lipopolysacchharide (LPS) and periplasmic constituents [10]. These vesicles are made up of five major proteins. Besides, there is the protein NadA and, depending on the conditions of cultivation, the iron regulated proteins (IRP) [11–13].

Furthermore, it is worth mentioning that OMV are also employed as carriers of polysaccharides in conjugated vaccines against *Haemophilus influenzae* and in vaccines against pneumonia [14,15]. A common antimeningococcal vaccine project against meningitis B and C had proposed a vaccine containing outer membrane vesicles (OMV) from *Neisseria meningitidis* B expressing iron regulated proteins (IRP) from a strain with high incidence in Brazil (N 44/89). The lipooligosaccharide (LOS endotoxin) of OMV is high toxic. However residual LOS amounts are needed to maintain vesicle structure and adjuvate the immune response.

Many studies have been carried out previously on other aspects of vaccine development, such as: the production process of *N. meningitidis* C [16–18]; the evaluation of the importance of a second serogroup B strain as vaccine component [19]; the obtainment of vesicles with appropriate characteristics (with IRP expression and with low level of LOS) [20,21]; and the conjugation process

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Nomenclature

O.D.	optical density
P_{max}	maximum OMV concentration (mg/L)
$Prod_P$	OMV productivity (mg/(L h))
$Prod_X$	biomass productivity (g/(L h))
t	cultivation time (h)
X_{max}	maximum cell concentration (g/L)
$Y_{X/S}$	yield factor biomass/lactate (g/g)
$Y_{P/X}$	yield factor OMV/biomass (mg/g)
α	growth associated coefficient (g/g)
β	non-growth associated coefficient (g/(g h))
μ_P	specific product formation rate (g/(g h))
μ_X	specific growth rate (h^{-1})

of *N. meningitidis* C polysaccharide with *N. meningitidis* B OMV [22,23].

The objective of this study was to investigate the *N. meningitidis* serogroup B for bacterial growth, consumption of carbon and nitrogen as substrates, OMV production kinetics, and the composition and morphology of the released OMV. The study also aimed the increasing of OMV yield and the employment of the generated data for further experiments relative to the development and scaling up of the vaccine production process.

2. Materials and methods

2.1. Process conditions

The inoculum of *N. meningitidis* B strain N44/89 (Instituto Adolpho Lutz, São Paulo, Brazil) was prepared according to Gotschlich et al. [24]. The inoculum, Catlin medium without iron supplementation and 7-L bioreactor preparation were described in previous work [25].

2.2. Analytical methods

Cell concentration was expressed as optical density at 540 nm and dry biomass weight per liter (g/L) after centrifugation of a known-volume sample at 3220 × g for 30 min, followed by pellet drying at 60 °C for 48 h. Glycerol concentration measurement [26] was based on oxidation of glycerol by sodium periodate. The formic acid generated was titrated with a NaOH solution (0.125 N) and the volume consumed corresponded to the glycerol concentration. Glycerol concentrations were also confirmed by HPLC, model 10AVP (Shimadzu, Kyoto, Japan) using an HPX-87H column (Bio Rad, Hercules, CA, USA) after dilution of samples (1:5). A 5.0 mM sulfuric acid solution was used as mobile phase under

flow rate of 0.6 mL/min. Lactate concentrations were determined employing an automatic enzymatic analyzer (Yellow Spring, model YSI 2700 Select, Yellow Springs, OH, USA). OMV were separated from supernatant cultivation after ultracentrifugation (Beckman, L8-M Ultracentrifuge, Palo Alto, CA, USA) of 50 mL samples at 30,000 rpm for 3 h. The obtained OMV were resuspended in 0.5 mL of 0.02% sodium azide. The amino acids concentrations were determined by HPLC, model 10AVP (Shimadzu, Kyoto, Japan) employing Ultrasphere C-18 column (Beckman, Palo Alto, CA, USA). Protein concentrations in the OMV resupensions were estimated by Lowry's method [27]. In order to verify IRP presence electrophoresis method was employed [28]. OMV were separated by SDS-PAGE (10% acrylamide/bisacrylamide gel) and the gel was stained with 0.1% Coomassie blue. The expression of IRP in the fractionated OMV extracts was estimated by the presence of 70–108 kDa bands [29]. For electronic microscopy, the negative contrast technique was employed. An OMV suspension contained in 15 μL of PBS, pH 7.2 was applied onto a parlodium/carbon coated 300 meshes copper grids during 2 minutes. The excessive fluid was removed from the grids and negative staining was carried out employing phosphotungstic acid 2%, pH 7.2 during 10 seconds. The grids were then examined under a transmission electronic microscope LEO 906E (Zeiss, Germany) operated at 80 kV with digital image capture system coupled.

3. Results

The main results of the batch tests are summarized in Table 1. All the experiments were carried out without iron supplementation. The experiments were classified in four series, as shown in Table 1 – *Series A*: original Catlin medium; *Series B*: original Catlin medium with lactate and amino acids pulsed at 6th hour of cultivation; *Series C*: Catlin medium with double initial concentrations of lactate and amino acids; *Series D*: Catlin medium without glycerol and with double initial concentrations of lactate and amino acids.

Typical growth curves of runs 1–7 are shown in Fig. 1a and the corresponding produced OMV in Fig. 1b. The behavior of pH variation and glycerol concentrations during cultivation are presented in Fig. 1f and e, respectively. The lactate and L-glutamic acid consumptions are shown in Fig. 1c and d, respectively. From these behaviors, it is evident that substrate consumption exerted remarkable influence on growth kinetics and OMV production. The analysis of the related dry mass and optical density indicated an average value of 0.46 g/L for each unit of O.D. (SD 0.06). This coefficient was employed for estimating dry biomass values from the O.D. values and for calculating μ_P.

According to the kinetics parameters presented in Table 1, the assays of Series A and B (original Catlin medium and original Catlin medium with lactate and amino acids pulse at the 6th cultivation hour, respectively) presented similar values of OMV

Table 1

Values of maximum cell concentration (X_{max}), maximum OMV concentration (P_{max}), biomass productivity ($Prod_X$), and OMV productivity ($Prod_P$), yield factor biomass/lactate ($Y_{X/S}$), OMV/biomass ($Y_{P/X}$), specific product formation rate (μ_P) and non-growth associated empirical constant (β) for the experiments 1–7.

Series	Run number	Stationary phase beginning (h)	X_{max} (g/L)	P_{max} (mg/L)	$Prod_X$ (g/(L h))	$Prod_P$ (mg/(L h))	$Y_{X/S}$ (g/g)	$Y_{P/X}$ (mg/g)	$\beta = \mu_P$ (g/(g h))
A	1	6	0.58	55.2	0.10	3.06	0.24	95.4	2.63
	2	6	0.57	44.2	0.09	2.21	0.23	78.1	1.11
B	3	10	0.98	59.4	0.10	2.97	0.43	60.8	4.28
	4	10	1.39	65.9	0.14	3.86	0.61	47.3	8.99
C	5	10	1.67	162	0.17	8.12	0.78	97.3	22.41
	6	9	1.22	157	0.14	7.85	0.47	129	24.51
D	7	12	2.27	121	0.19	6.05	0.38	53.3	16.06

A – original Catlin medium; B – original Catlin medium with lactate and amino acids pulsed at 6th hour of cultivation; C – Catlin medium with double initial concentrations of lactate and amino acids; D – Catlin medium without glycerol and with double initial concentrations of lactate and amino acids.
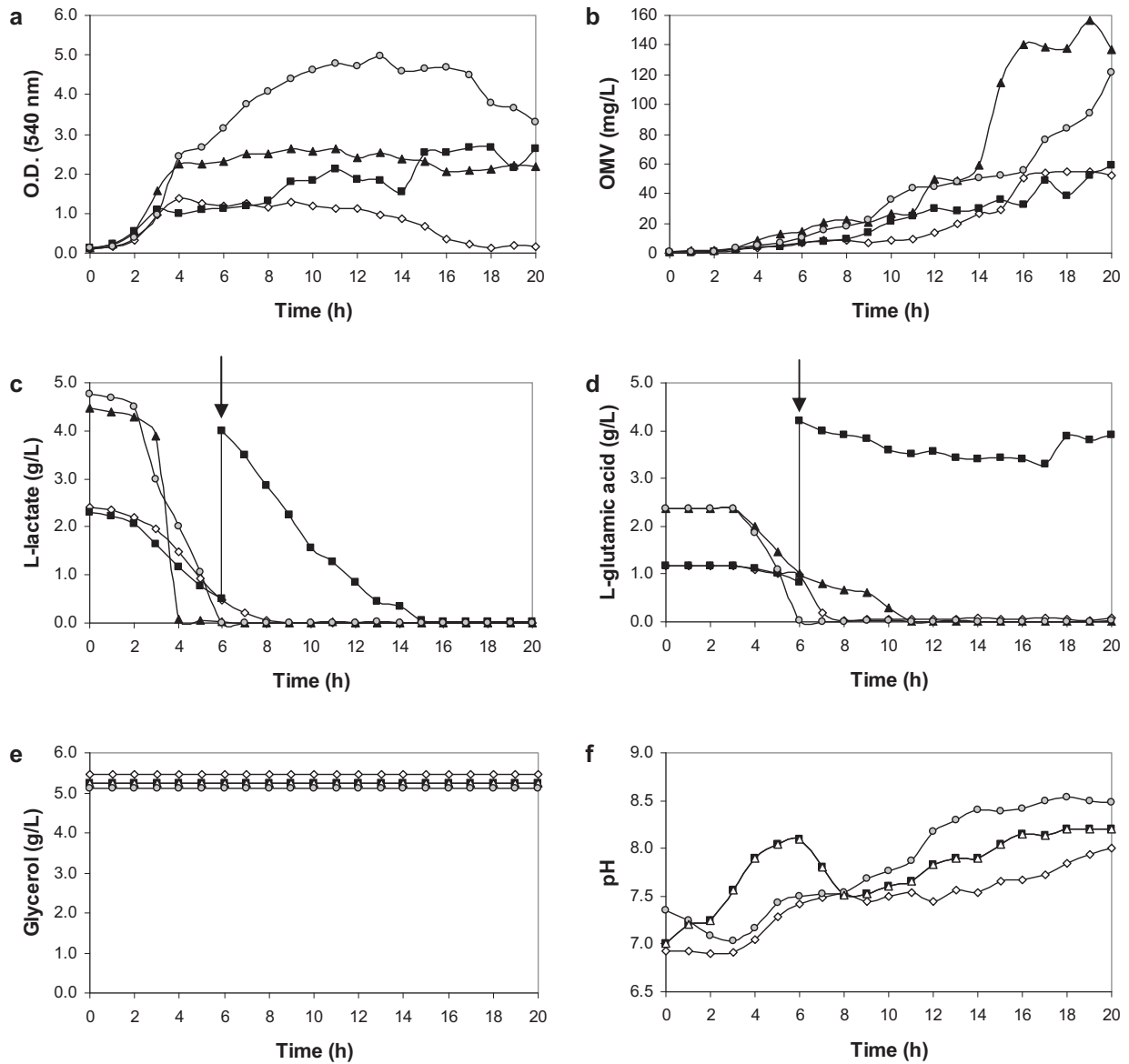
Fig. 1. Kinetics curves of bacterial growth (a), OMV production (b), L-lactate consumption (c), L-glutamic acid consumption (d), glycerol (e), and pH (f). *Run* 1 (◇): original Catlin medium; *run* 3 (■): original Catlin medium with lactate- and amino acids pulsed at 6th cultivation hour; *run* 6 (▲): Catlin medium with double initial concentrations of lactate and amino acids; and *run* 7 (○): Catlin medium without glycerol and with double initial concentrations of lactate and amino acids.

maximum concentration (P_{max}) and OMV productivity ($Prod_P$) for these two groups. However they were the lowest ones considering the overall experimental results. Series A and B presented, respectively, average values of $P_{max} = 56.2$ mg/L and $Prod_P = 3.03$ mg/(L h).

On the other hand, Series C experiments (Catlin medium, double initial concentrations of lactate and amino acids) presented the highest values of these parameters, namely $P_{max} = 162$ g/L and $Prod_P = 8.1$ mg/(L h). In all assays, glycerol was not consumed
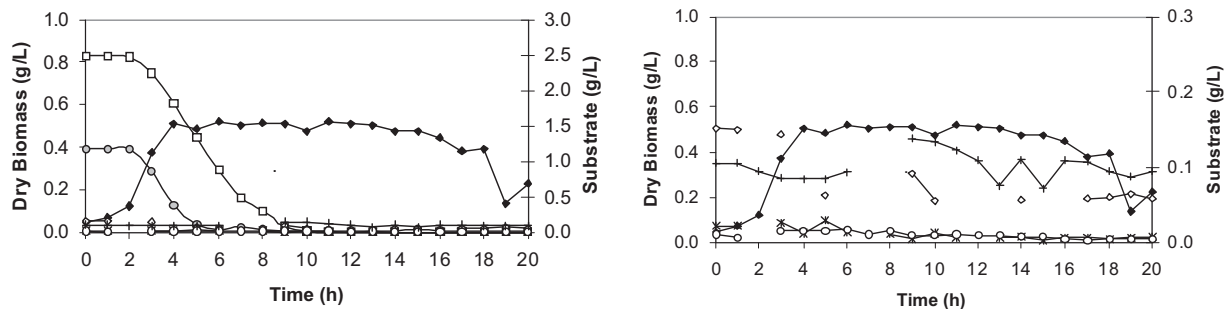

Fig. 2. Kinetics curves for Series A (original Catlin medium): dry biomass (♦), lactate (□), L-glutamic acid (⬤), L-glicine (◇), L-serine (×), L-cisteine (○) and L-arginine (Ɩ). The figure on the right represents substrate scale zoom-out of the figure on the left in order to visualize better marginal amino acids consumption.

(Fig. 1e). In Series D (Catlin medium, without glycerol, double initial concentrations of lactate and amino acids), the values of $P_{max} = 121$ g/L and $Prod_P = 6.0$ mg/(L h) were slightly better than other those from Series C. The highest OMV concentrations were obtained in Series C (where initial glycerol concentration was maintained and the initial concentrations of amino acids and lactate doubled) (Fig. 1c and d, run 6). Glycerol was not consumed in cultivations [25], so it has no direct influence on the OMV production. A plausible hypothesis is that glycerol could be the mechanical protector of the OMV released to the cultivation medium. Lactate is the main limiting carbon source while L-glutamic acid is the main limiting nitrogen source (Figs. 1c, d and 2). L-Glutamic acid consumption contributes to ammonia formation and pH rising in the course of the cultivation (Fig. 1f). By employing the original Catlin medium (Series A) lactate concentration decreased to zero at the 8th cultivation hour. At this moment, the cultivation reached the stationary growth phase (Fig. 1a). Thus, its consumption is directly related to cell growth. From Series A experiments amino acids were analyzed in order to estimate the specific amino acid yield factor to conduct further assays (Fig. 2). Thus, the yield factor determined for L-glutamic acid was $Yx/glu = 0.5$ g/g. L-Glicine ($Yx/gli = 4.8$ g/g) and L-arginine ($Yx/arg = 28.3$ g/g) were not limiting, since they were left over at the end of cultivation. L-Serine ($Yx/ser = 32.1$ g/g) and L-cisteine. HCl ($Yx/cis = 78.4$ g/g) could be limiting despite their small consumption, since they were not left over at the end of cultivation. The overall approximate relationship of carbon/nitrogen was 9.1 g/g. Results obtained from Series B–D indicated that all amino acids were left over at the end of cultivation in these experiments (data not shown). Therefore, these results suggest that the original Catlin medium composition must be reformulated in order to enhance antigen production from the N. meningitidis serogroup B cultivations.

OMV were released after the stationary growth phase beginning and, in almost assays, when all lactate was consumed (Fig. 1b and c). In all assays, the electrophoresis patterns revealed the presence of class proteins (major proteins). Iron regulated proteins (IRP) and high molecular weight proteins (NadA) are observed (Fig. 3). In the electronic microscopy images obtained for Series A–D, the contour, tubular and spherical shapes, cited formerly by
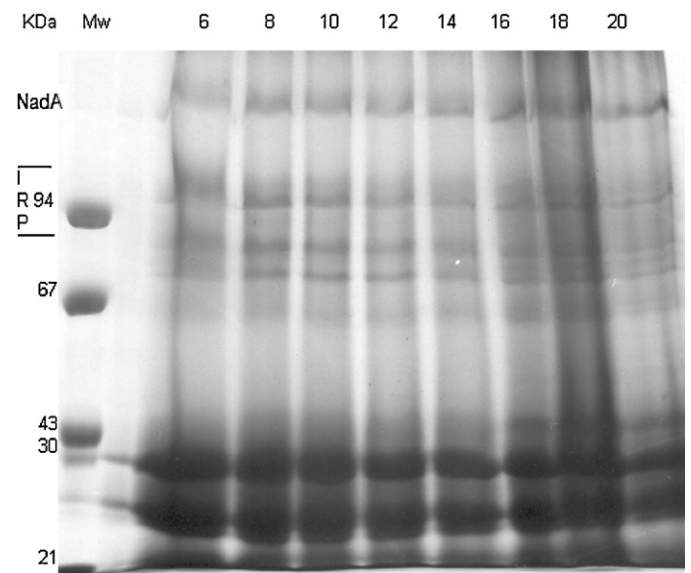

Fig. 3. SDS-PAGE gel electrophoresis of OMV samples obtained in experiment 5 of Series C (Catlin medium with double initial concentrations of lactate and amino acids). The columns numbered 6–20 correspond to the fractionated OMV extract from the corresponding cultivation hour. The representative bands of IRP expression appearing are between 70 and 108 kDa (indicated by arrows). The highest molecular weight bands indicate possible presence of NadA.

Devoe and Gilchrist [30], and the vesicle integrity were verified (Fig. 4).

4. Discussion

A kinetic correlation was established between cell growth and OMV production in cultivation of N. meningitidis serogroup B under different conditions employing lactate as the main carbon source. The growth of N. meningitidis requires pyruvate, or lactate, or glucose as the sole source of carbon and during cultivation in any of these carbon sources, secretion of acetate into the medium occurs
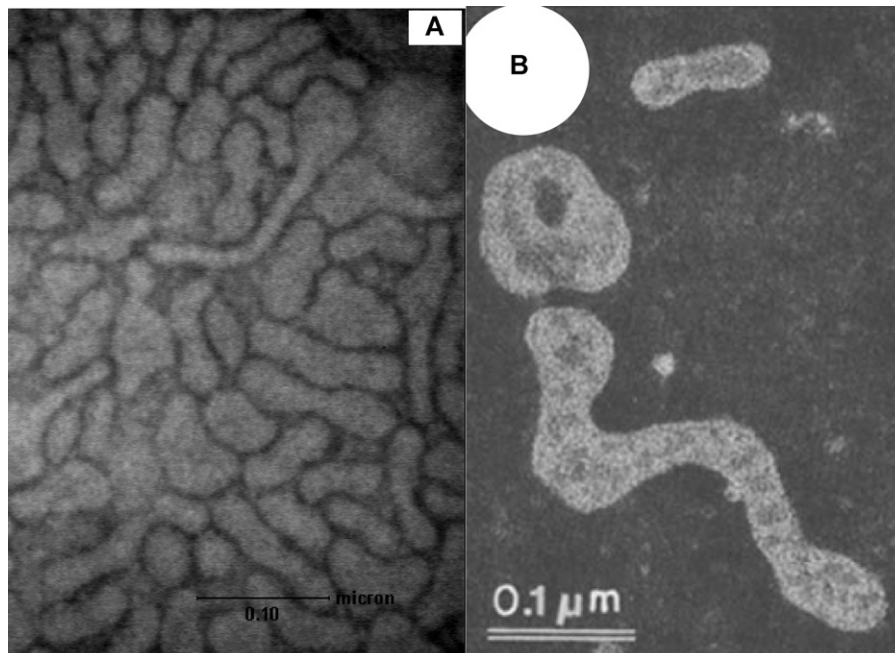

Fig. 4. (a) Electronic microscopy image of OMV shapes (tubular and spherical) of N. meningitidis B strain N 44/89 from experiment 5 of Series C (Catlin medium with double initial concentrations of lactate and amino acids) at 20th cultivation hour; (b) OMV shapes of N. meningitidis B SD1C strain reproduced from literature [30].

[31]. Employment of glucose can promote larger cell productivity according to a report by Fu et al. [32]. However, that study aimed mainly biomass generation and the OMV production was not investigated. They employed a synthetic medium (MC6), altering the original Catlin medium composition, with glucose as the main carbon source and iron supplementation. At the end of cultivation, they obtained almost 10 g/L of dry biomass. In such conditions, they observed that the main metabolic pathways for assimilation of the carbon source (glucose) would be Entner-Doudoroff (EDP), which would be responsible for about 80% of the consumption, and pentose-phosphate could have accounted for the remaining 20% of the glucose metabolized. Fu et al. [32] did not observe any activity of the Embden–Meyerhof–Parnas (EMP) pathway.

Recently Baart et al. [33,34] reported the modeling of *N. meningitidis* B metabolism at different specific growth rates in glucose cultivation medium. However, the authors did not present quantitative values for OMV production or the composition of their protein profile. The study described the influence of the growth rate of *N. meningitidis* on its macro-molecular composition and its metabolic activity, which was determined in chemostat cultures. In the applied range of growth rates, no significant changes in RNA content or in protein content with growth rate were observed in *N. meningitidis*. The DNA content of *N. meningitidis* was somewhat higher at the highest applied growth rate. The phospholipid and lipopolysaccharide contents in *N. meningitidis* varied with growth rate but no specific trends were identified. The cellular fatty acid composition and the amino acid composition did not vary significantly with growth rate. Additionally, the PorA content in the OMV was significantly lower at the highest growth rate. The metabolic fluxes at different growth rates were calculated using flux balance analysis. Errors in these calculations were detected using Monte Carlo Simulation. Thus the reliability of these calculated values of flux distribution could be specified, which are not reported for this type of analysis. The yield of biomass on the substrate ($Y(x/s)$) and the maintenance coefficient ($m(s)$) were determined as 0.44 ($\pm$0.04) g/g and 0.04 ($\pm$0.02) g/(g h), respectively. The growth associated energy requirement ($Y(x/ATP)$) and the non-growth associated ATP requirement for maintenance ($m(ATP)$) were estimated 0.13 ($\pm$0.04) mol/mol and 0.43 ($\pm$0.14) mol/mol h, respectively. These authors found the split ratio between the Entner–Doudoroff and the pentose phosphate pathways. The pathways utilizing glucose alone in *N. meningitidis*, had a minor effect on ATP formation rate but a major effect on the fluxes going through, for instance, the citric-acid cycle. Therefore, they presented flux values in ranges for the underdetermined parts of metabolic network rather than presenting single values, which is the more common practice in literature.

The studies aiming biomass or OMV production reported in previous literature and cited above were performed employing glucose as principal carbon source, instead lactate as in the present study. So no comparisons can be performed between them and the present one.

4.1. OMV production

The empirical expression proposed by Luedeking & Piret [35] was used for analysis of the main cultivation product. It relates the specific product formation rate (μ_P) with the specific growth rate of microorganism (μ_X) by the equation $\mu_P = \alpha \cdot \mu_X + \beta$. This equation, where α and β are empirical constants, indicates the existence of two mechanisms of production of the product. The first is associated with bacterial growth (represented by $\alpha \cdot \mu_X$) while the other is independent of the growth of microorganisms (represented by β) [36]. A computer program (*Logiciel du Lissage*), based on polynomial fit by the Spline method [37] was employed for OMV curve fitting and calculation of specific product formation rate. In the

present study, product formation is non-growth associated. The values of $\beta = \mu_P$ obtained for each assay are presented in Table 1. Series C assays presented the highest values of β, signifying the best cultivation condition among those studied for production of OMV.

This finding confirms that the strategy adopted for Series B assays (original Catlin medium with lactate and amino acids pulse at 6th cultivation hour) was less efficient than that adopted for Series C cultivations (Catlin medium with double initial concentrations of lactate and amino acids). The results showed that doubling the initial concentrations of lactate and amino acids in Series C assays did not promote any inhibitory effect in either growth or OMV production (Fig. 1a–d). On the contrary, it stimulated cell growth and OMV production. It is possible to speculate about the substrate storage capacity of cells. However, considering the severe iron restriction imposed on cultivation experiments, a hypothesis could be related with the larger residual quantities of iron present on doubling the initial lactate and amino acids concentrations in Series C experiments. If this limit on iron is less severe, small additional residual iron quantities could be used to stimulate cell growth kinetics and improve OMV production without compromising the appropriate protein pattern. This hypothesis is proposed to be studied in future experiments in order to further enhance Catlin medium composition.

4.2. Relationship between lactate consumption and OMV production

The growth of *N. meningitidis* requires pyruvate, or lactate, or glucose as the sole source of carbon [31]. As far as lactic acid consumption is concerned, there are three lactate-dehydrogenases (LDHs) responsible for the exclusive uptake of this carbon source. In the presence of NAD^+, the pyruvic acid produced by lactic acid oxidation is then used for gluconeogenesis, which is stimulated by lactic acid but inhibited by glucose. These three LDHs are also involved in bacteria virulence determinants [38]. In addition, an NMR and enzymatic study about carbon metabolism in *N. meningitidis* has shown that consumption of glucose, lactic acid and, especially, pyruvic acid, results in the excretion of significant amounts of acetic acid, via the phosphotransacetylase (PTA) acetate kinase (ACK) pathway [39]. Thus, the employ of lactate, which uptake is dependent to the LDHs activity and less associated to acetic acid formation, is most suitable for the culture of the *Neisseria meningitidis* serogroup B aiming at production of OMV for antigen vaccine. The OMV were released after the stationary phase beginning and, in almost assays, when all the lactate has been consumed (Fig. 1b and c). The preferential use of lactate as a carbon source agrees with the report of Tettelin et al. [40], who described the degradation of lactate by *N. meningitidis* B, its genome, and its functions. In addition, according to Pollard and Frasch [41] limiting the iron ion in Catlin medium is necessary to express the iron-regulated proteins (IRP).

4.3. OMV constitution and protein pattern

In all experiments, the OMV released contained IRP (Fig. 3) and NadA, a high molecular weight protein. The antigenic function of this protein was studied [8,42]; its presence could be considered a suitable complementary characteristic among the antigen properties needed for vaccine production.

It is worth of mention that prior to being formulated into the final product the OMV must be detoxified and analyzed for purity (chemical determination and pyrogenicity) and for potency. In this way, it is important to confirm whether the OMV obtained in production process satisfy the criteria of constitution and protein pattern and thereby their suitability as antigen for vaccine elaboration.

Satisfying these criteria, the images obtained of all the series investigated, the contour, tubular and spherical shapes, which were cited formerly by Devoe and Gilchrist [30], and the vesicles integrity were confirmed (Fig. 4).

5. Conclusion

The highest values of the maximum concentration of OMV, $Prod_P$, $Y_{P/X}$, and β were obtained in the experiments where the original Catlin medium without iron supplementation was formulated with double initial concentrations of lactate and amino acids and the original glycerol concentration maintained. The results indicated that lactate is the main source of carbon and the growth limiting factor. Results of amino acids analysis suggested that the original Catlin medium composition must be reformulated in order to enhance antigen production from *N. meningitidis* B cultivations. In all the experiments, glycerol was not consumed and could protect mechanically the released OMV. Further, the antigen (OMV) concentration in cultivation increased significantly during the stationary growth phase. In all the experiments, vesicle integrity was verified and the OMV released contained IRP. Thus, the OMV obtained satisfy the constitution and protein pattern criteria and are suitable for vaccine production. The cultivation medium composition, the effect of residual iron on growth and OMV production will be studied in future experiments.

Acknowledgments

References

[1] Giuliani MM, Adu-Bobie J, Comanducci M, Aricó B, Savino S, Santini L, et al. A universal vaccine for serogroup B meningococcus. Proc Natl Acad Sci USA 2006;103(29):10834–9.
[2] Sadarangani M, Pollard AJ. Serogroup B meningococcal vaccines–an unfinished story. Lancet Infect Dis 2010;10(2):112–24.
[3] Girard MP, Preziosi MP, Aguado MT, Kieny MP. A review of vaccine research and development: meningococcal disease. Vaccine 2006;24(22):4692–700.
[4] Pollard AJ, Moxon ER. The meningococcus tamed? Arch Dis Child 2002;87(1):13–7.
[5] Wang Q, Shao Z, Wang X, Gao Y, Li M, Xu L, et al. Genetic study of capsular switching between *Neisseria meningitidis* sequence type 7 serougroup A and C strains. Infect Immun 2010;78(9):3883–8.
[6] Holst J, Martin D, Arnold R, Huergo CC, Oster P, ÓHallahan J, et al. Properties and clinical performance of vaccines containing outer membrane vesicles from *Neisseria meningitidis*. Vaccine 2009;27(Suppl. 2):B3–12.
[7] Borrow R, Aaberge IS, Santos GF, Eudey TL, Oster P, Glennie A, et al. Interlaboratory standardization of the measurement of serum bactericidal activity by using human complement against meningococcal serogroup b, strain 44/76-SL, before and after vaccination with the Norwegian Menbvac outer membrane vesicle vaccine. Clin Diagn Lab Immunol 2005;12(8):970–6.
[8] Franzoso S, Mazzon C, Sztukowska M, Cecchini P, Kasic T, Capecchi B, et al. Human monocytes/macrophages are a target of *Neisseria meningitidis Adhesin* A (NadA). J Leukoc Biol 2008;83(5):1100–10.
[9] Yero D, Pajón R, Pérez Y, Fariñas M, Cobas K, Diaz D, et al. Identification by genomic immunization of a pool of DNA vaccine candidates that confer protective immunity in mice against *Neisseria meningitidis* serogroup B. Vaccine 2007;25(28):5175–88.
[10] Van de Waterbeemd B, Streefland M, van der Ley P, Zomer B, van Dijken H, Martens D, et al. Improved OMV vaccine against *Neisseria meningitidis* using genetically engineered strains and a detergent-free purification process. Vaccine 2010;28(30):4810–6.
[11] Frasch CE. Meningococcal vaccines: past, present and future. In: Cartwright K, editor. Meningococcal disease. Chichester, UK: John Wiley & Sons; 1995. p. 245–83.
[12] Rosenqvist E, Høiby EA, Wedege E, Kusecek B, Achtman M. The 5C protein of *Neisseria meningitidis* is highly immunogenic in humans and induces bactericidal antibodies. J Infect Dis 1993;167(5):1065–73.
[13] Sacchi CT, Gorla MC, de Lemos AP, Brandileone MC. Considerations on the use of *Neisseria meningitidis* class 5 proteins as meningococcal BC vaccine components. Vaccine 1995;13(1):112–8.
[14] Knuf M, Kowalzik F, Kieninger D. Comparative effects of carrier proteins on vaccine-induced immune response. Vaccine 2011;29(31):4881–90.
[15] Joshi VS, Bajaj IB, Survase SA, Singhal RS, Kennedy JF. Meningococcal polysaccharide vaccines: a review. Carbohydr Polym 2009;75(4):553–65.
[16] Baruque-Ramos J, Hiss H, Converti A, Gonçalves VM, Raw I. Accumulation of organic acids in cultivations of *Neisseria meningitidis* C. J Ind Microbiol Biotechnol 2006;33(10):869–77.
[17] Paz MF, Baruque-Ramos J, Hiss H, Leal MBB, Vicentin MA, Raw I. Polysaccharide production in batch process of *Neisseria meningitidis* serogroup C comparing Frantz, modified Frantz and Catlin 6 media. Braz J Microbiol 2003;34:27–32.
[18] van de Waterbeemd B, Streefland M, van Keulen L, van den Ijssel J, de Haan A, Eppink MH, et al. Identification and optimization of critical process parameters for the production of NOMV vaccine against *Neisseria meningitidis*. Vaccine 2012;30(24):3683–90.
[19] Corrêa AP. Formulation study of C anti-meningococcal polysaccharide vaccines, conjugated to B outer membrane vesicles of *Neisseria meningitidis*. Dissertation. São Paulo (SP): University of São Paulo; 2002.
[20] Fukasawa LO. Development of conjugated serogroup B/C anti-meningococcal vaccine. Thesis. São Paulo (SP): University of São Paulo; 2004.
[21] Byers HL, Campbell J, van Ulsen P, Tommassen J, Ward MA, Schultz-Knappe P, et al. Candidate verification of iron-regulated *Neisseria meningitidis* proteins using isotopic versions of tandem mass tags (TMT) and single reaction monitoring. J Proteomics 2009;73(2):231–9.
[22] Fukasawa LO, Schenkman RP, Perciani CT, Carneiro SM, Dias WO, Tanizaki MM. Optimization of the conjugation method for a serogroup B/C meningococcal vaccine. Biotechnol Appl Biochem 2006;45(3):141–6.
[23] Holmes JD, Martin D, Ramsay C, Ypma E, Oster P. Combined administration of serogroup B meningococcal vaccine and conjugated serogroup C meningococcal vaccine is safe and immunogenic in college students. Epidemiol Infect 2008;136(6):790–9.
[24] Gotschlich EC, Liu TY, Artenstein MD. Human immunity to the meningococcus: III. Preparation and immunochemical properties of the series A series B and series C meningococcal polysaccharides. J Exp Med 1969;129:1349–65.
[25] Santos S, Baruque-Ramos J, Tanizaki MM, Lebrun I, Schenkman RPF. Production of outer membrane vesicles (OMV) in batch cultivations of *Neisseria meningitidis* serogroup B. Braz J Microbiol 2006;37(4):488–93.
[26] American Oil Chemistry Society Glycerol. Method Ea 6-51.Official Methods and Recommended Practices of the American Oil Chemistry Society. 1987. p. 6–51.
[27] Lowry OH, Rosebrough NJ, Farr AL, Randall RH. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193(1):265–75.
[28] Sambrook J, Fritsch EF, Maniats T. Molecular cloning: a laboratory manual. 2nd ed. New York: Cold Spring Harbor Lab Press; 1989.
[29] Brandileone MC, Zanella RC, Vieira VS, Sacchi CT, Milagres LG, Frasch CE. Induction of iron regulated proteins during normal growth of *Neisseria meningitidis* in a chemically defined medium. Rev Inst Med Trop Sao Paulo 1994;36(4):301–10.
[30] Devoe IW, Gilchrist JE. Release of endotoxin in the form of cell wall blebs during in vitro growth of *Neisseria meningitidis*. J Exp Med 1973;138(5):1156–67.
[31] Baart GJ, Zomer B, de Haan A, van de Pol LA, Beuvery EC, Tramper J, et al. Modeling *Neisseria meningitidis* metabolism: from genome to metabolic fluxes. Genome Biol 2007;8(7):R136.
[32] Fu J, Bailey J, King JJ, Parker CB, Robinett RS, Kolodin DG, et al. Recent advances in the large scale fermentation of *Neisseria meningitidis* group B for the production of an outer membrane protein complex. Biotechnology 1995;13(2):170–4.
[33] Baart GJ, de Jong G, Philippi M, vannt Riet K, van der Pol LA, Beuvery EC, et al. Scale-up for bulk production of vaccine against meningococcal disease. Vaccine 2007;25(34):6399–408.
[34] Baart GJ, Willemsen M, Khatami E, de Haan A, Zomer B, Beuvery EC, et al. Modeling *Neisseria meningitidis* B metabolism at different specific growth rates. Biotechnol Bioeng 2008;101(5):1022–35.
[35] Luedeking R, Piret EL. A kinetic study of the latic acid fermentation. Batch process at controlled pH. Batch process at controlled pH. J Bioch Microb Techn Eng 1959;1(4):393–412.
[36] Hiss H. Cinética de processos fermentativos. In: Schmidell W, Lima UA, Aquarone E, Borzani W, editors. Engenharia Bioquímica – Biotecnologia Industrial. São Paulo: Edgard Blücher; 2001.
[37] Kahaner D, Moler C, Nash S. Numerical methods and software: computer methods for mathematical computations. Prentice Hall: Englewood Cliffs; 1989.
[38] Smith H, Yates EA, Cole JA, Parsons NJ. Lactate stimulation of gonococcal metabolism in media containing glucose: mechanism, impact on pathogenicity, and wider implications for other pathogens. Infect Immun 2001;69(11):6565–72.
[39] Leighton MP, Kelly DJ, Williamson MP, An Shaw JG. NMR and enzyme study of the carbon metabolism of *Neisseria meningitidis*. Microbiology 2001;147:1473–82.
[40] Tettelin H, Saunders NJ, Heidelberg J, Jeffries AC, Nelson KE, Eisen JA, et al. Complete genome sequence of *Neisseria meningitidis* serogroup B strain MC58. Science 2000;287(5459):1809–15.
[41] Pollard AJ, Frasch C. Development of natural immunity of *Neisseria meningitidis*. Vaccine 2001;19(11–12):1327–46.
[42] Capecchi B, Adu-Bobie J, Di Marcello F, Ciucchi L, Masignani V, Taddei A, et al. *Neisseria meningitidis* NadA is a new invasin which promotes bacterial adhesion to and penetration into human epithelial cells. Mol Microbiol 2005;55(3):687–98.