EVALUATION OF CARBON SOURCE CONSUMPTION AND ORGANIC ACID PRODUCTION BY *NEISSERIA LACTAMICA*

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ABSTRACT

The *Neisseria* genus stands out for its scalable production of outer membrane vesicles (OMVs). *Neisseria meningitidis* and *Neisseria lactamica* produce OMVs with significant immunogenicity. *N. meningitidis* OMVs play a crucial role in manufacturing meningococcal meningitis vaccines, particularly as adjuvants for serogroup B vaccines. *N. lactamica* OMVs offer similar immunological benefits with higher OMV yields and without pathogenicity, making them advantageous. However, the lack of investigation on a proper medium composition for *N. lactamica* impacts further developments. Our team has relied on media with lactic acid as carbon source, yet, recent medium formulations for *N. meningitidis* use glucose as a carbon source and media developed for the growth of other *Neisseria* genus bacteria combine both lactic acid and glucose as a carbon source. Our research aims to optimize cultivation parameters and conditions to maximize *N. lactamica* OMV production. In face of these questions, the present study utilized three media varying only on their carbon source composition. Lactic acid and glucose intake was analyzed separately and combined as well as organic acid production. Our results showed that *N. lactamica* is fully able to consume glucose, however the medium containing both carbon sources yielded lower cell density and directed the metabolism to a fermentation route with the production of malic and acetic acids.

Keywords: Medium formulation. OMVs. Neisseria. Carbon source.

1 INTRODUCTION

Neisseria is a genus of Gram-negative bacteria that exclusively inhabit humans. Among the species, *Neisseria meningitidis* and *Neisseria lactamica* hold prominence. *N. meningitidis* is a leading cause of bacterial meningitis, whereas *N. lactamica* is considered a commensal species[1]. Both species are capable of releasing outer membrane vesicles (OMVs), *Neisseria* OMVs possess high immunogenicity and are capable of modulating immune responses against pathogenic agents. Currently, *N. meningitidis* serves as the foundation for the development of two OMV-based vaccines, TRUMENBA and BEXSERO, both of which are licensed for human use. These vaccines effectively modulate immune responses specifically targeting serogroup B strains of *N. meningitidis*[2,3].

A significant comparative study conducted in a bioreactor has demonstrated that the yield of OMVs produced by *N. lactamica* (156 mg/L) surpasses that of *N. meningitidis* (27 mg/L) when utilizing yeast extract in the medium composition[4]. This underscores the potential of *N. lactamica* as a robust platform for the production of OMVs, serving as pivotal nanobiotechnological inputs in pharmaceutical applications. Notably, besides its enhanced OMV production capacity, *N. lactamica* lacks pathogenicity, thereby mitigating manipulation hazards and production costs[5]. However, for industrial-scale OMV production, the cultivation conditions of the microorganism must be established.

The literature regarding the use of culture media for cultivation of *N. lactamica* is sparse, with the most notable works having produced a defined agar medium for *Neisseria* (NEDA). However, tests conducted with this medium revealed that many components present in NEDA were not strictly utilized by the bacterium[6]. Furthermore, NEDA was not designed to serve as a minimal medium for the growth of *N. lactamica*, but rather as a general medium for all *Neisseria* species. Notably, a defined minimal medium developed for *N. meningitidis*, known as Meningococcal Defined Agar (MCDA), did not promote *N. lactamica* growth[6,7,8], and it was necessary the inclusion of yeast extract in the medium for bacterial growth[4,6]. Nevertheless, semi-solid culture media are not suitable for obtaining large quantities of OMVs. The development of a proper liquid medium for the growth of *N. lactamica* has been stagnant. The current MCDA medium uses only lactic acid as its major carbon source, while the causes for the omission of glucose from the final medium is not known, it is possible that lactic acid might have been originally used in the medium to identify colonies of *Neisseria* species harvested from biological sites. However, recent evidence shows that a glucose containing medium might be possible, or even desirable[9,10]. In face of these challenges, it is necessary to update *N. lactamica* methods of cultivation based on new evidence for further insights on this microorganism.

2 MATERIAL & METHODS

For this study, the microorganism *N. lactamica*, strain N.799/98, obtained from the culture bank of the Bacteriology Section of the Adolfo Lutz Institute, was used. The material was aliquoted into cryotubes, and frozen in liquid nitrogen. The working cell bank (WCB) was prepared from a cryotube which underwent a passage in Müller-Hinton agar medium[11] for a period of 18 h under an atmosphere of 6-8% CO_2 at 37°C. The colonies were resuspended in modified Catlin medium (MCM)[6,8,12] and finally, 30% glycerol was added. The suspension was then aliquoted into cryotubes and stored at -80ºC. The MCM composition is based on the meningococcal defined agar medium (MCDA), but with double the amino acid concentration and addition of 2 g/L of ultrafiltered yeast extract, Difco BD (Franklin Lakes, New Jersey, USA)[6]. To test the competence of *N. lactamica* in metabolizing each carbon

source, we have produced MCM with only lactic acid (MCL), only glucose (MCG) and with both lactic acid and glucose (MCLG) as carbon sources (Table 1).

Table 1 Media components

Compound	MCL (g/L)	MCG (g/L)	MCLG (g/L)
Sodium Lactate	15	-	3.860
Glucose	-	5.455	2.727

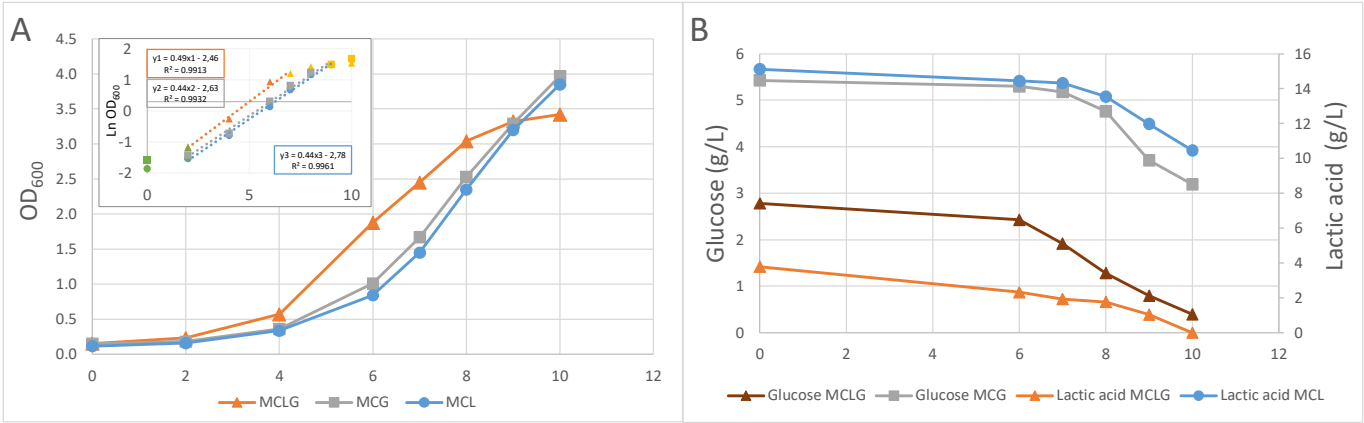
*Common components:

Compound	Concentration (g/L)
Sodium chloride	5.844
Ammonium chloride	0.401
Potassium chloride	0.186
Sodium citrate	0.568
Calcium chloride	0.0279
Magnesium sulfate	0.301
Manganese sulfate	0.0003
Sodium phosphate dibasic	1.062
Potassium phosphate monobasic	0.171
Glycerol	5.010
L-Glutamic acid	2.36
L-Arginine	0.174
Glycine	0.302
L-Serine	0.042
L-Cysteine	0.015
Yeast extract ultrafiltered	2.0

For cultivations, the content of one cryotube of WCB was suspended in a Tunair flask containing 100 mL of either MCL, MCG or MCLG. The suspension was maintained on a rotary shaker at 200 rpm and 37°C for 10 h and samples were collected. Cell density was measured on a spectrophotometer at 600 nm (WPA biowave CO8000, Cambridge, UK). After centrifugation at 10,000 rpm (15,417 x g) 10ºC for 10 min, the supernatant was analyzed using high-performance liquid chromatography (HPLC) (Agilent Technologies, model 1260 Infinity). The concentration of glycerol, glucose and organic acids was evaluated using the Aminex HPX-87H column (BioRad, California, USA), with a mobile phase of 5 mM H_2SO_4, and detection at 210 nm for organic acids and refractive index for glucose and glycerol.

3 RESULTS & DISCUSSION

In the present study, we have assessed carbon source consumption and organic acid formation by *N. lactamica* under three different compositions of medium. The MCL enabled growth until 9 h cultivation, reaching optical density 600 nm (OD_{600}) of 3.85 and μmax of 0.44 h^{-1} (**Figure 1A**). HPLC analysis showed that the lactic acid was consumed, but not depleted (**Figure 1B**). There also has been accumulation of acetic and malic acids, respectively 3.0 and 1.5 g/L, towards the end of the cultivation (**Figure 1C**). The MCG enabled growth up to 9 h cultivation, reaching OD_{600} of 3.97 and μmax of 0.43 h^{-1} (**Figure 1A**). HPLC analysis of the metabolites in the spent medium indicated the use of glucose as a carbon source (**Figure 1B**), and only acetic acid was produced, reaching 1.8 g/L (**Figure 1C**). In MCLG, the bacteria were able to grow up to 7 h, reaching OD_{600} of 3.42 and μmax of 0.49 h^{-1} (**Figure 1A**). Glucose and lactic acid concentration diminished from the start to the end of cultivation, lactic acid being completely depleted in 10 h, while glucose was not completely depleted (**Figure 1B**). Acetic and malic acids were produced reaching 3.5 and 2.0 g/L respectively (**Figure 1C**).



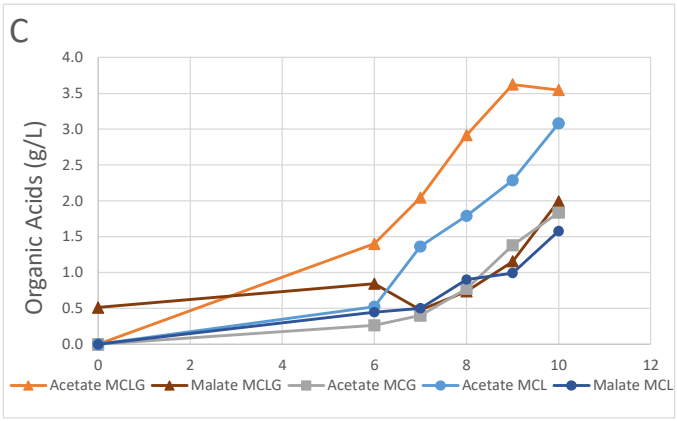


Figure 1: *N. lactamica* cultivation with various carbon sources. MCL: medium with lactic acid, MCG: medium with glucose, and MCLG: medium with lactic acid and glucose. A: optical density; B: carbon source consumption; and C: organic acid production. The inset graph in A represents the linear adjust used to calculate the maximum specific growth rate (μmax).

N. lactamica behavior in MCL and MCG was remarkably similar, with a slightly higher OD_{600} (3.97) with MCG than with MCL (3.85). In silico metabolic models for *N. meningitidis* have also predicted higher biomass yield by using glucose as a carbon source than using lactic acid[10]. Furthermore, the carbon source was utilized albeit did not deplete, suggesting that a lower concentration of carbon source might be used at least in flasks. Noticeably, the presence of lactic acid in MCL resulted in higher production of acetic acid and production of malic acid, while growth with MCG resulted in less accumulation of acetic acid and malic acid was not detected. The addition of both carbon sources together in MCLG resulted in lower cell density and even higher production of organic acids than just one carbon source alone. On the other hand, lactic acid and glucose were exhausted at the end of the culture in MCLG. The metabolic routes of *N. lactamica* available in the KEGG pathway database[9] may elucidate why the addition of both carbon sources led to fermentation. According to KEGG, *N. lactamica* is only able to convert acetate into acetyl-CoA and it also can only be regenerated by this route, while pyruvate can be converted into acetyl-CoA or malic acid. A possible explanation to higher production of organic acids in MCLG than in the other two media may rely on the fact that glucose and lactic acid are both being metabolized into pyruvate, however the enzymes that regulates the conversion of these two compounds might not compete for these carbon substrates, resulting in the observed metabolization of both carbon sources at the same time. The excess of acetyl-CoA is further converted into acetate, which then leaves the cell, while malic acid is converted to oxaloacetate, which is more widely used by the cell, since it plays a major role in the citric acid cycle, aspartate production and pyrimidine metabolism, hence malic acid is secreted in lower quantity than acetate in MCLG.

4 CONCLUSION

Our results suggest that glucose and lactic acid can be used interchangeably and glucose is a viable carbon source for the production of *N. lactamica* OMV.

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