I COBBIND
I CONGRESSO BRASILEIRO DE BIOTECNOLOGIA INDUSTRIAL
XXIV SINAFERM ·· XV SHEB ·· XV ENZITEC FLORIANÓPOLIS ·· 2024
Creating connections between biotechnology and industrial sustainability
August 25 to 28, 2024
Costão do Santinho Resort, Florianópolis, SC, Brazil
BIOPROCESS ENGINEERING

VIABLE CELL COUNT AND MAXIMUM SPECIFIC GROWTH RATE OF FED-BATCH CULTURES OF rBCG-PERTUSSIS

Sarah Mendes[1*], Maria C.P. Gonçalves[1] & Viviane M. Gonçalves[1]

[1] *Laboratory of Vaccine Development, Butantan Institute, São Paulo, Brazil*
** Corresponding author's email address: sarahmendes@outlook.com*

ABSTRACT

Tuberculosis remains a major global health concern, claiming 1.3 million lives in 2020, with a significant impact on children under 5 years old. The Bacillus Calmette-Guerin (BCG) vaccine, developed in 1921, is still the primary defense against tuberculosis, yet its production methods require modernization. The recombinant BCG-pertussis strain developed by our laboratory shows promise in providing dual protection against tuberculosis and whooping cough, especially for vulnerable newborns, and improved properties against bladder cancer. Submerged cultivation techniques for BCG offer higher productivity and standardization than the traditional static cultures for vaccine production. We have previously established rBCG-pertussis batch cultivation in bioreactor. Here, we aim to evaluate a fed-batch cultivation method using L-glutamic acid, the preferred substrate of rBCG-pertussis, with pH-stat control. This approach to enhance cell concentration and process yield, paving the way for more efficient vaccine production and improved public health outcomes, particularly for the most susceptible populations.

Keywords: *Mycobacterium bovis* BCG. Pertussis toxin. Onco-rBCG. pH-stat. Vaccine

1 INTRODUCTION

Tuberculosis is one of the main infectious diseases worldwide and caused 1.3 million deaths in 2020.[1] One in five children does not survive tuberculosis development,[1] and children under 5 years old are the main risk group.[2] Tuberculosis is caused by *Mycobacterium tuberculosis*, which is transmitted through respiratory droplets from infected hosts.[2]

In 1921, Calmette and Guerin attenuated *Mycobacterium bovis* through successive passages in culture medium and generated the Bacillus Calmette-Guerin (BCG) strain. Since *M. bovis* and *M. tuberculosis* species have 90% homology, BCG vaccine offers protection against human tuberculosis.[3] Currently, BCG is one of the most adopted vaccine worldwide, it is given at birth and the sole vaccine available against tuberculosis.[4] Since 1976, BCG has been also used as immunotherapeutic product (onco-BCG) to treat bladder cancer using a 10-times higher dose than the vaccine.[5] More recently, recombinant BCG (rBCG) strains have been investigated as antigen presenting systems for vaccination against other infectious diseases, because of BCG ability to stimulate innate immune response and deliver antigens directly to macrophages and dendritic cells.[6] Our laboratory developed the rBCG-pertussis strain, which expresses a nontoxic mutant gene of S1 subunit of pertussis toxin,[7] the most important antigen of *Bordetella pertussis*.[8] This bacterium infects the respiratory tract, can cause whooping cough in non-immunized individuals, principally newborns, which are the main risk group.[9]

The immunization with rBCG-pertussis protected neonatal mice against both tuberculosis and pertussis.[7] Currently, immunization against *B. pertussis* is achieved with the DTP vaccine, which prevents diphtheria, tetanus and whooping cough, and is administered at 2, 4, and 6 months of age. Thus, the new rBCG-pertussis vaccine would protect the age group under 6 months, which is the most vulnerable to *B. pertussis* infection. In addition, rBCG-pertussis showed better results against bladder cancer in terms of tumor weight reduction and survival time than the conventional BCG in mouse model,[10] which could contribute to reduce the number of patients resistant to onco-BCG treatment. Furthermore, there are shortage problems in onco-BCG and vaccine supply chains across the world,[11] which is mainly related to the labor intensive and not easily standardized BCG production process, which is achieved in static cultivation flasks for approximately 21 days[12]. BCG submerged cultivation in bioreactors would allow greater productivity and standardization, but the low price of the vaccine is one of the main barriers to invest in process modernization.[13] Therefore, a new and improved product as rBCG-pertussis could stimulate the investments necessary to modernize the production process.

Our laboratory has established submerged cultivation of rBCG-pertussis in bioreactors[14] and the study of feeding strategies could lead to an increase of cell concentration and process yield. Hence, the aim of this work is to evaluate fed-batch strategies using L-glutamic acid, which is the preferential substrate of rBCG-pertussis.

MATERIAL & METHODS

The rBCG-pertussis used here is a recombinant strain of the original BCG Moreau and lysine auxotrophic.[9] The strain was inoculated into a 250 mL Erlenmeyer flask containing 50 mL of 7H9Mfg medium, incubated in a shaker at 37°C and 125 rpm. After 96 h of cultivation, when the optical density (OD) reached approximately 4.0, the necessary inoculum volume was transferred to the bioreactor to give an initial OD around 0.2. The OD was measured using a Hitachi U-5100 spectrophotometer at 600 nm and culture samples were diluted in phosphate-buffered saline (PBS) when OD was higher than 0.6. The cultivations were done in 500 mL of medium, using a Ralf Plus Duet 1.0 L bioreactor (Bioengineering), equipped with three Rushton-type impellers. Prior

to inoculation, the bioreactor was sterilized with PBS at 120 °C for 30 min. Before cultivation, the PBS was drained and the 7H9Mfg medium, previously sterilized by filtration at 0.22 μm, was aseptically transferred into the reactor.

The 7H9Mfg medium was composed of Middlebrook 7H9 broth (Difco) supplemented with glycerol 20 g/L, glucose 2 g/L, ammonium sulfate 0.5 g/L, magnesium sulfate heptahydrate 0.25 g/L, zinc sulfate heptahydrate 4 mg/L and L-glutamic acid 2.5 g/L; tyloxapol 0.5 g/L was used as surfactant to prevent cell aggregation and the pH was adjusted to 6.7. Control cultures were done without feeding, and three pH values were evaluated in pH-stat by feeding 7.5 g/L L-glutamic acid solution complemented with tyloxapol (0.5 g/L) through the acid pump to control the pH. Two replicates were performed for each culture condition. All cultures were performed at 37 °C and 20% dissolved oxygen.

Daily samples were collected to determine the concentration of viable cells by plating and counting colony-forming units (CFU/mL). The samples were serially diluted in PBS+Tween 20% (1:10^3, 1:10^4, 1:10^5, and 1:10^6) and plated on Middlebrook 7H10 OADC (Difco) agar plates. The OD was also measured daily at 600 nm. The maximum specific growth rate (μ_{max}) was calculated by the angular coefficient of linear regression fit of Ln($OD/OD_{initial}$) versus time from day 0 to 4, and μ in the deceleration phase (μ_{dec}) after day 4 to the beginning of the stationary phase.

Tukey test for multiple comparisons (two-way ANOVA, α=0.05) was applied for statistical analysis using GraphPad Prism software.

RESULTS & DISCUSSION

L-glutamic acid was fed to control three different pH values, pH 7.0, pH 7.2 and pH 7.4, during the cultivation of rBCG-pertussis. Figure 1 shows the viable cell counts in these cultivations and in the control culture (no feeding of L-glutamic acid).

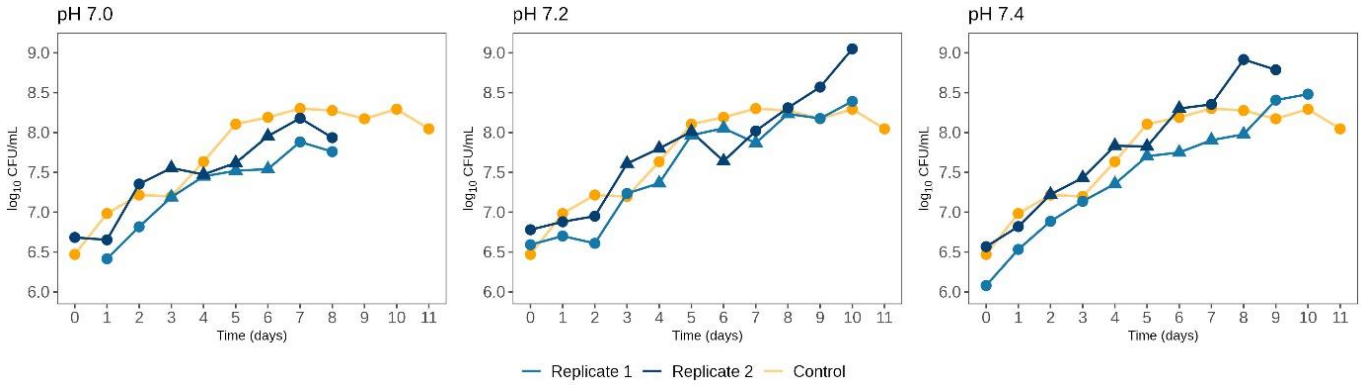

Figure 1 Viable cell counts at different pH values of fed-batch cultures of rBCG-pertussis in bioreactor with 0.5 L 7H9Mfg medium compared to control cultures without feeding (mean values represented in yellow). The circles represent the days without feeding and the triangles represent the days when L-glutamic acid was added and pH was controlled.

For the cultivation maintained at pH 7.0, the stationary phase was reached on day 9 (Figure 2). The feeding of L-glutamic acid started between day 2 and 3, the moment when the rBCG-pertussis culture naturally reached pH 7.0 due to glutamate consumption and ammonia release, and finished on day 6, when the feeding solution ended. The viable cell count for the fed-batch culture was in general lower for both replicates than the control curve, and the statistical analysis showed significant difference on day 8 ($p<0.05$). The maximum specific growth rate (μ_{max}) of this culture was 0.66 day^{-1}, lower than that of the control (0.76 day^{-1}), and μ_{dec} was the same as the control, 0.38 day^{-1} (Figure 2).

When the cultivation was controlled at pH 7.2, the L-glutamic acid feeding started in different periods, on day 3 for replicate 1 and day 4 for replicate 2 as this was the moment when pH 7.2 was naturally reached in each culture. The feeding ended 4 days later for both replicates. The mean viable cell count was similar to the control culture, except for day 10, when CFU/mL in the fed-batch at pH 7.2 was significantly higher than that in the control ($p<0.001$) and in the fed-batch at pH 7.4 ($p<0.05$). The values of μ_{max} (0.73 day^{-1}) and μ_{dec} (0.31 day^{-1}) were lower than the control (Figure 2).

Finally, when the pH was controlled at 7.4, the mean viable cell count showed a statically significant increase on day 8 compared to the fed-batch cultures at pH 7.0 ($p<0.0001$) and at pH 7.2 ($p<0.01$). The μ_{max} and μ_{dec} of the fed-batch controlled at pH 7.4 were higher than those of the control culture (Figure 2).

Although no statistically significant differences were found for μ values, it can be observed that the early feeding of L-glutamic acid at pH 7.0 lowered μ_{max}, while the late start of feeding at pH 7.4 increased μ_{dec} and reduced the cultivation time (Figure 2).

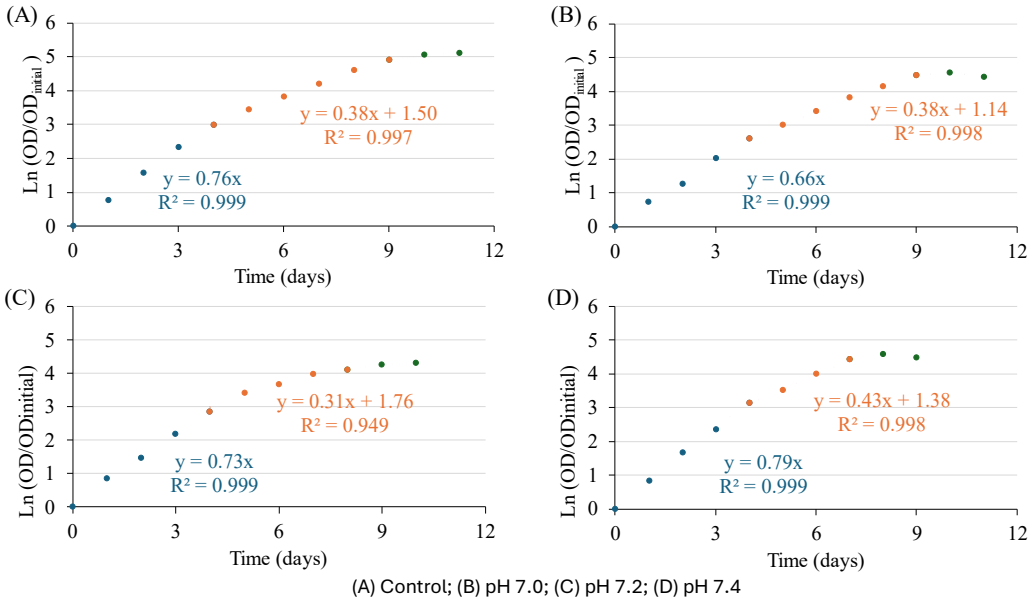

Figure 2 Growth profile of rBCG-pertussis in bioreactor with 0.5 L 7H9Mfg medium in simple batch control culture (A) and fed-batch cultures with pH-sat control (B-D). Average curves (n=2) are presented and specific growth rates (μ) are given by angular coefficients of linear regression equations: μ_{max} in blue and μ_{dec} in orange.

CONCLUSION

Although the rBCG-pertussis fed-batch cultures evaluated here showed higher viable cell count than the simple batch at specific time points of the cultures, no increase in μ_{max} was observed neither the exponential phase was extended. Therefore, there was no clear advantage of fed-batch cultures over the simple batch process and other feeding strategies should be evaluated, for example, adding all medium compounds to the feeding solution or using other feeding strategies.

REFERENCES

1 WORLD HEALTH ORGANIZATION. 2021. Global Tuberculosis Report, 2021. Available in: Global tuberculosis report 2021 (who.int)

2 CAMPBELL, J. R., BASTOS, M. L. 2020. No time to waste: preventing tuberculosis in children. The Lancet 395 (10228). 924-926.

3 FATIMA, S., KUMARI, A., DAS, G., DWIVEDI, V. P. 2020. Tuberculosis vaccine: a journey from BCG to present. Life Sciences 117594.

4 CERNUSCHI, T., MALVOLTI, S., NICKELS, E., FRIEDE, M. 2018. Bacillus Calmette-Guérin (BCG) vaccine: a global assessment of demand and supply balance. Vaccine 36 (4). 498-506.

5 HERR, H. W., MORALES, A. 2008. History of Bacillus Calmette-Guerin and bladder cancer: an immunotherapy success story. J. Urol. 179 (1). 53-56.

6 CONRADT, P., HESS, J., KAUFMANN, S. H. E. 1999. Cytolytic T-cell responses to human dendritic cells and macrophages infected with *Mycobacterium bovis* BCG and recombinant BCG secreting listeriolysin. Microbes Infect. I. 153-764.

7 NASCIMENTO, I. P., DIAS, W. O., QUINTILIO, W., HSU, T., JACOBS, W. R., LEITE, L. C. C. 2009. Construction of an unmarked recombinant BCG expressing a pertussis antigen by auxotrophic complementation: Protection against *Bordetella pertussis* challenge in neonates. Vaccine 27 (52). 7346-7351.

8 NICOSIA, A., BARTOLONI A., PERUGINI M., RAPPUOLI R. 1987. Expression and immunological properties of the five subunits of pertussis toxin. Infect. Immun. 55. 963-967.

9 WARD, J. I., CHERRY, J. D., CHANG, S.-J., PARTRIDGE, S., LEE, H., TREANOR, J., EDWARDS, K. 2005. Efficacy of an acellular pertussis vaccine among adolescents and adults. New Eng. J. Med. 353 (15). 1555-1563.

10 Andrade, P. M., Chade, D. C., Borra, R. C., Nascimento, I. P., Villanova, F. E., Leite, L. C., Andrade, E., Srougi, M. 2010. The therapeutic potential of recombinant BCG expressing the antigen S1PT in the intravesical treatment of bladder cancer. Urol Oncol. 28 (5). 520-525.

11 Harvey, M., Chislett, B., Perera, M., Lawrentschuk, N., Bolton, D., Jack, G. 2022. Critical shortage in BCG immunotherapy: How did we get here and where will it take us? Urol Oncol. 40 (1). 1-3.

12 Pascoe, J., Hendon-Dunn, C. L., Birch, C. P. D., Williams, G. A., Chambers, M. A., Bacon, J. 2020. Optimisation of *Mycobacterium bovis* BCG fermentation and storage survival. Pharmaceutics. 12. 1–14.

13 Black, S., Bloom, D. E., Kaslow, D. C., Pecetta, S., Rappuoli, R. 2020. Transforming vaccine development. Seminars in Immunology. 50. 101413.

14 Borban, L. S. 2023. Estratégias para monitoramento e controle do cultivo de BCG-pertussis recombinante. Dissertação de mestrado. Universidade de São Paulo. 108 p.

ACKNOWLEDGEMENTS

Acknowledging the support of FAPESP (2017/24832-6 and 2022/15269-4), CNPq (310973/2022-8) and Fundação Butantan for our project.