EVALUATING THE SHELF LIFE OF BIOINOCULANTS FORMULATED WITH AG6 SOIL-ISOLATED STRAIN ENCAPSULATED WITH ALGINATE

Adrielle A. P. Ribeiro[1], Iara Rossi[1], Fernanda M. Gomes[1*], Arthur C. Severino[1], Amanda C. da Rocha[1], Mayara T. da Silva[1], Patrisia de O. Rodrigues[1], Fabricio M. G. Porto[1], Vicelma L. Cardoso[1] & Miriam M. de Resende[1]

[1] College of Chemical Engineering/Department of Bioprocesses/Federal University of Uberlandia, Uberlandia, Brazil.
**fernanda.merola@ufu.br*

ABSTRACT

An alternative approach to prolonging the lifespan of bacteria involves encapsulating them once this method can protect them from both biotic and abiotic factors that can compromise their survival. In this study, we developed the Encapsulation of the bioinoculant AG6 in alginate. The extruded solution was prepared in a ratio of (2:1) sodium alginate solution (3% and 1.5%) with bacterial suspension. Survival rates were determined over nine storage periods (1, 4, 6, 8, 15, 20, 25, 30, and 60 days), and morphometric aspects were measured. Results showed a decrease in the quality of shelf life in all treatments; however, granules composed of 3% alginate could sustain the growth of AG6 for 60 days at $6{,}80.10^{9}$ CFU·g^{-1}, maintaining the integrity of the spheres.

Keywords: Microorganism encapsulation. Shelf-life of inoculants. Organic fertilizers.

1 INTRODUCTION

Bioinoculants are essential for promoting plant growth, nutrition, and health. However, their effective application is often compromised by their short lifespan, especially due to the environmental and storage conditions required for the microorganisms. Encapsulation has been explored as a promising strategy to protect and prolong bioinoculant viability.[1]

Due to its physical properties and biocompatibility, a biodegradable polymer derived from seaweed, sodium alginate, has been widely used in encapsulating living cells. Encapsulating bioinoculants in alginate matrices protects against adverse conditions such as temperature variations, pH, humidity, and leaching. It also allows the controlled release of microorganisms into the soil environment.[2]

However, despite the promising potential of alginate encapsulation, it is still necessary to investigate its effectiveness and stability over storage time for each microorganism of interest. This research evaluates the shelf life of encapsulated AG6 bioinoculants with alginate. Understanding these aspects is crucial for developing effective formulations and efficient storage conditions, contributing to a more sustainable and productive agriculture.

2 MATERIAL & METHODS

For encapsulation, a strain isolated from agave (AG) harvested in Sumé-Paraíba was used, and the isolation was carried out according to Baldani et al. (2014)[3] with modifications. Rhizosphere material was collected and diluted in saline (NaCl) solution (0.9%) with Tween 80 (1 g·L^{-1}). Dilutions were plated on nitrogen-free culture media, following a serial dilution up to 10^{-5}. The isolates were plated on N_2-free Jensen's media containing bromothymol blue at pH 6.8.[4] and nitrogen-free malate medium (NFMM) at pH 7.3.[5] After streaking, they were cultured for five days at 30°C. Gram staining technique was performed for microscopic examination of the isolates.[6]

The Nessler reagent method was applied to assess nitrogen-fixing capacity, where iodide and mercury ions react with ammonia under alkaline conditions to produce a brown-red complex.[7] According to Cappucino & Sherman (2019)[8], the Nessler reagent was prepared in ammonia-free distilled water, stored, and filtered before use. Triaged isolates were plated and inoculated in 10mL of peptone water, incubated at 28 ± 2°C, 120 rpm for 72 h. After incubation, the supernatant was centrifuged, and an aliquot was treated with 0.5 mL of the Nessler reagent. The color change of the samples from yellowish to brown indicates the presence of ammonia, whose concentration was determined in a UV-VIS spectrophotometer at 450 nm.[7] The same analysis was performed with a widely known microorganism used in agriculture, *Rhizobium tropici,* provided by Satis Indústria e Comércio Ltda.

For obtaining AG6 cells, the pre-inoculum was prepared with NaCl (0.9%) at a concentration of 0.5 g dry biomass·L^{-1}. After 12 h of incubation, 1 mL of pre-inoculum was transferred to a 250 mL Erlenmeyer containing 75 mL of Luria Bertani (LB) broth, and it was maintained for 74 h at 120 rpm and 32°C. Cell growth was quantified by turbidimetry at 600 nm using a UV-VIS spectrophotometer, pH was evaluated using a pH meter, and residual glucose concentration was measured using high-performance liquid chromatography (HPLC) with a Shimadzu LC-20A Prominence model and a SUPELCOGEL C-610H column. The cell culture was centrifuged for 10 minutes at 8000 rpm. The cell pellet was carefully washed with NaCl solution (0.9%) and resuspended in peptone solution (1%) at a concentration of 34 g dry biomass·L^{-1}.

The encapsulation of AG6 cells was performed according to Reetha et al. (2014)[9] with modifications. The ratio was 2:1 for sodium alginate solution (3% and 1.5%) and bacterial suspension (34 g biomass·L^{-1}). The mixture was homogenized for 30 minutes, then

extruded through a peristaltic pump into plates containing $CaCl_2$ solution (0.1 M). The spheres were kept in the solution for 2 hours and then washed with water. The spheres were incubated in an LB broth for 24 h at 120 rpm and 32°C to allow growth within the spheres. Subsequently, they were washed, collected, and left under airflow for 30 minutes. Approximately 5 g aliquots of spheres were packed for each treatment. The alginate solution was extruded to serve as a control. The packages containing the different formulations (granule-alginate 1.5% + AG6; control-alginate 1.5%; granule-alginate 3% + AG6; and control-alginate 3%) were stored at 7 ± 2°C.

For each treatment, the survival ($CFU \cdot g^{-1}$) of AG6 cells was evaluated at nine different storage periods (1, 4, 6, 8, 15, 20, 25, 30, and 60 days of storage) as indicated by Cappucino & Sherman (2019)[8]. Visible bacterial colonies count on nutrient agar plates were performed after 12 h. The diameters were measured with an electronic micrometer and weighed on an analytical balance in triplicate. The external and internal surfaces of the spheres were scanned using scanning electron microscopy (SEM). The sphere-shaped samples were fixed with 0.1 M sodium phosphate buffer (pH = 7.2), followed by dehydration in an oven at 36°C for three hours. The samples were placed on the sample holder, which contained a double-sided carbon adhesive tape, and subsequently dried and metalized with a thin layer of gold on the surface to allow for a photograph to be captured. The data were subjected to analysis of variance (ANOVA), and the means were compared using Tukey's test ($p < 0.05$) using Statistic 7 software. The data were expressed as mean ± SD (n=3).

3 RESULTS & DISCUSSION

Ten microorganisms were isolated from the agave rhizosphere, and six were gram-negative bacilli. AG6 could fix nitrogen, yielding 15.24±4.51 $mg \cdot L^{-1}$, whereas the *R. tropici* strain fixed 3.75±2.47 $mg \cdot L^{-1}$. Therefore, the AG6 strain has potential for application in agriculture settings, as it exhibited a satisfactory value compared to the commercial strain. Figure 1 depicts the variations of parameters (pH, biomass, glucose) during fermentation in the LB broth. Cell growth reached 1.9 $g \cdot L^{-1}$, with maximum growth observed after 60 hours, reaching 1.99 $g \cdot L^{-1}$. The pH initially fluctuated due to intense growth activity, with a peak in acid production at 8 hours. Following glucose consumption, the acids decreased, suggesting their utilization as a carbon source.

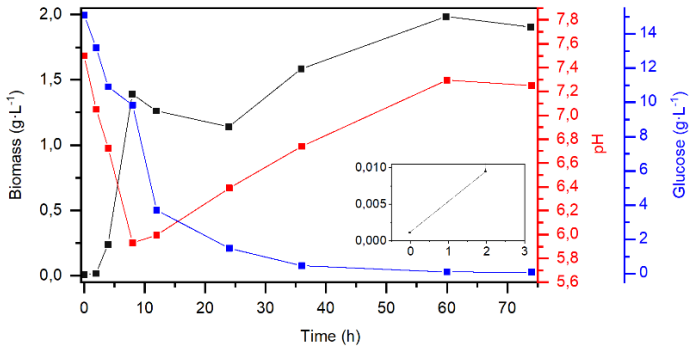

Figure 1 Fermentation by AG6 cultures grown in LB broth at 32°C at 120 rpm for 74 hours. A. Dry biomass, pH, and residual glucose.

The morphometric data obtained are presented in Table 1. The sphere dimensions exhibited uniform sizes with low standard deviation, confirming that the protocol efficiently generated standardized spheres. The granule dimensions decreased when the alginate concentration was 1.5%. The average yield was 16.82±0.02 spheres per mL of extruded solution, with losses during the production process due to the formation of non-conforming spheres. The morphometric data were similar to Zago et al. (2019)[2], which produced granules with *Azospirillum brasilense* using various alginate formulations via a Pasteur pipette, with sphere weights ranging from 17 to 38 mg and diameters ranging from 3.30 to 4.30 mm.

Table 1 Morphometric analyses yield and cell survival at 60 days.

Treatments	Weight (mg)	Diameter (mm)	Yield ($unit \cdot mL^{-1}$)	Survival ($CFU \cdot g^{-1}$) initial/final
Granule - alginate 1.5%	36,55±1,26^{a}	3,42±1,22^{a}	16,80±0,5^{a}	$6{,}53.10^{11}/1{,}3.10^{9}$ ^{a}
Control-alginate 1,5 %	30,10±0,90^{b}	3,49±0,11^{a}	16,85±0,6^{a}	0/0^{c}
Granule – alginate 3,0%	54,79±1,53^{c}	4,31±0,13^{b}	16,83±0,2^{a}	$1{,}54.10^{12}/6{,}8.10^{9}$ ^{b}
Control-alginate 3,0 %	52,58±1,71^{c}	4,47±0,04^{b}	16,79±0,3^{a}	0/0^{c}

Treatments followed by different letters differ significantly by the Tukey test ($p < 0.05$).

Despite a significant decrease, the unit of cells in the granule remained high; at sixty days, we observed that the granules still retained a considerable amount of CFU within them, close to 10^{9}; however, the granule composed of 1.5% alginate experienced a partial loss of its physical integrity. On the fifteenth day, the granule with 3% alginate grew 900% compared to the previous five days. On the twenty-fifth day, the granule with 1.5% alginate grew by 6760% compared to five days earlier, and this showed that the polymeric matrix did not hinder the growth of cells inside it.

When evaluating the structure through SEM (Scanning Electron Microscopy) (Figure 3), we observed that the dehydration treatment compromised the structure of the spheres. However, it was possible to observe the presence of cells in Figure 3 (F), while Figure 3 (E) does not show cells. AG6 cells were not found on either surface of the spheres. The control sphere appeared more rigid than the test one and more damage-resistant in both treatments. Upon handling, a significant fragility was observed in the 1.5% alginate + AG6 sphere.

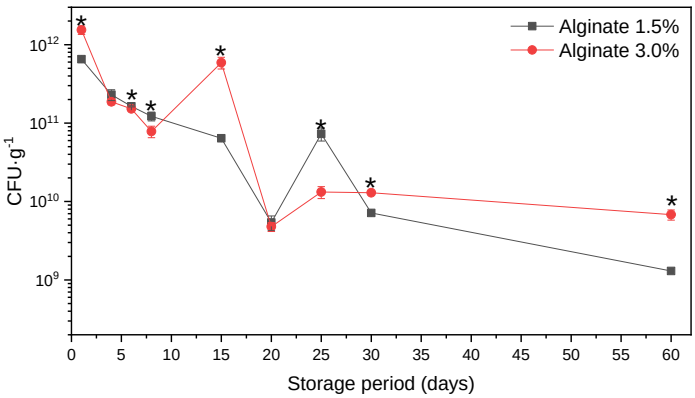

Figure 2 Survival of AG6 on different concentration carrier matter during storage. *Treatments differ significantly by ANOVA ($p < 0.05$).

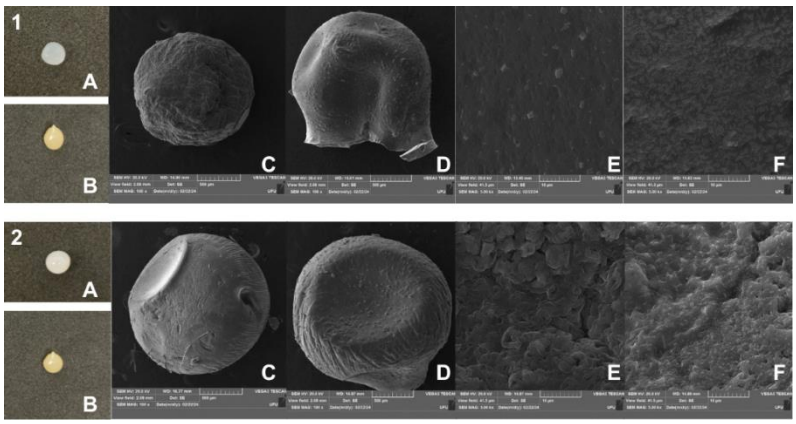

Figure 3 Morphological aspects of granules. 1. Alginate 1,5%; 2. Alginate 3,0%. A. Control sphere; B. Test sphere; C. External surface of control, an increase of 100x; D. External surface of the test, an increase of 100x; E. Internal surface of control, an increase of 5000x; F. Internal surface of the test, an increase of 5000x.

4 CONCLUSION

The study revealed that the AG6 strain isolated from the agave rhizosphere exhibits promising potential for application in the agricultural industry, demonstrating nitrogen fixation capabilities comparable to commercial strains. Additionally, the investigation into fermentation dynamics in LB broth revealed significant insights into the growth and metabolic activity of the AG6 strain. We observed fluctuations in pH, indicating intense growth activity and providing valuable information for optimizing fermentation processes and understanding microbial behavior. Evaluation of bacterial population dynamics showed that although there was a significant decrease in population concentration, especially in the treatment with 1.5% alginate, overall cell viability remained relatively high, suggesting the robustness of the encapsulation matrix. The substantial growth observed in certain treatments further emphasizes the compatibility of the polymeric matrix with cell growth and proliferation.

REFERENCES

[1] FLORENCIO, C., BORTOLETTO-SANTOS, R., FAVARO, C. P., BRONDI, M. G., VELLOSO, C. C. V., KLAIC, R., MATTOSO, L. H. C. 2022. Quimica Nova. 45. (9). 1133-1145

[2] ZAGO, S. L., SANTOS, M. F., KONRAD, D., FIORINI, A., ROSADO, F. R., MISSIO, F. R., VENDRUSCOLO, E. C. G. 2019. J. of Agricultural Science 11. (6). 269-280.

[3] BALDANI, J. I., REIS, V. M., VIDEIRA, S. S., BODDEY, L. H., BALDANI, V. L. D. 2014. Plant and Soil 384 (1-2). 413-431.

[4] SHOMI, F. Y., UDDIN, M. B., ZERIN, T. 2021. Stamford J. of Microbiology 11 (1). 11-13.

[5] SHAMEEM, M. R., SONALI., J. M. I., KUMAR, P. S., RANGASAMY, G., GAYATHRI, K. V., PARTHASARATHY, V. 2023. Environmental Research. 220. 115200.

[6] VERMELHO, A. B., PEREIRA, A. F., COELHO, R. R. R., SOUTO-PADRÓN, T. C. B. S. 2019. Práticas de microbiologia. GUANABARA KOOGAN (ed). 2nd ed. Rio de Janeiro. 97-97.

[7] ZHAO, Y., SHI, R., BIAN, X., ZHOU, C., ZHAO, Y., ZHANG, S., WU, F., WATERHOUSE, G. I. N., WU, L. Z., TUNG. C. H., ZHANG, T. 2019. Advanced Science 6 (8). 1802109.

[8] CAPPUCCINO, J. C., SHERMAN, N. 2019. Microbiology: A Laboratory Manual. 12th ed. Pearson, New York. 542.

[9] REETHA, D., KUMARESAN, G., JOHN MILTON, D. 2014. International J. of Recent Scientific Research 5. (12). 2178-2182.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the financial support provided by the Brazilian funding agencies: “Coordenação de Aperfeiçoamento de Pessoal de Nível Superior” (CAPES), the “Conselho Nacional de Desenvolvimento Científico e Tecnológico” (CNPq), and the “Fundação de Amparo à Pesquisa do Estado de Minas Gerais” (FAPEMIG). It also acknowledges the financial support provided by Satis Indústria e Comércio Ltda.