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FORMULATION DEVELOPMENT FOR MICROBIOLOGICAL CONTROL OF PESTS AND ANTAGONISM TESTS

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ABSTRACT

Due to the damage caused by chemical pest control, the use of biological agents emerges as a sustainable alternative. However, this practice still faces technological challenges related to obtaining a more efficient and stable product. In this context, a formulation of the fungus *Trichoderma asperellum* in alginate beads is developed. Viability analysis shows a concentration, after 103 days, of 4.86×10^8 CFUs/gds, a value 5.8 times higher than that obtained for a sample without formulation and 1.2 times higher than another formulated with a lower amount of liquid extract containing the microorganism. Antagonism tests reveal a greater dominance of the studied fungus over other fungi responsible for causing diseases in crops, through competition mechanisms. The formulation promotes an increase in product viability, while maintaining the fungus in the form of conidia and without the spread of mycelium, a characteristic of interest in the product. Thus, the developed formulation presents itself as a sustainable alternative to be used for biological pest control.

Keywords: Biocontrol. Fungus. Sustainability. Viability. Antagonism.

1 INTRODUCTION

Synthetic or chemical compounds provide short-term control of target pests; however, they often exhibit high or moderate toxicity to mammals. Additionally, it is common for several pests to develop resistance to synthetic agents over time, necessitating increasingly concentrated formulations and consequently, more toxic ones. The use of these products results in a series of damages to the environment and human health. Among the main impacts caused by them, some examples are phytosanitary problems resulting from ecological imbalance, negative alteration of soil microbial communities, contamination of aquatic ecosystems, air, water, and soil contamination. In humans, they can cause severe food poisoning, organ injuries, and even death.[1]

Considering this, the acceptability and utilization of biological agents have been increasing over the years, despite their costs being higher than those of chemical agents, due to their environmental and health benefits. Thus, the use of biologically derived inputs is enhancing its importance worldwide, as it represents one of the most sustainable agricultural management strategies to be employed, playing a significant role in the pursuit of more environmentally friendly practices in the field. However, its industrial process still faces technological challenges, such as the need to increase the efficiency of active agent recovery and formulation stability. Furthermore, the vast biodiversity in Brazil can be leveraged in the discovery and production of new biologically derived products, aiming to reduce dependence on imported chemical inputs, which currently constitute most of the products used[2]. In this sense, the present study pursues on obtaining a formulation that is viable and stable.

2 MATERIAL & METHODS

The raw material used, consisting of dried rice on which the fungus *Trichoderma asperellum* was cultivated (in a solid-state), was kindly provided by Agrivalle Brazil Ltda. For the extraction of the biological agent using distilled water as a vehicle, a ratio of 15 mL of water per gram of material (gram of dry solid) was respected. Four Erlenmeyer flasks containing 3 grams of dry solid (gds) and 45 mL of distilled water each were used and subjected to agitation on a shaker at 200 rpm and 25 °C for 5 hours. After the agitation period, a vacuum filtration system was employed to separate the liquid extract containing the spore of the active agent from the solid residue (residual rice). The concentrations of this sample were determined by conidia counting using a Neubauer chamber and by plating and counting colony forming units (CFUs). The liquid extract obtained after filtration was used in the next step of formulating the microorganism into alginate beads.

The formulation of the microorganism into Alginate-Based beads[3] was obtained using a 1% (w/w) sodium alginate solution containing the fungal conidial suspension obtained in the extraction step. This solution was dropped into a coagulation solution, which consisted of 0.25 M calcium chloride ($CaCl_2$). For the sodium alginate solution containing the liquid extract, samples using 10% (Formulation 1) and 50% (Formulation 2), by volume, of the extract were prepared. The formulated product underwent a viability analysis, conducted by determining the concentration of CFUs/gds. The methodology involved serial dilution of the liquid extract and the alginate beads (which were dissolved in an 8% (w/v) sodium citrate solution, at a ratio of 100 mg of beads per mL of solution, with the aid of a vortex) to approximately 10^{-6}. Then, 100 µL of the diluted solution was transferred onto Petri dishes containing a growth medium, PDA (Potato Dextrose Agar), supplemented with Triton X-100, in triplicate. The plates were then incubated for 72 hours at 25 °C to allow colony growth, followed by counting. This process was repeated six times on the following dates: 10/25/2023 (start), 11/10/2023 (16 days), 11/23/2023 (29 days), 12/07/2023 (43 days), 01/18/2024 (85 days), and 02/05/2024 (103 days).

Following the viability analysis, the sample that showed the best results was used in antagonism tests, where the formulated product competed against two species that cause diseases in crops used in biofuel production: the fungi *Sclerotinia sclerotiorium* and *Colletotrichum*. For this purpose, six Petri dishes containing PDA were prepared for each fungus. Three control plates were set up (containing only the fungus to be countered), and another three plates were prepared with the formulated product on one side and the phytopathogen on the other side. These plates were then incubated at 30 °C until the fungi grew across the entire control plate, at which point the experiment was terminated. It is important to note that for all methodologies, all materials used were autoclaved and handled only in a biological safety cabinet to prevent contamination by other microorganisms.

3 RESULTS & DISCUSSION

The concentrations obtained in the extraction analysis showed satisfactory results, reaching values on the order of 10^9, with good coefficients of variation (Table 1). It is important to note that higher variations are expected in this case, as these are values with very high orders of magnitude.

Table 1 Concentration of conidia and CFU in the extraction sample performed.

Concentration	Average	Standard deviation	Coefficient of variation
(conidia/gds)	$1,37x10^9$	$6,47x10^7$	4,72
(CFUs/gds)	$1,57x10^9$	$4,32x10^8$	27,60

In respect to the formulations, the analysis of samples with and without formulation over 103 days allowed the evaluation of the concentration (C) in CFUs/gds over time, as evidenced by Table 3 and Figure 1. Observing the concentrations obtained throughout the entire period, it was possible to notice that the results for the sample without formulation are in accordance with the literature, with the concentration decreasing exponentially. Additionally, it is evident that the formulations were effective in maintaining the viability of the samples, presenting higher concentrations than the sample without formulation at all time points. It is also noteworthy that Formulation 2 showed higher concentration values compared to Formulation 1, thus being the most effective in maintaining viability. The standard deviation (σ) and coefficient of variation (CV) values were also represented (Table 3) to facilitate a better interpretation of the results. The representation of these deviations in the graph (Figure 1) further highlighted the behavior of the curves and allowed for an analysis of which sample had the best viability.

Comparing the concentration values obtained for the three samples, it was observed that after 103 days, Formulation 2 showed a concentration 5.8 times higher than the sample without formulation, and 1.2 times higher than Formulation 1. Once again, it becomes clear that Formulation 2 achieved the best results in the viability analysis. The high deviation values were a consequence of what could be considered a visual and manual method, which involves counting CFUs, resulting in some imprecision. Additionally, these are high-order magnitude values, which contributed to the increase in the deviations observed. Furthermore, the formulation prevented the spread of mycelium, keeping the fungus in the form of conidia, which is a characteristic of interest for the product, but was not observed in samples without formulation.

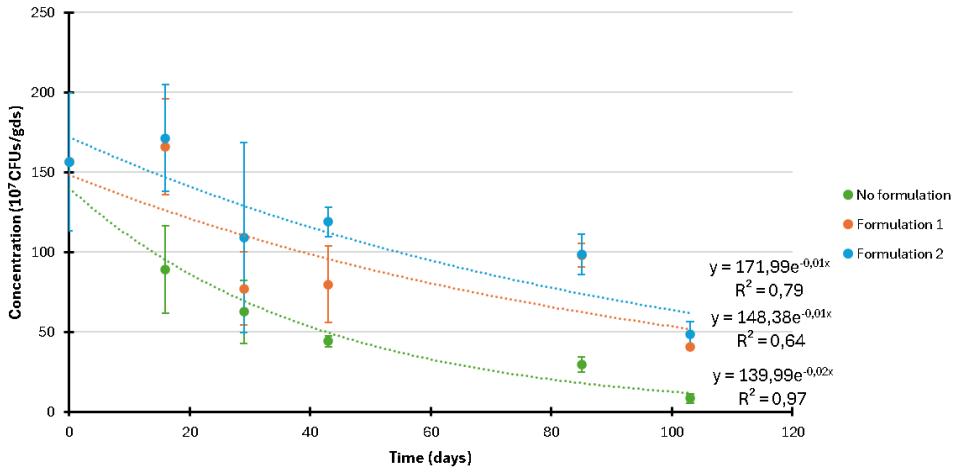

Figure 1 Concentration (CFUs/gds) over time (days).

Table 2 Parameters for exponencial fitting.

Parameters	No formulation	Formulation 1	Formulation 2
Pre-exponential factor	139.9903	148.3826	171.9902
Decay factor	-0.0242	-0.0102	-0.00995
R^2	0.9655	0.6396	0.7914

From the analysis of the pre-exponential factor (Table 2), it was observed that Formulation 2 presented the highest value, indicating an initial concentration, for the fit, higher than the other two samples. Additionally, the lower decay factor, in module, for Formulation 2 resulted in a smaller loss of viability, demonstrating that Formulation 2 afforded better results.

Table 3 Viability of samples with and without formulation over 103 days.

Time	No formulation			Formulation 1			Formulation 2		
(days)	C (CFUs/gds)	σ	CV (%)	C (CFUs/gds)	σ	CV (%)	C (CFUs/gds)	σ	CV (%)
0	$1.57x10^9$	$4.32x10^8$	27.60	$1.57x10^9$	$4.32x10^8$	27.60	$1.57x10^9$	$4.32x10^8$	27.60
16	$8.90x10^8$	$2.73x10^8$	30.63	$1.66x10^9$	$3.02x10^8$	18.21	$1.71x10^9$	$3.36x10^8$	19.59
29	$6.25x10^8$	$1.96x10^8$	31.32	$7.70x10^8$	$2.29x10^8$	29.72	$1.09x10^9$	$5.96x10^8$	54.69
43	$4.40x10^8$	$3.46x10^7$	7.87	$7.98x10^8$	$2.39x10^8$	29.93	$1.19x10^9$	$9.19x10^7$	7.73
85	$2.95x10^8$	$4.82x10^7$	16.35	$9.79x10^8$	$7.44x10^7$	7.60	$9.85x10^8$	$1.25x10^8$	12.69
103	$8.38x10^7$	$2.90x10^7$	34.59	$4.08x10^8$	$1.04x10^7$	2.55	$4.86x10^8$	$7.52x10^7$	15.47

Finally, the antagonism tests showed that *T. asperellum* was efficient in competing with the two fungi tested. Against *S. sclerotiorium* (Figure 2), it showed an inhibition percentage (Equation 1) of 68.52%, covering a significant portion of the Petri dish and indicating competition for nutrients and space. In contrast, against *Colletotrichum* (Figure 2), this percentage was 100%, indicating complete inhibition of the phytopathogen, with mycoparasitism as the main competition mechanism. This shows that the Formulation 2 is efficient to be used in biological control.

$$\%inhibition = \frac{D_c - D_t}{D_c} \cdot 100 \quad (1)$$

Where Dc and Dt represents, respectively, the control Petri dish diameter and the phytopathogen growth diameter.

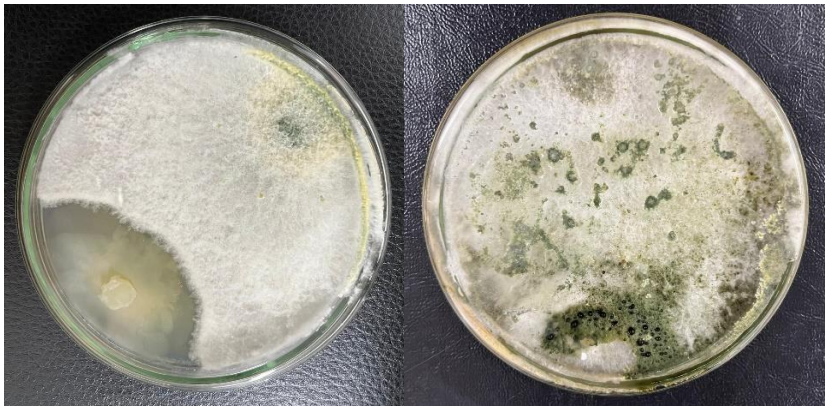

Figure 2 Antagonism tests using Formulation 2. On the left, test with *S. sclerotiorium*. On the right, test with *Colletotrichum*.

4 CONCLUSION

In summary, this study was responsible for the development of two Alginate-Based beads formulations, one containing 10%, by volume, extract (Formulation 1) and the other containing 50% (Formulation 2). Based on the results obtained, it was possible to conclude that both formulations were effective in maintaining the viability of the samples over the 103-day period. However, Formulation 2 proved to be particularly effective, showing a concentration 5.8 times higher than the sample without formulation and 1.2 times higher than Formulation 1. These results are supported by the good coefficients of variation, which, although slightly high in many cases, the data aligns with expectations given their very high order of magnitude. Additionally, due to the use of manual and visual analysis method, the data is consequently less precise. Furthermore, the behavior of the curves over time and the exponential fits, as evidenced by Figure 1, was also crucial in the analysis of the results. Thus, Formulation 2 demonstrated to be the most suitable for preserving the viability of the samples, providing a solid foundation for future studies and practical applications, in addition to helping to maintain the product without the spread of mycelium. Finally, the antagonism test showed that the Formulation 2 was efficient against both of phytopathogen tested.

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