Diversitas Journal
ISSN 2525-5215
Volume 9, Número 1 (jan./mar. 2024) p. 0183 – 0191
https://diversitasjournal.com.br/diversitas_journal

Immobilization of *Trichoderma* spp. in alginate and viability under different storage conditions

Imobilização de *Trichoderma* spp. em alginato e viabilidade sob diferentes condições de armazenamento

SILVA, Maria Clariana da[1]; SILVA, Lívia Ribeiro da[2]; ALVES, Heberth Gustavo Ferreira[3]; JESUS, Évelly Vitória Oliveira de[4]; SILVA, Paula Cibelly Vilela da[5]; SANTOS, Tania Marta Carvalho dos[6]

[1] 0009-0001-7405-7830; Federal University of Alagoas (*Universidade Federal de Alagoas*), student and researcher, Brazil, Email: maria.clariana@ceca.ufal.br
[2] 0009-0000-6850-7383; Federal University of Alagoas (*Universidade Federal de Alagoas*), student and researcher, Brazil, Email: livia.silva@ceca.ufal.br
[3] 0009-0002-8799-5970; Federal University of Alagoas (*Universidade Federal de Alagoas*), student and researcher, Brazil, Email: zootec18.gustavo@gmail.com
[4] 0009-0009-4115-0420; Federal University of Alagoas (*Universidade Federal de Alagoas*), student and researcher, Brazil, Email: vitoriaevelly61@gmail.com
[5] 0000-0003-2157-4698; Federal University of Alagoas (*Universidade Federal de Alagoas*), student and researcher, Brazil, Email: paulacibelly17@gmail.com
[6] 0000-0002-1816-7840; Federal University of Alagoas (*Universidade Federal de Alagoas*), student and researcher, Brazil, Email: tmcs@ceca.ufal.br

A B S T R A C T

The microencapsulation cell immobilization approach stands out as a highly effective alternative in conducting bioprocesses. By encapsulating cells in microcapsules, a three-dimensional matrix is created that provides prolonged cell retention. Based on this context, the objective of this study was to evaluate the immobilization of *Trichoderma* isolates in calcium alginate, the viability and to determine the ideal temperature to store the immobilized fungi. Starch and sodium alginate were used to produce the granules, which together with each isolate was dripped in a calcium chloride solution. To evaluate the viability of the encapsulated fungi and the best storage condition, tests were carried out with the granules stored at room temperatures, refrigerator and freezer. It was possible to obtain intact alginate capsules, and the evaluation of the concentration of conidia during 21 days of storage in different environments showed that they remained viable (107 conidia g^{-1}).

R E S U M O

A abordagem de imobilização celular por microencapsulação se destaca como uma alternativa altamente eficaz na condução de bioprocessos. Ao encapsular as células em microcápsulas, cria-se uma matriz tridimensional que proporciona retenção celular prolongada. Com base nesse contexto, objetivou-se avaliar a imobilização de isolados de *Trichoderma* em alginato de cálcio, a viabilidade e determinar a temperatura ideal para armazenar os fungos imobilizados. Para produção dos grânulos foram utilizados amido e alginato de sódio, que juntamente com cada isolado foi gotejado em solução de cloreto de cálcio. Para avaliar a viabilidade dos fungos encapsulados e a melhor condição de armazenamento, foram realizados testes com os grânulos armazenados nas temperaturas ambiente, geladeira e freezer. Foi possível obter cápsulas de alginato íntegras, e a avaliação da concentração dos conídios durante 21 dias de armazenamento em diferentes ambientes mostrou que eles se mantiveram viáveis (107 conídios g^{-1}).

INFORMAÇÕES DO ARTIGO

Histórico do Artigo:
Submetido: 01/11/2023
Aprovado: 06/12/2023
Publicação: 23/02/2024

Keywords:
biosolubilization, biofertilizers, growth promoting microorganisms.

Palavras-Chave:
biossolubilização, biofertilizantes, micro-organismos promotores de crescimento.

DOI: 10.48017/dj.v9i1.2848

Introduction

Trichoderma spp. are free living fungal species that inhabit tropical and temperate soils, and are one of the main microorganisms of importance for increasing plant growth. This fungus can positively influence seed germination, crop development and yield due to the production of growth-promoting substances and improved plant nutrition, mainly through phosphorus solubilization (OLIVEIRA et al., 2012; SILVA et al., 2012; GUZMAN et al., 2019; SÁNCHEZ-MONTESINOS et al., 2019) and indole-acetic acid synthesis (OLIVEIRA et al., 2012; CHAGAS et al., 2016), having great economic importance for agriculture because they are able to act as disease control agents of various cultivated plants and inducers of disease resistance in plants (CHAGAS et al., 2016; CHAGAS et al., 2017).

The microencapsulation of biological agents has been pointed out as a valuable alternative to produce effective bioformulations in agriculture, which mainly ensure longer shelf life and stability of products (BRAGA et al., 2019; BRAGA et al., 2022). Microencapsulation is a process that can provide a physical barrier between the core compound and the other components of the product or external environment. It is a technique in which liquid droplets, solid particles, or gas compounds are trapped in thin films, which may present a homogeneous or heterogeneous matrix (GHARSALLAOUI et al., 2007). The core and wall material can be composed of just one or several ingredients and the retention of these cores is determined by their chemical functionality, solubility, polarity, and volatility (FAVARO-TRINDADE, 2008).

The purpose of the encapsulation or microencapsulation process is to provide protection to the biological agent against abiotic and biotic stress, prolonging its viability and promoting a slow release of spores as it progresses degrading the microcapsule (VEMMER; PATEL 2013).

Ionic gelation involves an aqueous polymeric solution, with low molecular weight ions, in which polyelectrolytes of opposite charges interact to form a complex. Low-methoxylation alginate or pectin capsules are widely used as a covering material, with calcium ions being the most commonly used crosslinking agent (MESTDAGH; AXELOS, 1998).

The concentration of polysaccharide and cations, ionic strength and pH determine the kinetics of gel formation, as well as the volume, stability and porosity of the capsules, which may influence the diffusion of solutes into and out of the polymeric matrix (MESTDAGH; AXELOS, 1998).

The method consists of the physicochemical process, suspending the active ingredient in a solution of sodium alginate (insert the chemical formula of the product) for 1.5%, including an ideal concentration of the biological organism, suspension that is pulverized in a liquid solution of calcium acetate at 2%, as a result of which pearlescent or encapsulated particles are

obtained, which solidify in seconds when the cations react with biopolymer chains and form rigid structures (VEMMER & PATEL 2013).

According to Batista et al. (2013), the use of biopolymers, particularly alginates, is justified because they are easily hydrolytic and enzymatic degraded, biocompatible, and non-toxic to the environment. Alginate has the ability to form water-insoluble gels, although the gels have high porosity, which increases the diffusion levels of the active ingredient contained in the gel, without dissolving.

Therefore, the objective of this study was to promote the immobilization of cells of *Trichoderma* spp. isolates in calcium alginate and to evaluate the viability and safety of these fungi and to determine the best temperature for storage.

Methodological Procedures

The present work was carried out at the Microbiology Laboratory of the Campus of Engineering and Agrarian Sciences (CECA) of the Federal University of Alagoas (*Universidade Federal de Alagoas* - UFAL) in Rio Largo, AL. B1, B2 and M2 isolates of *Trichoderma* spp. were obtained from soil samples cultivated with vegetables, with the capacity to solubilize phosphate and synthesize phytohormones, and selected for tolerance to abiotic factors (salinity, temperature and water stress) belonging to the repository of the Microbiology Laboratory of CECA-UFAL.

For the mass production of *Trichoderma*, cultivation discs in Potato Dextrose Agar (BDA) culture medium were chopped to 500 ml Erlenmeyers, containing 250 ml of liquid potato-dextrose (BD) medium, followed by incubation at room temperature (±28 °C), under rotary agitation, at 120 rpm for 10 minutes. The mycelium mass was stored at 4oC for 24 hours before its use.

For the granular formulation and immobilization of *Trichoderma* spp. isolates, the methodology adapted from Lewis and Papavizas (1985) was adopted. In the preparation of the trapping matrices, 4g of alginate and 20g of soluble starch were added to 200 ml of sterilized distilled water. This formulation was homogenized for 20 minutes in a mechanical shaker, followed by the inclusion of 35 ml of *Trichoderma* spore suspension standardized to 10^6 CFU mL^{-1}, and homogenized for another 10 minutes. The mixture was dripped into a calcium chloride solution (2%) under light agitation, which allowed the formation of spherical granules of regular diameter (Figure 1).

Figure 1.

Isolates of *Trichodema* spp. immobilized in calcium alginate.

Source: Authors (2022).

After the end of the dripping, the material remained for another 30 minutes under agitation and then was sieved and washed with sterile distilled water to remove residues from the calcium chloride solution. The formed capsules were stored in sterile distilled water in the refrigerator.

To evaluate the viability of the encapsulated fungi and the best storage condition, tests were carried out with the granules stored at room temperatures (between 18 and 27 ºC), refrigerator (between 4 and 7 ºC) and freezer (between -3 and -6 ºC), where plating was done at 7, 14 and 21 days in BDA culture medium. After 6 days of plating, the diameter of the colonies (mm) was measured with a ruler and the conidia were counted in a Neubauer chamber. Culture without immobilization was used for control. The experiments were conducted in a completely randomized experimental design, with three replications, in a factorial design in the following arrangement: Three treatments (*Trichoderma* isolates), three storage time (7, 14 and 21 days) and three conservation conditions (environment, refrigerator and freezer). The assumptions for ANAVA were verified, assessing normality and homogeneity by the Lilliefors and Cochran tests, respectively, and the data were submitted to analysis of variance with the aid of the SISVAR software (FERREIRA, 2000) applying the F test ($p \leq 0.05$) and the means were compared by Tukey's test ($p \leq 0.05$) or polynomial regression.

Results and Discussion

After the preparation of the sodium alginate matrices with the addition of soluble starch and the inclusion of *Trichoderma* spp., capsules with an average size of 5 mm and viable consistency were obtained for the feasibility study of the encapsulated material.

No significant differences were identified (F test, p≤0.05) in mycelial growth among the isolates, regardless of the temperature and storage period they were subjected to. In the sowing in Petri dishes with PDA medium of the alginate capsules, it was possible to verify the radial growth of the fungi present in them, for the storage environments tested, however for refrigerator the mycelial growth was more vigorous.

On the surface of the granule, it was not possible to visualize hyphae or spores of the immobilized fungi, thus showing that the immobilization of microbial cells in alginate is a safe way to preserve the microbial structures, avoiding contamination, since they remain only inside the capsule.

Regarding the number of conidia, the analysis of variance detected significant differences (F test, p≤0.05) among the isolates. No significant interaction was observed between temperature and storage time, demonstrating that temperature did not influence sporulation. In the breakdown of the mean values of the storage conditions, higher values were obtained in refrigerators and freezers, which differed significantly from the environment. The average was only different for isolate B1, with 7 and 14 days of storage, and for M2 with 21 days (Tukey p≥0,05) (Table 1).

Table 1.

Viability of conidia (CFU number log) isolates of *Trichoderma* spp. immobilized in sodium alginate under different storage conditions.

Isolated	Time (days)	Incubation		
		Environment	Refrigerator	Freezer
B1	7	7.80b	7.84a	7.80a
B2	7	7.84a	7.85a	7.84a
M2	7	7.88a	7.86a	7.85a
B1	14	7.73b	7.84a	7.84a
B2	14	7.83a	7.85a	7.83a
M2	14	7.83a	7.87a	7.88
B1	21	7.83a	7.80a	7.82a
B2	21	7.84a	7.84a	7.84a
M2	21	7.75b	7.83a	7.86a
Average		7.82b	7.85a	7.84a

*Average followed by the same letter do not differ from each other by Tukey's test (p≤0,05).

Source: Authors (2023).

The observed results show that the temperatures of the storage environments did not interfere in the viability of the isolates, maintaining the rates of growth speed of the fungus, indicating that the immobilization conferred protection to them. However, the isolates showed better development when stored at lower temperatures, the reduction in the amount of water associated with low temperatures slows down the fungal metabolism, which results in the viability of microbial cells for long periods.

The amount of conidia does not necessarily express the viability of the conidia, because during the process of making the formulations the conidia went through several processing steps, including temperature variation, the results of the amount of conidia alone could not reflect the viability of the conidia after each step (LOCATELLI et al, 2018). Thus, the colony-forming unit (CFU/g) is a more appropriate method to demonstrate the viability of conidia.

Locatelli et al. (2018) studied the development of formulations of *Trichoderma* sp. in encapsulated granules containing sodium alginate modified with different polymers and evaluated the viability of conidia during storage. The authors found that, starting from an initial concentration of 10^{10} CFU^{-1}, it was possible to maintain the viability of conidia stored at 28 °C for up to 14 months at cell concentrations above 106 CFU/g for granules containing alginate, sodium polyphosphate, pectin, and glycerol.

No significant differences were detected for the storage time, and after 21 days, the conidia remained viable (10^7 conidia/g) (Table 1), with no reduction in viability during the evaluation period of the assay.

The efficacy, practicality and safety of the methods for the application and maintenance of fungi are fundamental, both for the success of biocontrol in cultivation systems, and for the acceptance of biocontrol by farmers and society (MACHADO et al., 2012).

Among the polymeric matrices used in the encapsulation of fungi, sodium alginate has been most frequently used. This polymer allows the formation of gels quickly in the presence of calcium ions, without drastic changes in temperature, pH and osmotic pressure, preserving the activity and viability of encapsulated microorganisms (CARVALHO et al., 2006).

The immobilization process occurs through the simple coacervation method, in which a homogeneous solution of charged macromolecules undergoes a separation of liquid phases, giving rise to a colloid rich and dense phase and a less dense, colloid poor phase; both are kept in balance and immiscible (MOURA, 2011).

The protection mechanism occurs because of the formation of the capsule, cover or wall by the encapsulating material, which surrounds the encapsulated material called the filling or core (BARRETO et al., 2015; XING et al., 2014).

In the evaluation of the visual aspect of the alginate capsules, stored in the three environments, it was observed that the capsules remained intact, spherical, opaque, and the

particles containing the fungal isolates presented a vast number of “nuclei” (conidia of the isolates) in all samples, demonstrating high encapsulation efficiency.

Products formulated based on *Trichoderma* spp. contain live spores, making it crucial to observe storage conditions such as refrigeration at temperatures below 28 °C. In addition, field applications should be carried out in conditions of high relative humidity (MACHADO et al., 2012). As observed in the conditions of the present study, the conidia maintained their viability, indicating that the fungus present in the capsules may still be able to grow for a longer period than the one evaluated.

Immobilization methods have both advantages and disadvantages. Although the stability of the cells is not assured, some notable advantages include: The retention of the inoculum in the holder, allowing a more effective control of the actual properties of the medium, higher purity and increased yield (PRADELLA, 2001). In addition, it presents the possibility of continuous use of the cells and the protection of the alginate (VILELA et al., 2012).

Locatelli et al. (2018) studied the development of *Trichoderma* spp. formulations in encapsulated granules containing sodium alginate modified with different polymers and evaluated the viability of conidia during storage. The authors found that, starting from an initial concentration of 10^{10} CFU/g, it was possible to maintain the viability of conidia stored at 28 °C for up to 14 months at cellular concentrations above 10^{6} CFU/g for granules containing alginate, sodium polyphosphate, pectin and glycerol.

Conclusion

Under the conditions in which the assay was conducted, the technique used for immobilization of *Trichoderma* isolates in calcium alginate was adequate for the isolates studied.

REFERENCES

BARRETO, A. R.; RAMÍREZ-MÉRIDA, L. G.; ETCHEPARE, M. A.; JACOB-LOPES, E.; MENEZES, C. R. Materiais de revestimento usados na microencapsulação de probióticos. Ciência e Natura, v. 37, p. 164-174, 2015. http://dx.doi.org/10.5902/2179-460X19747

BRAGA, A. B. A. C.; COSTA, C. J. M.; POMELLA, A. W. V.; RIBEIRO, E. J.; SANTOS, L. D.; ZOTARELLI, M. F. Evaluation of lethality temperature and use of different wall materials in the microencapsulation process of *Trichoderma asperellum* conidias by spray drying. Powder Technol, v. 347, p. 199–206, 2019. https://doi.org/10.1016/j.powtec.2019.02.037

BRAGA, A. B. A. C.; COSTA C. J. M.; RIBEIRO, E. J.; ZOTARELLI, M. F.; SANTOS, L. D. Evaluation of the microencapsulation process of conidia of *Trichoderma asperellum* by spray drying. Braz Journal Microbiol v. 53, 1871–1880,022. https://doi.org/10.1007/s42770-022-00832-z

CARVALHO, W.; CANILHA, L.; SILVA, S. S. D. Uso de biocatalisadores imobilizados: uma alternativa para a condução de bioprocessos. Revista Analytica, v. 23, pp. 60-70, 2006. https://www.researchgate.net/publication/334330145_Uso_de_biocatalisadores_imobilizados_Uma_alternativa_para_a_conducao_de_bioprocessos

CHAGAS, L. F. B.; CASTRO, H. G.; COLONIA, B. S. O.; CARVALHO FILHO, M. R.; MILLER, L. O.; CHAGAS JUNIOR, A. F. Efficiency of *Trichoderma* spp. as a growth promoter of cowpea (*Vigna unguiculata*) and analysis of phosphate solubilization and indole acetic acid synthesis. Brazilian Journal of Botany, São Paulo-SP, v. 38, n. 4, p. 1-11, 2016. https://doi.org/10.1007/s40415-015-0247-6

CHAGAS, L. F. B.; CHAGAS JUNIOR, A. F.; SOARES, L. P.; FIDELIS, R. R. *Trichoderma* na promoção do crescimento vegetal. Revista de Agricultura Neotropical, v. 4, p. 97-102, 2017. https://doi.org/10.32404/rean.v4i3.1529

FÁVARO-TRINDADE, C. S.; PINHO, S. C.; ROCHA, G. A. Revisão: Microencapsulação de ingredientes alimentares. Revista Brasileira de Tecnologia de Alimentos, v. 11, n. 2, p. 103-112, 2008. https://www.researchgate.net/publication/237521407_Revisao_Microencapsulacao_de_ingredientes_alimenticios

FAVARO-TRINDADE, C. S.; SANTANA, A. S.; MONTERREY-QUINTERO, E. S.; TRINDADE, F. M.; NETTO, M. A. Uso da tecnologia de secagem por pulverização para redução do sabor amargo do hidrolisado de caseína. Food Hydrocolloids, v. 24, p. 336-340, 2010. https://doi.org/10.1016/j.foodhyd.2009.10.012

FERREIRA, D. F. Análises estatísticas por meio do SISVAR para Windows® versão 4.0. In: REUNIÃO ANUAL DA REGIÃO BRASILEIRA DA SOCIEDADE INTERNACIONAL DE BIOMETRIA, 45, 2000, São Carlos, SP. Programas e Resumos. São Carlos: UFSCar, 2000. p. 235.

GHARSALLAOUI, A.; ROUDAUT, G.; CHAMBIN, O.; VOILLEY, A.; SAUREL, R. Aplicações da secagem por atomização na microencapsulação de ingredientes alimentícios: uma visão geral. Food Research International, 40, p. 1107–1121, 2007. https://doi.org/10.1016/j.foodres.2007.07.004

GUZMÁN-GUZMÁN P, PORRAS-TRONCOSO MD, OLMEDO-MONFIL V, HERRERA-ESTRELLA A. *Trichoderma* Species: Versatile Plant Symbionts. Phytopathology. V.1, p. 6-16, 2019. https://doi.org/10.1094/PHYTO-07-18-0218-RVW

LEWIS, J.A.; PAPAVIZAS, G.C. Características de pellets de alginato formulados com *Trichoderma* e *Gliocladium* e seu efeito na proliferação de fungos no solo. Plant Pathology, London, v.34, n.6, p.571-577, June 1985. https://doi.org/10.1111/j.1365-3059.1985.tb01409.x

LOCATELLI, G. O.; SANTOS, G. F.; BOTELHO, P. S.; FINKLER, C. L. L.; BUENO, L. A. Desenvolvimento de *Trichoderma* sp. formulações em grânulos encapsulados (GC) e avaliação da vida útil dos conídios. Controle biológico, v. 117, p. 21-29, 2018. https://doi.org/10.1016/j.biocontrol.2017.08.020

MACHADO, D. F. M.; PARZIANELLO, F. R.; SILVA, A. C. F.; ANTONIOLLI, Z. I. *Trichoderma* no Brasil: o fungo e o bioagente. Revista de Ciências Agrárias, v. 35, n. 1, p. 274-288, 2012. https://doi.org/10.19084/rca.16182

MESTDAGH, M. M.; ANEXELOS, M. A. V. Physic-chemical properties of polycarboxylate gel phase and their incidence on the retention/release of solutes. Biopolymer science: Food and Non-food Applications, p. 303-314, 1998.

MOURA, D. S.; Estudo espectroscópico de esferas à base de alginato contendo *Trichoderma harzianum* visando o controle de fitopatógenos. (Dissertação de Mestrado). Departamento de Física, Universidade Federal Rural de Pernambuco, Recife, 2011. https://oasisbr.ibict.br/vufind/Search/Results?lookfor=Estudo+espectrosc%C3%B3pico+de+esferas+%C3%A0+base+de+alginato+contendo+Trichoderma+harzianum+visando+o+controle+de+fitopat%C3%B3genos&type=AllFields

OLIVEIRA, A. G.; CHAGAS JÚNIOR, A. F.; SANTOS, G. R.; MILLER, L. O.; CHAGAS, L. F. B. Potencial de solubilização de fosfato e produção de AIA por *Trichoderma* spp. Revista Verde de Agroecologia e Desenvolvimento Sustentável, v. 7, p. 149-155, 2012. https://dialnet.unirioja.es/servlet/articulo?codigo=7419841

PRADELLA, J. G. C. Reatores com células imobilizadas. *In:* SCHMIDELL, W.; LIMA U. A.; Aquarone E.; BORZANI, W. Biotecnologia industrial. Editora Edgard Blücher Ltda., São Paulo. 2001. P. 355–371.

SÁNCHEZ-MONTESINOS B.; DIÁNEZ F.; MORENO-GAVIRA A.; GEA F. J.; SANTOS M. Plant Growth Promotion and Biocontrol of *Pythium ultimum* by Saline Tolerant *Trichoderma* Isolates under Salinity Stress. Int J Environ Res Public Health, v. 16, p. 2053, 2019. doi: 10.3390/ijerph16112053.

VEMMER, M.; PATEL, A.V. Revisão de métodos de encapsulamento adequados para agentes de controle biológico microbiano. Biological Control, v. 67, p. 380-389, 2013. https://doi.org/10.1016/j.biocontrol.2013.09.003

VILELA, A.; SCHULLER, D.; MENDES-FARIA, A.; CÔRTES-REAL, M. Redução da acidez volátil de vinho por células de *Saccharomyces cerevisiae* imobilizadas em esferas de alginato-quitosano. Revista Enologia, p. 38-42, 2012. https://doi.org/10.1007/s00253-013-4719-y

XING, Y.; XU, Q.; MA, Y.; CHE, Z.; CAI, Y.; JIANG, L. Efeito das concentrações de amido poroso nas características microbiológicas de *Lactobacillus acidophilus* microencapsulado. Comida e função, v. 5, n. 5, p. 972-983, 2014. https://doi.org/10.1039/C3FO60438A.