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Preformulation study as a basis for the development of *Streptomyces* sp. PNM-9 microparticles for biological control

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ABSTRACT

The use of biocontrollers such as *Streptomyces* sp. PNM-9, which is effective against *Burkholderia glumae* and *Colletotrichum gloeosporioides*, the phytopathogens of yam and rice crops, serves as a promising alternative to pesticides. However, its effectiveness may be compromised by environmental stress. The preformulation of a microparticulate system that could contain the biocontroller was conducted to improve its stability. *Streptomyces* sp. PNM-9 was cultivated on rice husk and kaolin solid supports, subjecting them to temperatures of 60°C and 80°C for 10 minutes and desiccation stress for 6 hours at 25°C. To evaluate compatibility with wall materials, selected and inoculated solid support was used to prepare mixtures with maltodextrin, soy protein, and gum arabic. Each of these mixtures was subsequently spray-dried under predefined conditions. Solid supports demonstrated a protective effect on *Streptomyces* sp. PNM-9 against high temperature and drying conditions, in contrast to the broth. No statistically significant differences were found in the viability of *Streptomyces* sp. PNM-9 between the two supports after exposure to different temperatures and drying conditions. The rice husk, selected as the solid support, showed no incompatibilities with the wall materials. Therefore, subsequent studies focusing on microparticle formulation with rice husk and the evaluated polymer as wall material are strongly recommended.

INTRODUCTION

The indiscriminate use of chemical pesticides has caused several problems. These include environmental pollution, negative effects on human and animal health, and the development of pest resistance. This resistance forces the use of higher concentrations of pesticides [1,2]. In response to the adverse effects arising from chemical pesticide application, diverse alternative strategies, particularly the employment of biocontrol agents utilizing organisms or their secondary metabolites, have been pursued to ameliorate phytopathogen-mediated damage [3,4].

In the search for biocontrol agents, *Streptomyces* spp. can be used as potential biocontrol agents against a wide range of phytopathogens, due to the large number of secondary metabolites with biological activity that have been attributed to them in various fields such as medicine and agriculture [5,6]. Studies conducted by Betancur *et al*. [6,7] analyzed the abilities of *Streptomyces* strains (PNM-161 and PNM-9) to produce secondary metabolites with biocontrol potential. As a result, some compounds, such as 2-methyl-N-(2′-phenylethyl)-butanamide and 3-methyl-N-(2′-phenylethyl)-butanamide, were identified that showed activity against relevant phytopathogens in rice and yam crops, such as *Burkholderia glumae* (ATCC 33617), *B. gladioli* (3704−1-FEDEARROZ), and *Colletotrichum gloeosporioides*.

Although biocontrol agents are a viable alternative to pesticides, their formulation as agricultural products is crucial for their application and commercial success [8]. In the market for agricultural products containing biocontrollers, most of

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the formulations marketed are in granulated and liquid form. Nevertheless, they reveal difficulties linked to stability and the maintenance of cell viability, which leads to a considerable decrease in product yield [9]. Due to the difficulties that these formulations can exhibit, the use of microencapsulation techniques has been proposed, which provide solutions to the problems in conventional formulations and may guarantee better maintenance of cell viability [10,11]. Mancera-Lopez *et al.* [12] employed an extrusion and desiccation method for the microencapsulation of a *Streptomyces* strain. Their encapsulation approach yielded viable formulations, demonstrating an 87% success rate.

Microencapsulation is a technology based on coating the active ingredient or biocontroller with an encapsulating agent that acts as a barrier (wall material), completely isolating the core material from the external environment. This process protects the viability of microorganisms and facilitates its incorporation into various formulations, prolonging its stability and optimizing its functionality [13]. Before starting the development of microencapsulation, it is crucial to perform preformulation studies. These are understood as a phase in bioproduct development where the biocontroller's stability under various conditions (e.g., temperature and humidity) and the compatibility of the core with the wall materials are examined and defined [14].

Various techniques exist for microparticle development, with physical methods being the most common. These methods are also considered safest for producing small particles with high viability. Freeze-drying, a prominent drying method widely used in probiotic microencapsulation, presents disadvantages such as high cost and prolonged processing time [15]. Conversely, spray drying is notable for its versatility, low production costs, speed, and reproducibility, enabling scalable microparticle production [16]. Small, well-formed particles can be achieved by establishing crucial conditions in this method, such as air flow, supply temperature, and air inlet and outlet temperatures.

However, other factors influence microparticle outcomes, notably the choice of wall material. Natural polymers such as maltodextrins, gum arabic, and vegetable proteins such as isolated soy protein have proven suitable for microbial microencapsulation, yielding promising results individually or in combination [9,17]. Furthermore, the efficacy of this immobilization technique can be improved through a combination with other methods, such as adsorption on solid supports [18]. As demonstrated by Baena-Aristizábal *et al.* [19], combining microencapsulation and immobilization on a solid support yielded enhanced microparticles containing *Rhizobium* strains.

Having understood the benefits offered by the spray drying method, this research aimed to study the preformulation of microparticles containing *Streptomyces* sp. PNM-9, which has been proven to act as an antibiotic against the phytopathogens *C. gloeosporioides* and *B. glumae*, which have an impact on yam and rice crops, respectively [6,7].

MATERIALS AND METHODS

Streptomyces sp. and encapsulation materials

The *Streptomyces* sp. PNM-9 was isolated from marine environments along the Colombian Caribbean region [7]. Samples were collected under the collecting permit granted by the Ministerio de Ambiente y Desarrollo Sostenible (Permiso N°4 10/02/2010 Anexo 2; Contrato de Acceso a recursos Genéticos y sus productos derivados N°108; Contrato Marco de Acceso a Recursos Genéticos y sus productos derivados No. 121, otrosí No. 6). Reactivation of the *Streptomyces* sp. PNM-9 was performed by streaking colonies preserved on ISP-2 agar plates, a culture medium belonging to the International Streptomyces Project (ISP) series [7], previously described by Betancur *et al.* [20], and frequently used for the growth of this type of microorganism. The ISP-2 medium was prepared with the following composition: yeast extract 4 g/l, malt extract 10 g/l, dextrose 4 g/l, and agar 20 g/l. The inoculated plates were incubated at 28°C for seven days.

Rice husks (RH) were obtained and milled from a commercial supplier (Jardineros de la Sabana, Bogotá, Colombia), the kaolin was provided by Cavisa (La Coruña, Spain), maltodextrin was obtained from the Grain Processing Corporation (Iowa, USA), soy protein was purchased from Liaoning Pharmaceutical Foreign Trade Corporation (Shenyang, China), and acacia gum was provided by Willy Benecke (Hamburg, Germany). All solid support and wall materials were sterilized.

Preparation of *Streptomyces* sp. inoculums

Activated colonies of *Streptomyces* sp. PNM-9 were inoculated into 10 ml of Luria Bertani (LB) medium (composed of 10 g/l tryptone, 5 g/l yeast extract, and 0.5 g/l sodium chloride) and incubated at 28°C in an orbital shaker (THZ-300, BOYN) at 110 rpm for 72 hours. Subsequently, the preinoculum concentration was adjusted to 1×10^8 cells/ml by comparing its optical density at 625 nm to the McFarland 0.5 standard, using a UV/VIS spectrophotometer model S2100 UV+ (Cole Parmer).

Inoculum preparation followed the method described by Baena-Aristizabal *et al.* [7,19]. Briefly, 200 ml of LB broth was prepared and divided into two screw-cap bottles. The adjusted pre-inoculum was then transferred to these bottles. Both inoculated broths were incubated at 28°C in a shaking incubator (THZ-300, BOYN) at 110 rpm for 72 hours. Bacterial cell concentration was determined by measuring the optical density at 625 nm until reaching 1×10^8 CFU, using a UV/VIS spectrophotometer model S100 UV+ (Cole Parmer).

Support materials were inoculated following the inoculation process described by Baena-Aristizábal *et al.* [19] and Albareda *et al.* [21], with minor modifications to sample quantity and sterilization method. Fifty grams of each support material (rice husk and kaolin) were weighed in duplicate and placed into 250 ml screw-cap bottles, which were then steam sterilized (Sturdy SA-300H). Each support material was inoculated with 100 ml of culture broth in a class II A1 laminar flow cabinet and incubated at 28°C for 15 days.

Temperature tolerance

Temperature tolerance tests were performed by suspending 1 g of each support material in phosphate buffer solution (pH 7.0), fresh LB broth was included as a control. The samples and control were exposed to temperatures of 60°C and 80°C in a preheated water bath (Memmert WB-7), with their

respective replicates. These temperatures were selected based on the outlet temperatures of the spray dry equipment used by Baena-Aristizabal *et al.* [19]. Duplicate samples were taken after 5 and 10 minutes to assess bacterial survival. Serial dilutions (fourfold) of the samples were prepared in phosphate buffer (pH 7.0), followed by microdroplet seeding. Thirty microliters of aliquots were then dispensed in parallel lines onto ISP 2 medium. The colonies formed were counted after incubation at 28°C for four days in incubator plates (Memmert UL50), and viability was expressed in CFU/ml or CFU/g. The experiment was performed twice following the protocols described by Baena-Aristizabal *et al.* [19].

Drying tolerance

Twenty-five grams of each of the previously inoculated supports (rice husk and kaolin) were taken and deposited on two trays sterilized using UV light and heat. The trays were placed inside a laminar flow cabinet at room temperature (25°C ± 1°C, 50% ± 2% RH) for 6 hours, following the protocols described by Baena-Aristizabal *et al.* [19]. Every 2 hours, 1.5 g of each support was extracted from each tray and suspended in 10 ml of pH 7.0 phosphate buffer in 15 ml Falcon tubes. These suspensions were diluted four times, and subsequently, different 30 µl aliquots were taken and inoculated via microdroplet seeding (a total of six drops) onto ISP-2 media. The colonies formed were counted after incubation at 28°C for four days in incubator plates (Memmert UL50), and viability was expressed in CFU/g. A moisture analyzer balance (MA 50/1.R) was used to measure the moisture content at 130°C, and the moisture percentage of each support was quantified.

Compatibility study

The core of the microparticles was formed by *Streptomyces* sp. PNM-9 inoculated in the previously selected solid support (rice husk). Maltodextrin, isolated soy protein, and gum acacia were used as wall materials, commonly employed in microparticle production via the spray drying method [22,23]. During the test, 200 ml suspensions of the different previously sterilized and hydrated wall materials were prepared in distilled water with the core materials at a 1:1 ratio. These suspensions were then mixed using a high-speed stirrer (IKA T18 ULTRA TURRAX) at 11000 rpm for one minute. Subsequently, the suspensions were dried using spray drying equipment (Mini Spray Dryer Buchi B-290) with the following parameters: inlet temperature of 130°C, aspirator speed of 35 m³/hour, constant airflow of 819 l/hour, and a pumping speed of 2.5 ml/minutes, as described by Baena-Aristizábal *et al.* [19]. For the samples in which soy protein and acacia gum were used as wall material, the feed flow was increased to 5 ml/minutes, because the suspensions were highly viscous. The compatibility of the core and wall material was evaluated according to microorganism viability; for this purpose, 1 g of the dry powder obtained from each sample was taken and rehydrated in 9.9 ml of phosphate buffer pH 7.0, which was previously sterilized and stirred for 10 minutes. Four serial dilutions of the suspensions were performed. They were inoculated in an ISP-2 medium at 28°C for 5 days to count the number of viable cells.

Statistical analysis

Temperature tolerance and drying tolerance assays were analyzed using a viability data linear regression model expressed logarithmically versus time. Interactions between factors were determined by analysis of covariance (ANCOVA) using SPSS® 30.0 statistical software (IBM). Mean viability in compatibility assays was analyzed by way of an ANOVA test and Tukey's post hoc test (95%) using Minitab® 19.1 software (MiniTab Inc).

RESULTS AND DISCUSSION

Temperature tolerance

The results obtained at different temperatures (60°C and 80°C) and using different supports (rice husk and kaolin) showed no statistically significant differences in the cell viability of *Streptomyces* PNM-9 for any of the main variables or factors. However, multifactor interactions revealed by the inter-subject test in the analysis of covariance (ANCOVA) indicated that only the interaction between time and support was statistically significant with respect to bacterial survival ($p < 0.001$) (Table 1).

In the liquid medium (fresh LB broth), cell viability at the tested temperatures and time points showed a gradual decrease, as expected (Fig. 1A). Starting with an initial cell concentration of 1×10^6 CFU/ml, viability decreased to 5×10^3 CFU/ml after 5 minutes at both temperatures. No viable cells were detected after 10 minutes at the same temperatures. However, when incubated with the supports, viability was not significantly affected by temperature ($p = 0.619$). These results confirm the protection provided by the supports, which has been previously reported in other studies using spray drying (with high temperatures) for the microencapsulation of microbial cells [24].

On the other hand, the initial cellular load in rice husk was 3.0×10^5 CFU/g, subsequently when exposed to temperatures of 60°C and 80°C over the course of 5 and 10 minutes, it was observed that cell concentrations were almost constant throughout the different exposure times (Fig. 1B). The preservation of viability at high temperatures could be the reason why the interaction between time and supports has a statistically significant difference with respect to cell viability

Table 1. Significance of factors and interaction during the evaluation of temperature tolerance of *Streptomyces* PNM-9 inoculants based on two carriers (rice husk and kaolin). Data were fit to a linear regression model using log-transformed data.

Factors and interactions	SE	*p*-value
Time	0.690	0.660
Supports	0.138	0.263
Temperature	3.473	0.619
Time: Temperature		0.347
Time: Support		< 0.001*
Support: Temperature		0.167

p-values determined by covariance analysis (ANCOVA) with 95% confidence. The values are means from two independent experiments, each one with two replicates ($n = 4$). Values marked with an asterisk (*) are statistically significant.

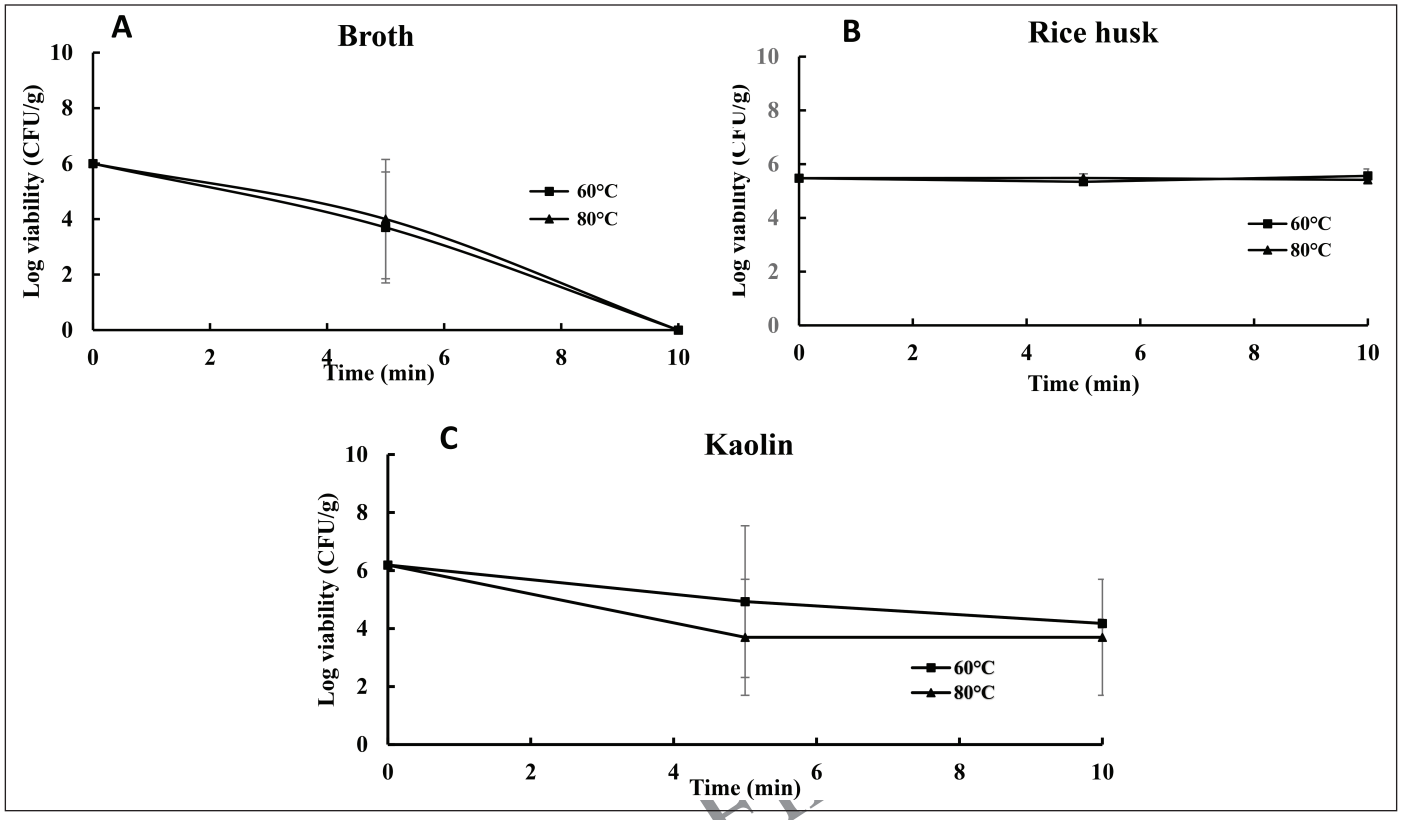

Figure 1. Survival of *Streptomyces* PNM-9 in fresh broth and cultivated on rice husk and kaolin when exposed to different temperatures (60 °C and 80 °C). The values are the average of two independent experiments each with two replicates (n = 4). The bars in the figure correspond to the SEM.

(Table 1). Temperature tolerance and the maintenance of cell viability in rice husk can be attributed to its composition, specifically its 50% cellulose, 25%–30% lignin, 15%–20% silicon, and 10% water content, which facilitates favorable interactions. Furthermore, its porosity aids in the adsorption and entrapment of microbial cells, contributing to this protection. Cellulose and lignin can be used as carbon sources, and the presence of functional groups in these molecules such as hydroxy, methoxy, and carboxyl facilitates interactions with the external structures of microbial cells. This attachment to a support has been documented as a strategy for improving tolerance under unfavorable conditions. It is suggested that this mechanism has maintained the viability of *Streptomyces* PNM-9 in this study [25–27]. In addition to adsorption, rice husk has been used as a support in the production of secondary metabolites of interest through solid-state fermentation [28]. In other studies, rice husk was used for the production of enzymes, such as xylanases from *Streptomyces olivaceoviridis* [29]. Therefore, rice husk should be considered as a solid support due to its ability to preserve cell viability by providing temperature tolerance, potential to induce the production of valuable metabolites, and cost-effectiveness, which supports its growing use in agriculture [30].

Similarly, the initial cellular load in kaolin (1×10^6 CFU/g) was equal to that obtained in the fresh broth and higher than that initially obtained in the rice husk. Subsequently, exposure to 60°C and 80°C resulted in a decline in cell viability; however, cells remained viable even at the longest exposure time (Fig. 1C). At 60°C, the cell concentration decreased to approximately 8.5×10^4 CFU/g after 5 minutes and further decreased to 1.5×10^4 CFU/g after 10 minutes. Likewise, at 80°C, cell viability also declined, but alive cells were still detectable at both 5 and 10 minutes, with a concentration of 5.0×10^3 CFU/g for both time points. Despite showing lower results than the rice husk at different temperatures and exposure times, viability was maintained. Kaolin is a set of clay minerals in which kaolinite ($Al_2Si_2O_5(OH)_4$) is most commonly known and utilized. It is spread across different sheets to aid water retention and favor the adsorption of microorganisms on its surface. The interactions that form microbial cell membranes with the surface of the kaolin are mostly electrostatic, non-electrostatic, and hydrogen bonds. External structures such as fatty acids, proteins, and polysaccharides mainly favor interactions that occur with silane and hydroxyl groups and with the rest of the kaolin structure that is negatively charged [31,32]. Its use in solid formulations is recognized in agriculture for maintaining product viability during storage and as a low-cost support [33]. For instance, a study by Myo *et al.* [34] showed that a granulate containing *Streptomyces fradiae* NKZ-259 maintained significant viability for four months at 4°C and 27°C. Similarly, Zacky and Ting [35] found that the biocontrol activity of a *Streptomyces griseus* strain improved. Furthermore, kaolin has been used as a core material with polymers such as chitosan and alginate as wall materials [33]. While its role in maintaining viability during storage is documented, information on how it can enhance the temperature tolerance of inoculated microorganisms is lacking.

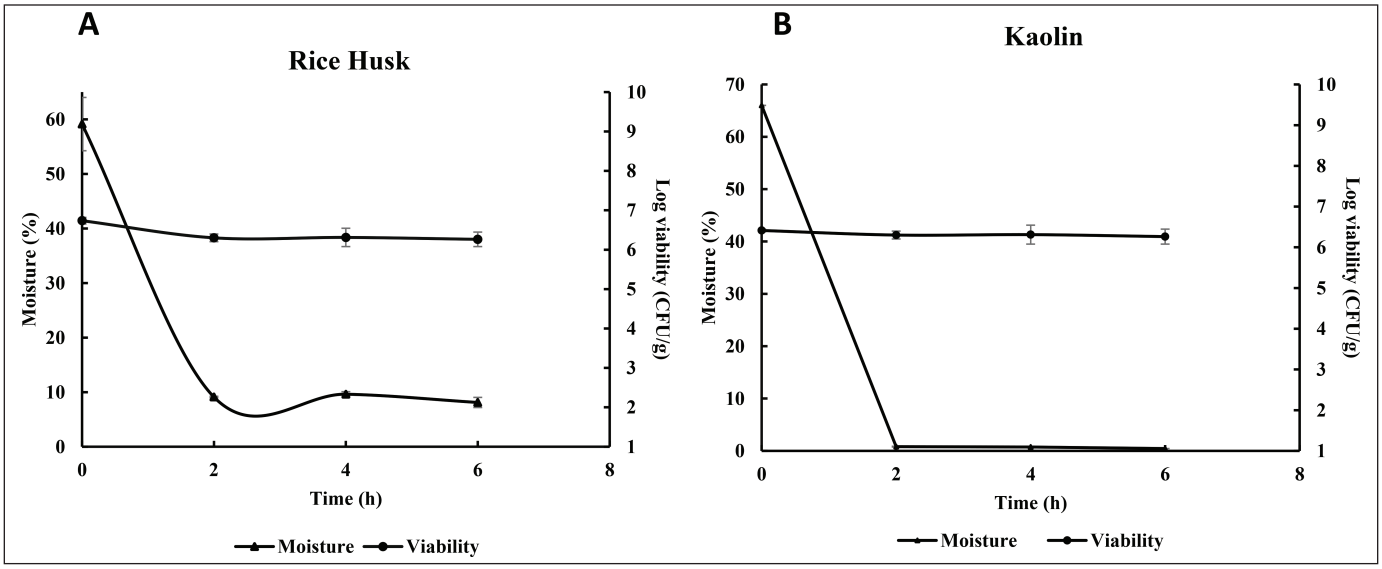

Figure 2. *Streptomyces* sp. PNM-9 growth in a solid medium (ISP-2) when exposed to different drying times (2, 4, and 6 hours). The bars in the figure refer to the SEM.

Drying tolerance

The drying rate significantly impacts the quality of microbial spray formulations [36]. While drying promotes cell inactivation and stability through dehydration, it also induces desiccation stress, potentially reducing the formulation's cell load, particularly for non-spore-forming bacteria. Therefore, drying processes should consider other formulation parameters affecting cell viability, such as the inclusion of osmoprotectants or support materials [8,19].

The initial moisture content of the inoculated kaolin and rice husk supports before drying was 66% ± 8% and 59% ± 7%, respectively. Following 6 hours of drying, a slight decrease in cell viability was observed for both carriers, with no significant statistical difference in viability conservation between them (p = 0.561). This minimal reduction indicates that the decrease in moisture content did not significantly impact the cell viability of *Streptomyces* sp. PNM-9 under the experimental conditions (p = 0.890), despite the faster drying rate of kaolin compared to rice husk (Fig. 2). Furthermore, the interaction between drying time and support type showed no statistically significant effect on cell viability conservation (p = 0.936) (Table 2). Nevertheless, retaining a certain level of moisture in the solid supports, as seen in rice husk with 8%, is essential for ensuring the long-term viability, stability, and functionality of the final product [37].

The sustained cell viability observed during drying exposure could be attributed to the formation of uninucleoid endospores. These structures, characteristic of some microorganisms, are known for their significant resistance to challenging environmental conditions, including desiccation. Their resilience is due to their complex morphology involving multiple surface layers, their low internal water content, and the accumulation of osmoprotective compounds such as trehalose. This compound provides protection not only to the spores but also to the vegetative mycelium phase under desiccation stress

Table 2. Factors and interactions during the evaluation of drying tolerance of *Streptomyces* sp. PNM-9 based on two solid supports (rice husk and kaolin). The data were adjusted to a linear regression model expressed logarithmically versus time.

Factors and interactions	SE	*p*-value
Moisture	0.022	0.890
Supports	0.293	0.561
Time	1.462	0.975
Support: Time		0.936

p-values were determined by the way of analysis of covariance (ANCOVA) with 95% confidence. Values marked with an asterisk (*) are statistically significant.

[38–40]. The process of spore production in *Streptomyces* sp. when faced with unfavorable conditions involves intricate mechanisms, such as the activity of RNA polymerase promoters known as sigma (σ) factors. These factors play a role in the expression of genes that modulate responses to stress conditions that induce osmotic stress, including desiccation [41].

The interaction between the microorganism and the support materials did not exert a considerable influence on the maintenance of cell viability. The final moisture percentage obtained in the rice husk (8%) is close to that reported by other investigators who have used rice husk as a potential support in microorganism-based formulations. Its low moisture content and high capacity for water absorption make the use of rice husk as a support material suitable, in addition to displaying a long shelf life [42]. However, no documented information exists regarding the protective mechanisms of rice husk for *Streptomyces* spp. specifically during drying processes. Consequently, the data obtained in this study could be valuable for future research exploring the use of rice husk as a support material. On the other hand, kaolin has been employed as a support material in studies involving drying techniques, yielding inconsistent results concerning the maintenance

Table 3. Compatibility results of the inoculated rice husk (core) with the wall materials.

Wall material + core material	Viability (log CFU/g)
RH	5.5 (0.022)
MD+RH	5.3 (0.11)
SPI+RH	5.0 (0.27)
GA+RH	5.0 (0.21)

The values are presented as the mean ± SEM (n = 3). Values marked with an asterisk (*) indicate significant differences according to Tukey test (95%). MD = Maltodextrin; SPI = Soy protein isolate; GA = Gum acacia; RH = Rice husk.

of cell viability. Notably, in the research conducted by Mancera-Lopez *et al.* [12], the incorporation of kaolin as a protective agent in alginate capsules containing *Streptomyces* sp. CDBB1232 failed to preserve or enhance cell viability following the drying process.

In accordance with the abovementioned, and although no significant differences in cell viability maintenance were observed during drying for both of the used supports, rice husk was selected for further evaluation in the spray drying process. This choice was made due to the temperature tolerance test results, which indicated that the microorganism inoculated in rice husk exhibited superior tolerance to the applied temperatures.

Compatibility study

For this test, suspensions of the microorganism inoculated in the rice husk were prepared with the different wall materials commonly used in the preparation of microparticles for the spray drying method, such as maltodextrin, isolated soy protein, and gum acacia. The compatibility of the different wall materials with the inoculated microorganism was determined by analyzing the maintenance of cell viability once each of the prepared suspensions was dried in the equipment. Among the tested materials, maltodextrin exhibited the highest conservation of cell viability, resulting in an average cell load of 5.33 log CFU/g. Isolated soy protein and gum acacia showed comparable cell loads of 5.0 log CFU/g. Notably, no statistically significant differences ($p = 0.137$) in viability were found between the combinations of various wall materials with the core and the core used individually (Table 3). This indicates that the rice husk-inoculated *Streptomyces* sp. was compatible with the evaluated wall materials.

CONCLUSION

The results obtained from the temperature tolerance test showed better thermal compatibility of the *Streptomyces* strain PNM-9 in rice husk, suggesting its potential use as a solid support. Regarding tolerance to drying, there were no statistically significant differences between the two solid supports used (rice husk and kaolin), indicating that they both can be resistant to drying. Finally, the compatibility study with wall polymers (maltodextrin, soy protein isolate, and gum acacia) showed that they did not significantly affect the viability of *Streptomyces* sp. PNM-9 and could be used as wall materials in the formulation of the microparticles.

Future studies should focus on establishing the formulation of microparticles containing *Streptomyces* sp. PNM-9 inoculated in rice husk. Field trials are necessary to evaluate their biocontrol efficacy. This present preformulation study lays the groundwork for the future development of *Streptomyces* microencapsulation for biocontrol, a promising alternative that faces key challenges such as establishing clear regulatory guidelines on its stability and release for registration. Future formulation studies will need to optimize application methods to protect the microorganism and evaluate its efficacy in the field. Additionally, developing cost-effective scale-up strategies will be crucial to ensure product quality. Commercial success will depend on demonstrating superior field performance and longer shelf life, consolidating *Streptomyces* as a competitive alternative in pest management.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

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