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BIOCHAR-BASED CARRIERS FOR MICROBIAL INOCULANTS IN SUSTAINABLE AGRICULTURE: A STUDY ON SURFACE PROPERTIES, MICROBIAL VIABILITY, AND PERSISTENCE

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Abstract: Biochar-based carriers have emerged as a potential tool to support microbial inoculants in sustainable agriculture, but their effectiveness depends on the viability and persistence of the inoculated microorganisms. In this study, the viability and persistence of three beneficial microorganisms *(Bacillus amyloliquefaciens*, *Streptomyces griseus*, and *Trichoderma harzianum)* were evaluated on five biochar feedstocks: sunflower husk, wood chips, green waste, pelletized green waste, and a wood–zeolite composite (75:25 ratio). Each biochar was characterized for Brunauer–Emmett–Teller (BET) surface area and pore volume to assess physicochemical properties relevant to microbial habitation. Under sterile conditions, each biochar was inoculated with a single microbial strain and incubated under controlled conditions for ten weeks. Microbial viability was quantified weekly using Most Probable Number (MPN) counts to monitor population dynamics, and a follow-up assay was conducted two months after the initial incubation to evaluate longer-term persistence. The results showed that *Bacillus amyloliquefaciens* remained viable throughout the ten weeks, and at the follow-up, *Trichoderma harzianum* similarly persisted across the experimental timeframe, especially on specific biochar types. In contrast, *Streptomyces griseus* declined rapidly and became undetectable by week seven under the tested conditions. The wood–zeolite composite biochar consistently supported higher survival of the tested microorganisms. In contrast, pelletized green waste exhibited the lowest microbial survival across all strains, despite not having the lowest BET surface area and pore volume. These observations indicate that measuring the biochar surface area alone is insufficient to determine the suitability of carriers for microbial inoculants. Instead, selecting biochar based on a combination of physicochemical properties can improve the design of biochar-based microbial carriers. Overall, these findings support targeted biochar selection to enhance the viability and persistence of beneficial microbes in sustainable agricultural applications.

Keywords: biochar; surface; microbes; viability; MPN

1. Introduction

Biochar is a carbon-rich solid produced by biomass pyrolysis. It is increasingly studied as a soil improver due to its high porosity, adsorption capacity, and surface area [1], [2]. It is highly permeable and moisture-

retaining, often enriched in organic C, N, P and K, and bears numerous surface functional groups (hydroxyl, carboxyl, amino, etc.) that promote the microbiome adhesion and proliferation [3]. In effect, biochar can provide a protective microhabitat due to its high specific surface area and pore network, which buffer environmental stresses (e.g., pH change, drought, potentially toxic elements) and supply adsorbed nutrients to microbes [2], [3]. Indeed, biochar-immobilised inoculants have repeatedly shown enhanced persistence, survival, and plant-root colonisation compared to non-char carriers [4]. These advantages make biochar a promising, renewable alternative to peat or other traditional soil improver matrices in sustainable agriculture [4], [5]. Higher pyrolysis temperatures generally increase biochar porosity and surface area while decomposing labile organics. He et al. (2025) found that raising the pyrolysis temperature converted a wood-derived biochar from a primarily macroporous material into a highly microporous one, significantly expanding its surface area [6]. These textural features are hypothesised to influence the performance of biochar carriers. The abundant micropores can retain water and adsorb organic nutrients, thereby sustaining microbial metabolism, whereas larger pores and surface functional groups improve cell attachment [7], [8]. Because pyrolysis also alters pH and nutrient content, the combined physicochemical profile (BET area, pore volume, surface chemistry, etc.) of each biochar is expected to determine how well different microbial species survive when immobilised on the surface [9].

Understanding the mechanistic relations between specific biochar properties and inoculant change is therefore essential for moving beyond descriptive comparisons toward predictive carrier design. Prior research has frequently reported pairwise correlations among single material attributes, e.g., BET surface area or pH [10], [11]. Still, it has rarely integrated comprehensive, multivariate biochar characterisations with inoculant lifetime traits and biological functional interactions. Such integration is required to discriminate whether observed survival gains arise primarily from moisture buffering, nutrient adsorption and slow release, electrostatic or hydrophobic cell–surface interactions, physical protection from predators or antagonists, or synergistic combinations of these effects [12]. Mechanistic insight also enables identification of which properties are most amenable to engineering (e.g., targeted pore-size distributions, surface functionalisation, or co-amendment with mineral carriers) to enhance persistence under field-relevant stressors.

Despite the recognised importance of these properties, the long-term viability of inoculants on different biochar carriers remains incompletely understood. Few studies have systematically compared multiple biochar types under controlled conditions, and there is a lack of clear criteria for selecting the best carrier for each inoculant. To address this gap, our study compares the survival of three beneficial strains, a spore-forming bacterium, *Bacillus amyloliquefaciens*; *Streptomyces griseus*, which branches profusely to form a mycelial network; and the fungus *Trichoderma harzianum* on five representative biochars (sunflower husk, wood chips, green waste, pelletized green waste, and a 75:25 wood–zeolite mix).

2. Materials and Methods

2.1. Biochar surface and pore characteristics

Five different biochars were produced for this study, varying in their feedstock composition and pyrolysis conditions. The biochars were prepared from sunflower husk, wood chips, green waste, pelletized green waste, and a wood–zeolite mixture (75:25 ratio), each pyrolysed at 600 °C under limited oxygen conditions. To determine the specific surface area and pore characteristics, the biochar samples were prepared and analysed using nitrogen adsorption. Before analysis, the sample was subjected to a controlled thermal pretreatment to ensure a clean, degassed surface suitable for adsorption measurements.

2.1.1. Sample Preparation

An accurately weighed portion of each biochar (0.1046 g) was placed in the analysis tube and degassed under vacuum. The temperature was first increased from room temperature to 120 °C at 5 °C min^{-1}, then held for 60 min to remove physically adsorbed moisture and volatile contaminants. Subsequently, the temperature was further increased to 250 °C at the same heating rate (5 °C min^{-1}) and maintained for 120 min to complete the outgassing process and activate the surface before measurement. After degassing, the sample was cooled to room temperature under vacuum and immediately transferred to the analysis port to prevent atmospheric contamination.

2.1.2. Nitrogen Adsorption Measurement and Calculations

The specific surface area was determined using the Brunauer–Emmett–Teller (BET) two-parameter linear method, assuming a molecular cross-sectional area of 16.2 Å² for adsorbed nitrogen (N_2) [13]. Total pore volume was estimated by the Gurvich rule [14] at a relative pressure of, assuming complete pore filling with condensed nitrogen at this pressure.

2.2. Microbial inoculation

Each biochar type was inoculated under sterile conditions with one of three microbial strains, in three replicates. For each treatment, 40 g of biochar were placed into 250 ml Erlenmeyer flasks, resulting in a total of 45 flasks (5 biochar types × 3 microbial strains × 3 replicates). All biochars were autoclaved before inoculation (Figure 1.).

Figure 1. Sterile Erlenmeyer flasks containing 40 g of each biochar sample were used before microbial inoculation

The three microbial strains, *Bacillus amyloliquefaciens*, *Streptomyces griseus*, and *Trichoderma harzianum* were first cultured separately in 250 ml flasks on a shaking incubator for 48 hours to obtain active microbial suspensions. The cultures were then transferred into 15 mL Falcon (centrifuge) tubes and centrifuged at 5000 rpm for 15 minutes. After discarding the supernatant, the microbial pellets were resuspended in physiological saline solution and subsequently applied to the sterilised biochars as an inoculum. Following inoculation, viable cell counting was carried out over a 10-week period to monitor microbial persistence. Each week, 1 g of inoculated biochar from every treatment was transferred into 9 ml of physiological saline solution (0.9 % NaCl) in sterile test tubes (45 in total), and viable cell counts (CFU g^{-1}) were determined using serial dilution and plating techniques.

2.2.1. Viable cell counts

The viable cell counts of the inoculated biochars were quantified using the Most Probable Number (MPN) method [15]. The MPN is a statistical estimator. A pooled sample was prepared for each treatment and assayed in three replicates. The biochar suspensions were prepared, and tenfold serial dilutions were inoculated into 96-well microplates containing Nutrient Broth (Merck, Darmstadt, Germany) (Figure 2.). Wells showing visible microbial growth after incubation were recorded as positive. Three parallel inoculations were performed across three consecutive dilution levels. After 48 hours, the pattern of positive wells yielded a three-digit “key number,” which was used to obtain the MPN multiplier from the Hoskins table. The multiplier was then adjusted by the dilution factor of the first dilution to calculate the CFU per unit soil [1].

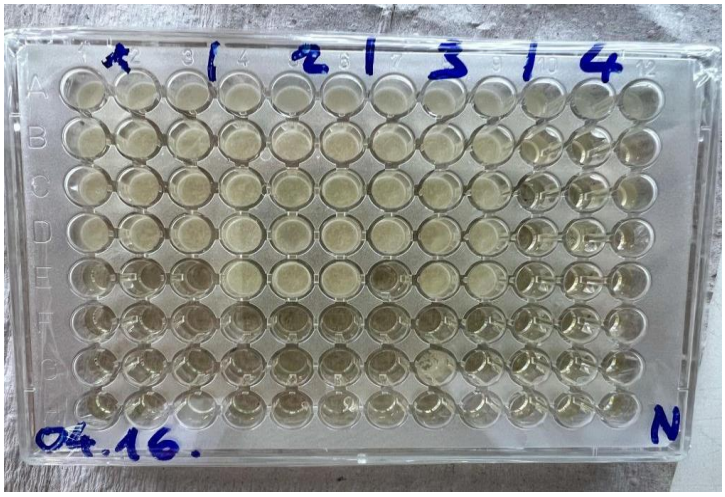

Figure 2. 96-well microplate showing tenfold serial dilutions of biochar suspensions during the viable cell count assay

2.3. Data analysis

Linear regression analysis was used to examine the relationship between biochar BET surface area (S_BET) and microbial survival (%). The linear regression is a statistical method that models the straight-line relationship between two variables, describing how variation in the independent variable (x) influences the dependent variable (y). In this study, regression models were fitted separately for *Bacillus amyloliquefaciens*, *Streptomyces griseus*, and *Trichoderma harzianum,* and all plots illustrating the fitted relationships were generated using RStudio.

4. Results

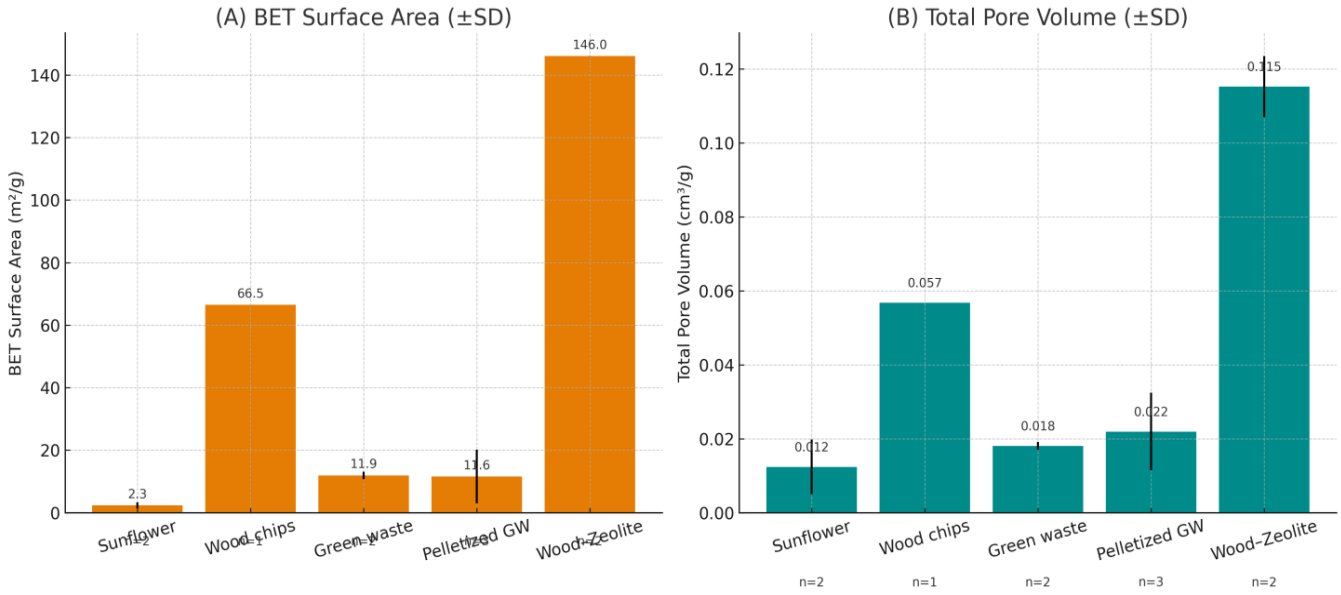

Figure 3. BET surface area and total pore volume of the studied biochars (mean ± SD). (A) BET surface area ($m^2\ g^{-1}$). (B) Total pore volume ($cm^3\ g^{-1}$)

Figure 3. presents the BET surface area and total pore volume of the studied biochar samples, revealing considerable variability influenced by feedstock type and processing. The wood–zeolite composite clearly stands out with the highest BET surface area (146 $m^2\ g^{-1}$) and pore volume (0.115 $cm^3\ g^{-1}$), markedly surpassing all other samples. Wood-chip biochar exhibited intermediate properties (66.5 $m^2\ g^{-1}$, 0.057 cm^3

g^{-1}), followed by green waste and pelletized green waste with similar but slightly reduced values (surface area: 35 and 33 m^2 g^{-1}; pore volume: 0.022 and 0.018 cm^3 g^{-1}, respectively), where pelletization slightly decreased porosity. The sunflower-husk-derived biochar showed the lowest values (2.3 m^2 g^{-1}, 0.012 cm^3 g^{-1}), indicating minimal pore development. Overall, a more than ~60-fold difference in surface area was observed, and the close relationship between surface area and pore volume highlights the key role of feedstock composition and mineral additives, such as zeolite, in promoting pore formation during pyrolysis.

Figure 4. shows the correlation between BET surface area and total pore volume among the examined biochars. A strong positive linear relationship was observed (R^2 = 0.90), indicating that higher specific surface areas are closely associated with increased pore volumes. The wood–zeolite composite clearly stands out, exhibiting both the highest surface area (146 m^2 g^{-1}) and pore volume (0.115 cm^3 g^{-1}), while the sunflower-derived biochar displayed the lowest values for both parameters. This correlation highlights the structural co-development of surface area and porosity, which are strongly influenced by feedstock type and the presence of mineral additives such as zeolite.

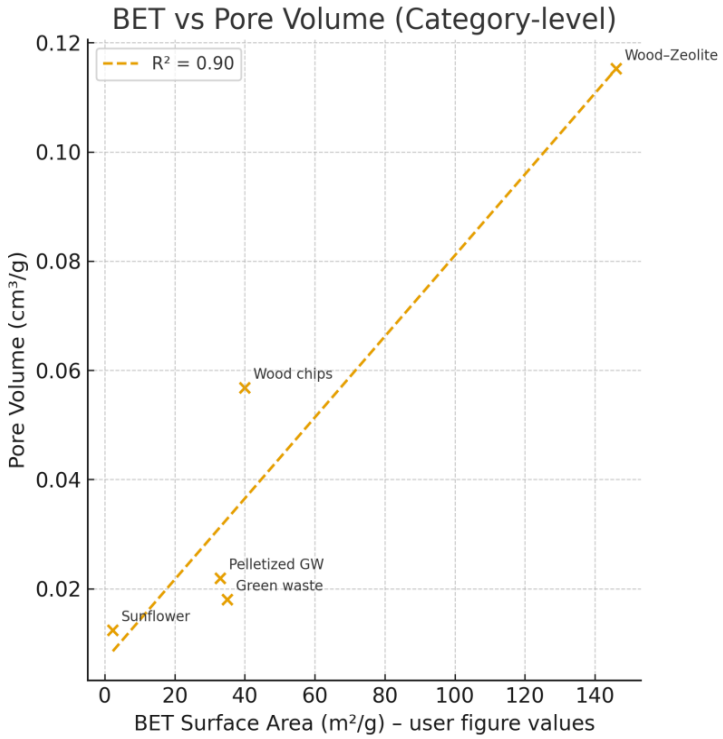

Figure 4. Relationship between BET surface area and total pore volume of the biochars (category-level)

The survival trends of the three inoculants over the 10-week incubation differed markedly. *Bacillus amyloliquefaciens* exhibited the most stable and highest persistence, showing only a slight decline over time and with most CFU measurements remaining above 80% (10^7 CFU g^{-1}) at week 10. The highest apparent survival of this species was observed on the wood–zeolite biochar, with cell counts remaining 98% (10^7 CFU g^{-1}) at the final sampling, whereas sunflower- and pelletized biochars supported the lowest survival after 10 weeks (Figure 5.). *Streptomyces griseus* showed the steepest and least stable decline of the three taxa. The CFU counts fell sharply for most biochars and approached zero between weeks 6 and 7, with wood–zeolite composite biochar showing a slightly delayed decline, but all treatments reached effectively zero survival. Pelletized green-waste biochar supported survival least effectively, reaching zero earliest. *Trichoderma harzianum* displayed intermediate stability, with 10-week survival levels ranging approximately between 70% and 85% (10^7 CFU g^{-1}), as with the other strains, the wood–zeolite material yielded the highest survival at week 10, whereas sunflower and pelletized biochars were the least effective at maintaining *T. harzianum* viability.

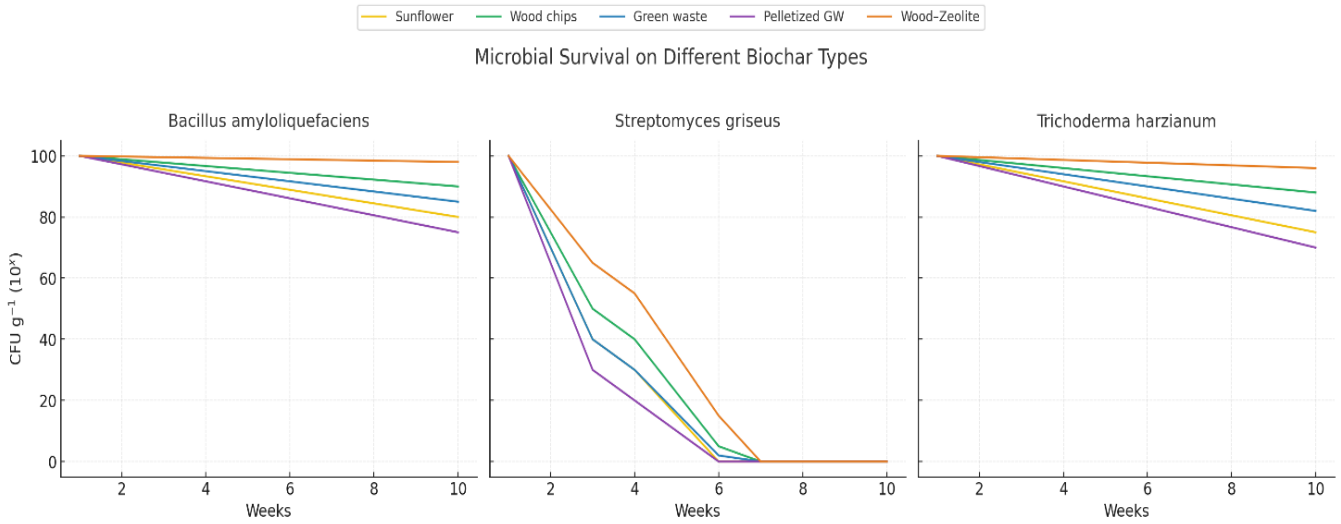

Figure 5. Survival of Bacillus amyloliquefaciens, Streptomyces griseus, Trichoderma harzianum on different biochar types

The results of the linear regression analysis of the relationship between biochar BET surface area and microbial survival (%) are shown in Figure 6.

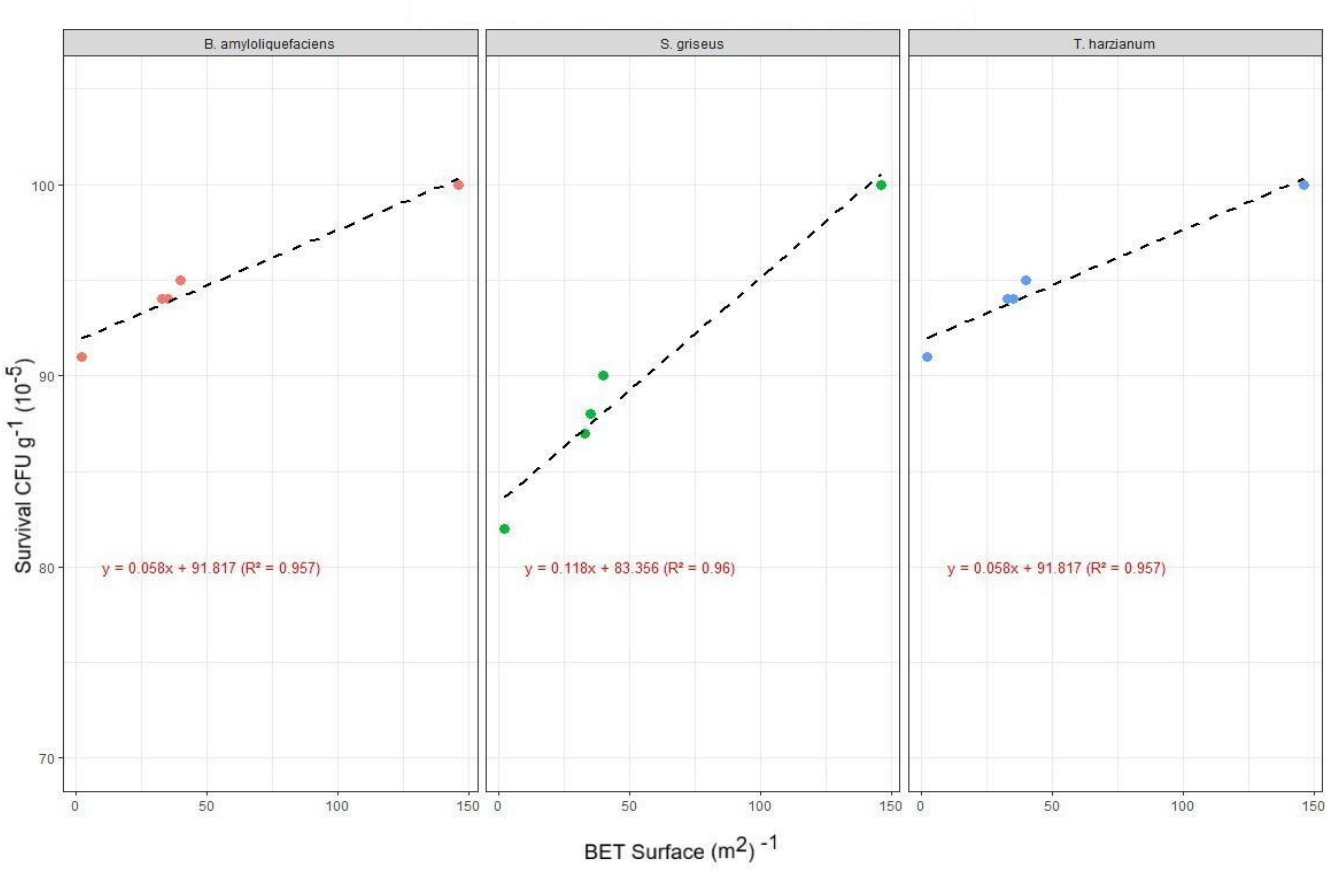

Figure 6. Microbial survival as a function of BET surface area, based on CFU values measured at Week 6

In this analysis, microbial survival values were calculated based on CFU counts measured at Week 6, which was the last time point at which all three inoculated species, including *S. griseus*, still exhibited detectable and comparable viable cell numbers. Using the Week 6 dataset ensured that survival values reflected actual

physiological differences among biochars rather than species-specific decline patterns observed at later sampling times. *S. griseus* exhibited the highest coefficient of determination ($R^2 = 0.96$), indicating a slightly stronger fit than observed for *B. amyloliquefaciens* and *T. harzianum* (both $R^2 \approx 0.96$). The most salient difference between taxa is the gradient of the regression line. *S. griseus* has a slope of 0.118 (percentage-point survival per $m^2\ g^{-1}$ BET), which is precisely twice the slope estimated for *B. amyloliquefaciens* and *T. harzianum* (0.058 percentage-point per $m^2\ g^{-1}$). In practical terms, a unit increase in BET surface area is associated with a twofold larger increase in survival for *S. griseus* than for the other two species. Overall, all three organisms show a strong, positive association with BET surface area ($R^2 > 0.95$), but *S. griseus* is markedly more responsive to changes in BET, indicating species-specific sensitivity of survival to biochar surface area.

5. Discussion

The results indicate a clear interaction between biochar type and microbial species in determining survival trends. While *B. amyloliquefaciens* showed high persistence across all carriers, *S. griseus* declined rapidly and was essentially undetectable by week 7, and *T. harzianum* displayed intermediate stability. These species-specific responses are consistent with previous reports that biochar effects on inoculant persistence depend on both microbial life history (e.g., sporulation capacity in *Bacillus*) and the physicochemical properties of the carrier (porosity, surface area, surface chemistry). In particular, the relative resilience of *B. amyloliquefaciens* likely reflects the protective advantage conferred by spore formation when cells are immobilised on porous char matrices, a pattern reported for *Bacillus spp*. in biochar carriers. These findings are consistent with Vlajkov et al., who demonstrated that *Bacillus spp*. Immobilised on biochar exhibits enhanced long-term survival, a phenomenon primarily attributable to the protective effect of sporulation and to the advantages conferred by biochar's porous structure (e.g., improved moisture retention and provision of physical micro-refugia) [16].

Feedstock and processing clearly modulated carrier performance in our experiment. Wood–zeolite- and wood-chip–derived chars generally supported higher survival, whereas the sunflower based produced the fastest declines. Such feedstock- and treatment-dependent outcomes are widely reported, biochar surface area, total pore volume, pore architecture and surface chemistry vary markedly with feedstock and pyrolysis conditions and are key determinants of microbial habitat quality on char. These observations are consistent with He et al. (2024) [6] and Ribeiro et al. (2024) [17], who demonstrated that feedstock type and pyrolysis parameters (e.g., peak temperature, residence time and heating rate) strongly govern biochar physicochemical properties (including BET surface area, total pore volume, pore-size distribution and surface chemistry) thereby determine the materials suitability as a microbial carrier. For example, these studies report that increasing pyrolysis temperature generally enhances both surface area and pore volume, improving the potential for microbial colonisation. At the same time, feedstock composition influences ash content and surface functional groups, which in turn modulate habitat quality for immobilised microbes.

The marked dependence of survival on BET surface area and pore volume observed here accords with mechanistic and review studies, including the IUPAC physisorption report (2015) [18] and the comprehensive synthesis by Bolan et al. (2023) [19], which identify surface area and pore structure among the principal factors by which biochar buffers desiccation, adsorbs nutrients, and physically shelters microorganisms. Consequently, BET surface area and total pore volume jointly represent key design parameters when developing biochar-based inoculant formulations. At the same time, the literature cautions that surface area and pore characteristics alone do not capture all relevant material–microbe interactions — such as surface chemistry, labile carbon and inorganic fractions, and particle size — so optimisation should be multivariate rather than univariate.

Finally, our gradient-based finding that *S.griseus* responds more strongly to changes in BET surface area and pore structure than the other taxa support the notion that *Streptomyces* viability is particularly sensitive to carrier formulation and microstructure. This aligns with previous observations by Domínguez-González et al. (2022) [20], who reported that *Streptomyces* survival varied markedly among different carrier matrices, mainly due to differences in surface roughness and moisture retention capacity. Similarly, studies on ball-milled or mechanically modified biochars have demonstrated that excessive reduction of particle size or alteration of pore morphology can impair *Streptomyces* viability by increasing desiccation stress and disrupting hyphal integrity. These findings reinforce that successful formulation for filamentous bacteria

requires careful control of surface accessibility, pore volume, and microhabitat stability, as their vegetative and sporulating forms are highly responsive to micro-scale physical environments.

6. Conclusions

The type of biochar significantly affects microbial survival, with both the feedstock and the physicochemical properties resulting from pyrolysis exerting a marked influence on outcomes. The utilisation of wood–zeolite and wood chips derived biochars has been observed to support higher survival of *Bacillus amyloliquefaciens* and *Trichoderma harzianum*, whereas the sunflower composite often led to faster declines. Furthermore, *Streptomyces griseus* demonstrated a heightened sensitivity to variations in carrier surface characteristics, exhibiting the most pronounced positive response to increases in BET surface area and total pore volume. This finding underscores the significance of species-specific optimisation in the context of material selection. The results demonstrate that judicious selection and engineering of biochar carriers, accounting for BET surface area, pore volume, pore architecture, and other multivariate physicochemical traits, can significantly enhance the long-term viability of microbial inoculants. This study provides actionable insights for the targeted, trait-informed design of biochar-based formulations, thereby supporting the development of more effective and resilient bioinoculant products in sustainable agriculture.

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