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Design of a Clinoptilolite-Zeolite Mineral Carrier to Prolong the Shelf Life of Tea-Root Endophytic Inoculants Under Cold Storage

Tatang Padmawidjaja[1*], Budhy Agung Supriyanto[1], Eko Pranoto[2], Franto Navico[1], Rina Zuraida[1], Tarsono Tarsono[1]

[1] National Research and Innovation Agency, Bandung 40135, Indonesia
[2] Tea and Quinine Research Center, Gambung 40972, Indonesia

Corresponding Author Email: tata016@brin.go.id

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ABSTRACT

Bioinoculants derived from endophytic microbes can improve sustainable agriculture; nevertheless, their use is constrained by limited shelf life and inconsistent viability during storage. This research formulates and assesses a natural clinoptilolite–zeolite carrier to maintain the viability of endophytic bacteria isolated from healthy tea (*Camellia sinensis*) roots obtained at the Tea and Quinine Research Center in Gambung, West Java, Indonesia. The clinoptilolite was sieved to 45–80 mesh, thermally conditioned at either 100 °C or 200 °C, adjusted to approximately 4% moisture, and inoculated at three microbial loadings (12.5%, 25%, and 50% w/w). The formulations were maintained at 0 °C and evaluated by plate counts (plate count agar (PCA)) at 0, 30, 90, and 150 days. On day 0, the initial viable counts ranged from 3.35×10^9 to 6.70×10^9 CFU g^{-1}, depending on the loading conditions. The highest initial population (6.70×10^9 CFU g^{-1}) was achieved at 50% loading, whereas the lowest (3.35×10^9 CFU g^{-1}) occurred at 12.5% loading. The results demonstrated that all zeolite-based formulations maintained viable counts of approximately 10^9 CFU g^{-1} throughout the 150-day storage period. This was significantly higher than the control group without zeolite, which exhibited a final count of only 5.0×10^5 CFU g^{-1} at day 150 ($p < 0.05$). Relative to their initial populations (CFU_0), the zeolite formulations retained 33%–60% of viable cells by the end of the storage period, with higher retention observed at lower initial loadings. The findings indicate that specific carrier design factors (particle size, thermal treatment, and moisture content) can enhance viability stability and yield more resilient bioinoculant formulations for tea agroecosystems. Statistical analysis confirmed that microbial loading had a significant effect on cell survival ($p < 0.01$), with lower initial loadings resulting in higher percentage retention.

1. INTRODUCTION

A prevalent issue in commercial and field applications is the swift deterioration of viable cells during storage and transportation, which impacts product consistency and reliability [1]. Because they provide chemical fertilizer substitutes and promote soil health, nutrient cycling, and plant resilience, biofertilizers and microbial inoculants are increasingly recognized as essential tools for sustainable agriculture. However, delivering the ideal quantity of living microbial cells at the time of application is crucial to its efficacy. Along with flaws in quality assurance and regulatory systems, these major issues are also affected by developments in formulation, preservation, environmental adaptability, and application techniques. To achieve consistent field performance and broader adoption in sustainable farming systems, these problems must be addressed [2]. Carrier-based formulations are essential in the design of inoculants. Conventional organic carriers, such as peat or compost, can enhance microbial viability but face challenges due to variability, moisture instability, and quality assurance [3]. A recent study underscores the significance of carriers possessing well-defined physical and chemical characteristics. In this regard, natural zeolites, particularly clinoptilolite, have emerged as promising inert, porous substrates. Their aluminosilicate structure and ion-exchange capability establish microhabitats that control moisture and ionic conditions [4]. Clinoptilolite has been thoroughly researched for agricultural applications, particularly as a stabilizing matrix for microbial inoculants. Endophytic bacteria derived from tea (*Camellia sinensis*) roots are increasingly recognized for their potential to improve plant health and enhance stress resilience [5]. Although these tea plant endophytic bacteria have great potential, the lack of a stable carrier formulation with clear process parameters suitable for their characteristics limits their commercialization and large-scale application [6]. Moreover, current research on zeolite-based carriers frequently omits specific processing characteristics (e.g., mesh size, heat conditioning, and moisture content), complicating comparisons of results [7]. From a formulation design

standpoint, regulating these factors is crucial for consistent results. The recommended mesh size and moisture content were selected to optimize microbiological protection while ensuring practical handling: particles are tiny enough to create protective niches, yet coarse enough to allow flowability, and low moisture to reduce microbial activity during storage. This study seeks to (i) create a clinoptilolite carrier (45–80 mesh; heat-treated at 100 °C and 200 °C; moisture adjusted to approximately 4% w/w) for an endophytic bacterial inoculant derived from tea roots in Gambung, West Java; and (ii) assess viability retention during cold storage at 0 °C for up to 150 days across various microbial loadings (12.5%, 25%, and 50% w/w) using a factorial design and total plate counts [8]. This study delineates design parameters and information on plant samples, carrier preparation, and enumeration methodologies, thereby enabling reproducible comparisons and facilitating the translation of endophyte-based inoculants into practical applications [9].

2. MATERIALS AND METHODS

2.1 Study area, host plant, and root sampling

Endophytic microbes were isolated from healthy tea plants (*Camellia sinensis*) cultivated at the Tea and Quinine Research Center in Gambung, West Java, Indonesia. Plants exhibiting no signs of illness were selected. Fine roots were excavated because of their critical roles in endophyte recruitment and plant growth [10]. Roots were collected using sterilized instruments, placed in sterile sampling bags, transported in a chilled container, and processed promptly to minimize alteration of the microbial community [11].

2.2 Isolation of endophytic bacteria

The collected roots were rinsed with sterile water to eliminate dirt. Surface bacteria were eradicated by sequential immersion in 70% ethanol for 1 min and 2% sodium hypochlorite for 3 min, followed by multiple rinses in sterile distilled water [12]. The concluding rinse water was cultured on nutrient agar (NA) to verify sterility. Roots that passed the sterility assessment were dissected aseptically and homogenized in sterile saline. Aliquots (100 µL) were spread on NA and incubated at 28 °C–30 °C for 24–48 hours [13]. Separate colonies were consistently subcultured to obtain pure isolates, which were preserved on NA slants at 4 °C until required. The isolates constitute the microbial consortium used for formulation and possess plant-beneficial (potential bioinoculant candidate) characteristics, such as phosphate solubilization, inferred from their origin but not individually verified in this investigation [14].

2.3 Preparation of the clinoptilolite–zeolite carrier

Natural clinoptilolite–zeolite was initially hand-sorted to remove visible impurities. The material was then oven-dried at 105 °C for 24 h to a constant weight. The dried zeolite was coarsely crushed using a jaw crusher, followed by fine grinding in a disk mill until it passed through a 45-mesh sieve. The ground material was then sieved to obtain the 45–80 mesh fraction, which was used for all subsequent experiments to optimize packing density, pore accessibility, and manageability. The selected mesh size was intended to maximize surface area and porosity for microbial interactions while maintaining the material's free-flowing, manageable characteristics [15].

The sieved zeolite was heat-treated in a drying oven at 100 °C or 200 °C for 2 h to reduce adsorbed water and improve porosity. Heating the clinoptilolite samples until 200 °C will not change the mineral structure, including porosity, considering structural change starts at 600 °C [16]. After cooling in a desiccator, the carrier's moisture content was calibrated to approximately 4% (w/w). A zeolite sample was oven-dried at 105 °C until constant weight to determine the initial moisture content; then, sterile distilled water was added using a sterile spray bottle with continuous mixing, or further drying was conducted to reach the 4% objective [17]. This reduced moisture level was chosen to minimize water activity and metabolic stress during storage, preventing total desiccation of the microbial cells. The conditioned zeolite was preserved in sterile, airtight containers until inoculation [18].

2.4 Formulation design and storage conditions

This study employed a two-factor factorial design with three replications (Table 1), with factor A being thermal treatment temperature (100 °C and 200 °C) and factor B being microbial loading (12.5%, 25%, and 50% w/w) [19].

The bacterial culture used for inoculation was grown in nutrient broth at 30 °C for 48 h on an orbital shaker (150 rpm), corresponding to the late-exponential/early-stationary phase ($OD_{600} \approx 1.2–1.5$). The culture was concentrated by centrifugation at 5,000 × g for 15 min at 4 °C, and the pellet was resuspended in sterile physiological saline (0.85% NaCl) to achieve a final cell density of approximately 1.0×10^{10} CFU mL^{-1}, as determined by preliminary plate counts. This concentrated suspension was used immediately to prepare the formulation [20].

Formulations were prepared by aseptically combining the conditioned clinoptilolite carrier with the concentrated endophytic bacterial culture to achieve microbial loadings of 12.5%, 25%, and 50% (w/w) relative to the total formulation mass [21]. An adequate volume of liquid culture was combined with the carrier to achieve the desired weight loading; the resultant paste-like mixture was thoroughly homogenized using a sterile spatula [22].

A non-zeolite control was prepared by dispensing 1 mL of the concentrated bacterial suspension (approximately 1.0×10^{10} CFU mL^{-1}) into sterile 2 mL microcentrifuge tubes without any carrier material. The initial bacterial count of the control was determined immediately after preparation (day 0) by serial dilution and plate counting, yielding a baseline value for comparison with the carrier formulations. These control samples were stored under identical conditions and sampled at the same time points as the zeolite-based formulations. Each formulation (approximately 1 g per sample) was subsequently enclosed in sterile, sealed containers and maintained at 0 °C in a refrigerated incubator. To minimize moisture exchange, containers remained sealed during sampling. Samples were collected after 30, 90, and 150 days of storage (Table 2) [23].

2.5 Viable counts (plate count method)

Viable cells were enumerated as colony-forming units per gram of formulation (CFU g^{-1}) using standard plate-count procedures.

Immediately after formulation preparation (day 0), triplicate samples from each treatment were analyzed to determine the

initial viable count (CFU_0). These day-0 values served as the baseline for all subsequent calculations [24].

At each sampling interval (30, 90, and 150 days), 1 g of the formulation was suspended in 9 mL of sterile physiological saline (0.85% NaCl) and homogenized by vigorous vortexing for 2 min. For formulations that did not completely disperse, additional gentle grinding with a sterile glass rod was performed to ensure thorough homogenization [25]. Decimal serial dilutions were prepared in the same saline solution, and 100 µL aliquots from appropriate dilutions were spread onto plate count agar (PCA) plates. Plates were incubated at 30 °C for 48 h, and colonies in the countable range (30–300 colonies per plate) were counted. Results are expressed as CFU g^{-1} of formulation. The mean and standard deviation (SD) of CFU g^{-1} were computed from triplicates for each treatment at each time point [26].

For the non-zeolite control, 1 mL of the stored bacterial suspension was withdrawn at each sampling interval, serially diluted in sterile saline, and plated on PCA following the same procedure. Results are expressed as CFU mL^{-1}.

2.6 Data processing and analysis

Retention (%) for each treatment at each sampling time was calculated as (CFU_t / CFU_0) × 100, where CFU_0 is the viable count determined immediately after formulation (day 0) and CFU_t is the count at 30, 90, or 150 days of storage [27]. Descriptive statistics (mean ± SD) and graphical representations were produced in Microsoft Excel. Statistical analysis was performed using two-way analysis of variance (ANOVA) followed by Tukey's Honestly Significant Difference (HSD) test ($\alpha = 0.05$). All analyses were conducted using ANOVA [28]. All microbiological techniques adhered to pertinent standards, and endophyte management complied with published guidelines [29].

Table 1. A two-factor factorial experimental design: Thermal treatments (factor A) and microbial loadings (factor B), with sampling times

Thermal Treatment (°C)	Microbial Loading (% w/w)	Replicates (n)	Sampling Times (days)
100	12.5	3	30, 90, 150
100	25	3	30, 90, 150
100	50	3	30, 90, 150
200	12.5	3	30, 90, 150
200	25	3	30, 90, 150
200	50	3	30, 90, 150

Table 2. Summary of key experimental parameters and conditions

Design Parameter	Specification
Host plant/tissue	Tea (*Camellia sinensis*) – healthy root tissues
Sampling location	Tea and Quinine Research Center, Gambung, West Java, Indonesia
Isolation medium	Nutrient Agar (NA)
Isolation incubation	28 °C–30 °C, 24–48 h
Carrier material	Natural clinoptilolite zeolite
Particle size fraction	45–80 mesh
Thermal conditioning	100 °C and 200 °C (drying oven)
Target carrier moisture	~4% (w/w), gravimetric control
Microbial loading	12.5%, 25%, and 50% (w/w)
Storage condition	0 °C, sealed sterile containers
Viable count medium	Plate Count Agar (PCA)
TPC incubation	30 °C, 48 h
Sampling times	30, 90, and 150 days

3. RESULTS AND DISCUSSION

3.1 Initial bacterial counts

We counted viable cells for all treatment combinations immediately after formulation (day 0) to obtain reliable baseline values for the retention calculations that followed. Table 3 shows the first set of data, which shows the viable counts (CFU g^{-1}) for each combination of microbial loading levels (12.5%, 25%, and 50% w/w) and thermal treatment temperature (100°C and 200°C) [28]. In this case, w/w means the ratio of microbial mass to the overall mass of the formulation. This shows the real loading percentage based on weight, not volume. These baseline measurements are important because they ensure that microbial retention measurements during the observation period are accurate and consistent [30].

Table 3. Initial viable counts (CFU g^{-1}) on day 0 for all treatment combinations

Thermal Treatment	Microbial Loading	Mean CFU g^{-1}	SD ($\times 10^9$)
100 °C	12.5%	3.40×10^9	± 0.28
100 °C	25%	4.85×10^9	± 0.42
100 °C	50%	6.70×10^9	± 0.65
200 °C	12.5%	3.35×10^9	± 0.31
200 °C	25%	4.80×10^9	± 0.38
200 °C	50%	6.65×10^9	± 0.58

3.2 Viability retention during cold storage: Factorial analysis

Viable counts were assessed at 30, 90, and 150 days of storage at 0 °C. Table 4 displays the comprehensive dataset for all treatment combinations, categorized by thermal treatment

temperature and microbial loading [30].

Across all clinoptilolite treatments, viable counts at day 30 consistently measured approximately 10^9 CFU g^{-1} (Table 5), suggesting that mixing and the moisture adjustment to ~4% did not exert undue stress on the endophytic culture [2, 31-36]. The mineral carrier successfully maintained viability throughout storage. On day 90, mean counts varied from 2.85 × 10^9 to 5.85 × 10^9 CFU g^{-1}, contingent upon loading and temperature (Table 5). Conversely, the non-zeolite control significantly declined to roughly 5.0 × 10^5 CFU g^{-1} by day 90, illustrating the protective effectiveness of the clinoptilolite carrier under the same conditions. Zeolite-based carriers likely improve survival by regulating microscale water availability and offering physical protection against desiccation and osmotic stress [2, 3].

By day 150, viable counts in all clinoptilolite formulations consistently approximated 10^9 CFU g^{-1}, with retention rates varying with initial loading (Table 6 and Figure 1). The 12.5% loading maintained 59.7% of its initial population (day 0 baseline), while the 25% and 50% loadings kept 100 °C, respectively (Table 6). This density-dependent trade-off indicates that heightened initial loads may exacerbate competition for limited pore space or nutrients within the carrier matrix, thereby hastening population decline. Similar density effects have been observed in other carrier-based inoculants [2, 36].

A two-way analysis of variance was conducted for each sampling time point to statistically assess the impacts of thermal treatment temperature, microbial loading, and their interaction on viable counts. Table 4 encapsulates the ANOVA findings.

To statistically evaluate the effects of thermal treatment temperature, microbial loading, and their interaction on viable counts, a two-way analysis of variance was performed for each sampling time point.

Table 4. Two-way analysis of variance (ANOVA) results for viable counts at days 30, 90, and 150

Source of Variation	Day 30		Day 90		Day 150	
	F-value	p-value	F-value	p-value	F-value	p-value
Temperature (A)	0.32	0.58	0.28	0.61	0.35	0.56
Loading (B)	245.6	< 0.001	187.3	<0.001	8.92	0.003
A × B Interaction	0.15	0.86	0.12	0.89	0.18	0.84

Table 5. Viable counts (CFU g^{-1}) at days 30, 90, and 150 for all treatment combinations

Temp.	Loading	Day 30 (× 10^9)	SD	Day 90 (× 10^9)	SD	Day 150 (× 10^9)	SD
100 °C	12.5%	3.38	± 0.30	2.89	± 0.25	2.03	± 0.18
100 °C	25%	4.80	± 0.45	4.12	± 0.40	2.18	± 0.22
100 °C	50%	6.65	± 0.60	5.85	± 0.55	2.22	± 0.20
200 °C	12.5%	3.32	± 0.28	2.85	± 0.24	1.98	± 0.17
200 °C	25%	4.75	± 0.42	4.08	± 0.38	2.15	± 0.20
200 °C	50%	6.60	± 0.55	5.80	± 0.52	2.18	± 0.19

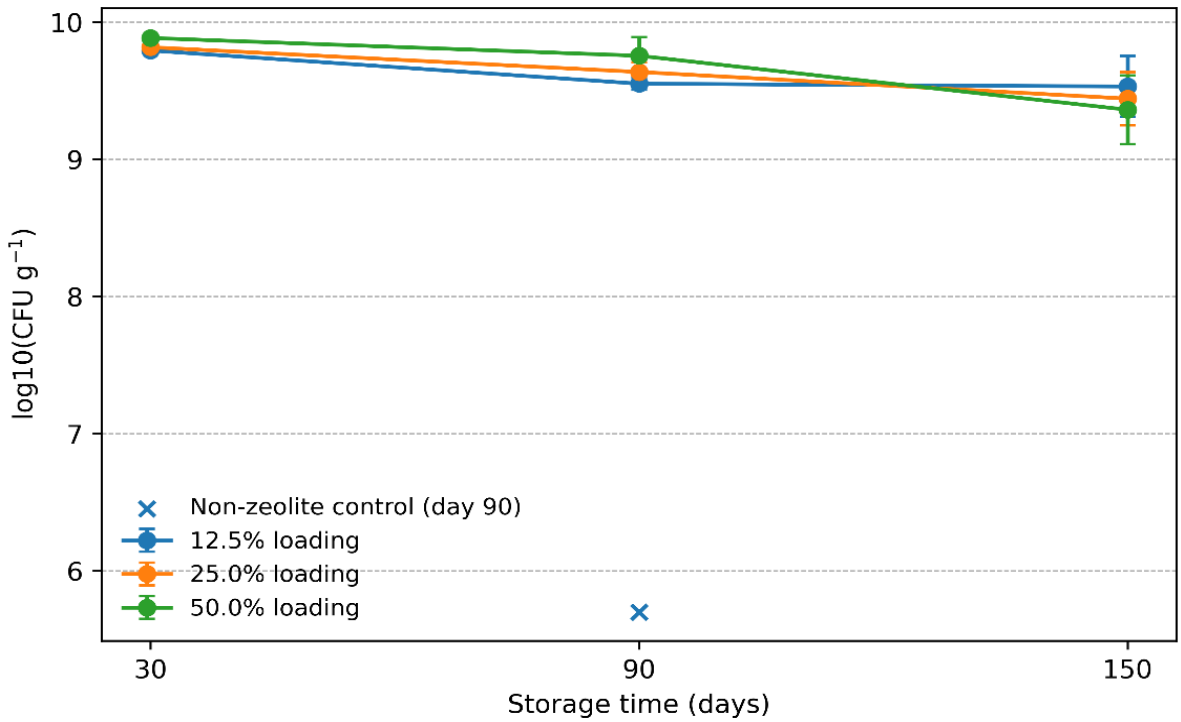

Figure 1. Endophytic bacteria on clinoptilolite carriers survive for 150 days at 0 °C due to microbial loading
All zeolite formulations maintained viability at about 10^9 CFU g^{-1} after five months of cold storage, despite starting counts varying from 3.35 to 6.70 × 10^9 CFU g^{-1} (day 0). Lower initial loading (12.5% w/w) resulted in a much greater retention rate (~59%) compared to 25% (~45%) and 50% (~33%) loadings ($p < 0.05$, Tukey's HSD). The data from 100 °C and 200 °C thermal treatments (n = 6, mean ± SD) indicated no significant effect ($p > 0.05$). All zeolite formulations significantly outperformed the non-zeolite control, reducing the CFU per sample to 5.0 × 10^5 by day 150, highlighting the carrier's beneficial role.

Table 6. Retention (%) relative to day-0 baseline for all treatment combinations

Temp.	Loading	Day 30	Day 90	Day 150
100 ℃	12.5%	99.4	85.0	59.7
100 ℃	25%	99.0	84.9	45.3
100 ℃	50%	99.3	87.3	33.1
200 ℃	12.5%	99.1	85.1	59.1
200 ℃	25%	99.0	85.0	44.8
200 ℃	50%	99.2	87.2	32.8

The ANOVA revealed that microbial loading had a highly significant effect on viable counts at all time points ($p < 0.001$ at days 30 and 90; $p = 0.003$ at day 150). In contrast, thermal treatment temperature (100 ℃ vs. 200 ℃) showed no significant effect at any sampling time ($p > 0.05$), and the interaction between temperature and loading was also non-significant ($p > 0.05$) [33]. These results indicate that the two heat treatment temperatures performed similarly in preserving viability, and their effects were consistent across all loading levels. Figure 1 presents viable counts at day 150 as bar charts with error bars (mean ± SD), with significant differences among loading levels indicated by lowercase letters ($p < 0.05$, Tukey's HSD post hoc test) [34]. The non-zeolite control decreased dramatically during storage, from an initial count of approximately 1.0×10^{10} CFU per sample to 5.0×10^{5} CFU per sample on day 90, and became undetectable on day 150. This sharp decline underscores the protective efficacy of the clinoptilolite carrier under identical storage conditions [33].

3.3 Retention rates and density-dependent effects

Retention rates were calculated relative to the day-0 baseline (CFU_0) for each treatment combination. Table 6 presents the percent retention at days 30, 90, and 150. By day 150, retention rates showed a clear inverse relationship with initial loading: the 12.5% loading preserved approximately 59% of its initial population, whereas the 25% and 50% loadings retained only 45% and 33%, respectively (Table 6) [35]. This density-dependent decline was consistent across both temperature treatments. Several interconnected mechanisms may explain this observation:

First, spatial constraints within the carrier matrix are fundamental. The microstructural basis for this density-dependent effect becomes evident when examining the carrier architecture (Figure 2). With pore dimensions of 1–10 μm and bacterial cell diameters of 1–5 μm, each pore can accommodate only a limited number of cells. At 12.5% loading (~3.4×10^9 CFU g^{-1}), cells likely occupy pores in a dispersed pattern with adequate spacing. However, at 50% loading (~6.7×10^9 CFU g^{-1}), pore occupancy approaches or exceeds the optimal carrying capacity, forcing cells into closer proximity. This overcrowding intensifies competition for available microhabitats, leading to accelerated mortality when initial loading exceeds the carrier's finite pore volume [36].

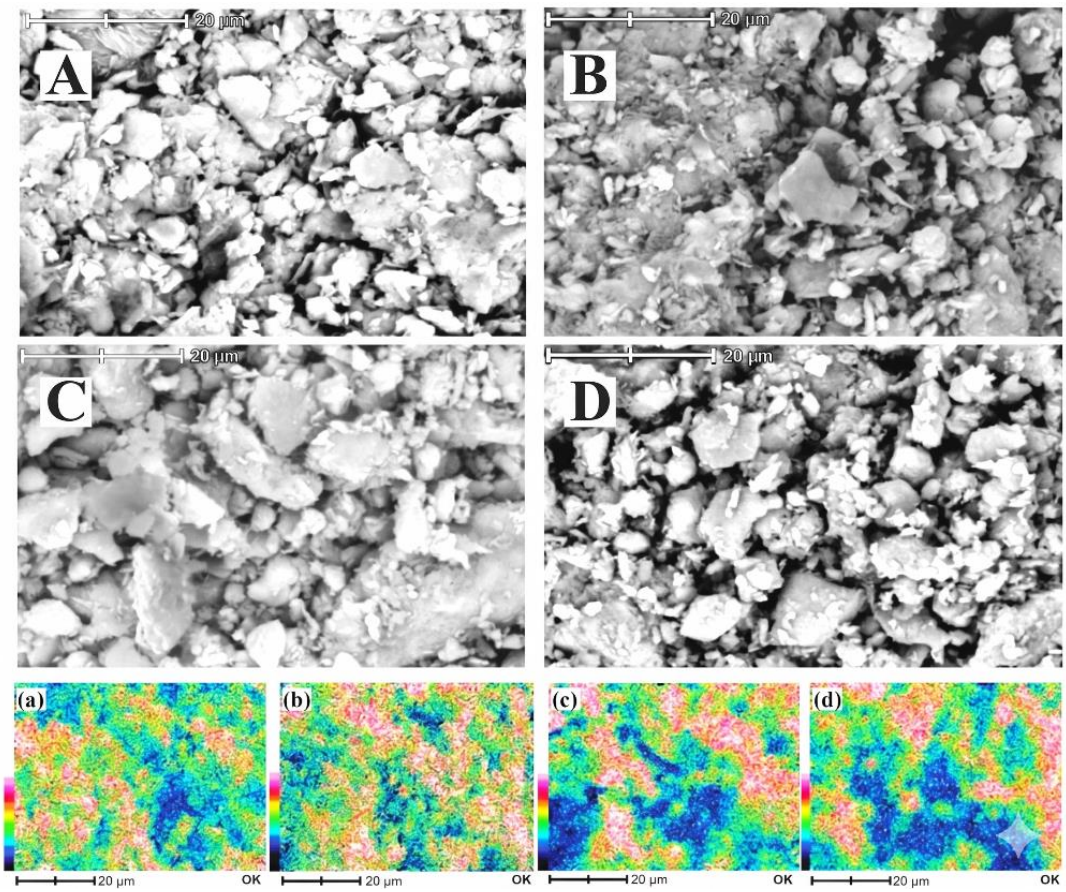

Figure 2. Scanning electron microscopy (SEM) images and corresponding energy-dispersive X-ray spectroscopy (EDS) mapping of the clinoptilolite carrier microstructure. (A–D) Morphology of clinoptilolite particles at 500× magnification, revealing irregular, angular grains with surface roughness and fracture planes that facilitate bacterial adhesion and colonization; (a–d) Corresponding elemental distribution maps for samples A–D, highlighting surface compositional variations at a scale of 20 μm
Quantitative imaging analysis (n = 50 pores) shows a mean pore diameter of 4.2 ± 2.1 μm, ideal for rod-shaped endophytic bacteria (1–3 μm in length). This pore-size distribution maintains a steady moisture content (~4% w/w) and prevents full desiccation while also providing physical sequestration to protect cells from ice-crystal injury during 0°C storage. Scale bars: 20 μm.

Second, nutrient limitations become more acute at higher cell densities. The confined pore spaces contain limited nutrients, derived either from residual organic matter in the zeolite or from carryover from the culture medium. At higher initial loadings, these nutrients are consumed more rapidly, potentially leading to early starvation stress [37].

Third, the accumulation of metabolic waste products may reach inhibitory levels sooner in densely populated microenvironments, creating localized toxicity. Comparable density-dependent effects have been reported in other carrier-based inoculants, where moderate initial loadings often yield better long-term survival than excessively high loadings. These findings suggest that optimizing microbial load rather than simply maximizing initial cell numbers is critical for formulation design.

3.4 Carrier microstructure and design implications

Scanning electron microscopy (SEM) coupled with energy-dispersive X-ray spectroscopy (EDS) was used to characterize the microstructure of the clinoptilolite carrier. Figure 2 presents representative SEM micrographs at 500× and 5000× magnification, revealing angular, porous particles with irregular surfaces [10, 18]. At higher magnification, numerous microscale voids and channels are visible, with pore sizes predominantly in the 1–10 μm range (estimated from image analysis). These dimensions are particularly significant given that bacterial cells typically measure 1–5 μm in diameter, indicating that individual pores can accommodate only a limited number of cells [2]. This microstructural observation provides direct physical evidence supporting the density-dependent mortality mechanism discussed in Section 3.3: at 50% loading (~6.7 × 10^9 CFU g^{-1}), pore occupancy likely exceeds optimal carrying capacity, intensifying spatial competition compared to 12.5% loading (~3.4 × 10^9 CFU g^{-1}).

The observed pore architecture is well-suited for bacterial colonization, as it creates protective microhabitats that shield cells from shear forces during handling while still allowing nutrient diffusion and waste exchange [14]. This pore size range is consistent with previous reports on clinoptilolite microstructure [8, 10, 16] and supports its potential as a microbial carrier matrix.

The EDS spectrum (Figure 3) confirms the expected elemental composition, showing predominant signals of silicon (Si) and aluminum (Al) characteristic of the clinoptilolite aluminosilicate framework, along with minor peaks corresponding to exchangeable cations (Ca^{2+}, K^+, Mg^{2+}). This elemental profile corroborates the carrier's moisture-buffering and ion-exchange capabilities, which are relevant to maintaining stable microenvironments for microbial survival during storage [9, 10]. The aluminosilicate framework can adsorb water and exchange cations, potentially helping to buffer against osmotic stress and maintain favorable ionic conditions within pores—factors that likely contributed to the sustained viability (> 10^9 CFU g^{-1} for 150 days) observed across all formulations.

From a formulation design standpoint, the selected parameters, 45–80 mesh particle size, thermal conditioning at 100 °C–200 °C, and approximately 4% moisture content, produced a consistent and effective carrier. Our results support the scientific basis for these choices:

- Particle size (45–80 mesh): This fraction provides an optimal balance between surface area for microbial colonization and interparticle porosity for gas exchange and flowability. The SEM images confirm the presence of pores in the 1–10 μm range, which are accessible to bacteria (typical cell size 1–5 μm) while providing physical protection from shear forces during handling [14, 21].
- Moisture content (~4%): This reduced water activity level was chosen to minimize metabolic activity during cold storage while preventing complete desiccation that could cause cell membrane damage. The successful maintenance of approximately 10^9 CFU g^{-1} over five months validates this selection [2, 5].
- Thermal treatment (100 °C–200 °C): Heat conditioning was intended to remove bound water and potentially increase pore volume [16]. However, the lack of a significant difference between 100 °C and 200 °C treatments suggests that the lower temperature (100 °C) is sufficient to achieve the desired effects and that the additional energy input at 200 °C provides no further benefit for microbial preservation. This finding has practical implications for industrial scalability, as lower temperatures reduce energy consumption and processing costs without compromising product quality.

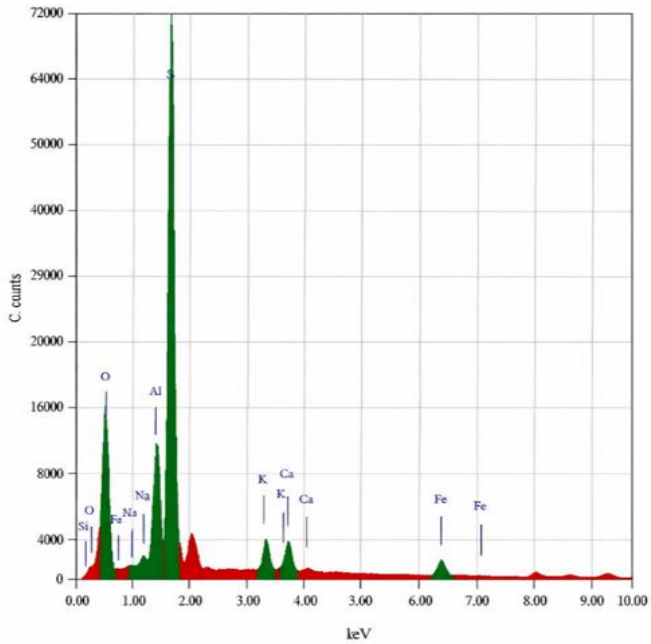

Figure 3. Clinoptilolite carrier elemental composition confirmed by energy-dispersive X-ray spectroscopy (EDS)
Aluminosilicate framework peaks for oxygen (O), silicon (Si), and aluminum (Al) are seen in the spectrum. Minor peaks for exchangeable cations (Ca^{2+}, K^{2+}, Mg^{2+}) are also observed. This elemental profile supports the carrier's moisture buffering and ion exchange characteristics, ensuring bacterial survival during long-term storage.

The performance of clinoptilolite-based carriers can be contextualized by comparison with other common carrier materials reported in the literature. Peat-based formulations, while widely used, often suffer from batch-to-batch variability and inconsistent moisture retention [2, 5]. Studies using peat carriers have reported declines in viability of 1–2 log units over 3–6 months at ambient temperatures (Q). In contrast, our clinoptilolite formulations maintained counts within the same log order (10^9 CFU g^{-1}) throughout 150 days at 0 °C. Vermiculite, another mineral carrier, has shown good moisture

retention but lower mechanical stability [15]. The rigid crystalline structure of clinoptilolite offers advantages in terms of physical stability and reproducible pore architecture, as evidenced by the consistent performance across replicate samples in this study.

3.5 Limitations and future directions

This study has several limitations that should be acknowledged and addressed in future research: First, the endophytic bacterial isolates were not identified at the species or strain level. While their plant-beneficial potential is inferred from their origin (healthy tea roots), specific functional traits such as phosphate solubilization, nitrogen fixation, or phytohormone production were not verified. Future studies should include molecular identification (e.g., 16S rRNA sequencing) and functional characterization of the isolates to enable strain-specific optimization of carrier formulations [24-27]. Second, although thermal treatment was applied at two temperatures (100 ℃ and 200 ℃), the lack of a significant difference between them suggests that more extreme temperature variations or different treatment durations might be needed to observe effects. Alternatively, the benefits of heat treatment may already be saturated at 100 ℃, making 200 ℃ unnecessary. Future optimization studies could explore a wider range of temperatures and include untreated controls to quantify the baseline effect of heat conditioning. Third, the study was conducted under controlled laboratory conditions (0 ℃ storage) without field validation. While cold storage is practical for many agricultural applications, real-world performance may be affected by fluctuating temperatures and humidity, as well as interactions with soil or plant environments. Field trials are needed to evaluate whether the viability preserved during cold storage translates into effective plant colonization and growth promotion under actual growing conditions. Fourth, the absence of significant temperature × loading interactions indicates that these factors act independently, however, mechanistic studies using microscopic observation (e.g., confocal microscopy or fluorescence in situ hybridization) could provide direct evidence of how cells distribute within carrier pores and how spatial competition evolves. Fifth, the study focused solely on viability preservation and did not assess the functional integrity of the preserved cells. Future work should include bioassays to confirm that cells recovered from storage retain their plant growth-promoting activities (e.g., indole acetic acid production and phosphate solubilization) [27, 35]. Finally, while the 45–80 mesh particle size and 4% moisture content were effective, systematic optimization studies (e.g., response surface methodology) could identify the precise parameter combination that maximizes long-term survival for specific endophyte strains.

4. CONCLUSION

A clinoptilolite-zeolite carrier with regulated characteristics (45–80 mesh particle size, thermal conditioning at 100 ℃, and approximately 4% moisture) successfully preserved the viability of endophytic bacteria originally isolated from tea roots during cold storage, supporting their potential as bioinoculants. Following 5 months at 0 ℃, viable counts remained around 10^9 CFU g^{-1} across all treatments, exhibiting retention of 33%–60% relative to the baseline at day 0. The non-zeolite control showed significantly lower numbers by day 90, confirming that the mineral carrier provided substantial protection. Key findings from this study include:

- Initial viable counts were directly proportional to microbial loading, ranging from 3.35×10^9 to 6.70×10^9 CFU g^{-1};
- All zeolite-based formulations maintained approximately 10^9 CFU g^{-1} throughout storage, significantly outperforming the control ($p < 0.001$);
- Microbial loading had a significant effect on viability ($p < 0.01$), with higher loadings leading to lower percent retention;
- Thermal treatment at 100 ℃ was sufficient, as 200 ℃ provided no additional benefit.
- SEM-EDS analysis confirmed the microporous structure (1–10 μm pores) and aluminosilicate composition that underpins the carrier's protective function.

Notably, thermal treatment temperature did not significantly affect viability preservation at any time point ($p > 0.05$), indicating that the lower temperature (100 ℃) is sufficient—a finding with practical implications for industrial scalability, as lower temperatures reduce energy consumption and processing costs without compromising product quality. The inverse relationship between initial loading and percentage retention suggests that optimizing microbial load—balancing initial population with long-term survival—is critical for formulation design. Furthermore, optimizing parameters such as carrier activation, moisture content, and strain-specific physiology will be crucial for advancing the application of these findings in agronomic practices within tea systems. These results support the use of clinoptilolite as a customizable, natural carrier material for durable bioinoculant compositions.

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NOMENCLATURE

CFU	Colony-forming units
CFU_0	Baseline viable count at day-0 (CFU g^{-1})
CFU_t	Viable count at time t (CFU g^{-1})
SD	Standard deviation