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Research Article

Efficiency of *Bacillus pseudomycoides* RAY21 and *Bacillus subtilis* CYA27 Endospore Formulation on Biochar and Oil Spill Dispersant

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ABSTRACT

Bacillus sp. is well known for its functional capabilities such as solubilizing phosphorus (P) and potassium (K), and fixing nitrogen (N_2). These bacteria can form endospores under stressed conditions, allowing long-term survival and application in biotechnological fields. This study aims to isolate *Bacillus* sp. capable of forming endospores and evaluates their viability on different carriers, specifically biochar and oil spill dispersant (OSD), to enhance biodegradation in contaminated environments. Soil samples from the bamboo rhizosphere were heat-shocked to isolate endospore-forming *Bacillus* strains, with the isolate identified as *Bacillus pseudomycoides* RAY21 through 16S rRNA sequencing. This strain exhibited Gram-positive characteristics, formed endospores, and demonstrated potential on various media such as Pikovskaya, Alexandrov, and N-Free Mannitol. The physiological characterization indicated optimal growth in a pH range of 6-8, salinity up to 3.5%, and thermophilic properties. Endospores from *B. pseudomycoides* RAY21 and *B. subtilis* CYA27 were tested on biochar and OSD as carriers. The results showed that endospores adhered better to biochar, but their viability was more stable in OSD over time. Notably, *B. pseudomycoides* RAY21 on OSD degraded 23.43% of total petroleum hydrocarbons (TPH), outperforming *B. subtilis* CYA27 (21.62%). In conclusion, the study demonstrates the potential of using *Bacillus* endospores on OSD as an effective carrier for bioremediation, particularly in degrading petroleum hydrocarbons. Future research should focus on optimizing carrier materials and exploring field-scale applications for enhanced environmental cleanup.

1. Introduction

Functional microbes are microorganisms with capabilities such as phosphate $(PO_4)^{3-}$ solubility, potassium (K) solubility, nitrogen (N_2) fixation, biocontrol, heavy metal leaching, sulfate reducer, plant growth promotion, decomposition, and petroleum degradation. Among these, *Bacillus* sp. is a well-known functional microbe with diverse abilities. Recent studies have confirmed that *Bacillus* sp. possesses the ability to solubilize phosphate, enhancing its potential as a biofertilizer (Mazylyte *et al.* 2022). Cahyani (2021) also reported that the strain of Bacillus subtilis CYA27 has the ability to degrade petroleum and soluble P and K. *Bacillus* sp. has been widely commercialized under various trademarks and formulations for agricultural applications.

One of the methods to cultivate *Bacillus* sp. for the production of biological fertilizers is by converting vegetative cells into endospores. According to Borris (2020), *Bacillus* sp. are Gram-positive, aerobic, rod-shaped bacteria, and capable for produce spherical endospores centrally positioned within the cell. Endospores are known for their thick-walled structure and low water content (Abbate *et al.* 2023), making

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them highly resistant to physical stresses such as heat (Setlow & Christie 2023), dryness (Zammuto & Gugliandolo 2019), and UV radiation (Checinska *et al.* 2015), as well as chemical stresses including disinfectants, antibiotics (Paul *et al.* 2019), and extreme pH levels (Schottroff *et al.* 2019). Endospore formation in *Bacillus* sp. is induced by environmental stresses such as nutrient deprivation (Khanna *et al.* 2020) and drought (Mendoza-Alatore *et al.* 2024), conditions unfavorable for the survival of vegetative cell (Logan & Vos 2015). Endospores can survive in dormant conditions for years or even hundreds of years. These characteristics can provide an advantage to maintain the stability of cell viability of *Bacillus* sp.

Carrier material is a component that supports the stability of the endospore viability. Ardyani (2021) reported biochar as a carrier material could maintain the viability of vegetative cells over a certain period. The nature of the carrier material needs to be compatible with endospores, abundant quantity, and affordable, so that it does not significantly affect production input costs. In addition, the carrier material can be aligned to the purpose of the formulation, such as using Oil Spill Dispersant (OSD) as carrier for *B. subtilis* CYA27 endospore which can degrade petroleum.

Biochar is a fine grain of porous charcoal resulting from combustion without oxygen (pyrolysis) at a temperature of 300-500°C. The carbon content of the biochar is approximately 70-80% can improve stability of soil aggregate, pH, cation exchange capacity (CEC), aeration, and soil nutrient availability. According to Saxena *et al.* (2013) application of biochar to the soil together with *Bacillus* sp. can increase plant growth, N uptake, and P uptake. Case *et al.* (2012) mentioned that biochar increases the population of phosphate solubilizing bacteria (PSB) and improves soil chemical properties through the adsorption of ammonium (NH_4^+) in the soil. Biochar has also been shown to increase the nitrogen intake efficiency (Miller *et al.* 2011).

OSD is a mixture of surfactants to decompose waste oil into small-dispersed particles, so OSD is used as a carrier for *B. subtilis* CYA27 endospore. Surfactants are agents that reduce surface tension and can be synthesized from either petroleum or vegetable oils, such as palm oil. OSD formulation consist of anionic surfactants and non-ionic surfactants. Anionic surfactants are surfactants that have negative ions in their hydrophilic groups e.g., methyl ester sulfonate (MES), while non-ionic surfactants are surfactants that do not contain ions in their hydrophilic groups e.g., diethanolamide (DEA) (Yuan *et al.* 2014; Aziz *et al.* 2020).

Humic acid is an organic compound that has a role in soil to increase cation exchange capacity (CEC), increase soil carbon (Anwar & Sudadi 2013), and increase phosphorus (P) solubility from aluminium (Al) and ferrum (Fe) metal bonds. Humic acid comprises roughly 41-57% carbon, 33-46% oxygen, and 2-5% nitrogen. Additionally, it includes aromatic, aliphatic, and phenolic hydroxyl groups .

Recent studies highlight the critical role of functional microbes in sustainable agriculture and environmental remediation. For instance, Demir *et al.* (2023) and Khan *et al.* (2021) demonstrated that the application of *Bacillus* sp. in biofertilizers significantly improves plant growth and yield, while also enhancing soil health. Moreover, recent advancements in microbial technology, as reviewed by Umesh *et al.* (2021), emphasize the potential of endospore-forming bacteria in bioremediation, particularly in degrading persistent organic pollutants such as petroleum hydrocarbons. Given the increasing concerns over soil degradation and pollution, the development of effective microbial formulations is essential for sustainable agricultural practices and environmental conservation.

Despite the known benefits of *Bacillus* sp., there are gaps in understanding the viability and effectiveness of *Bacillus* sp. endospores on different carrier materials. This study aims to isolate *Bacillus* sp. that capable of forming endospores and to evaluate their viability on biochar and OSD carriers. Specifically, it seeks to isolate and identify *Bacillus* sp. from the bamboo rhizosphere using the heat shock method; characterize the physiological and metabolic properties of the isolates; assess the viability and stability of *Bacillus* sp. endospores on biochar and OSD carriers; and evaluate the effectiveness of *Bacillus* sp. endospores in degrading total petroleum hydrocarbons (TPH) in contaminated environments. By addressing these objectives, this research aims to provide a comprehensive understanding of the potential applications of *Bacillus* sp. endospores in sustainable agriculture and environmental remediation.

2. Materials and Methods

2.1. Sampling Location

Soil samples were taken from forest land at the IPB University, Bogor, Indonesia (6°33'24.5"S 106°43'02.0"E). Soil samples were compositely taken

at a depth of 0-20 cm in the rhizosphere of bamboo stands. Soil samples were stored in plastic containers and placed in a cool box, then taken to the laboratory for isolation of *Bacillus* sp.

2.2. Isolation and Culturing of *Bacillus subtilis* CYA27 Isolate

A 10 g of soil sample was mixed in 90 ml of physiological 0.85% NaCl solution for sample preparation. Heat shock treatment was carried out by heating the mixture at 80°C for 15 minutes to eliminate vegetative cells, leaving only the heat-resistant endospores (Setlow & Christie 2023). The treated sample was diluted to 10^{-5}, 10^{-6}, and 10^{-7} levels and cultured on a 10% Nutrient Agar (NA) medium. The colonies obtained were then subjected to morphological observations as an initial screening of *Bacillus* sp. Initial characterization of *Bacillus* sp. was conducted by testing the isolates through Gram staining, dissolving 3% KOH, and observing the shape of bacterial cells (Tuhumury *et al.* 2021). The Gram stain test was carried out by a multilevel staining technique using crystal violet, Lugol's iodine, safranin, alcohol, and aquades (Sutedjo *et al.* 1996). The color of Gram-positive bacteria would be purple, while The Gram-negative bacteria would be red.

2.3. Potential Test

Pure isolates of *Bacillus* sp. were cultured on Pikovskaya medium to observe their ability to solubilize phosphate, and Alexandrov's medium to determine their potency in dissolving K (Setiawati *et al.* 2019). The ability to dissolve phosphate and potassium were indicated by the presence of a clear zone (holozone) around the bacterial colony (Feng *et al.* 2019). The pure isolates of *Bacillus* sp. were also cultured on nitrogen-free mannitol (NFM) medium to see their potential in fixing free N_2 (Mir *et al.* 2022).

2.4. Physiology Characterization (pH, Temperature, Salinity, Carbohydrate Fermentation, Oxidase, and Catalase)

The pH test was carried out by growing one bacterial colony in Nutrient Broth (NB) medium with a pH concentration of 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and control. pH adjustments were made using 0.01 N HCl or 0.01 N NaOH. The pH concentration was measured with a hand electrode pH meter, and bacterial growth was observed by turbidity changes using a spectrophotometer.

The temperature test was carried out by growing one bacterial colony in NB medium. The incubation process is carried out at different temperatures, such as at freezer temperatur, -18°C, cold storage at 2-4°C, an ambient temperature of 25-28°C, and an incubator at 50°C. The period of incubation is 48 hours. The bacterial growth was observed by turbidity change of the medium in each treatments using spectrophotometer.

The salinity test was carried out by growing single bacterial colony in NB medium at different salinity concentrations of NaCl (Ansar *et al.* 2023), such as 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0%. The bacterial growth was observed by turbidity change of the medium in each treatments using spectrophotometer.

Carbohydrate fermentation test was carried out by growing a colony of bacteria in NB medium with added carbohydrates, such as glucose, lactose, and sucrose. Each cultured was added with 1 ml bromothymol blue 1% at pH 7.0. The period of incubation was for less than 48 hours at 37°C. Bacterial growth was detected by a reddish-yellow color change, indicating an acidic reaction from sugar decomposition. Absence of this color change indicated that the bacteria were unable to decompose sugar (Apriani 2015).

The oxidase test involved scratching a colony of bacteria onto Oxidase Test Strip paper (Sigma-Aldrich) and allowing it to stand for approximately 60 seconds. Researchers then observed the color change: a shift from white to blue-violet indicated the bacteria's ability to oxidize, while no color change indicated a negative result .

The catalase test aims to determine the catalase enzyme produced by bacteria through dripping a 3% hydrogen peroxide (H_2O_2) solution on bacterial colonies on a sterile glass object. If the results are bubbly, the bacteria are positive for the catalase enzyme (Oluwaferanmi & Ogwu 2021).

2.5. Isolate Identification

Bacterial identification was carried out by determining the 16S rRNA sequence and the results were match with data in the NCBI GeneBank database. Identification was carried out in the laboratory of the Indonesian Center for Biodiversity and Biotechnology (ICBB). Meanwhile, *B. subtilis* CYA27 samples from Cahyani (2021) were freshened and cultured on NA medium. The kinship of isolates was made using a phylogenetic tree using the maximum-likelihood method in MEGA 11 software.

2.6. Culture of *Bacillus* sp. and Endospore Observation

Bacillus sp. obtained through the screening process is cultured in Tryptic Soy Broth (TSB) medium for 48

hours under stirring conditions at 100 rpm in a shaker to achieve a density of >10^7 CFU ml^{-1}. The suspension of *Bacillus* sp. TSB cultured were heated at 80°C for 24 hours to induce sporulation. Furthermore, endospores were counted using the Aerobic Plate Count (APC) to determine the population (CFU ml^{-1}).

Endospore staining was done by suspension of *Bacillus* sp. dripped onto a glass slide. Furthermore, malachite green is dripped and heated over boiling water for 10 minutes. After that, the glass slides were rinsed using distilled water and then added with safranin, allowed to stand for 2 minutes (Hadioetomo 1993). Endospores observations were carried out using a microscope, the color of endospores was green while vegetative cells were red. Endospores subjected on a biochar carrier were observed using a scanning electron microscope (SEM).

2.7. Pathogenicity Test

Suspension of *Bacillus* sp. with a density of ±10^7 CFU ml^{-1} in NB medium was injected using a syringe into the lower surface of healthy tobacco leaves (*Nicotiana tabacum* L.) and observed for one week (Umesha *et al.* 2008). Necrosis was observed for 5 to 7 days with an indicator of a change in leaf color to brownish yellow. A haemolysis test was carried out by streaking bacterial colonies on a blood agar medium to test for pathogenicity in humans and animals (Darmawati *et al.* 2021). The bacterial growth was positively observed by looking at the clear zone formed in the blood agar medium.

2.8. Formulation and Viability Test

The endospore suspension of *Bacillus* sp. was extracted by centrifugation at a speed of 12,000 rpm for 3 minutes using 2 ml tubes. The volume of endospore pellets was accumulated as much as 0.5 ml then washed twice with physiological solution, then added with OSD and culture medium until it reached 1 ml. The endospore suspension solution was formulated with a carrier material in a ratio of 1/100 (v/v) OSD and 1:100 (v/w) biochar.

A viability test was performed by performing APC on days 0, 5, 10, 15, 20, 25, and 30 to determine cell viability (Mazylyte *et al.* 2022). The population of endospores were calculated by:

$$\text{Population} = x / (p * v)$$

where:
x: number of colonies at a certain dilution
p: dilution factor
v: volume of dispersed suspension (ml)

2.9. Total Petroleum Hydrocarbon (TPH) Analysis

TPH analysis was carried out by adding 5 g of petroleum-contaminated sand sample and 20 ml of *n*-hexane into an flask, then addition of anhydrous Na_2SO_4 to remove the water content. The mixture was shaken for 30 minutes and separated by filter paper. After that, silica gel was added to remove polar compounds and then separated. The extract obtained was then transfered into a boiling flask and concentrated with a rotary evaporator, until it was concentrated and separated from the solvent. The extracted sample was transferred to a porcelain dish that had been weighed, then dried in an oven at 70°C for 45 minutes and cooled in a desiccator, and then weighed. The measured weight is oil residue (TPH) (Behera *et al.* 2021; Li *et al.* 2021; Mostafa *et al.* 2021). The TPH concentration is calculated by the formula:

$$\text{TPH} = (\text{residual weight}) / (\text{sample weight}) \times 100\%$$

While the percentage of oil degradation is calculated by the formula:

$$\%\ \text{TPH degradation} = \left(\text{Initial TPH} - \text{Final TPH}\right) / (\text{Initial TPH}) \times 100\%$$

3. Results

3.1. Isolation, Characterization, and Identification

The results of screening obtained the best isolate, namely RAY21. Then, isolate was identified using 16S RNA gene.

Figure 1A shows the result of visualization of the 16s rRNA gene from the RAY21 isolate that is successfully amplified to a band of ±1500 bp long, once Figure 1B figuring the relationship of *B. pseudomycoides* RAY21 is displayed using a phylogenetic tree with the maximum-likehood method in MEGA 11 software.

3.2. Pathogenicity Test

The results of the hypersensitivity test of *B. pseudomycoides* RAY21 on tobacco plants showed no symptoms of necrosis on Day 6 and no change in leaf color (Figure 2B). In addition, the RAY21 strain was tested for hypersensitivity. As a result, the strain did not show a clear zone and no color change (Figure 2A). Both hypersensitivity tests showed that the bacterial strain of *B. pseudomycoides* RAY21 was not pathogenic to plants, animals, and humans. The *B. pseudomycoides* RAY21 and *B. subtilis* CYA27 could be safe as biofertilizer agent.

3.3. Metabolic Characteristic

The results of the physiological characteristic of *B. pseudomycoides* RAY21 were compared with the physiological characteristic of *B. subtilis* CYA27 in Figure 3. *B. subtilis* CYA27 was used as a positive control in the carrier formulation test, viability test, and validation of endospore ability. *B. subtilis* CYA27 can survive at pH 5 and range 7-10, salinity concentration 2% and 4.0 to 12%, and survive at both low and high temperature (range from -18 to 50°C).

B. pseudomycoides RAY21 is able to be active in the pH range of 6-8, salinity concentration of 0.5 to 3.5%, and thermophilic temperature (50°C). *B. pseudomycoides* RAY21 grows on NFM medium and is able to produce clear zones on Pikovskaya and Alexandrov media, so it is worth considering that RAY21 isolate has a potential as biofertilizer because it is able to solubilize P and K.

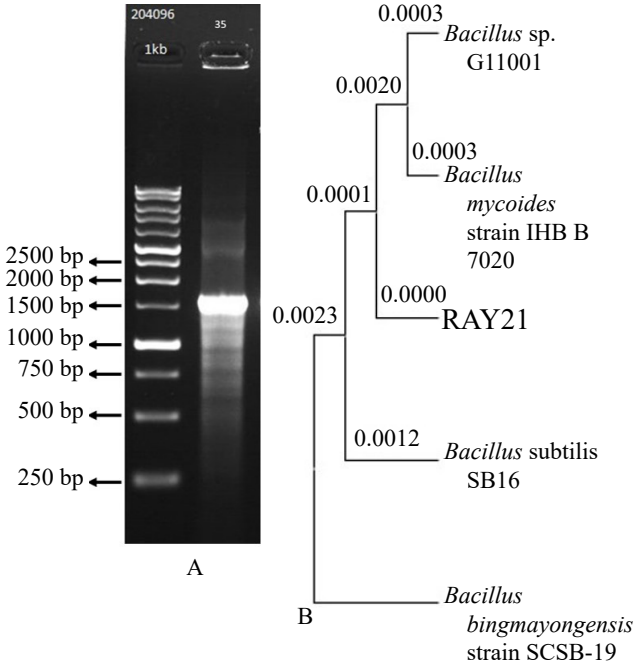


Figure 1. (A) Visualization of PCR amplification results in agarose gel (Lane 1-Ladder 1 kb; Lane 2-Gene 16S rRNA isolate RAY21), and (B) phylogenetic tree of isolate *B. pseudomycoides* strain RAY21

3.4. Endospore Production

The endospore (Figure 4A) shows *B. pseudomycoides* RAY21 and endospores (Figure 4B) shows *B. subtilis* CYA27 at 400 times magnification.

3.5. Carrier Formulation

The endospore formulation of *B. pseudomycoides* RAY21 and *B. subtilis* CYA27 in carrier were shown on Figure 5. The composition ratio between endospore and carrier material is 1:100 (w/w). The density of bacterial cells in the biochar carrier material on Day 0 adjusts to the standard requirements for biological fertilizers based on the Indonesian Minister of Agriculture Regulation No. 1 year 2019, which has a cell density of 10^7 CFU g^{-1}. In addition, the formulation of the liquid carrier is also adjusted to the solid carrier so that

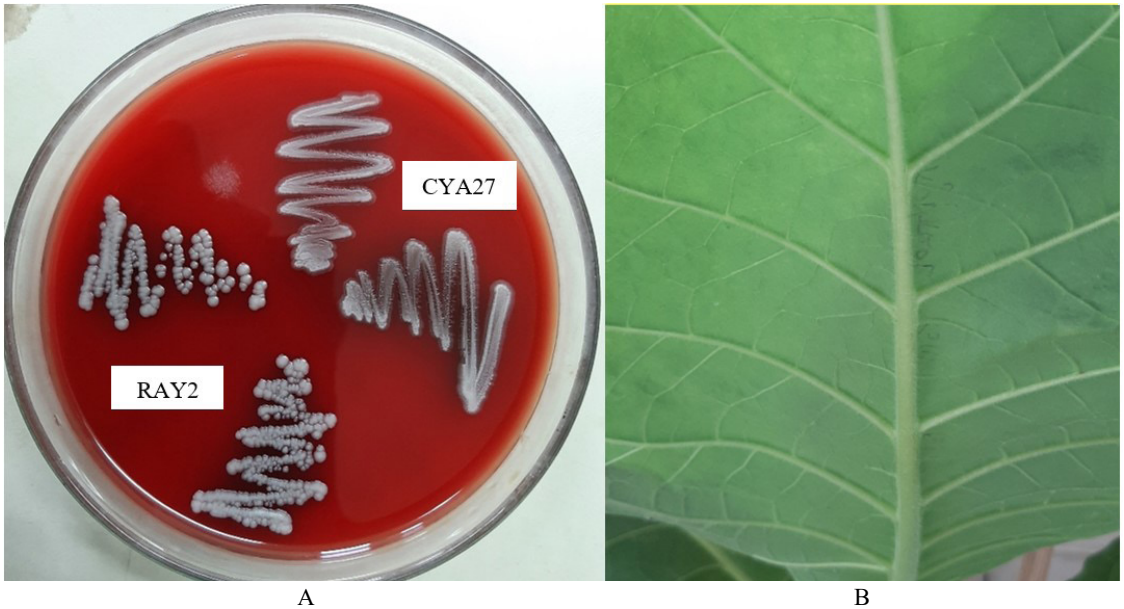
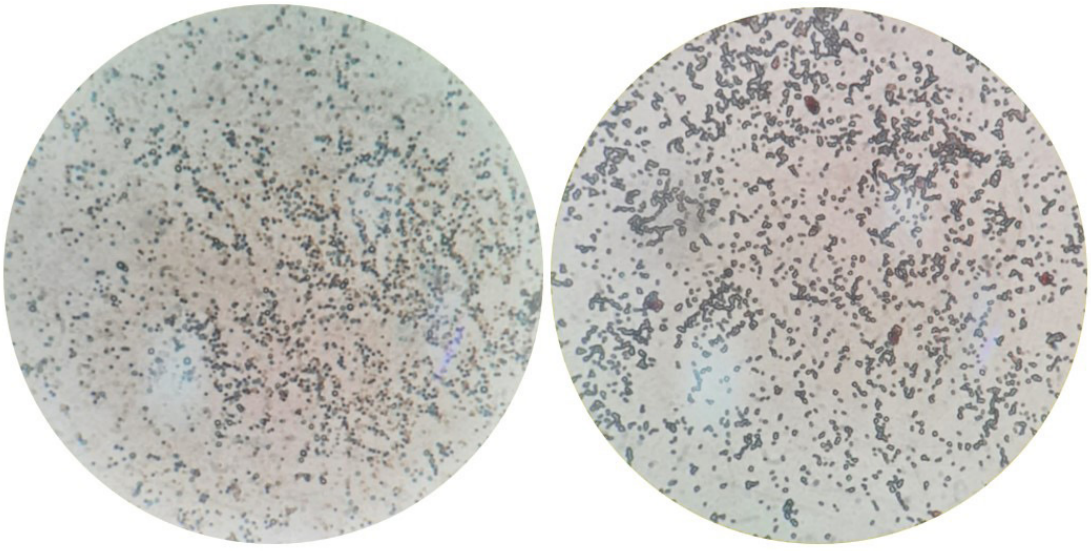


Figure 2. (A) Blood agar test, (B) hypersensityvity test

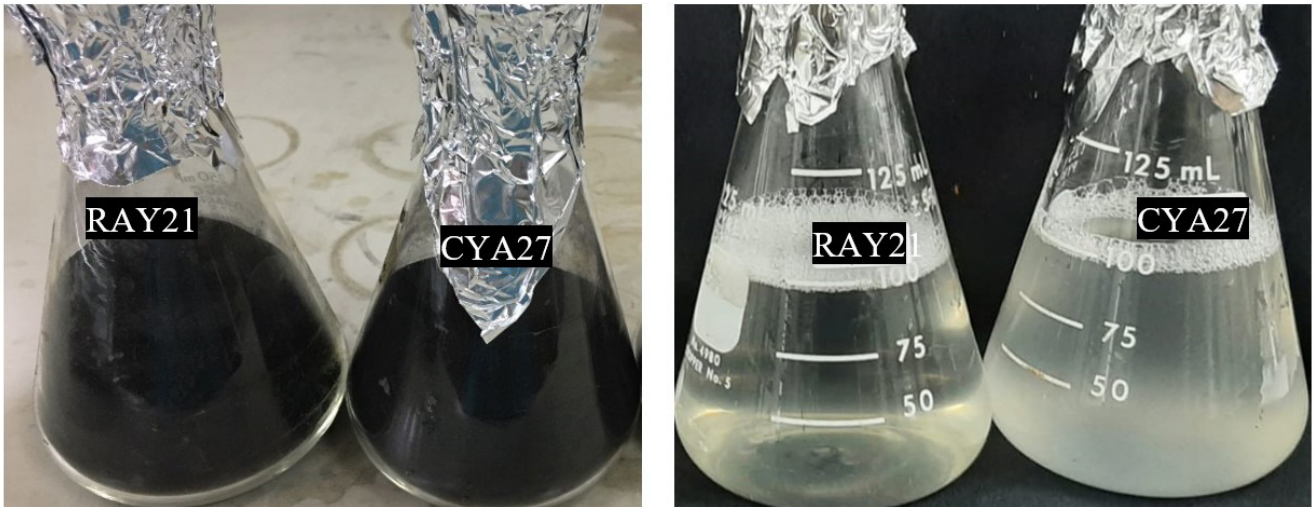
Strain	Endospore	Rod-shaped	N Fixing	P Solubilizing	K Solubilizing	Gram	Hyper Sensitivities: Blood Agar	Hyper Sensitivities: Tobacco	Oxidase	Catalase	Carbohydrate Fermentation: Glucose	Mannitol	Lactose	Sucrose	pH: 3	4	5	6	7	8	9	10	Salinity (%): 0	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	6.0	8.0	10	12	Temperature (°C): -18	-1	4	10	20	28	37	50
***B. pseudomycoides* RAY21**	+	+	+	+	-	+	-	-	●	●	●	●	◐		◐	◐	◐	●	●	●	◐			◐	●	●	●	●	●	●						◐		◐			●		●
***B. subtilis* CYA27**	+	+	+	+	+	+	-	-	●	●	●		●	●	●		●		●	●	●	●	●				●				●	●	●	●	●	●	◐	◐	●	●	●	●	●

Figure 3. Physiological characteristic of *B. pseudomycoides* RAY21 and *B. subtilis* CYA27



A B

Figure 4. Endospores of (A) *B. pseudomycoides* RAY21 and (B) *B. subtilis* CYA27; with malachite green staining



A B

Figure 5. Endospore formulation of *B. pseudomycoides* RAY21 and *B. subtilis* CYA27 in carrier: (A) biochar and (B) oil spill dispersant

it has a population density of 10^7 CFU ml^{-1}. The carrier material that has been inoculated by the endospores must be shaken to achieve homogeneity.

3.6. Viability Test

Endospore viability in the carrier formulation was analysed every 5 days from Day 0 (inoculation) to Day 30 of storage condition so that 6 viability data were obtained. Each viability data was obtained from 3 replicates at the dilution level used in each formulation.

Figure 6 in the article shows the viability of *B. pseudomycoides* RAY21 and *B. subtilis* CYA27 endospores in two types of carrier media, namely biochar and oil spill dispersant (OSD). The viability of endospores in biochar media showed a steady decline over the 30-day storage period. Both *B. pseudomycoides* RAY21 and *B. subtilis* CYA27 exhibited consistent decreases in the number of surviving colonie. Endospore viability in OSD media was more stable compared to biochar. This is due to the liquid phase nature of OSD, allowing more homogeneous distribution of endospores. However, the composition of OSD does not support the life of vegetative cells, thereby not triggering endospore germination.

3.7. Endospore Observation in Carriers

After a period of storage on the biochar and OSD carrier materials, the formulations were observed to confirm the presence of endospores. The endospores observation was done through a scanning electron microscope (SEM) photo on biochar and endospore staining on OSD. Figure 7 is an SEM photo using JSM

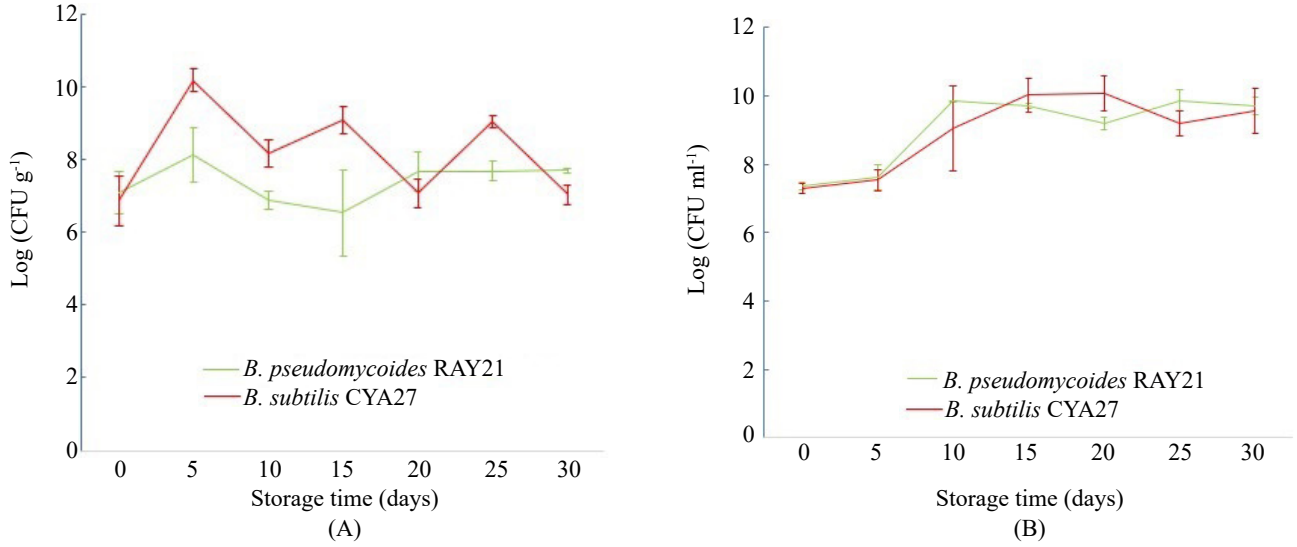


Figure 6. Endospore viability of *B. pseudomycoides* RAY21 and *B. subtilis* CYA27 on (A) biochar carrier and (B) oil Spill dispersant carrier for 30 days of storage

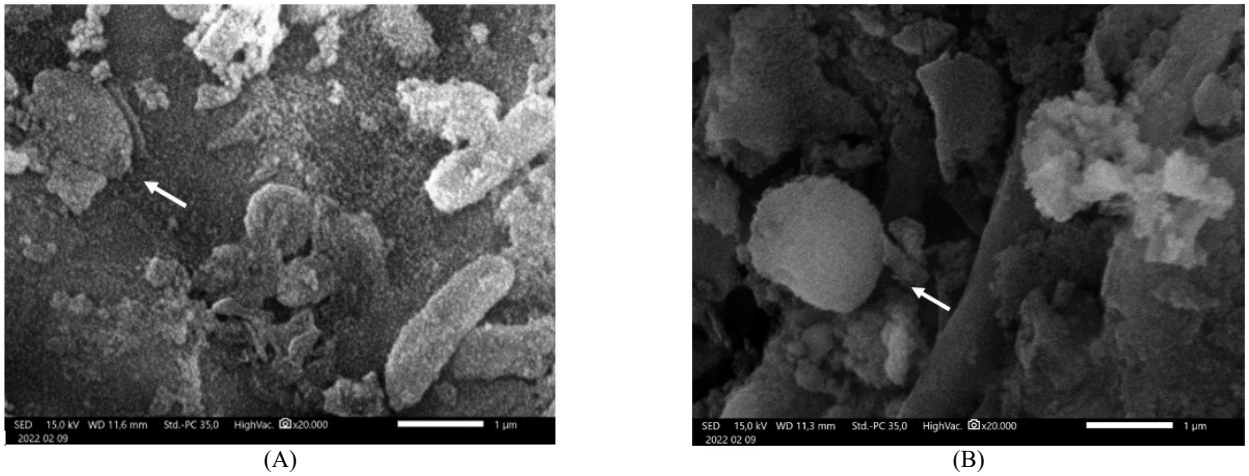


Figure 7. SEM photos of endospores of (A) *B. pseudomycoides* RAY21 and (B) *B. subtilis* CYA27 on a biochar carrier

IT 200 with a magnification of 20,000 times which shows the presence of endospores of *B. pseudomycoides* RAY21 and *B. subtilis* CYA27 that are still attached to the surface of the biochar after 90 days of storage. This is because biochar is a relatively conducive material for use as an inoculant carrier, including for endospores.

3.8. Endospore Ability Validation

Validation was carried out to confirm if there was a change in the ability of *B. pseudomycoides* RAY21 and *B. subtilis* CYA27 when transformed into endospores and formulated into a carrier material. The validation carried out was to re-test the ability of both isolates on various medium and TPH test.

The TPH test on OSD carrier material was not done to *B. subtilis* CYA27, but also on *B. pseudomycoides* RAY21 with the aim of testing its potential to degrade crude oil. After 30 days storage of both isolates in OSD carrier, the formulated solutions were inoculated to petroleum contaminated sand and incubated for 14 days.

The results of the TPH test showed that both strains were able to degrade TPH to decrease of 21.62% by *B. subtilis* CYA27 and 23.43% by *B. pseudomycoides* RAY21 (Figure 8A). Furthermore, reduction of TPH during 14 days from 71.333 mg/L to 55.913 mg/L by *B. subtilis* CYA27 and TPH reduction from 68.433 mg/L to 52.423 mg/L by *B. pseudomycoides* RAY21 (Figure 8B).

4. Discussion

Bacillus sp. successfully isolated from the rhizosphere soil sample of bamboo plants. Isolation was carried out using a heat shock method at temperature of 80°C for 15 minutes. Screening of isolates is directed to characterize the morphological of *Bacillus* sp. which has Gram-positive, rod-shaped cells, round colonies, and can produce endospores. The screening was also carried out by testing as functional microbes on Pikovskaya, Alexandrov, and NFM medium. The isolates The isolates were able to form holozone on Pikovskaya and Alexandrov media, indicating their ability to solubilize phosphate and potassium. This occurs due to organic acids excreted by the bacteria that bind with calcium (Ca) ions from $Ca_3(PO_4)_2$ in Pikovskaya medium, releasing H_2PO_4 and forming a clear area (Rawat *et al.* 2021). Once, the holozone formed on Alexandrov medium are caused by the release of organic or inorganic acids by the bacteria that solubilize potassium compounds from an unavailable form to a form that can be absorbed by plants. These bacteria are capable of mobilizing potassium from minerals through the production of chelating compounds and exopolysaccharides (EPS), which help increase the potassium content in the soil and support plant growth, especially in soils affected by salinity (Feng *et al.* 2019). Biochemical tests on *B. pseudomycoides* RAY21 also showed this strain had

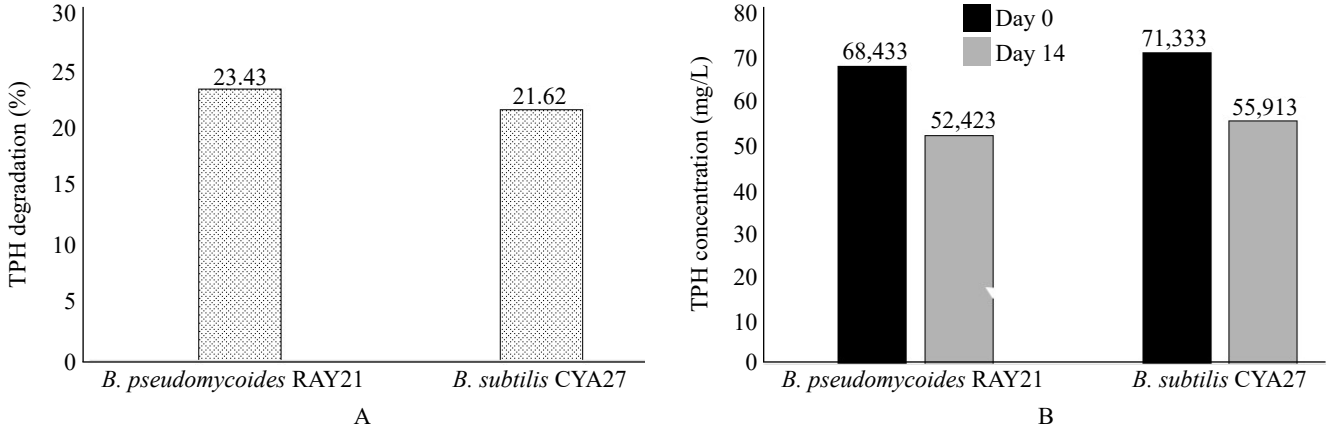


Figure 8. (A) Percentage of degradation of total petroleum hydrocarbons by formulated endospores of *B. subtilis* CYA27 and *B. pseudomycoides* RAY21 in OSD carrier after 14 days storage (B) concentration of total petroleum hydrocarbons by formulated endospores of *B. subtilis* CYA27 and *B. pseudomycoides* RAY21 in OSD carrier at Day 0 and Day 14

physiological characteristics related to its potency when applied to the soil environment. This strain was able to ferment several carbon sources such as glucose and mannitol, and produce catalase and oxidase enzymes. *B. pseudomycoides* RAY21 is able to be active in the pH range of 6-8, salinity concentration of 0.5 to 3.5%, and thermophilic temperature (50°C) (Figure 3).

The isolation and identification of *Bacillus* sp. from the bamboo rhizosphere demonstrated the presence of endospore-forming bacteria capable of surviving extreme conditions. This aligns with previous findings that *Bacillus* sp. is commonly found in diverse environments, showcasing its adaptability and resilience (Akinsemolu *et al.* 2024). The use of the heat shock method effectively selected for *Bacillus* species, confirming its reliability for isolating endospore-forming bacteria (Song *et al.* 2016). Physiological and metabolic characterization revealed that the isolated *Bacillus* sp. exhibited significant traits beneficial for agricultural applications, such as phosphate solubilization and nitrogen fixation. These traits are critical for promoting plant growth and improving soil health (Khan *et al.* 2021). Moreover, the ability of *Bacillus* sp. to degrade petroleum hydrocarbons underscores its potential for bioremediation, a crucial innovation in managing environmental pollution (Umesh *et al.* 2021).

The RAY21 isolate, which had been morphologically characterized and proven to produce endospores, was further identified at the molecular level by 16S rRNA analysis. The 16S rRNA gene has a conserved region so it is appropriate to be used as a universal primer in the PCR process (Rinanda 2011). Figure 1A shows the result of visualization of the 16s rRNA gene from the RAY21 isolate that is successfully amplified to a band of ±1500 bp long, the result is in accordance with the length of the 16S rRNA gene sequence, which is 1550 bp (Noer 2021). Next, the samples were sequenced to analyse the sequence of nucleotide bases. The results of gene sequences from isolate RAY21 analysed by BLASTN showed that it was 99.64% identical to isolate *Bacillus pseudomycoides* strain IHB B 7147 [KJ767330.1]. *B. pseudomycoides* isolate RAY21 was identified as having the closest relationship with *Bacillus* sp. G11001 [AB531397.1] and *Bacillus mycoides* IHB B 7020 [KJ721201.1] based on 16S rRNA sequences in the NCBI GeneBank database.

The formulation of endospores of *B. pseudomycoides* RAY21 and endospores of *B. subtilis* CYA27 with solid carrier of biochar and liquid carrier of OSD were combined using a completely randomized design (Figure 4). The specification of the biochar carrier material is made of rice husk charcoal, passed a 35-mesh screener, pH 7.5, moisture content 1%, and added humic substance as much as 2% (w/w). Meanwhile, the composition of the OSD carrier used is a formulation of 5% diethanolamide and 7.5% MES with a ratio of 7:3 and has a pH of 8-9. The *Bacillus* sp. are bacteria that can form endospores and require a carrier material to maintain their viability. Endospore carrier materials made of biochar and OSD need to be appropriately formulated so that the viability of the strains remains stable and maintains the ability of the strains.

Endospore production was carried out on strains of *B. pseudomycoides* RAY21 and *B. subtilis* CYA27 isolated by Cahyani (2021) which known to have the ability to degrade crude oil. Endospores are produced by transforming vegetative cells through treatment at 80°C for 24 hours in TSB medium that has been cultured for 48 hours on 100 rpm. Figure 4 illustrates the endospores of two bacterial strains under malachite green staining, observed at 400 times magnification. (Figure 4A) displays the endospores of *B. pseudomycoides* RAY21, while panel (Figure 4B) shows the endospores of *B. subtilis* CYA27. The endospores of *B. pseudomycoides* RAY21 (Figure 3A) exhibit a characteristic oval shape with a central or terminal position within the vegetative cells. These endospores are designed to withstand extreme environmental conditions, enabling the bacteria to survive unfavorable circumstances. On the other hand, the endospores of *B. subtilis* CYA27 (Figure 4B) also demonstrate a similar oval morphology but might show slight variations in size and position within the cells compared to *B. pseudomycoides* RAY21. The staining process with malachite green allows for the clear visualization of the endospore structure, highlighting their robust nature. Both types of endospores are known for their resilience, capable of enduring harsh conditions such as high temperatures, desiccation, and exposure to chemicals. This ability is crucial for their application in bioremediation and as biofertilizers, as it ensures the longevity and effectiveness of the bacterial strains in various environmental settings. According to Gauvry *et al.* (2019), temperatures at above 49°C are not optimal conditions for vegetative cells of *B. subtilis*. The sporulation parameters indicated that at suboptimal temperatures (e.g., 49°C), vegetative cells initiate sporulation more synchronously, but in smaller quantities and with a delay, compared to the optimal

temperature (e.g., 40°C). Bacterial endospores are one of the most resistant forms of life known to date, highly tolerant of various stresses such as heat, chemicals, and harsh physical conditions. One of the characteristics of endospores is heat resistance (Zammuto & Gugliandolo 2019). Generally, endospores are resistant to temperatures around 40-45°C higher than vegetative cells. One of the key factors is the unusual endospore structure formed during sporulation. This results in a dehydrated endospore nucleus surrounded by an inner endospore membrane, a peptidoglycan cortex, and an outer protein layer, where the cortex plays an important role in the maintenance of resistance and dormancy by maintaining low water content in the central protoplast. Endospores will only germinate into vegetative cells when the environment supports their survival. Therefore, strains capable to modify themselves into endospores can provide the advantage of maintaining shelf life when mixed into the carrier (Setlow & Christie 2023).

The endospore viability results indicate that OSD as a carrier has advantages in maintaining endospore viability during long-term storage compared to biochar. This stability is crucial for field applications where bioremediation agents must remain effective over extended periods. Biochar is a solid carrier material and, although it shows a faster decline in viability, it remains relevant in some applications, particularly in soil that requires bioremediation agents with slower and more controlled release characteristics. Biochar proved to be an effective carrier, supporting prolonged cell viability due to its porous structure and favorable physicochemical properties (Ngo *et al.* 2024). This finding corroborates the potential of biochar as a sustainable carrier material for microbial formulations in agriculture (Ajeng *et al.* 2021). OSD offers a more homogeneous carrier medium for endospores, allowing for more uniform distribution and longer maintenance of endospore viability. The use of OSD as a carrier not only supports microbial viability but also enhances the degradation of petroleum hydrocarbons, offering a dual benefit (Kleindienst *et al.* 2015). This innovation demonstrates the versatility of *Bacillus* sp. formulations in addressing both agricultural and environmental challenges.This is important for applications in oil-contaminated environments where rapid and effective distribution of bioremediation agents is crucial for efficiently addressing oil pollution. Both carrier types demonstrate that *B. pseudomycoides* RAY21 and *B. subtilis* CYA27 can survive and remain active under different conditions, highlighting their potential as effective bioremediation and biofertilizer agents. The success in maintaining this viability indicates the adaptability of both bacterial strains in various carrier media, which is important for diverse field applications. By understanding endospore viability in various carrier media, more effective strategies can be designed for field applications in bioremediation and biofertilizer, enhancing the efficiency and impact of these biological agents in addressing environmental and agricultural issues.

Table 1 shows the stability of the ability of the two strains which apparently remained the same between before transforming into endospore and the end of the storage period of the formulations of the two carriers. *B. pseudomycoides* RAY21 and *B. subtilis* CYA27 had the potential to dissolve P and K as seen from the formation of a clear zone on Pikovskaya and Alexandrov medium and were able to grow on NFM medium. Several previous studies reported that the *Bacillus* sp. are a multifunctional bacterium in the soil, Mahmud *et al.* (2021) stated that *B. pseudomycoides* was a functional bacterium capable of dissolving K in the soil, and Ulfiyati and Zulaika (2015) reported their exploration results that *Bacillus* sp. can dissolve phosphate. Sondang *et al.* (2021) reported that *B. pseudomycoides* was able to act as PGPR in the rhizosphere of rice plants. Validation test showed that *B. pseudomycoides* RAY21 isolate on biochar carrier had greater P and K dissolving ability than OSD carrier, this was indicated by the diameter of the clear zone formed.

The TPH test was carried out on both strains in the OSD carrier material. OSD is a mixture of surfactants to decompose waste oil into small-dispersed particles (Adlina *et al.* 2017). Li *et al.* (2016) in their research results reported that *B. pseudomycoides* BS6 explored from soybean waste can produce biosurfactants that play a role in the degradation process of household industrial oil waste. Table 2 shows more studies according to the potential ability of *Bacillus* sp. that already known.

The effectiveness of *Bacillus* endospores in degrading total petroleum hydrocarbons (TPH) was evident in contaminated environments. The study's findings align with recent research indicating that endospore-forming bacteria play a pivotal role in the biodegradation of persistent organic pollutants. This highlights the potential of *Bacillus* sp. as a bioremediation agent, providing a sustainable solution to environmental contamination (Das *et al.* 2024).

Table 1. Endospore ability test after 30 days storage on both carriers

Parameters test	Isolate ability			
	B. pseudomycoides RAY21		*B. subtilis* CYA27	
	Vegetative cells	Endospore after 30 days storage	Vegetative cells	Endospore after 30 days storage
P solubilizing	(+)	(+)	(+)	(+)
K solubilizing	(+)	(+)	(+)	(+)
N_2 fixing	(+)	(+)	(+)	(+)

Table 2. Potential *Bacillus* sp. from different sources in worldwide

Sites	Country	Isolates	Potency	References
Bamboo rhizosphere	Indonesia	*Bacillus pseudomycoides* RAY21	Phosphate and potassium solubilizing bacteria, N_2 fixer, and PAH degrader	This study
Crude oil contaminated coastal area	Indonesia	*Bacillus subtilis* CYA27	Phosphate and potassium solubilizing bacteria, N_2 fixer, biosurfactant, and PAH degrader	This study; Cahyani (2021)
Edible oil contaminated soil	China	*Bacillus pseudomycoides* BS6	Biosurfactant	Li *et al.* (2016)
Rhizosphere of tea plantation	India	*Bacillus pseudomycoides* O-5	Potassium solubilizing bacteria	Pramanik *et al.* (2019)

This study presents several key innovations. First, the successful isolation and identification of *Bacillus* sp. from the bamboo rhizosphere expand the understanding of its ecological niches and potential applications. Second, the characterization of physiological and metabolic traits underscores the multifunctionality of *Bacillus* sp. in agriculture and bioremediation. Third, the assessment of carrier materials provides critical insights into optimizing microbial formulations for practical use.

The relevance of this study is underscored by the increasing need for sustainable agricultural practices and effective bioremediation strategies. With soil degradation and environmental pollution posing significant global challenges, the development of robust microbial formulations is essential. This research contributes to this goal by demonstrating the viability and effectiveness of *Bacillus* endospores on innovative carrier materials.

Future research should focus on large-scale field trials to validate the findings of this study under real-world conditions. Additionally, exploring the synergistic effects of *Bacillus* sp. with other beneficial microbes could further enhance the effectiveness of microbial formulations. Investigating the long-term impacts of these formulations on soil health and plant productivity will also be crucial for their successful implementation in sustainable agriculture and environmental management.

In conclusion, the *B. pseudomycoides* RAY21, isolated from the rhizosphere of bamboo plants, demonstrated the ability to form endospores. Physiologically, *B. pseudomycoides* RAY21 thrived in a pH range of 6-8, salinity concentrations up to 3.5%, and thermophilic conditions (50°C). The viability of *B. pseudomycoides* RAY21 and *B. subtilis* CYA27 on OSD carriers was more stable compared to biochar + humic 2% (w/w) carriers after 30 days of storage. Both endospores showed good survival and stable performance during this period. Notably, *B. pseudomycoides* RAY21 on OSD carriers achieved a higher TPH degradation ability of 23.43%, while *B. subtilis* CYA27 degraded TPH by 21.62%. These results suggest that *Bacillus* endospore formulations are promising for bioremediation, especially in breaking down petroleum hydrocarbons. Future research should optimize carrier formulations and conduct field trials to confirm their effectiveness. Additionally, exploring interactions with other beneficial microbes could enhance their application in sustainable agriculture and environmental management.

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