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BIOREMEDIATION FORMULA: SPORULATION INDUCTION AND ENDOSPORE STABILITY OF *Bacillus pseudomycoides* RAY21 IN BIO-OSD

Pengembangan Formula Bioremediasi: Induksi Sporulasi dan Stabilitas Endospora *Bacillus Pseudomycoides* RAY21 dalam Bio-OSD

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ABSTRACT

Oil-based soil pollution is a pressing global environmental issue and has serious impacts on ecosystems and human health. Hydrocarbon compounds, especially heavy fractions, are persistent and difficult to decompose naturally in a short time, thus requiring intervention to accelerate the environmental recovery process. This research focuses on developing strategies to increase the effectiveness of oil-contaminated soil degradation. The development of a palm oil-based oil spill dispersant (Bio-OSD) combined with bacterial endospores of *Bacillus pseudomycoides* RAY21 offers an innovative approach to bioremediation. This particular bacterial endospore was selected for its capacity to sustain the viability of a potent biological agent until the point of application. The bacterial growth process was monitored to determine the optimal harvesting time by measuring optical density (OD600) at hourly intervals using a spectrophotometer until the stationary phase was reached. Following the harvesting of bacterial cells, various stressors were applied to stimulate sporulation, which included high-temperature incubation (80°C), nutrient deprivation, and their synergistic combination. The efficacy of the sporulation process was validated through endospore staining with *malachite green*. The resultant endospore pellets were integrated into the Bio-OSD and subjected to viability assessments every three days over a 24-day period using the *Total Plate Count* (TPC) method. The biodegradative capacity of the Bio-OSD containing endospores was evaluated through the analysis of Total Petroleum Hydrocarbon (TPH) degradation in artificially contaminated soils (3%) across a 28-day incubation. Findings indicated that optimal bacterial cell production occurred in Nutrient Broth, with peak harvesting at 40 hours. Moreover, the most effective sporulation was attained under combined stress conditions specifically, exposure to high temperature (80 °C for 24 hours) concomitant with nutrient deprivation (NaCl 0,85%) as demonstrated by the presence of green-stained endospores. This study provides evidence that Bio-OSD functions effectively as a stable carrier medium for bacterial endospores over a period of up to 24 days. The Bio-OSD formulation incorporating Bacillus pseudomycoides RAY21 endospores demonstrated the capability to degrade 78% of Total Petroleum Hydrocarbons (TPH) in petroleum-contaminated soil after 28 days of incubation. This degradation efficiency was significantly superior to that of the control treatment, which achieved a mere 32% degradation under the same laboratory conditions. To enhance the understanding and application of this approach, further investigations are recommended to be conducted on a larger scale, along with optimization analyses, to facilitate the development of more sustainable environmental remediation.

Keywords: *Bacillus pseudomycoides, bioremediation, contaminated soil, oil spill dispersant (OSD), petroleum*

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ABSTRAK
Pencemaran tanah oleh minyak bumi merupakan isu lingkungan global yang mendesak dan menimbulkan dampak serius terhadap ekosistem serta kesehatan manusia. Senyawa hidrokarbon, terutama fraksi berat, bersifat persisten dan sulit terurai secara alami dalam waktu singkat, sehingga memerlukan intervensi untuk mempercepat proses pemulihan lingkungan. Penelitian ini berfokus pada pengembangan strategi peningkatan efektivitas degradasi tanah tercemar minyak bumi. Pengembangan oil spill dispersant berbahan kelapa sawit (Bio-OSD) yang dikombinasikan dengan endospora bakteri *Bacillus pseudomycoides* RAY21 menawarkan pendekatan inovatif untuk bioremediasi. Endospora bakteri ini dipilih karena kemampuannya menjaga potensi agen biologis yang efisien hingga saatnya akan digunakan. Proses produksi sel diamati untuk memperoleh waktu panen sel optimal dengan interval pengamatan setiap 1 jam dengan spektofotometer (OD600) hingga kurva pertumbuhan menunjukkan fase puncak stasioner. Setelah sel dipanen, diberikan kombinasi cekaman untuk memicu terjadinya sporulasi, diantaranya inkubasi suhu tinggi (80 ºC), krisis nutrisi, dan kombinasi. Keberhasilan tahap sporulasi dikonfirmasi dengan pewarnaan endospora mengunakan *malachite green.* Pelet endospora kemudian dipanen dan dimasukan kedalam Bio-OSD untuk pengujian viabilitas setiap 2 hari hingga hari ke 24 dengan metode *Total Plate Count* (TPC). Kemampuan Bio-OSD yang telah mengandung endospora dikonfirmasi dengan analisis degradsi Total Petrolium Hidrokarbon (TPH) pada tanah tercemar buatan (3%) selama 28 hari inkubasi. Hasil menunjukkan produksi sel bakteri tumbuh optimal pada Nutrient Broth dan dapat dipanen dalam waktu 40 jam, dan proses sporulasi terbaik terdapat pada pemberian cekaman kombinasi yaitu suhu tinggi 80 ºC selama 24 jam dan tanpa nutrisi (NaCl) yang dibuktikan dengan hasil pewarnaan endospora menampilkan warna hijau. Penelitian ini membuktikan bahwa Bio-OSD mampu berperan sebagai media pembawa endospora yang stabil selama 24 hari. Formulasi Bio-OSD menandung endospora *B pseudomycoides* RAY21 ini juga terkonfirmasi mampu mendegradasi 78% TPH pada tanah tercemar minya bumi dalam 28 hari inkubasi. Peningkatan degradasi yang dicapai signifikan dibandingan kotrol perlakuan yang hanya mencapai 32% pada skala laboratorium. Penelitian lanjutan diharapkan dapat dilakukan dalam skala yang lebih besar dengan analisis optimasi untuk membuka jalan bagi solusi lingkungan yang lebih berkelanjutan.

Kata Kunci: *Bacillus pseudomycoides, bioremediasi minyak bumi, tanah tercemar, oil spill dispersant (OSD)*

INTRODUCTION

Petroleum hydrocarbon contamination of soil resulting from oil spills represents a significant global environmental challenge, posing severe repercussions for both ecosystems and human health (Haris 2009; Koshlaf and Ball 2017). Hydrocarbon compounds, particularly those classified within the heavier fractions, exhibit notable persistence and are inherently resistant to natural degradation processes within a short time frame. Consequently, the implementation of intervention strategies is essential to expedite environmental recovery (Handrianto 2018). In this context, bioremediation emerges as a more eco-friendly and sustainable approach, leveraging the natural capabilities of microorganisms to transform toxic hydrocarbon contaminants into innocuous compounds (Vidali,2001: National Research Council 1993). Nevertheless, a significant limitation of biological remediation methods lies in their typically protracted time frames (Xu et al. 2025).

To enhance the efficacy of bioremediation processes, a synergistic approach that combines biological and chemical methods has been proposed. Oil Spill Dispersants (OSDs), composed of surfactants and solvents, function by emulsifying oil layers into finely dispersed droplets (Mulligan et al. 2001). This emulsification increases the surface area of the oil, thereby facilitating more efficient bacterial degradation, and empirical evidence has demonstrated that the application of OSDs significantly enhances oil biodegradation performance (Silva et al. 2014). In alignment with the quest for sustainable remediation solutions, the Surfactant and Bioenergy Research Center (SBRC) at IPB University has pioneered the development of an environmentally benign OSD derived from palm oil (Vitra Meizar et al. 2017; Leo 2021). Previous studies that integrated microbial strains, such as Pseudomonas aeruginosa, with OSDs, have reported marked reductions in Total Petroleum Hydrocarbon (TPH) concentrations (Elvina et al. 2016). Despite these advancements, a pivotal challenge persists: the stability and shelf-life of biological agents used in remediation. Conventional formulations predominantly utilize vegetative bacterial cells, which exhibit vulnerability and a rapid decline in viability, thereby constraining practical field applications.

This limitation delineates a critical research gap: the necessity for an integrated bioremediation product characterized by high efficacy and enhanced stability for prolonged storage and transport. Research has substantiated the oil-degrading potential of bacterial species from the genus *Bacillus* (Cahyani et al. 2022), specifically their ability to form resilient endospores (Rahmatullah et al. 2025). These dormant forms are capable of withstanding extreme environmental conditions, including elevated temperatures, desiccation, and nutrient deprivation. The proposition to amalgamate robust biological agents in spore form within a Bio-OSD framework offers a promising avenue for the development of more effective remediation products (Zafira 2021). However, to date, no investigation has addressed the formulation of a cohesive, stable product that directly integrates bacterial endospores into a sustainable, palm oil-based Bio-OSD.

Hence, this study aims to conceptualize and evaluate a novel bioremediation agent that synergizes heat-stable endospores of Bacillus pseudomycoides RAY21 with a palm oil-based Bio-OSD. The research will prioritize assessments of the endospores' stability under thermal stress, alongside evaluations of the formulation's efficacy in degrading petroleum hydrocarbons within contaminated soil matrices. The successful outcome of this investigation is anticipated to yield a practical, efficient, and sustainable strategy for oil spill remediation, contributing significantly to the broader field of environmental microbiology and bioremediation technologies.

MATERIALS AND METHODS

Location and time of the stud

This research was conducted from June to August 2024 at the Laboratory of Bioindustry, Faculty of Agricultural Technology, IPB University, Bogor, Indonesia

Material

The bacterial strain used was *Bacillus pseudomycoides* RAY21, an isolate from the collection of the Laboratory of Bioindustry, IPB University.

The primary equipment included an autoclave, oven, shaking incubator, laminar air flow cabinet (LAFC), vortex mixer, light microscope, colony counter, centrifuge, and a UV-Vis spectrophotometer. General laboratory glassware, petri dishes, micropipettes, inoculating needles, and microscope slides were also used.

The chemical reagents and media included deionized water, 75% ethanol, Nutrient Broth (NB), Yeast Extract, Nutrient Agar (NA), 0.85% (w/v) NaCl solution, and malachite green stain, crude petroleum oil. The palm oil-based Bio-OSD was provided by the Surfactant and Bioenergy Research Center (SBRC), IPB University.

Method

This study was designed to develop a bioremediation formula by evaluating the sporulation induction method and the endospore stability of *B. pseudomycoides* RAY21 in Bio-OSD. This research was conducted through several main stages, which consisted of bacterial cell production, induction and confirmation endospore formation, testing of endospore viability in Bio-OSD, and confirmation of degradation ability of the Bio-OSD formula containing *B. pseudomycoides* RAY21 endospores.

Growth Curve *of B. pseudomycoides* RAY21

The growth curve of *B. pseudomycoides* RAY21 was determined to identify the optimal harvest time for sporulation induction. The bacterial culture was grown in Nutrient Broth supplemented with 1% (v/v) crude oil to maintain its hydrocarbon-degrading capability. The culture was incubated at 30°C with agitation at 120 rpm. Bacterial growth was monitored every hour by measuring the optical density at 600 nm (OD 600) using a UV-Vis spectrophotometer until the culture reached the early stationary phase. All measurements were performed in triplicate.

Sporulation and Confirmation

Bacterial cells harvested in the early stationary phase were subjected to stress treatments for 24 hours to induce sporulation. The treatments consisted of temperature stress (50°C and 80°C) and nutritional stress (incubation in 0.85% NaCl solution to deprive cells of a carbon source). The experimental design for sporulation induction is detailed in Table 1.

Table 1. combination of sporulation treatments

Code	Temperature	Nutrition
P0 (control)	30 ºC (normal)	Nutrient broth (normal)
P1	50 ºC	Nutrient broth
P2	50 ºC	NaCl 0,85 %
P3	80 ºC	Nutrient broth
P4	80 ºC	NaCl 0,85 %

After incubation, cells were harvested by centrifugation (4000 rpm, 20 min, 4°C), and the resulting pellets were washed with sterile 0.85% NaCl solution. Endospore formation was confirmed using the Schaeffer-Fulton staining method with malachite green. The treatment that yielded the highest number of endospores was selected for subsequent experiments.

Analysis of Endospore Viability in Bio-OSD

To ascertain their viability, endospore pellets obtained from the optimal sporulation treatment were aseptically resuspended in a palm oil-based Bio-OSD formulation. The resultant endospore suspension was subsequently maintained under ambient temperature conditions (approximately 25-28°C) for a 24 days observation period.

The quantitative assessment of endospore viability was conducted at predetermined every 2 days until 24 days. For this purpose, the plate count method was employed, with all analyses performed in triplicate for statistical robustness. This rigorous methodology was established to comprehensively evaluate the capacity of the endospores to remain viable within the formulation, a key factor for their potential application in oil spill bioremediation.

Confirmation of degradation capacity in petroleum-contaminated soil

A laboratory-scale experiment was conducted to confirm the degradation capability of the endospore Bio-OSD formula. Artificial soil was prepared by mixing sterile soil with crude oil to a final concentration of 3% (w/w). The contaminated soil was divided into four treatment groups as described in Table 2. Each treatment was performed in triplicate. The samples were incubated at room temperature for four weeks. The residual petroleum concentration was measured using the gravimetric method for Total Petroleum Hydrocarbon (TPH) analysis.

Table 2. Treatment group analysis TPH

Code	Treatment
T0 (control)	Control
T1	Bio-OSD
T2	*B. pseudomycoides* RAY21
T3	Bio-OSD containg endospore *B. pseudomycoides* RAY21

The results of sporulation induction indicate that *B. pseudomycoides* RAY21 responds significantly to different levels of environmental stress. The qualitative results in Table 3 clearly distinguish the effectiveness of each treatment. Treatments that applied only one type of stress, such as nutritional stress at 50°C (P2) or high-temperature stress with available nutrients (P3), produced only a small number of endospores less. Conversely, under conditions approaching optimal without high temperature or nutrient stress (P0 and P1), no endospore formation was observed at all. This pattern underscores that the most effective trigger for sporulation is not merely the presence of unfavorable conditions, but the accumulation of multiple severe stress factors simultaneously. The dual stress condition (P4), high temperature 80°C and nutrient deprivation, creates an extremely hostile environment that forces the bacteria to enter a dormant stage as a self-defense mechanism, resulting in optimum endospore formation (Santos 2015). The ability to efficiently induce these conditions is the key focus of this research, as it ensures the production of biological agents in their most stable and robust form, which is crucial for their viability during long-term storage within the Bio-OSD.

RESULTS AND DISCUSSION

Growth Curve *of B. pseudomycoides* RAY21

The growth curve analysis showed that *B. pseudomycoides* RAY21 reached the early stationary phase after 40 hours, with a peak optical density of 1.16 (

Figure *1*). This phase is marked by a balance between cell division and cell death, indicating a stable environment for the cells. At this crucial time point, *B. pseudomycoides* RAY21 was successfully cultivated in 1 liter of nutrient broth medium, resulting in a substantial biomass that will be utilized in the subsequent stages of experimentation. This initial growth lays the groundwork for further investigations into the metabolic and physiological characteristics of the strain.

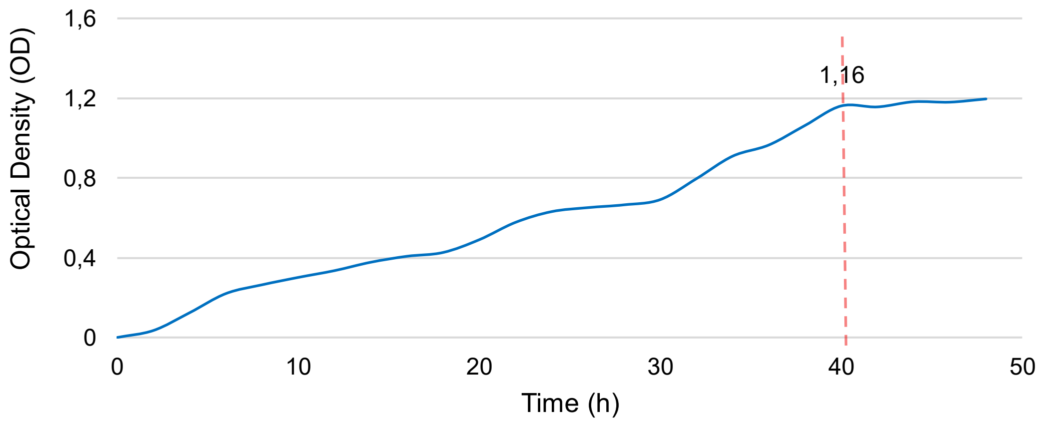

Figure 1. Growth curve of *B. pseudomycoides* in nutrient broth medium

Sporulation and Confirmation

Successful sporulation is critical for creating a stable bioremediation agent. The results confirmed that inducing endospore formation in *B. pseudomycoides* RAY21 requires significant environmental stress. As shown in Table 3 and Fig. 2, the optimal yield of endospores was achieved only under a dual-stress condition: high temperature (80°C) combined with nutrient deprivation (P4). In contrast, single-stress conditions (P2, P3) or near-optimal conditions (P0, P1) failed to produce a significant number of spores. This finding aligns with the established understanding of sporulation as a survival mechanism in *Bacillus* species, which is triggered by an accumulation of harsh environmental signals rather than a single stressor (Errington 2003). The ability to efficiently produce these robust endospores is a key step in developing a product with a long shelf-life.

Table 3. Qualitative analysis based on endospore staining (Schaeffer-Fulton method)

Code	Endospore Confirmation
P0 (control)	None
P1	None
P2	Less
P3	Less
P4	Optimum

This aspect is vital as endospores represent a remarkably stable and resilient form of the organism, thereby facilitating its viability during long-term storage in applications such as Bio-OSD, which is a primary focus of this research. Moreover, the endospore staining results provide compelling evidence of the sporulation process and underscore the ability of *B. pseudomycoides* RAY21 to adapt to and thrive under extreme environmental conditions. Fig.2 illustrates the endospore staining results, further elucidating the successful induction of sporulation under the examined conditions. Overall, this analysis contributes to a deeper understanding of the sporulation mechanisms in thermophilic bacteria and their potential biotechnological applications.

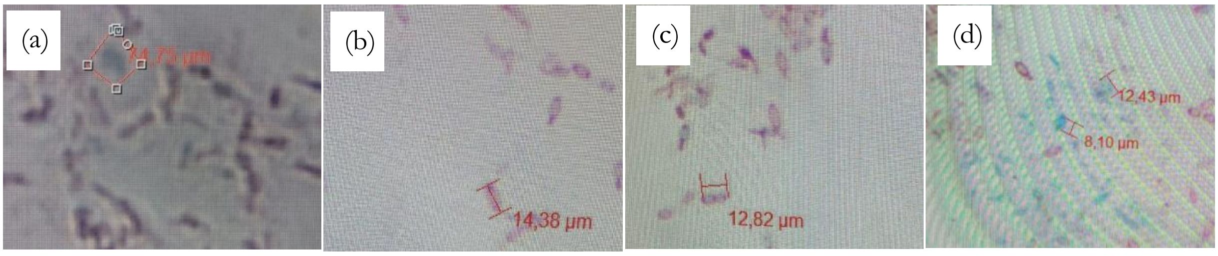

Figure 2. The endospore pellet staining using malachite green with 100× magnification of (a) P1; (b) P2; (c) P3: dan (d) P4.

Analysis of Endospores in Bio-OSD

The results of endospore viability testing in Bio-OSD for 24 days showed that endospore viability was stable. The minimal decrease in viability indicates that Bio-OSD is not toxic to endospores and may even provide an environment that supports their survival. These results are a key indicator confirming that Bio-OSD is capable of acting as a stable endospore carrier, as shown in Fig.3.

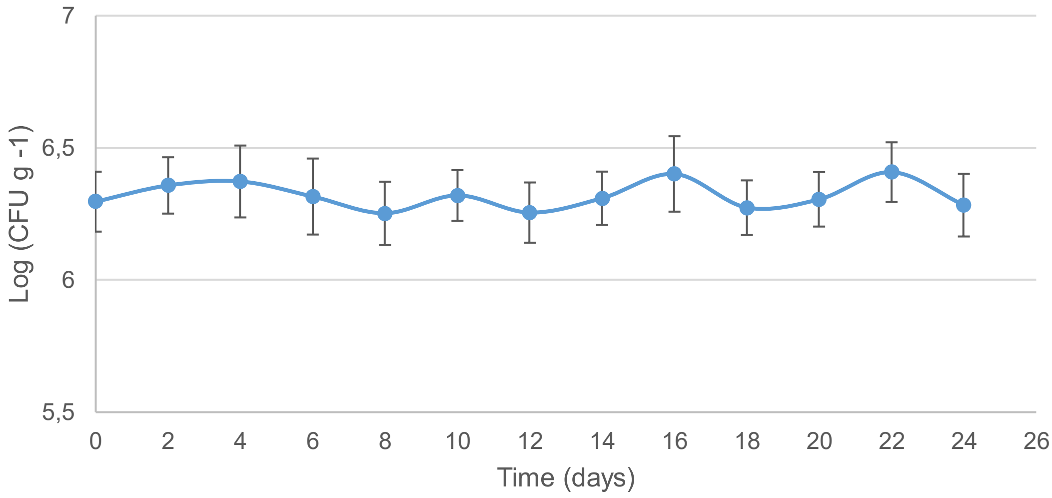

Figure 3. The viability analysis of *B. pseudomycoides* endospores in Bio-OSD for 24 days

Confirmation of degradation capacity in petroleum contaminated soil

The analysis into petroleum degradation capabilities demonstrated that the Bio-OSD formulation, which incorporates *B. pseudomycoides* RAY21 endospores, significantly outperformed both the control group and the individual treatment approaches. Initially, the total petroleum hydrocarbons (TPH) concentration was quantified at 3%. Subsequent measurements revealed a marked reduction in TPH levels across the various treatment conditions, with final values recorded as 2.03% for the control group (T0), 1.31% for the single treatment (T1), 1.12% for the dual treatment (T2), and an impressive 0.66% for the combined Bio-OSD application with *B. pseudomycoides* RAY21 endospores (T3). The degradation efficiency calculated for the Bio-OSD treatment utilizing *B. pseudomycoides* RAY21 endospores was found to be 78%, indicating a robust enhancement in the degradation process. These findings suggest that employing a strategic combination of Bio-OSD and *B. pseudomycoides RAY21* endospores can considerably augment the effectiveness of bioremediation efforts aimed at oil-contaminated soils. Detailed results supporting these conclusions are illustrated in Figure 3.

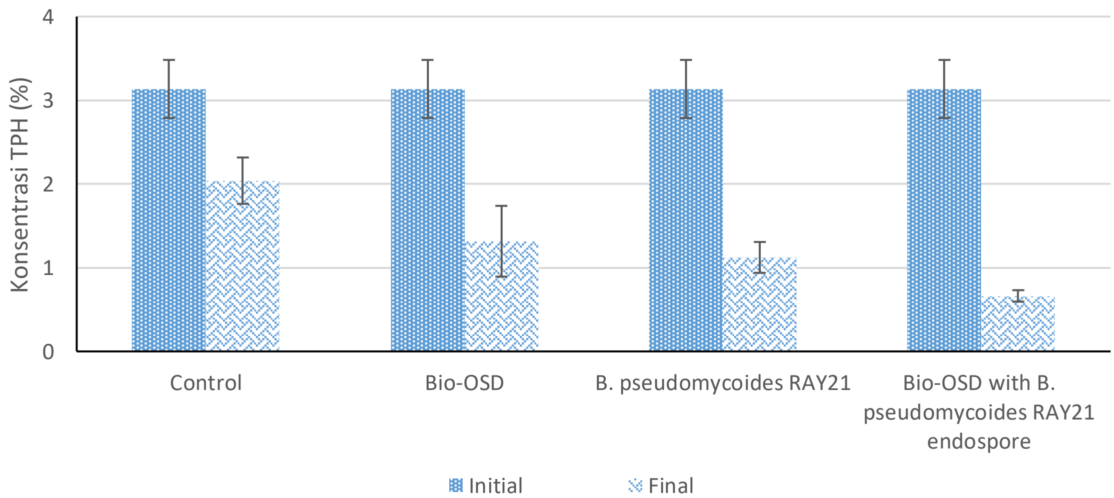

Figure 4. Total Petroleum Hydrocarbon (TPH) concentrations in 28 days bioremediation

The central finding of this study is the powerful synergistic effect between the Bio-OSD and B. pseudomycoides RAY21 endospores in degrading petroleum hydrocarbons. After 28 days, the combined treatment (T3) reduced TPH concentration from

3% to 0.66%, achieving a degradation efficiency of 78% (Fig.4). This rate is substantially higher than the control (T0, 32% degradation via natural attenuation) and the individual treatments of Bio-OSD alone (T1, 56%) or endospores alone (T2, 63%).

The observed synergistic effect can be elucidated through a two-step mechanistic framework. Initially, the Bio-OSD functions as a physical agent, facilitating the disruption of large hydrocarbon globules into microscopic droplets through a process of emulsification. This significantly enhances the oil water interfacial area, thereby increasing the bioavailability of hydrocarbons for microbial degradation. In the subsequent phase, the augmented bioavailability environment promotes the germination of dormant endospores into metabolically active vegetative cells. These active cells are capable of efficiently metabolizing the dispersed hydrocarbons, which would otherwise remain largely inaccessible. Notably, our findings indicate a degradation efficiency of 78%, surpassing rates documented in analogous studies that employed either microbial consortia or chemical dispersants independently (Cahyani et al., 2022). This outcome underscores the efficacy of a remediation strategy that synergistically integrates the physical dispersive action of a sustainable agent with the biological capabilities of resilient endospores.

CONCLUSION

The results of this study have significant practical implications. The development of a stable, storable Bio-OSD containing endospores offers a practical solution for rapid response to oil spills. The use of palm oil as a base for the OSD is particularly relevant for countries like Indonesia, promoting a sustainable, cost-effective, and locally sourced product. This formula simplifies logistics for field applications, as it does not require separate transport and mixing of dispersant and microbial cultures.

However, this study has several limitations. The experiments were conducted in a controlled laboratory setting using artificially contaminated soil. Real-world conditions involve complex factors like fluctuating temperatures, microbial competition, and varying soil compositions, which were not simulated here. Furthermore, the 28-day timeframe provides a snapshot of degradation but does not assess long-term ecosystem recovery.

Future research should focus on bridging the gap between laboratory and field. We recommend conducting pilot-scale field trials to validate the effectiveness of the formula under real-world environmental conditions. Further studies could also investigate the formula's performance on different types of crude oil and aged contaminants, as well as assess the long-term ecological impact on the native soil microbiome.

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