



RESEARCH ARTICLE

# Assessment of biosurfactant-producing bacteria from enriched shola forest soil and its oil degradation potential

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## Abstract

This study engineered a highly synergistic bacterial consortium for enhanced bioremediation of marine oil spills. The consortium was designed from 5 distinct biosurfactant-producing strains, isolated from a novel niche: shola forest rhizosphere soil enriched with petroleum-contaminated sediments and cocopeat. Through systematic screening under marine-relevant conditions such as pH, temperature and moisture stress induced by NaCl and PEG 6000, 5 strains were identified with exceptional halotolerance and hydrocarbon-degrading functions. Among isolates, *Ochrobactrum tritici* demonstrated direct hydrocarbon adhesion via 82 % cell surface hydrophobicity and a *Rhizobium* sp., a potent emulsifier, maintaining 76 % emulsification index (E24) even at 3 % NaCl. The consortium, comprised of 5 strains, exhibited strong synergistic interactions, achieving an exceptional 94 % degradation of 5 % (v/v) crude oil under standard laboratory conditions. Under simulated marine stress conditions, it maintained a high degradation efficiency of 88 %, outperforming individual strains by 1.5 to 2.5-fold. This work provides a marine-adapted, functionally synergistic microbial strategy for effective oil remediation in challenging coastal and ocean environments.

**Keywords:** bioemulsifier; biodegradation; crude oil; forest rhizosphere; halotolerance; microbial consortium

## Introduction

The global dependence on petroleum continues to render marine ecosystems vulnerable to pollution from spills, operational discharges and runoffs, with an estimated 1.3 million tons of hydrocarbons entering oceans annually (1, 2). Catastrophic events, such as the deepwater horizon spill, underscore the limitations of conventional response strategies. Chemical dispersants can introduce secondary toxicity, while physical methods are often inefficient against dispersed pollutants and logistically challenging in dynamic marine settings (3–5). This critical gap necessitates sustainable, effective and environmentally compatible solutions.

Bioremediation, leveraging microbial metabolism, can detoxify pollutants and is a promising alternative (6, 7). However, its success in marine environments is constrained by multifactorial stresses, including high salinity (~3.5 % NaCl), alkaline pH (~8.1), nutrient limitation and hydrocarbon toxicity (8, 9). These conditions can inhibit microbial inoculants, leading to frequent bioaugmentation failures (10). Overcoming the primary rate-limiting step, hydrocarbon bioavailability in aqueous environments, is crucial. Biosurfactants, amphiphilic molecules produced by microbes, play a pivotal role in emulsifying oil and dramatically increasing the interfacial area for microbial attack (11, 12). Natural microbial communities in oil-impacted sites exhibit resilience

through metabolic cooperation, a principle that can be harnessed via a rationally designed synthetic consortium (13, 14). Combining specialists with complementary traits, such as hydrocarbon degradation, biosurfactant production and stress tolerance, can yield more robust and efficient bioremediation than single-strain approaches (15, 16).

Despite this promise, a translational gap persists. Many studies focus on single-strain performance under idealised conditions, without engineering and validating a consortium tailored to specific, combined environmental stressors such as saline-alkaline pH (17, 18). Furthermore, systematic investigations linking environmental parameters to biosurfactant functional efficacy in degradation are scarce (19, 20). Moreover, the microbial sourcing strategy itself remains underexploited. Conventional isolation from chronically oil-polluted sites often selects specialists with narrow metabolic ranges, which may lack the adaptive resilience needed for dynamic marine conditions (21). In contrast, we propose that undisturbed, ecologically complex environments, such as the shola forest rhizosphere, harbor microbial communities pre-adapted to multifactorial stresses, including fluctuating nutrient availability, pH shifts and biotic interactions. The rhizosphere is a hotspot for microbial diversity and metabolic exchange, fostering traits such as biofilm formation, cross-feeding and stress resilience (22). By enriching this native community with petroleum-contaminated

sediment and cocopeat (a natural biostimulant), we aimed to simultaneously select for hydrocarbon degraders while preserving and enhancing innate stress-tolerance and synergistic potential. This approach leverages ecological principles of microbial assembly and niche partitioning, yielding a consortium with broader functional versatility and enhanced environmental robustness, a critical advantage over isolates from conventional contaminated sites.

To address these gaps, this study was designed with three interconnected objectives: (1) to characterise biosurfactant-producing bacteria from a novel enriched niche (shola forest rhizosphere soil amended with petroleum-contaminated sediment and cocopeat); (2) to comprehensively profile the functional niches of these isolates by evaluating their biosurfactant production, emulsification and cell surface hydrophobicity across a spectrum of environmental stresses (pH, salinity, moisture potential and temperature); (3) to critically compare the crude oil biodegradation efficacy of individual strains versus a constructed consortium under both standard and simulated marine conditions. By moving from individual characterisation to consortium validation, this work aims to provide a mechanistic understanding and a high-performance, marine-optimised microbial blueprint for effective bioremediation.

## Materials and Methods

### Bacterial isolates, enrichment strategy and growth conditions

Five bacterial strains with distinct hydrocarbon-degrading potential were isolated from a selective enrichment niche. In brief, we constructed a microcosm by amending the rhizosphere soil of a shola forest ecosystem (250 g) with petroleum-contaminated coastal sediment (250 g) and cocopeat (50 g), followed by a 60-day incubation. From this enrichment, 5 morphologically distinct colonies were purified and identified through 16S rRNA sequencing as *Ochrobactrum tritici* (PX666869; 99.64 % similarity), *Achromobacter xylosoxidans* (PX769224; 100 % similarity), *Rhizobium* sp. (PX666870; 97.05 % similarity), *Novosphingobium organovorum* (PX767048; 99.53 % similarity) and *Sphingobium yanoikuyae* (PX767047; 99.71 % similarity).

The crude oil used throughout the study, with an API gravity of 27.8°, was sourced from the Chennai Petroleum Corporation Limited (CPCL). A defined mineral salt agar medium (MSM) was used as the base medium for all experiments. Its composition was: 1.0 g/L  $\text{KH}_2\text{PO}_4$ , 1.0 g/L  $\text{K}_2\text{HPO}_4$ , 2.0 g/L  $\text{Na}_2\text{HPO}_4$ , 1.5 g/L NaCl, 0.15 g/L  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.04 g/L  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.15 g/L  $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5 g/L  $\text{NH}_4\text{Cl}$ , 0.05 g/L  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.5 g/L  $\text{NH}_4\text{NO}_3$ , 0.02 g/L  $\text{FeCl}_3 \cdot 3\text{H}_2\text{O}$  and 0.03 g/L  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ . The pH was adjusted to  $7.0 \pm 0.2$  unless otherwise specified. All chemicals used were of analytical grade (Sigma-Aldrich, purity  $\geq 99.0\%$ ).

### Assessment of biosurfactant activity under environmental stressors

All 5 bacterial strains were tested for biosurfactant production under various environmental conditions across different pH levels ranging from acidic to alkaline (pH 4–10), temperatures from 25–40 °C, salt concentrations (1–4 % NaCl) and polyethylene glycol (PEG 6000) levels (1–30 %) (-0.01 MPa to -1.0 MPa) to simulate different environmental conditions. The following methods were used for biosurfactant characterisation.

### Oil spreading assay

The oil spreading assay was employed to assess the surface-active properties of the cell-free supernatants (23). Briefly, 100  $\mu\text{L}$  of crude oil was added to a Petri dish containing 25 mL of distilled water. Subsequently, 100  $\mu\text{L}$  of cell-free supernatant (obtained by centrifuging culture broth at 5000 rpm for 20 min) was gently placed onto the center of the oil film. The presence of potent biosurfactants causes the oil film to be dispersed, forming a concentric circle. Triton X-100 and distilled water served as positive and negative controls respectively.

### Emulsification activity (E24)

Emulsification activity of the cell-free supernatant was quantitatively determined (24). In Brief, an equal volume of crude oil and cell-free supernatant was taken. The mixture was vortexed at maximum speed for 2 min and then allowed to stand at room temperature for 24 hr. The MSM broth without bacterial inoculum served as the negative control. The E24 index was calculated as:

$$\text{E24 (\%)} = (\text{Height of emulsion layer} / \text{Total height of liquid column}) \times 100$$

### Cell surface hydrophobicity

Bacterial adherence to hydrocarbons, a direct measure of cell surface hydrophobicity (CSH), was determined using standard method (25). Cultures were grown in MSM broth supplemented with glucose (8 g/L) for 72 hr at 30 °C. Cells were harvested by centrifugation (5000 rpm, 20 min), washed twice with phosphate-buffered saline (PBS, 50 mM, pH 7.0) and resuspended in the same buffer. Optical density ( $\text{OD}_{600}$ ) was standardised to 0.6–0.8 (A1). An equal volume of this cell suspension and crude oil was taken and vortexed for 2 mins. The MSM broth without bacterial culture was served as a control. The mixture was left for 24 hr, after which the  $\text{OD}_{600}$  of the aqueous phase (A2) was carefully measured. The percentage of bacterial adherence to hydrocarbons was calculated as:

$$\text{CSH (\%)} = [(A1 - A2) / A1] \times 100$$

### Crude oil degradation efficacy of individual strains and consortium

The crude oil biodegradation assay was performed (26). Briefly, the isolates were grown in MSM broth with glucose (8 g/L) for 24 hr at 30 °C with a pH of 7.0 at 100 rpm. Cells were harvested by centrifuging at 5000 rpm for 10 min, washed twice with 50 mM PBS to remove residues and resuspended in fresh PBS (5 mL) or carbon-free MSM broth.  $\text{OD}_{600}$  was adjusted to  $10^8$  CFU/mL. For the consortium, equal volumes (1 mL) of each bacterial suspension were combined. A flask containing fresh MSM broth without glucose was inoculated with 5 % of the bacterial suspension. Two conditions were tested: 1. A standard environment with MSM broth at neutral pH 7 without salt stress. 2. Simulated marine conditions where MSM broth was amended with 3.5 % (w/v) NaCl and an alkaline pH of 8.7. In addition, abiotic control containing only MSM broth and crude oil was included. Sterile crude oil was added as the sole carbon source at concentrations of 0, 1, 3 and 5 % (w/v). Flasks were then incubated at 30 °C for a period shaking at 100 rpm. At predetermined time intervals, residual crude oil was extracted using n-hexane. The organic solvent evaporated to dryness at low temperature and the weight of the residual oil (%) was determined gravimetrically and calculated as follows:

$$\text{Residual crude oil (g)} = (\text{Weight of beaker} + \text{extracted crude oil}) - \text{Weight of empty beaker.}$$

Amount degraded (g) = (Weight of crude oil initially added) - Residual crude oil

Degradation (%) = (Amount degraded / Amount of crude oil initially added) × 100

### Statistical analysis

All experiments were performed in triplicate (n=3) and the results are expressed as mean ± standard deviation (SD). Statistical significance was determined using one-way analysis of variance (ANOVA), followed by Duncan's multiple range test (DMRT) performed via XLSTAT. Differences were considered statistically significant at  $p < 0.05$ . Data visualisation and preliminary statistical computations were performed using Microsoft Excel 2019.

## Results

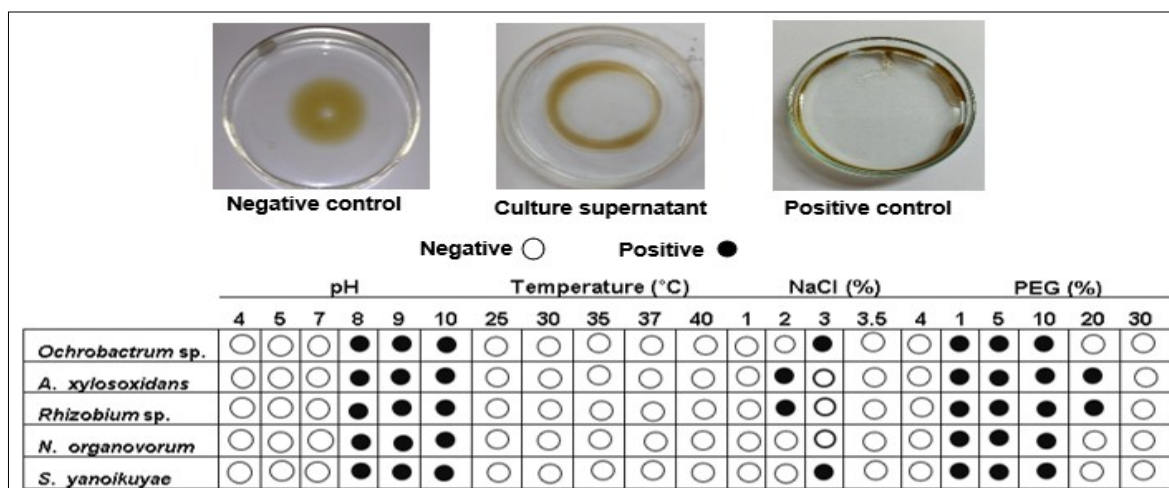
### Functional profiling reveals distinct marine-relevant niches

The oil displacement assays revealed a consistent environmental dependence for biosurfactant activity across all 5 bacterial isolates. Activity was strictly observed under alkaline conditions (pH 8–10), with no positive detection in acidic or neutral ranges (Fig. 1). PEG 6000-induced moisture stress (1–10 % w/v) was critical for triggering activity, simulating osmotic stress akin to low water availability in hydrocarbon-polluted coastal sediments. Differential salt tolerance was also evident: *A. xylosoxidans* and *Rhizobium* sp. exhibited biosurfactant activity at 2 % NaCl, while *O. tritici* and *S. yanoikuyae*

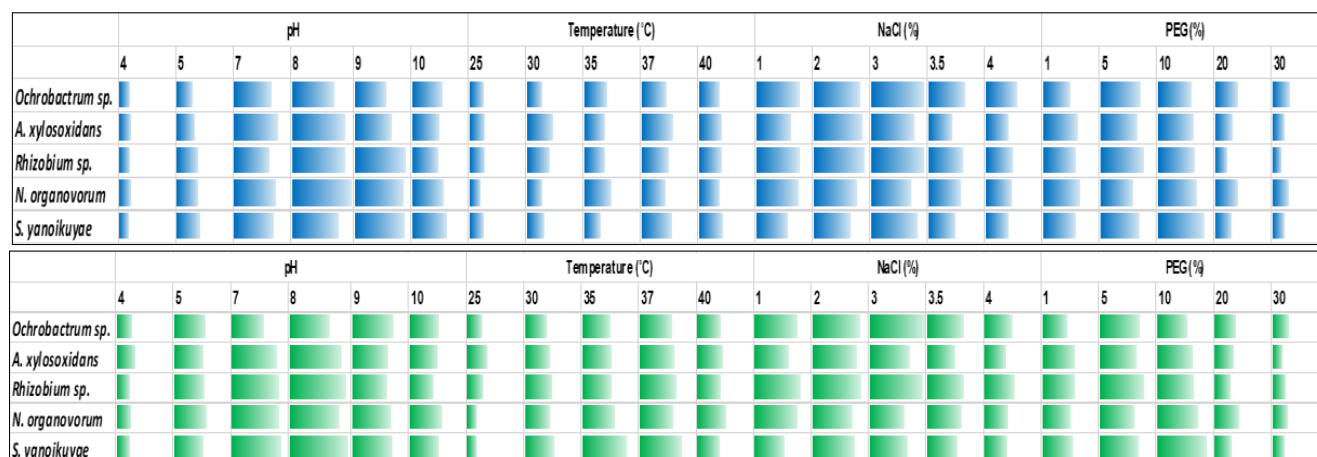
remained active at 3 % NaCl. Temperature variations (25–40 °C) did not significantly affect biosurfactant functionality. These findings indicate that biosurfactant production is specifically adapted to alkaline, osmotically stressed and moderately saline environments.

### Emulsification and cell adhesion: Uncovering hidden potential

The quantitative assays for E24 and cell surface hydrophobicity of the 5 bacterial strains were significantly influenced by variations in pH, temperature, salinity and PEG concentration, revealing distinct environmental optima and strain-specific adaptations. For emulsification, all strains exhibited low activity under acidic conditions (pH 4–5), with performance peaking at neutral to slightly alkaline pH (7–9) (Fig. 2a). Notably, *N. organovorum* showed the highest E24 value (78 % at pH 8). At the same time, *Rhizobium* sp. and *S. yanoikuyae* performed best at pH 9 (73 % and 71 % respectively). Activity declined sharply at pH 10, indicating reduced biosurfactant efficacy under strong alkalinity. Under varying temperatures, emulsification was generally moderate, with optimal activity observed at 37 °C for most strains, particularly *A. xylosoxidans* (45 %). Salinity tolerance was notable, with maximum E24 values recorded at 2–3 % NaCl for *O. tritici* (76 %), *Rhizobium* sp. (76 %) and *S. yanoikuyae* (66 %), though activity decreased at higher salt concentrations. PEG-induced moisture stress revealed that moderate PEG levels (5–10 %) sustained emulsification, with *S. yanoikuyae* achieving the highest activity at 10 % PEG (67 %), whereas higher concentrations (20–30 %) strongly inhibited all strains.



**Fig. 1.** Qualitative assessment of biosurfactant production under varied environmental conditions: pH (4–10), temperature (25–40 °C), NaCl concentration (1–4 %) and PEG-induced water potential (-0.01 MPa to -1.0 MPa) (1–30 %) via oil displacement assay.



**Fig. 2.** Functional characterisation of bacterial isolates for biosurfactant production and cell surface properties. (a) Emulsification activity (E24 %) of cell-free supernatants and (b) Cell surface hydrophobicity (CSH %) of the bacterial cells tested across a range of pH, temperature, salinity and water stress.

**Table 1.** Growth rate (OD<sub>600</sub>) of 5 isolates and their consortium in crude oil-enriched MSM broth over a periodic interval under standard laboratory conditions

	1 % oil					3 % oil					5 % oil				
	5 days	10 days	20 days	5 days	10 days	20 days	30 days	40 days	5 days	10 days	20 days	30 days	40 days	60 days	
<i>O. tritici</i>	1.07 ± 0.03 <sup>c</sup>	1.66 ± 0.04 <sup>c</sup>	1.89 ± 0.03 <sup>b</sup>	0.93 ± 0.05 <sup>c</sup>	1.42 ± 0.06 <sup>c</sup>	1.73 ± 0.04 <sup>d</sup>	2.01 ± 0.05 <sup>b</sup>	2.21 ± 0.04 <sup>b</sup>	0.84 ± 0.06 <sup>d</sup>	1.13 ± 0.03 <sup>d</sup>	1.52 ± 0.07 <sup>d</sup>	2.03 ± 0.17 <sup>b</sup>	2.33 ± 0.16 <sup>a</sup>	2.49 ± 0.15 <sup>a</sup>	
<i>A. xylosoxidans</i>	1.36 ± 0.02 <sup>b</sup>	1.92 ± 0.05 <sup>b</sup>	2.13 ± 0.05 <sup>ab</sup>	1.12 ± 0.04 <sup>b</sup>	1.54 ± 0.08 <sup>bc</sup>	1.84 ± 0.06 <sup>c</sup>	2.17 ± 0.07 <sup>b</sup>	2.32 ± 0.06 <sup>ab</sup>	0.93 ± 0.08 <sup>c</sup>	1.23 ± 0.05 <sup>d</sup>	1.64 ± 0.06 <sup>c</sup>	1.91 ± 0.08 <sup>c</sup>	2.16 ± 0.11 <sup>c</sup>	2.45 ± 0.13 <sup>a</sup>	
<i>Rhizobium</i> sp.	1.12 ± 0.04 <sup>b</sup>	1.53 ± 0.03 <sup>c</sup>	1.71 ± 0.07 <sup>b</sup>	0.98 ± 0.03 <sup>c</sup>	1.45 ± 0.04 <sup>c</sup>	1.62 ± 0.07 <sup>d</sup>	1.96 ± 0.06 <sup>c</sup>	2.28 ± 0.06 <sup>b</sup>	0.88 ± 0.06 <sup>d</sup>	1.34 ± 0.06 <sup>c</sup>	1.46 ± 0.08 <sup>d</sup>	1.84 ± 0.19 <sup>c</sup>	2.28 ± 0.17 <sup>b</sup>	2.39 ± 0.11 <sup>b</sup>	
<i>N. organovorum</i>	1.32 ± 0.03 <sup>b</sup>	2.05 ± 0.06 <sup>a</sup>	2.27 ± 0.07 <sup>a</sup>	1.25 ± 0.05 <sup>ab</sup>	1.69 ± 0.05 <sup>b</sup>	1.93 ± 0.09 <sup>c</sup>	2.26 ± 0.08 <sup>a</sup>	2.39 ± 0.12 <sup>ab</sup>	1.10 ± 0.06 <sup>bc</sup>	1.39 ± 0.05 <sup>c</sup>	1.61 ± 0.09 <sup>c</sup>	1.98 ± 0.09 <sup>bc</sup>	2.22 ± 0.12 <sup>b</sup>	2.46 ± 0.13 <sup>a</sup>	
<i>S. yanoikuyae</i>	1.46 ± 0.03 <sup>b</sup>	2.08 ± 0.05 <sup>a</sup>	2.42 ± 0.08 <sup>a</sup>	1.33 ± 0.03 <sup>ab</sup>	1.78 ± 0.08 <sup>b</sup>	2.06 ± 0.10 <sup>b</sup>	2.25 ± 0.08 <sup>a</sup>	2.46 ± 0.10 <sup>b</sup>	1.22 ± 0.08 <sup>b</sup>	1.52 ± 0.09 <sup>b</sup>	1.76 ± 0.10 <sup>b</sup>	2.17 ± 0.12 <sup>a</sup>	2.38 ± 0.14 <sup>a</sup>	2.54 ± 0.14 <sup>a</sup>	
Consortium	2.15 ± 0.04 <sup>a</sup>	1.91 ± 0.06 <sup>b</sup>		1.66 ± 0.06 <sup>a</sup>	1.91 ± 0.07 <sup>a</sup>	2.42 ± 0.10 <sup>a</sup>			1.49 ± 0.09 <sup>a</sup>	1.76 ± 0.10 <sup>a</sup>	2.35 ± 0.13 <sup>a</sup>	2.11 ± 0.14 <sup>a</sup>			

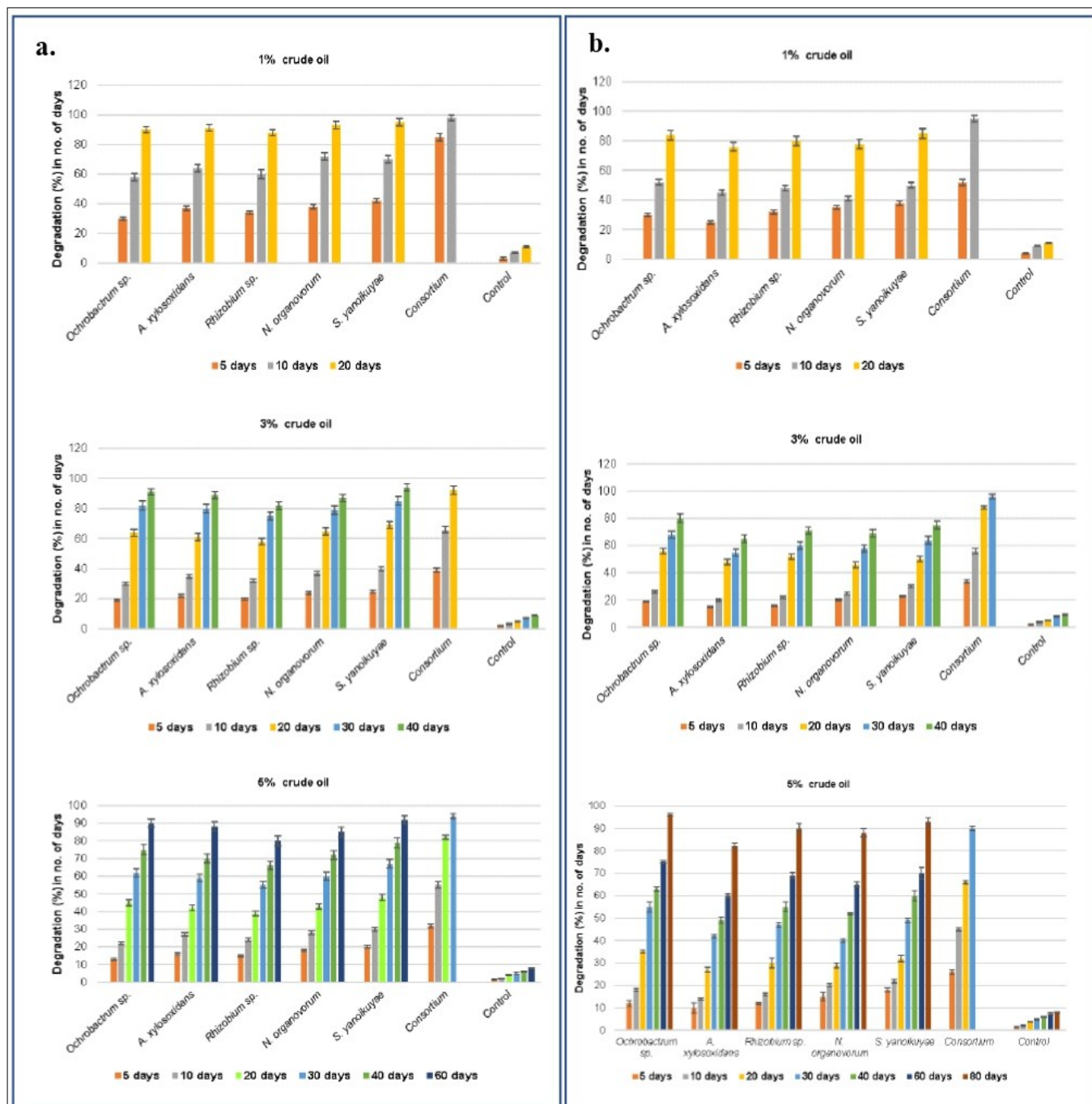
Cell surface hydrophobicity followed a similar trend, with the highest values observed at neutral to slightly alkaline pH (7–9), where *S. yanoikuyae* reached a peak of 81 % at pH 8 (Fig. 2b). Hydrophobicity generally increased with temperature up to 37 °C, with *A. xylosoxidans* and *Rhizobium* sp. showing strong affinity (53 % and 55 %, respectively). Salinity also enhanced the hydrophobic nature, particularly at 3 % NaCl for *O. tritici* (82 %) and *Rhizobium* sp. (79 %). Under PEG-induced stress, hydrophobicity was maintained at lower to moderate concentrations, with *S. yanoikuyae* again exhibiting resilience at 10 % PEG (74 %) but diminished sharply at higher water pressures.

Notably, strong emulsification did not always correlate with high surface tension reduction, underscoring the functional distinction between bioemulsifiers and classical biosurfactants. *Rhizobium* sp. and *S. yanoikuyae* exhibited the most robust and consistent performance across multiple stresses, highlighting their potential for synergistic consortium design.

### Crude oil degradation efficiency via gravimetric analysis

Under standard laboratory conditions, degradation efficiency varied with oil concentration and incubation time (Fig. 3a and Table 1). At 1 % oil, individual isolates achieved degradation between 30 % (OD<sub>600</sub>, 1.07) and 42 % (OD<sub>600</sub>, 1.46) within 5 days, with *S. yanoikuyae* performing best (42 %), increasing to 58–72 % by day 10 (*N. organovorum* highest at 72 %) and reaching a maximum of 84–95 % after 20 days. In contrast, the bacterial consortium demonstrated markedly faster degradation, attaining 85 % in 5 days, representing a 2.0-fold increase over the best individual (*S. yanoikuyae*) and 98 % within 10 days. At 3 % oil, individual strains exhibited slower progress, reaching 19–25 % in 5 days, 58–69 % in 20 days and 82–91 % only after 40 days, while the consortium achieved 39 % in 5 days (1.6-fold increase over *S. yanoikuyae*), 66 % in 10 days and 92 % by day 20 (1.3-fold increase). At 5 % oil, isolates showed further reduced rates, with only 13–20 % degradation in 5 days, 39–48 % in 20 days and 80–92 % (OD<sub>600</sub>, 2.03–2.54) after 60 days. The consortium again outperformed them, degrading 32 % in 5 days (1.6–2.5-fold increase), 55 % in 10 days, 82 % in 20 days (1.7–2.1-fold increase) and 94 % in 30 days, achieving near-complete removal nearly twice as fast as the best individual strain.

Under simulated marine conditions, degradation was slower and less efficient across all oil concentrations, though fold increases in consortium performance remained evident (Fig. 3b and Table 2). At 1 % oil, individual isolates degraded 25–38 % in 5 days (*S. yanoikuyae* highest at 38 %), 41–52 % in 10 days (*Ochrobactrum tritici*) and 76–85 % after 20 days, while the consortium reached 52 % in 5 days (1.4–2.1-fold over the top individuals) and 95 % (OD<sub>600</sub>, 2.32) in 10 days, representing near-complete degradation in half the time required by individual strains. At 3 % oil, individual strains achieved 15–23 % degradation in 5 days, 46–56 % in 20 days and 65–80 % after 40 days, whereas the consortium attained 34 % in 5 days (1.5–2.3-fold increase), 56 % in 10 days, 88 % in 20 days (1.6–1.9-fold increase) and 96 % (OD<sub>600</sub>, 2.28) in 30 days. At 5 % oil, individual performance was further limited, with only 10–18 % degradation in 5 days, 27–35 % in 20 days and 60–75 % after 60 days, eventually requiring up to 80 days to reach 82–96 %. The consortium achieved 26 % in 5 days (1.4–2.6-fold increase), 45 % in 10 days, 66 % in 20 days (1.9–2.4-fold increase) and 90 % in 30 days, demonstrating a consistent 1.5- to 2.5-fold enhancement in degradation rate over the best-performing strain across all stress conditions.



**Fig. 3.** Crude oil biodegradation efficiency of individual bacterial strains and their constructed consortium. (a) Degradation performance under standard laboratory conditions (pH 7.0, no NaCl). (b) Degradation performance under simulated marine stress conditions (pH 8.7, 3.5 % NaCl). Efficiency was tested at 1 %, 3 % and 5 % (w/v) crude oil concentrations. Error bars represent the standard deviation (n=3). Control represents abiotic loss.

Optical density ( $OD_{600}$ ) was monitored as an indirect indicator of microbial growth, but its interpretation in oil-containing media is limited due to interference from oil droplets and cell aggregation. Therefore, degradation efficiency was primarily assessed gravimetrically, with  $OD_{600}$  values provided as supplementary growth trends rather than precise biomass measurements.

## Discussion

The results of this study reveal a robust and environmentally adapted microbial system engineered for enhanced marine oil spill bioremediation. The oil displacement assays established a clear environmental dependence for surface-active compound production across all 5 isolates, with activity strictly confined to alkaline conditions (pH 8–10) and induced under PEG-mediated osmotic

stress. This functional adaptation aligns with the known physiological response of many hydrocarbon-degrading bacteria, which upregulate biosurfactant synthesis under conditions of environmental stress to enhance substrate accessibility (27). Biosurfactants are defined as amphiphilic molecules that significantly reduce surface and interfacial tension, thereby increasing the solubility and bioavailability of hydrophobic compounds. In contrast, bioemulsifiers are high-molecular-weight extracellular polymers that stabilise oil-in-water emulsions but may not substantially lower surface tension (28). It is crucial to maintain this functional distinction, as the 2 classes of molecules play complementary yet mechanistically distinct roles in hydrocarbon degradation.

In this study, we observed that while all strains produced surface-active compounds, their functional profiles diverged. For instance, *Rhizobium* sp. demonstrated strong emulsification

**Table 2.** Growth rate (OD<sub>600</sub>) of 5 isolates and their consortium in crude oil-enriched mineral salt agar medium (MSW) broth over a periodic interval under simulated marine conditions

	1 % oil					3 % oil					5 % oil				
	5 days	10 days	20 days	5 days	10 days	20 days	30 days	40 days	5 days	10 days	20 days	30 days	40 days	60 days	80 days
<i>O. tritici</i>	0.98 ± 0.05 <sup>d</sup>	1.66 ± 0.08 <sup>b</sup>	1.98 ± 0.08 <sup>b</sup>	0.91 ± 0.09 <sup>d</sup>	1.45 ± 0.05 <sup>b</sup>	1.68 ± 0.06 <sup>c</sup>	1.93 ± 0.06 <sup>c</sup>	2.12 ± 0.07 <sup>b</sup>	0.86 ± 0.11 <sup>c</sup>	1.18 ± 0.09 <sup>d</sup>	1.53 ± 0.05 <sup>c</sup>	1.86 ± 0.06 <sup>d</sup>	2.03 ± 0.12 <sup>ab</sup>	2.24 ± 0.05 <sup>b</sup>	2.31 ± 0.09 <sup>a</sup>
<i>A. xylooxidans</i>	1.22 ± 0.06 <sup>c</sup>	1.59 ± 0.09 <sup>c</sup>	1.88 ± 0.09 <sup>c</sup>	1.05 ± 0.08 <sup>c</sup>	1.28 ± 0.06 <sup>d</sup>	1.55 ± 0.09 <sup>d</sup>	1.78 ± 0.08 <sup>d</sup>	1.96 ± 0.08 <sup>c</sup>	0.81 ± 0.09 <sup>c</sup>	1.10 ± 0.10 <sup>d</sup>	1.49 ± 0.04 <sup>c</sup>	1.77 ± 0.07 <sup>e</sup>	1.92 ± 0.13 <sup>c</sup>	2.08 ± 0.09 <sup>c</sup>	2.22 ± 0.12 <sup>ab</sup>
<i>Rhizobium</i> sp.	1.18 ± 0.06 <sup>c</sup>	1.62 ± 0.10 <sup>b</sup>	2.01 ± 0.11 <sup>a</sup>	1.12 ± 0.06 <sup>c</sup>	1.35 ± 0.09 <sup>c</sup>	1.61 ± 0.10 <sup>c</sup>	1.88 ± 0.06 <sup>d</sup>	2.03 ± 0.09 <sup>c</sup>	0.85 ± 0.10 <sup>c</sup>	1.22 ± 0.11 <sup>c</sup>	1.62 ± 0.03 <sup>b</sup>	1.88 ± 0.04 <sup>d</sup>	2.07 ± 0.10 <sup>ab</sup>	2.33 ± 0.08 <sup>a</sup>	2.15 ± 0.13 <sup>b</sup>
<i>N. organovorum</i>	1.26 ± 0.08 <sup>b</sup>	1.44 ± 0.09 <sup>d</sup>	1.92 ± 0.14 <sup>b</sup>	1.21 ± 0.07 <sup>b</sup>	1.31 ± 0.08 <sup>c</sup>	1.72 ± 0.07 <sup>bc</sup>	1.95 ± 0.09 <sup>c</sup>	2.18 ± 0.10 <sup>b</sup>	0.98 ± 0.12 <sup>b</sup>	1.32 ± 0.12 <sup>b</sup>	1.68 ± 0.06 <sup>b</sup>	1.91 ± 0.06 <sup>c</sup>	1.98 ± 0.11 <sup>c</sup>	2.14 ± 0.10 <sup>c</sup>	2.36 ± 0.14 <sup>a</sup>
<i>S. yanoikuyae</i>	1.34 ± 0.07 <sup>b</sup>	1.53 ± 0.10 <sup>c</sup>	2.09 ± 0.15 <sup>a</sup>	1.26 ± 0.08 <sup>b</sup>	1.49 ± 0.06 <sup>b</sup>	1.84 ± 0.08 <sup>b</sup>	2.09 ± 0.10 <sup>b</sup>	2.23 ± 0.09 <sup>a</sup>	1.05 ± 0.13 <sup>b</sup>	1.29 ± 0.09 <sup>b</sup>	1.61 ± 0.06 <sup>b</sup>	2.03 ± 0.06 <sup>b</sup>	2.12 ± 0.09 <sup>a</sup>	2.38 ± 0.08 <sup>a</sup>	2.09 ± 0.11 <sup>b</sup>
Consortium	1.64 ± 0.05 <sup>a</sup>	2.32 ± 0.11 <sup>a</sup>		1.56 ± 0.04 <sup>a</sup>	1.71 ± 0.07 <sup>a</sup>	2.39 ± 0.06 <sup>a</sup>	2.28 ± 0.06 <sup>a</sup>		1.35 ± 0.10 <sup>a</sup>	1.69 ± 0.10 <sup>a</sup>	2.07 ± 0.12 <sup>a</sup>	2.44 ± 0.13 <sup>a</sup>			

Column values followed by different letters are significantly different from each other as determined by Duncan's multiple range test (DMRT) and column values followed by the same letters are not significant at 5 % LSD ( $p < 0.05$ ).

(E24 > 70 %) even at 3 % NaCl yet exhibited only moderate activity in the oil displacement assay, a pattern consistent with bioemulsifier production. Conversely, *O. tritici* displayed high cell surface hydrophobicity (82 %) but comparatively lower emulsification under certain conditions, suggesting a greater reliance on biosurfactant-mediated adhesion and direct interfacial activity. This functional specialisation underscores the importance of precise terminology: where biosurfactants enhance mass transfer by reducing interfacial barriers, bioemulsifiers increase the interfacial area through the formation of stable microdroplets (29, 30). The constructed consortium was therefore designed to incorporate both functional types, creating a system capable of both solubilising (via biosurfactants) and stabilising (via bioemulsifiers) crude oil in aqueous environments, a dual strategy that maximises bioavailability and degradation kinetics (31).

The pH-dependent activity profiles further support the ecological relevance of the isolates. Marine and coastal environments typically exhibit pH values between 7.5 and 8.5, with alkalinity increasing in oil-impacted sediments due to microbial metabolic activity. The peak emulsification and hydrophobicity observed within pH 7–9 indicate that the produced surface-active compounds are functionally attuned to these conditions. The sharp decline in activity at pH 10 suggests a limit to alkaline adaptation, possibly due to denaturation of the proteins or polysaccharides constituting the bioemulsifiers, or inactivation of biosurfactant molecules beyond their structural stability range (19). Salinity tolerance emerged as a critical trait, with sustained or even enhanced E24 and CSH at 2–3 % NaCl for several strains. This is of direct relevance to marine bioremediation, where salinity averages 3.5 % and can fluctuate in coastal zones. The ability of *O. tritici* and *Rhizobium* sp. to maintain high hydrophobicity and emulsification under saline conditions likely involves modifications to cell envelope architecture, such as increased production of hydrophobic outer membrane proteins or changes in lipopolysaccharide composition, which facilitate direct hydrocarbon adhesion, a key mechanism when emulsification is physiologically costly or environmentally constrained (32). This aligns with previous reports on halotolerant hydrocarbon degraders such as *Dietzia* species CN-3, which also demonstrates enhanced cell surface hydrophobicity under saline conditions (3). The use of PEG 6000 to simulate moisture stress requires explicit ecological justification. In real-world marine spill scenarios, osmotic stress arises not only from salt concentration but also from desiccation in tidal and supratidal zones, hydrocarbon-induced water exclusion in sediments and fluctuating hydration in oil-polluted soil matrices (10, 12). PEG 6000, a non-ionic osmolyte, lowers water potential without introducing ionic effects, thereby mimicking the matric stress experienced in low-moisture environments (33). While PEG does not replicate the specific ion–microbe interactions of seawater, it provides a controlled and reproducible means of assessing microbial resilience to low water availability, a common constraint in surface and subsurface oil-contaminated habitats (23). Our observation that emulsification and CSH were maintained under moderate PEG stress (5–10 %) but inhibited under severe stress (20–30 %) indicates a threshold of osmotic tolerance beyond which cellular metabolism or exopolymer synthesis is compromised (33).

A crucial methodological consideration is the use of OD<sub>600</sub> measurements in oil-amended media. Turbidity is a convenient but often misleading indicator of microbial growth in systems containing hydrophobic substrates, as light scattering by oil

droplets, cell–oil aggregates and extracellular polymeric substances can inflate absorbance readings independent of biomass (26). Although OD<sub>600</sub> values provide a comparative growth trend, we explicitly acknowledge this limitation. Degradation efficiency was therefore quantified gravimetrically, a direct and robust measure of hydrocarbon removal. All 5 isolates demonstrated intrinsic crude oil degradation capability, but with markedly different efficiencies and kinetics. Under standard conditions, *S. yanoikuyae* emerged as the most effective degrader, consistent with its high CSH and resilient emulsification across stresses. However, under simulated marine conditions, all individual strains exhibited prolonged lag phases and reduced degradation rates, highlighting the inhibitory effects of combined salinity and alkalinity. Notably, *A. xylosoxidans* and *N. organovorum* were particularly sensitive, with degradation efficiencies declining by more than 40 % under stress, a decrease correlated with their diminished biosurfactant yields in saline-alkaline media.

In stark contrast, the constructed consortium not only maintained high degradation efficiency under marine stress but accelerated the degradation timeline, achieving near-complete oil removal in half the time required by the best individual strain. This synergistic enhancement can be attributed to several interconnected mechanisms: [1] Metabolic cross-feeding, where intermediates generated by one strain serve as substrates for another, preventing the accumulation of inhibitory metabolites; [2] Functional partitioning, whereby different consortium members specialise in degrading different hydrocarbon fractions (alkanes, aromatics, resins), thereby broadening the catabolic spectrum (13); and [3] Complementary surface activity, where biosurfactants from some members increase substrate solubility while bioemulsifiers from others stabilise oil–water interfaces, collectively optimising bioavailability (29). Performance of the consortium under combined saline-alkaline stress is particularly noteworthy when compared to previous bioremediation studies. For instance, natural beach sand communities from the Deepwater Horizon spill required 35–50 days to achieve 65–80 % degradation (13), while halotolerant pure cultures such as *Brevibacterium* sp. achieved 82–89 % degradation of 2 % oil over 14–21 days (30). Our consortium degraded 5 % crude oil to 90 % within 30 days under simulated marine conditions, demonstrating not only higher tolerance to oil concentration but also greater resilience to environmental stressors. This depicts that the consortium is a promising candidate for field applications in coastal and open-ocean environments, where salinity, pH and osmotic fluctuations are major constraints on conventional bioremediation agents.

## Conclusion

This study successfully engineered a highly synergistic bacterial consortium tailored for marine oil spill remediation. The consortium demonstrated enhanced crude oil degradation under simulated marine conditions, significantly outperforming individual isolates. The observed functional synergy, hydrocarbon adhesion, emulsification and metabolic cross-feeding underscore the ecological and practical advantages of designed microbial communities over single-strain approaches. This work provides a scalable and environmentally resilient microbial blueprint for bioremediation in saline, alkaline and osmotically stressed environments. Future research should focus on field validation in

real marine ecosystems, long-term ecological impact assessments and the integration of omics technologies to elucidate consortium stability, metabolic networks and regulatory mechanisms under dynamic environmental conditions.

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## Authors' contributions

SK executed the research work, drafted the manuscript including figures and tables, data analysis and result interpretation. TK supervised the whole research work, provided the methodology and reviewed the manuscript. AB, EK, DU and RS participated in and monitored the research work and reviewed the manuscript. All authors read and approved the final manuscript.

## Compliance with ethical standards

**Conflict of interest:** Authors do not have any conflict of interest to declare.

**Ethical issues:** None

**AI Declaration :** During the preparation of this work, the authors used Chat GPT by Open AI to enhance language clarity and readability. After using this tool/service, the authors reviewed and edited the content as needed and takes full responsibility for the publication's content.

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