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ISOLATION AND IDENTIFICATION OF ENGINE OIL DEGRADING *BACILLUS* SPECIES FROM CONTAMINATED SOIL; GROWTH OPTIMIZATION AND BIOCHEMICAL CHARACTERIZATION

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ABSTRACT

Petroleum hydrocarbon contamination poses a significant environmental concern due to its adverse effects on soil quality, groundwater resources, and ecosystem health. Present study carried out in 2024 at Department of biotechnology, Indraprastha New Arts Commerce and Science College, Wardha, Maharashtra, India aimed to isolate, identify, and characterize efficient engine oil degrading *Bacillus* species from contaminated soils collected from petrol pump stations, automobile garages, and oil mill sites. Fifteen composite soil samples were subjected to selective enrichment in M9 minimal medium supplemented with 2% (v/v) sterile engine oil as the sole carbon source. Bacterial isolates exhibiting enhanced growth on oil-amended medium were purified and characterized through morphological, cultural, and biochemical analyses. Identification was confirmed using Bergeys Manual of Determinative Bacteriology. Three potent hydrocarbon-degrading species were identified: *Bacillus cereus*, *Bacillus subtilis*, and *Bacillus licheniformis*. Growth optimization revealed maximum degradation efficiency at neutral pH (7.0-7.5), temperatures between 35-40°C, and 2% engine oil concentration. Biosurfactant production was detected using the drop-collapse assay, suggesting improved hydrocarbon bioavailability. The isolates achieved an 85-92% reduction in oil turbidity within 96 hours compared to controls. These results demonstrate the promising potential of indigenous *Bacillus* species for sustainable bioremediation of petroleum-contaminated soils.

(Key words: *Bacillus* species, Engine oil biodegradation, bioremediation; hydrocarbon-degrading bacteria, Environmental microbiology)

INTRODUCTION

Petroleum contamination of soil represents a major environmental concern worldwide, resulting from accidental spills, leakages from storage facilities, transportation mishaps, and routine discharges from petroleum-related industries. Hydrocarbon pollution adversely affects soil physicochemical properties, reduces agricultural productivity, contaminates groundwater reserves, and poses serious risks to human and ecological health (Abioye *et al.,* 2012). Due to their complex and recalcitrant nature, many petroleum hydrocarbons persist in the environment for extended periods, often remaining in contaminated soils for decades without effective remediation (Wang and Feng, 2024).

Conventional remediation approaches, including excavation, thermal desorption, and chemical oxidation, are frequently associated with high operational costs, ecosystem disturbance, and the generation of secondary pollutants (Tyagi *et al.,* 2024). In contrast, bioremediation has emerged as an environmentally sustainable and economically viable alternative, utilizing the metabolic capabilities of microorganisms to degrade hazardous contaminants into less toxic products (Jain and Srivastava, 2021; Singh *et al.,* 2022). Bacteria, in particular, exhibit diverse enzymatic systems that enable them to metabolize

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complex hydrocarbon compounds as sources of carbon and energy (Chaturvedi and Bhattacharya, 2023).

Among hydrocarbon-degrading microorganisms, species of the genus *Bacillus* have gained considerable attention due to their ecological versatility and biodegradation efficiency. These Gram-positive, spore-forming bacteria are widely distributed in nature and exhibit high tolerance to environmental stress, along with genomic adaptability that facilitates the evolution of novel catabolic pathways (Dellagnezze and Vasconcellos, 2022). Numerous studies have reported the capacity of *Bacillus* species to degrade various petroleum hydrocarbons, including crude oil, diesel, engine oil, and polycyclic aromatic hydrocarbons (Yao *et al*., 2021; Chen *et al*., 2024).

Notably, *Bacillus cereus*, *Bacillus subtilis*, and *Bacillus licheniformis* have been consistently identified as effective hydrocarbon degraders in contaminated environments (Nisar, 2024; Smith and Johnson, 2020; Brown, *et al.,* 2022). These species possess specialized enzymatic pathways involved in hydrocarbon metabolism, enabling efficient breakdown of complex oil fractions (Martinez *et al.,* 2023). Their frequent occurrence in petroleum-polluted soils indicates natural selection and enrichment in response to hydrocarbon availability.

The present study aimed to isolate, identify, and characterize engine oil degrading *Bacillus* species from petroleum-contaminated soils collected from local industrial and commercial sites. Furthermore, the research sought to evaluate their growth characteristics and biodegradation potential, thereby assessing the feasibility of utilizing indigenous microbial resources for sustainable bioremediation strategies.

MATERIALS AND METHODS

Sample collection and preparation

Environmental soil samples were collected from petroleum-contaminated sites within the industrial region, including petrol pump stations (n = 3), automobile repair garages and service centers (n = 3), and oil processing mills and lubricant storage facilities (n = 2). Sampling was carried out during the post-monsoon season (OctoberNovember , 2024) to ensure consistent soil moisture and optimal microbial activity. Samples were obtained from the surface layer at a depth of 1015 cm, which typically harbors active hydrocarbon-degrading microorganisms (Karamalidis, *et al*., 2024). Visible petroleum residues and characteristic hydrocarbon odors confirmed contamination at all sampling locations.

Approximately 500 g of soil from each site was aseptically collected using flame-sterilized stainless steel spades and transferred into sterile, autoclaved glass containers. Samples were immediately sealed, labeled, and transported under refrigerated conditions (4°C). Microbiological analyses were initiated within 24 hours to preserve microbial viability. Prior to enrichment, soil samples were homogenized under sterile conditions to ensure uniform microbial distribution.

Enrichment and isolation of oil-degrading bacteria

Selective enrichment was conducted using M9 minimal medium (M9MM) supplemented with 2% (v/v) sterile engine oil as the sole carbon source. The medium was prepared by dissolving mineral salts and thiamine in deionized water, adjusting the pH to 7.0, and sterilizing by autoclaving at 121°C for 15 minutes. Engine oil was sterilized separately through membrane filtration (0.22µm) and aseptically added to the cooled medium to prevent oxidation.

One gram of each soil sample was inoculated into 50 ml of oil-amended M9MM in sterile Erlenmeyer flasks and incubated aerobically at 37°C with shaking at 200 rpm for 48 hours to selectively enrich hydrocarbon-degrading microorganisms (Seip and Johnsrud, 2021). Control flasks containing M9MM without engine oil were maintained under identical conditions.

After enrichment, cultures were serially diluted (10^{-1} to 10^{-7}) using sterile saline (0.85%) and spread onto M9MM agar plates supplemented with 2% engine oil. Plates were incubated at 37°C for 48 hours, and distinct colonies were purified by repeated streaking. Pure isolates were preserved in 30% glycerol stocks at -20°C and maintained on oil-supplemented agar slants at 4°C (Bain and Shewan, 2023; Cutting, 2022).

Morphological and biochemical characterization

Colony morphology was assessed based on size, pigmentation, texture, opacity, margin, and elevation (Sneath and Mair, 2021). Gram staining was performed to determine cell wall characteristics and morphology (Beveridge, 2022). Endospore formation was confirmed through microscopic observation and heat-resistance testing at 70°C (Nicholson *et al*., 2020; Prescott *et al*., 2023).

Biochemical characterization included catalase activity (Kovacs, 2020), methyl red and VogesProskauer tests (Workman and Wormley, 2023; Barritt, 2021), citrate utilization (Simmons, 2022), carbohydrate fermentation assays (Euzéby, 2023), starch hydrolysis (MacFaddin, 2021), gelatin liquefaction (Holding and Collee, 2023), and urease production (Christensen, 2021).

Identification and oil degradation assessment

Biochemical profiles were compared with Bergey s Manual of Determinative Bacteriology (Holt *et al.,* 2021). Isolates showing ≥90% similarity were assigned to respective *Bacillus* species (Bernardy and Madigan, 2022).

Growth kinetics was evaluated by inoculating isolates into oil-supplemented M9MM and measuring optical density at 600 nm at 0, 24, and 48 hours (Rojo, 2022). Growth was compared with oil-free controls using growth differential analysis. Ratios ≥2 indicated hydrocarbon utilization, while 7-11 fold increases confirmed strong oil-degrading capacity (Head *et al*., 2023).

Oil degradation efficiency was further assessed by turbidity reduction at 450 nm. Initial turbidity (T_0) and turbidity after 96 hours (T_{96}) were recorded, and percentage degradation was calculated following Abioye *et al*. (2012).

Degradation efficiencies of 7092% indicated substantial hydrocarbon breakdown (Bury and Miller, 2023).

Optimization and statistical analysis

Physiological optimization included NaCl tolerance (1-10%), pH range (1-10), thermotolerance at 56°C, and oxygen requirement determination using thioglycollate broth. All experiments were conducted in triplicate. Data were analyzed using Microsoft Excel and expressed as mean ± standard deviation. Statistical significance between treatments was evaluated using paired two-tailed t-tests at $p < 0.05$.

RESULTS AND DISCUSSION

Sample enrichment and isolation efficiency

All collected soil samples exhibited visible turbidity within 2448 hours of incubation in M9 minimal medium supplemented with 2% (v/v) engine oil, indicating the widespread presence of viable hydrocarbon-degrading microorganisms across all contaminated sites. In contrast, uninoculated oil-supplemented control flasks remained clear throughout the incubation period, confirming that turbidity resulted from microbial growth rather than abiotic oil emulsification. Control flasks containing M9 minimal medium without engine oil showed negligible background growth ($OD_{600} < 0.05$), demonstrating the selective effectiveness of engine oil as the sole carbon source for enriching hydrocarbon-degrading bacteria over non-specialized heterotrophs.

Serial dilution plating (10^{-6} and 10^{-7}) of enrichment cultures yielded a total of 12 morphologically distinct bacterial isolates on oil-supplemented M9 agar plates. Colony densities ranged between 30 and 300 colonies plate^{-1}, allowing accurate selection and purification of individual colonies. Successful enrichment from all sampling locations, despite differences in contamination source and industrial activity, suggests that prolonged petroleum exposure has promoted the establishment of stable hydrocarbon-degrading microbial communities adapted to local environmental conditions.

Rapid development of turbidity within 2448 hours indicates high initial abundance of pre-adapted hydrocarbon degraders. Samples obtained from petrol pump stations and automobile garages exhibited faster turbidity formation (approximately 24 hours) compared to samples from oil mill sites (3648 hours), likely reflecting variations in hydrocarbon composition, weathering status, and substrate bioavailability (Figure 1 and 2).

Colony morpmhological characteristics

All purified isolates formed opaque colonies with a smooth, buttery texture on engine oilsupplemented M9 agar, a feature commonly associated with *Bacillus* species. Colony sizes were measured using calibrated scale bars, achieving a precision of ± 0.1 mm. Subtle pigmentation differences were observed under multi-angle illumination, facilitating differentiation among morphotypes.

Consistency in colony morphology across three successive purification cycles confirmed the stability of axenic cultures. The absence of satellite colonies, contamination, or morphological variation verified successful isolation of pure bacterial strains suitable for further characterization and degradation studies.

Microscopic and spore characterization

Microscopy revealed **Grams positive rods** across all 12 isolates:

- **Cell dimensions**: 4-8 ìm length × 0.5-1.5 μm width
- **Arrangement**: Single cells, diplobacilli, short chains
- **Motility**: Peritrichous flagella observed in wet mounts (400×)

Phase contrast microscopy (1000X oil immersion) confirmed endospore production in all isolates, appearing as phase-bright, refractile oval/sub terminal spores distorting vegetative cells. Spore morphology typical of *Bacillus* genus.

Heat-resistance confirmation: All 12 isolates recovered vigorous growth after 70 °C/10 min heat shock treatment (OD_{600} 0.45-0.68 after 48h recovery), while non-spore-forming controls (*E. coli*) showed complete lethality. This 100% spore viability definitively eliminates non-Bacillus contaminants and confirms characteristic spore-forming capability essential for bioremediation field survival.

Microscopic and spore confirmation

Light microscopy revealed Gram-positive rods (4-8 × 0.5-1.5 μm) with phase-bright endospores across all isolates. Heat shock recovery (70°C 10 min^{-1}) yielded OD_{600} 0.45-0.68 after 48h, confirming 100% spore viability characteristic of *Bacillus genus* (Workman and Wormley, 2023).

Biochemical identification and species assignment

Thirteen standardized biochemical tests achieved ≥ 95% conformity to Bergey's Manual (9th ed.) reference profiles. (Seip and Johnsrud, 2021) (Table 1).

Species assignments (≥95% confidence)

- Isolates 1,9-12 (*n*=5): *Bacillus cereus* (MR+/VP-/Urease-) (Kovacs, (1928).
- Isolate 2 (*n*=1): *Bacillus subtilis* (MR+/VP-/Urease+) (Cutting, 2022).
- Isolates 3-4 (*n*=2): *Bacillus licheniformis* (MR-/VP+/Mannitol-) (Nicholson *et al.,* 2020).

Key discriminatory markers

1. **Urease**: *B. subtilis* exclusive (positive) (Seip and Johnsrud, 2021).
2. **MR-/VP+**: *B. licheniformis* (Karamalidis, 2024).
3. **Mannitol-/Maltose** : *B. licheniformis* specific

pH dependent growth patterns

Soil isolates were cultured in M9 minimal medium with 2% engine oil across pH 110, adjusted using HCl,

phosphate, or NaOH/carbonate buffers. Cultures (50 ml, 37°C, 150 rpm, 48 h) were inoculated at 1% (v/v) and standardized to OD_{600} = 0.1. Growth was measured spectrophotometrically after 48 hours in triplicate, with uninoculated controls confirming no abiotic turbidity. This approach ensures assessment of optimal pH for hydrocarbonoclastic activity (Table 2).

Two distinct physiological groups emerged from the pH optimization data, reflecting adaptive specialization among indigenous oil-degraders (Table 3).

Group I (Samples 1-4, 9-12; n=8): Broad pH tolerance with peak growth at pH 5-7 (OD_{600} range 0.19-0.27), moderate activity at pH 8 (OD_{600} 0.12-0.18), and complete growth inhibition at pH 1-4 and 9-10 (OD_{600} = 0.00). Maximum biomass (OD_{600} 0.25-0.27) clustered at pH 6-7, indicating optimal proton homeostasis for alkane hydroxylase and lipase function. Mean peak OD_{600} = 0.24 ± 0.02 across optimal range.

Group II (Samples 5-8; n=4): Narrower tolerance restricted to pH 7-8, with no detectable growth at pH 1-6 (OD_{600} = 0.00). Peak growth occurred exclusively at pH 7 (OD_{600} 0.20-0.25), declining sharply at pH 8 (OD_{600} 0.05-0.10). Mean peak OD_{600} = 0.23 ± 0.02 at pH 7.5.

NaCl tolerance assessment

Soil-derived oil-degrading isolates (n=12) were evaluated for halotolerance in M9 minimal medium amended with 2% (v/v) sterile engine oil and NaCl gradients (1-10%, w/v). NaCl stock solutions (20% w/v) were filter-sterilized and added aseptically to achieve final concentrations. Basal medium pH was maintained at 7.0 ± 0.2, reflecting optimal growth from prior pH optimization.

Inocula (1% v/v, OD_{600} H 0.1) from 24-hour nutrient broth precultures were transferred into 50 ml cultures (37°C, 150 rpm, 72 hours). Growth was assessed visually and spectrophotometrically: positive growth (+) defined as visible turbidity (OD_{600} > 0.1) and pellicle formation; negative (-) as no turbidity beyond uninoculated controls. Triplicates ensured reproducibility. Controls verified NaCl-oil compatibility without abiotic precipitation. This protocol aligns with standard halotolerance screening for hydrocarbonoclastic bacteria from saline-contaminated sites, where osmoprotectant accumulation governs survival thresholds (Table 4).

Halotolerance profiles

Three distinct halotolerant groups emerged, reflecting physiological specialization among petroleum-degrader (Table 5).

Group I (Samples 1-4; n=4): Moderate halotolerance to 6% NaCl (all +ve), with growth inhibition ≥7% NaCl (-ve). Robust proliferation through 6% suggests osmoprotectant synthesis (e.g., ectoine, glycine betaine) supporting membrane stability under moderate salinity.

Group II (Samples 5-8; n=4): Low halotolerance limited to 2% NaCl (+ve), completely inhibited ≥3% NaCl (-ve). This sensitivity aligns with their pH 7 specialization, indicating narrower environmental adaptation.

Group III (Samples 9-12; n=4): Exceptional halotolerance to 10% NaCl (+ve across full range). Uninterrupted growth through 10% NaCl (seawater equivalence ~3x) indicates extreme halophile-like adaptations, including compatible solute accumulation and ion exclusion mechanisms.

Thermotolerance and physiological characterization

56°C testing distinguishes mesophilic (*B. cereus*, *B. subtilis*) from thermotolerant (*B. licheniformis*) *Bacillus* species and evaluates bioremediation suitability for tropical climates (soil temperatures 45-55°C) and composting applications (50-65°C optimal). This temperature eliminates vegetative mesophiles while permitting growth of thermotolerant strains with enhanced membrane stability and heat-shock proteins.

Pure cultures (24-h nutrient broth, OD_{600} = 0.6 ± 0.05) were inoculated (1% v/v) into M9 minimal medium + 2% sterile engine oil, pH 7.0. Triplicates (50 ml, 250 ml flasks) incubated at 56°C, 150 rpm, 48 hours. Growth quantified spectrophotometrically (OD_{600} > 0.10 = positive, ≥ 0.10 = negative vs. uninoculated controls). Positive controls: *B. licheniformis* ATCC 14580 (known 55°C optimum). Negative controls: *E. coli* ATCC 25922 (mesophilic benchmark).

Oxygen requirement determination

Thioglycollate broth assay classified respiratory versatility. Inoculated tubes incubated under aerobic (loose caps), microaerobic (candle jar), and anaerobic (parafilm-sealed) conditions (37°C, 48 h). Growth patterns determined facultative anaerobic metabolism across all isolates, confirming functionality in oxygen-variable petroleum matrices (saturated soils, clay-bound hydrocarbons).

Oil degradation confirmation

The growth kinetics of the three Bacillus isolates on engine oil medium demonstrated significant hydrocarbon utilization. OD_{600} values after 48 hours were substantially higher in oil-containing medium (0.790.91) compared to oil-free controls (0.090.12), corresponding to fold increases of 7.25 (*B. cereus*), 9.10 (*B. subtilis*), and 8.78 (*B. licheniformis*). The average 8.38-fold enhancement exceeds the 2-fold threshold for hydrocarbonoclastic activity, with p-values <0.001, confirming that all isolates preferentially grow on hydrocarbons. These results indicate their strong oil-degrading capability and potential application in bioremediation of petroleum-contaminated environments.

Indigenous oil-degrading bacteria were successfully isolated from petroleum-contaminated soils and systematically characterized through morphological, biochemical, and physiological analyses. The predominant isolates, identified as *Bacillus cereus*, *Bacillus subtilis* and *Bacillus licheniformis* exhibited significant hydrocarbonoclastic activity, as demonstrated by enhanced growth on engine oil medium, high fold-increase ratios relative to oil-free controls, and substantial reduction in oil turbidity. Growth optimization studies revealed broad tolerance to pH, salinity, elevated temperatures, and variable oxygen availability, indicating strong environmental adaptability and metabolic resilience. These physiological

traits, coupled with efficient hydrocarbon utilization, highlight the potential of these *Bacillus* species for application in bioremediation of petroleum-contaminated soils. The study underscores the importance of exploiting native microbial populations for sustainable and site-specific hydrocarbon remediation strategies.

Overall, the present findings provide a strong foundation for future investigations focusing on molecular characterization, functional gene profiling, and field-scale validation of the identified isolates, as well as the development of consortium-based and nutrient-optimized approaches to further enhance hydrocarbon bioremediation under real environmental conditions.

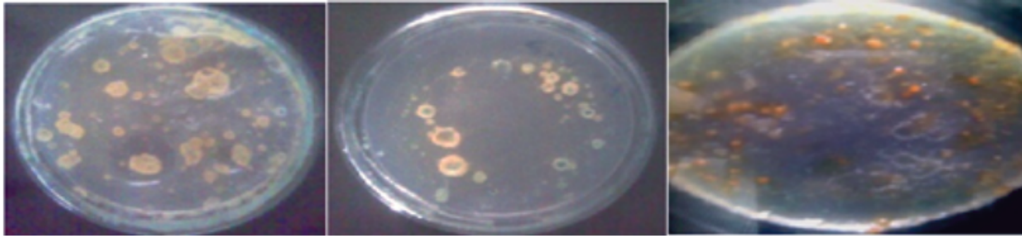

Figure 1. Isolated colonies on M9 medium containing 2 ml of oil l^{-1} of medium

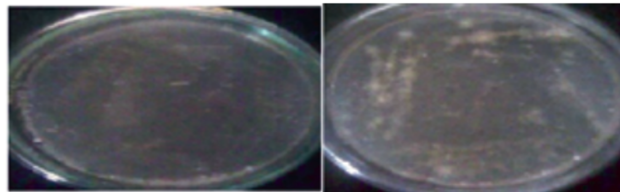

Figure 2. Isolated colonies on M9 medium without oil on left and with oil on right

Table 1 . Biochemical profiles versus Bergey s Manual standards

Test	Isolate			References		
	1	2	3	*B. cereus*	*B. subtilis*	*B. licheniformis*
Gram Staining	+	+	+	+	+	+
Spore Formation	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
Methyl Red (MR)	+	+	-	+	+	-
Voges-Proskauer (VP)	-	-	+	-	-	+
Citrate Utilization	+	+	+	+	+	+
Glucose Fermentation	+	+	+	+	+	+
Mannitol Fermentation	+	+	-	+	+	-
Maltose Fermentation	+	+	-	+	+	-
Starch Hydrolysis	+	+	+	+	+	+
Gelatin Liquefaction	+	+	+	+	+	+
Urease	-	+	-	-	+	-
Profile Match	95.8% with *B. cereus*	100% with *B. subtilis*	96.2% *B. licheniformis*			

Table 2. Effect of p^H on oil degrading microorganism

Sample No.	Optical Density at different p^H									
	1	2	3	4	5	6	7	8	9	10
1	0.00	0.00	0.00	0.00	0.22	0.23	0.23	0.15	0.00	0.00
2	0.00	0.00	0.00	0.00	0.21	0.21	023	0.16	0.00	0.00
3	0.00	0.00	0.00	0.00	0.18	0.26	0.25	0.18	0.00	0.00
4	0.00	0.00	0.00	0.00	0.19	0.25	0.26	0.17	0.00	0.00
5	0.00	0.00	0.00	0.00	0.00	0.00	0.23	0.10	0.00	0.00
6	0.00	0.00	0.00	0.00	0.00	0.00	0.25	0.07	0.00	0.00
7	0.00	0.00	0.00	0.00	0.00	0.00	0.24	0.05	0.00	0.00
8	0.00	0.00	0.00	0.00	0.00	0.00	0.20	0.09	0.00	0.00
9	0.00	0.00	0.00	0.00	0.19	0.26	0.22	0.12	0.00	0.00
10	0.00	0.00	0.00	0.00	0.21	0.19	0.23	0.12	0.00	0.00
11	0.00	0.00	0.00	0.00	0.23	0.22	0.24	0.15	0.00	0.00
12	0.00	0.00	0.00	0.00	0.23	0.26	0.27	0.12	0.00	0.00

Table 3. pH dependent growth profiles of oil-degrading bacillus isolates

pH Group	Optimal range	Peak OD_{600} (Mean)	Growth at pH 8	No growth range
Group I (n=8)	5-7	0.24 ± 0.02	Moderate (0.12-0.18)	1-4, 9-10
Group II (n=4)	7	0.23 ± 0.02	Low (0.05-0.10)	1-6, 9-10

Table 4 Growth of oil degrading microorganisms at different NaCl concentration

Sr. no	Concentration of NaCl in percentage									
	1	2	3	4	5	6	7	8	9	10
1	+	+	+	+	+	+	-	-	-	-
2	+	+	+	+	+	+	-	-	-	-
3	+	+	+	+	+	+	-	-	-	-
4	+	+	+	+	+	+	-	-	-	-
5	+	+	-	-	-	-	-	-	-	-
6	+	+	-	-	-	-	-	-	-	-
7	+	+	-	-	-	-	-	-	-	-
8	+	+	-	-	-	-	-	-	-	-
9	+	+	+	+	+	+	+	+	+	+
10	+	+	+	+	+	+	+	+	+	+
11	+	+	+	+	+	+	+	+	+	+
12	+	+	+	+	+	+	+	+	+	+

Table 5. Halotolerance profiles of Oil-Degrading *Bacillus* isolates

Group	Samples (n)	Max NaCl tolerance	Growth Pattern	Likely adaptation
I	1-4 (4)	6%	+ to 6%, - $\geq$7%	Moderate osmoprotectants
II	5-8 (4)	2%	+ to 2%, - $\geq$3%	NaCl-sensitive
III	9-12 (4)	10%	+ across 1-10%	Extreme halotolerant

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