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BIOREMEDIATION OF SPENT MOTOR OIL BY INDIGENOUS *PSEUDOMONAS* SPECIES

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ABSTRACT

Motor oil contains 80-90% of hydrocarbons, the most significant pollutant due to its recalcitrance and toxicology. The spent motor oils generated by mechanical workshops are released into the environment and become a threat to the soil and drinking water ecosystem. Bioremediation is one of the most promising technologies for removing environmental pollutants. This research aims to isolate the *Pseudomonas* species from spent motor oil-contaminated soil and screen its biodegradation capability. The degradation study was conducted in a Bushnell Hass Mineral Salts (BHMS) medium characterized by hydrocarbon and salt concentrations, surfactants, and carbon and nitrogen sources. The gravimetric analysis was used to calculate the degradation percentage. The two isolated *Pseudomonas* species ATK-01 and ATK-02 showed significant degradation of spent motor oil that is 93.96% and 89.65% by consuming 5% hydrocarbon, 2M salt concentration, 1.5% Tween 80, and 0.5% maltose and tryptone which exhibited great potential for the bioremediation of spent motor oil in minimum duration and will become the best remediation alternative.

Keywords: Hydrocarbons, Bioremediation, Environmental pollutants, *Pseudomonas* species, Degradation

INTRODUCTION

A thick mineral oil liquid applied in the engine or machine to reduce the friction between the moveable parts of the appliance is known as "Motor Oil" (Makut *et al.*, 2022). Motor oils can be used in vehicles, generators, and engine bicycles (Makut *et al.*, 2022; Okebalama *et al.*, 2024). They can be transformed during use due to the breakdown of additives, contamination with ignition products, and the accumulation of metals. It is considered as exploited greasing-up oils debarred from the crankcase of inner ignition motors. It comprises 80-90% of hydrocarbons and 10-20% of added substances by volume to enhance their performance (Makut *et al.*, 2022). These hydrocarbons contain oxygen, nitrogen, sulfur, alkanes, and aromatic compounds. Polycyclic aromatic hydrocarbons (PAH) are the most significant pollutant since they are recalcitrant, toxic, mutagenic (Sánchez Mata *et al.*, 2023), and carcinogenic (Nwachukwu and Anegbode, 2024; Sánchez Mata *et al.*, 2023). High concentrations of PAH can cause damage to bone marrow and diseases of the kidneys and liver after prolonged exposure (Ekanem *et al.*, 2017).

Day-to-day, a massive amount of used/spent motor oils are generated by mechanical workshops from changing the oils of automobiles and different engines (Makut *et al.*, 2022). Gallons of consumed motor oil are produced and released into the environment without treatment, threatening the soil and drinking water ecosystem due to toxic compounds (Ajiboye *et al.*, 2020). Yearly, 80,000 tons of used motor oils are processed in Pakistan for various applications (Naeem *et al.*, 2023). Bioremediation is one of the most promising technologies for removing environmentally harmful contaminants into harmless end products (Soumeya *et al.*, 2022) without any laborious, expensive mechanical and chemical interventions. This process can be executed by in-situ or ex-situ bioremediation (Sánchez Mata *et al.*, 2023). Globally, biostimulation and bioaugmentation are the (Soumeya *et al.*, 2022) most common in-situ or ex-situ methods for bioremediation of oil-contaminated soil (Sánchez Mata *et al.*, 2023; Soumeya *et al.*, 2022).

Microbial degradation and transformation are some of the finest approaches to eliminating the hydrocarbons from the polluted environment. Soil is the habitat of hydrocarbon-degrading microbes, which can break down complex molecules by adapting their degradative enzyme system (Ayandele, 2018). Many organisms were reported for the bioremediation of spent motor oils in consortium or pure cultures (Soumeya *et al.*, 2022). Bacteria have a higher biodegradation ability than other organisms, including *Ochrobactrum pseudintermedium*, *Bacillus licheniformis*, *Streptomyces ginkgonis*, *Acinetobacter* spp, *Pseudomonas* spp, and *Pseudoalteromonas* spp (Ajiboye *et al.*, 2020). In the last six years, the reported bacteria for the biodegradation of used motor oil include *Stenotrophomonas maltophilia* strain 5DMD (Larik *et al.*, 2019), *Staphylococcus hominis*, *Staphylococcus* sp., *Bacillus flexus*, *Bacillus oceanisediminis* (Javed *et al.*, 2022), *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Enterobacter* sp. (Hossain *et al.*, 2022). This research aimed to isolate the *Pseudomonas* species from spent motor oil-contaminated soil and screen its biodegradation capability.

MATERIALS AND METHODS

Samples Collection

Spent motor oil was collected from the LONCIN LC500D-A series generator after one week of combustion. The used motor oil-contaminated soil samples were collected from the adjacent local automobile workshop (near NIPA) in Karachi, Pakistan. These samples were collected in sterile conditions and transferred to the laboratory for further processing. The soil sample was stored at 4^0C to prevent the loss of moisture.

Isolation and Identification of Bacteria

0.5g of consumed motor oil-contaminated soil was added into 50 mL of Bushnell Hass Mineral Salts (BHMS) medium (Composition: $MgSO_4.7H_2O$: 0.200 g/L, $CaCl_2$: 0.020 g/L, KH_2PO_4: 1.00 g/L, NH_4NO_3: 1.00 g/L, $FeCl_3$: 0.050 g/L, pH 7.0$\pm$0.2) with 4% of spent motor oil (2 mL) as a carbon source. Incubated at 37^0C for one week for culture enrichment and observed daily as reported in Hussein (2018) with a slight alteration. After that, the serial dilutions were prepared in 10^{-1} to 10^{-5} tubes by taking 900µL of distilled water as a diluent. 100µL from 10^{-4} and 10^{-5} tubes were spread onto nutrient agar plates and incubated at 37^0C for 24 hours. The obtained colonies were further sub-cultured based on colony morphology to get pure representative isolates. Maintained the isolates at 4^0C and preserved them in 80% glycerol at -20^0C. Cultural characteristics of the isolates were observed on nutrient agar plates, the morphology of the bacteria was observed by gram staining, and biochemical characteristics were followed by performing catalase, indole, citrate, urease, and gelatin hydrolysis tests as described in Bergey's Manual of Determinative Bacteriology, 9th edition (Holt *et al.*, 1994).

Bioremediation of Spent Motor Oil by Pseudomonas species

The isolated strains designated ATK-01 and ATK-02 were subcultured and used for the bioremediation of spent motor oil. Selected colonies were dispensed into 100 mL of BHMS medium containing 4% of spent motor oil in separate flasks. The flasks were incubated at 35-37^0C and 120 rpm under shaking conditions for one week. The sample's optical density (OD) at 600 nm was taken regularly. It was used as a parameter to determine the degradation potential of the bacteria followed by the protocol of Younus *et al.* (2020) with trivial modifications.

Effects of Hydrocarbon Concentrations on Biodegradation of Spent Motor Oil

BHMS medium was supplemented with different motor oil concentrations of 1-5% with an increment of 1 unit. The isolated strains were inoculated into the medium. The flasks were incubated at 37^0C and 120 rpm under shaking conditions for 96 hours. The uninoculated medium was taken as a control. The OD at 600 nm of the samples was taken at regular intervals.

Effects of Salt Concentrations on Biodegradation of Spent Motor Oil

BHMS medium was supplemented with 5% consumed motor oil and different salt concentrations of 0.1 M, 0.5 M, 1.0 M, and 2.0 M. The isolated strains were inoculated into the medium and incubated at 37^0C and 120 rpm in a shaking incubator for 96 hours. The uninoculated medium was taken as a control. The absorbance of the samples was taken at 600 nm at regular intervals.

Effects of Surfactants on Biodegradation of Spent Motor Oil

25 mL BHMS medium containing 5% consumed motor oil and different concentrations of 0.5-2.5% of Sodium dodecyl sulfate (SDS), and Tween 80 with an increment of 0.5 unit was taken in 10 flasks. The isolates were inoculated into the flasks and incubated at 37^0C and 120 rpm under shaking conditions for 96 hours. The uninoculated medium was taken as a control. The OD at 600 nm of the samples was taken at regular intervals.

Effects of Carbon and Nitrogen Sources on Biodegradation of Spent Motor Oil

25 mL BHMS medium was added with 5% consumed motor oil, and 0.5% of each carbon and nitrogen source, maltose, dextrose, tryptone, and yeast extract were taken. The isolates were inoculated into the flasks and incubated at 37^0C and 120 rpm in a shaking incubator for 96 hours. The uninoculated medium was taken as a control. The absorbance of the samples was taken at 600 nm at regular intervals.

Study of Degradation

The above-optimized parameters were used to analyze the degradation of spent motor oil. 100 mL of the BHMS medium containing 5% consumed motor oil, 2M NaCl, 1.5% Tween 80, 0.5% maltose, and tryptone were taken in two flasks. Each isolate was inoculated into a separate flask and incubated at 37^0C and 120 rpm under shaking

conditions for 15 days. The uninoculated medium was taken as a control to observe the abiotic losses of the oil substrate.

Extraction of Residual Motor Oil

Liquid-liquid extraction (LLC) was used to estimate the rate of oil degradation by separating compounds into two immiscible liquids based on their relative solubilities. Then, the broths were centrifuged at 10,000 rpm for 10 minutes at room temperature. The pH of the supernatant was adjusted to 1 by 1N HCL. The residual oil in the supernatant was extracted using a 1:2 ratio of hexane in a pre-weighed flask. This step was repeated thrice to ensure a complete extraction procedure as testified by Salam (2016) with minor changes.

Gravimetric Analysis

After extraction, the beaker containing the residual oil was kept in a hot air oven at 55-75^{0}C to evaporate the organic layer of hexane and then cooled down at room temperature. The dried powder was weighed and stored at 4^{0}C in sterile Eppendorf. The following formulas calculated the percentage of degraded spent oil as stated by Collins *et al.* (2018) with insignificant variations.

Weight of the residual motor oil = Weight of beaker containing extracted motor oil - Weight of empty beaker.
Amount of motor oil degraded = Weight of residual motor oil added in the medium - Weight of residual motor oil.
Degradation % = Amount of motor oil degraded /Amount of motor oil added in the medium x 100.

RESULTS

Cultural, Morphological, and Biochemical Identification of Bacteria

The cultural identification of the designated ATK-01 and ATK-02 isolates was observed on nutrient agar plates incubated at 37^{0}C for 24 hours. The results of the cultural, morphological, and biochemical identification of the bacteria are shown in Table 1.

Table 1. Cultural, Morphological, and Biochemical Characterization of Bacteria.

Characteristics	**Bacterial isolates**	
	ATK-01	**ATK-02**
Cultural Characteristics		
Shape	Circular	Circular
Surface	Smooth	Smooth
Elevation	Convex	Convex
Margin	Entire	Entire
Color	Blue-green	Blue-green
Morphological Characteristics		
Gram reaction	-	-
Cell shape	Rods	Rods
Biochemical Characterization		
Catalase	+	+
Indole	-	-
Citrate	+	+
Urease	-	-
Gelatin hydrolysis	+	+
Tentative Identification	*Pseudomonas* spp.	*Pseudomonas* spp.

+ = Positive, - = Negative

Effects of Hydrocarbon Concentrations on Biodegradation of Spent Motor Oil

The effect of hydrocarbon degradation was evaluated by using a BHMS medium supplemented with 1-5% motor oil concentrations as a sole source of carbon. The maximum degradation was observed in 5% hydrocarbon concentration in 72 hours of incubation with the optical density of 0.75 in ATK-01 and 0.68 in ATK-02 **(Fig. 1)**. These results showed that motor oil could be used as a good carbon source for the growth of bacteria at high hydrocarbon concentrations.

Effects of Salt Concentrations on Biodegradation of Spent Motor Oil

The impact of salt concentration was studied on BHMS medium supplemented with 5% consumed motor oil and different salt concentrations of 0.1 M, 0.5 M, 1.0 M, and 2.0 M. The maximum degradation was observed in 2M

salt concentration in 48 hours of incubation with the optical density of 0.35 in ATK-01 and 0.32 in ATK-02 **(Fig. 2)**. These results showed that hydrocarbon degradation increases with the increase in salinity, and the bioremediation of oil in high salinity gradient areas like intertidal land is possible.

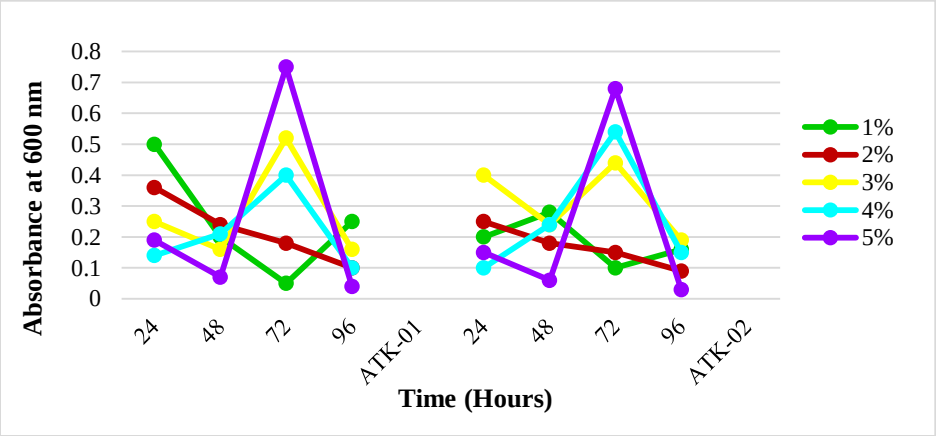

Fig. 1. Effects of Hydrocarbon Concentrations on Biodegradation of Spent Motor Oil

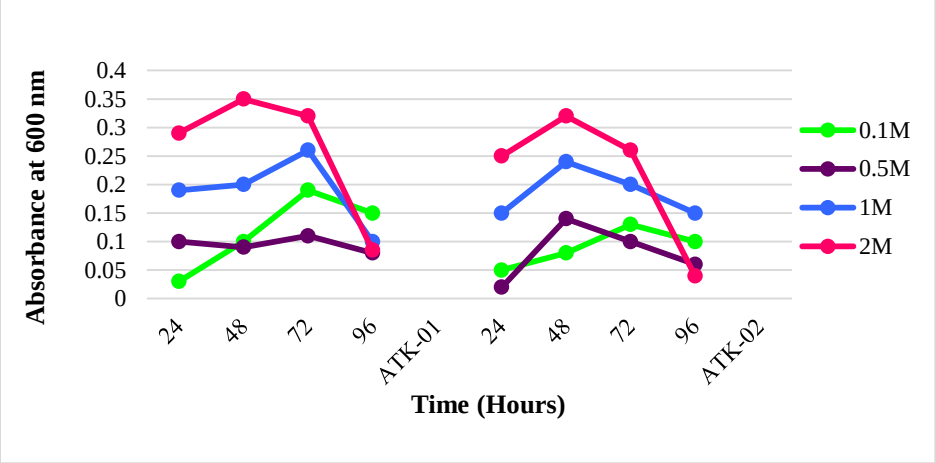

Fig. 2. Effects of Salt Concentrations on Biodegradation of Spent Motor Oil

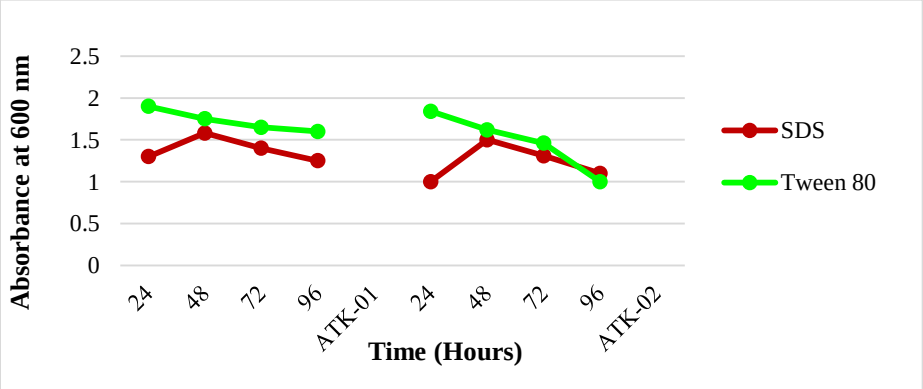

Fig. 3. Effects of Surfactants on Biodegradation of Spent Motor Oil

Effects of Surfactants on Biodegradation of Spent Motor Oil

The impact of surfactants on biodegradation was observed by taking a BHMS medium containing 5% consumed motor oil and different concentrations of 0.5-2.5% of SDS and Tween 80. The maximum degradation was

observed in a 1.5% concentration of Tween 80 in 24 hours of incubation with an optical density of 1.9 in ATK-01 and 1.84 in ATK-02 **(Fig. 3)**. These results exhibited that Tween 80, as a non-ionic surfactant, increases the degradation of motor oil.

Effects of Carbon and Nitrogen Sources on Biodegradation of Spent Motor Oil

The influence of carbon and nitrogen sources was determined by consuming BHMS medium with 5% consumed motor oil with 0.5% of each carbon and nitrogen source; maltose, dextrose, tryptone, and yeast extract were taken. The highest degradation potential of the carbon source was detected in maltose in 96 hours of incubation with an optical density of 2.1 in ATK-01 and 2.0 in ATK-02 **(Fig. 4)**. In contrast, the maximum degradation potential of the nitrogen source was detected in tryptone in 72 hours of incubation with an optical density of 2.2 in ATK-01 and 1.9 in ATK-02 **(Fig. 5)**. These results showed that adding supplementary carbon and nitrogen sources increases biodegradation.

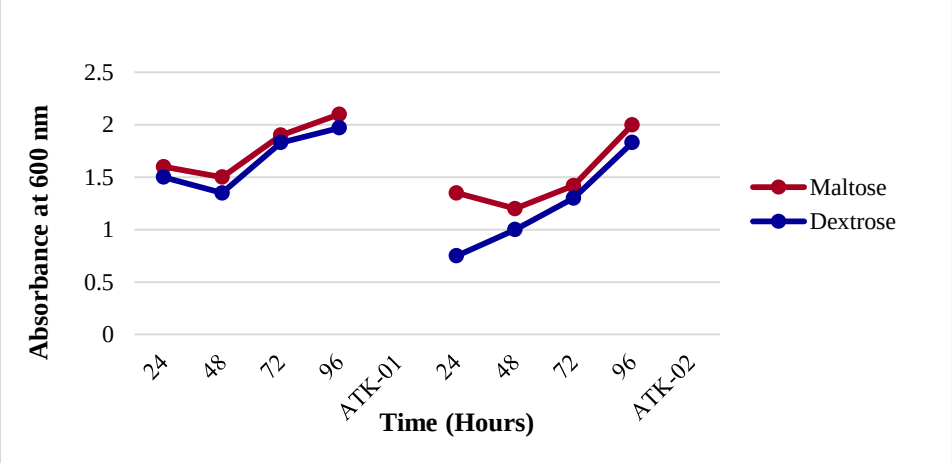

Fig. 4. Effects of Carbon Sources on Biodegradation of Spent Motor Oil

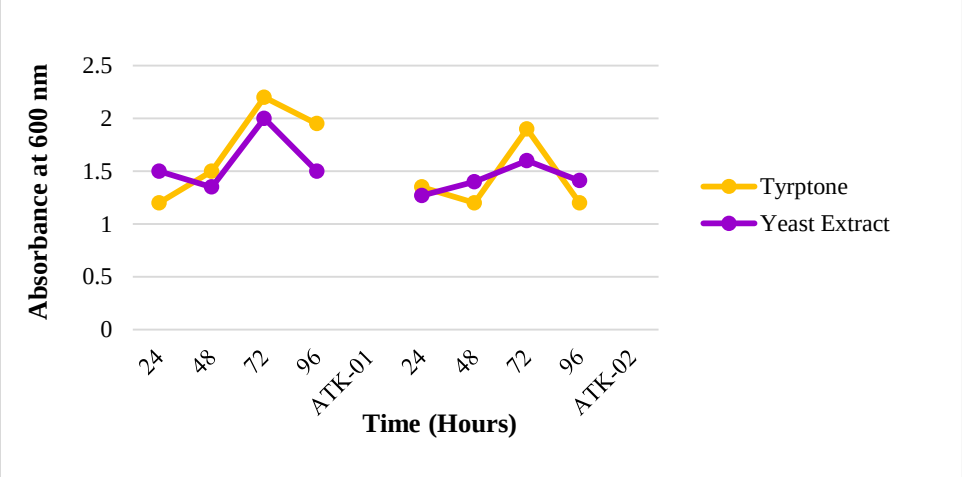

Fig. 5. Effects of Nitrogen Sources on Biodegradation of Spent Oil

Study of Degradation

The optimized conditions observed the degradation of spent motor oil using a BHMS medium supplemented with 5% consumed motor oil, 2M NaCl, 1.5% Tween 80, 0.5% maltose, and tryptone for 15 days of incubation at 37^0C. After degradation, the color of the broth is changed from brown-black to white **(Fig. 6)**.

Extraction of Residual Motor Oil

The LLC method was performed to estimate the rate of oil degradation. After centrifugation, the supernatant of the broths was taken, and the residual oil was extracted via a 1:2 ratio of hexane.

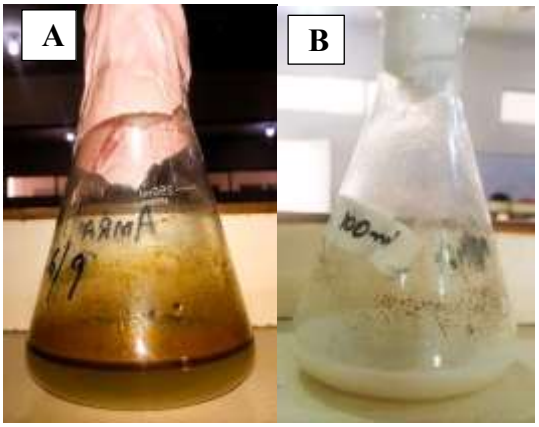

Fig. 6. (A) Spent Motor Oil Before Degradation (B) Spent Motor Oil After Degradation

Gravimetric Analysis

The amount of residual oil was measured after extraction from the medium by evaporating it to dryness at 55-75^{0}C in a hot air oven. The percentage of spent motor oil degradation was calculated using the following formulas.

Weight of the residual motor oil = Weight of beaker containing extracted motor oil - Weight of empty beaker

ATK-01	**ATK-02**
= 118.08g - 118.01g	= 118.13g - 118.01g
= 0.07g	= 0.12g

Amount of motor oil degraded = Weight of residual motor oil added in the medium - Weight of residual motor oil

ATK-01	**ATK-02**
= 1.16g-0.07g	= 1.16g-0.12g
= 1.09g	= 1.04g

Degradation % = Amount of motor oil degraded/Amount of motor oil added in the medium x 100

ATK-01	**ATK-02**
= 1.09/1.16 x 100	= 1.04/1.16 x 100
= 93.96%	= 89.65%

DISCUSSION

The increasing number of cars and machines used in industries and mechanical workshops is the major cause of environmental pollution, eventually reaching the soil and water. The leakage of substantial oil spills can modify soil's physical and chemical properties and threaten human life. The composition of 80% of motor oils is molybdenum, zinc disulfide, and lubricating oil. Due to the lack of an environmental control system, pollution increases in developing countries (Younus *et al.*, 2020). In this study, both isolates showed maximum degradation at

5% of motor oil consumption at 37^0C and 120 rpm incubation in 72 hours. Gogoi *et al*. (2022) reported that the *Pseudomonas otitidis* strain DU13 can degrade 2% of used motor oil at 37°C and 135 rpm in 28 days. Similarly, Salam (2016) described that the *Pseudomonas aeruginosa* strains RM1 and SK1 exhibited extensive degradation ability on 2% waste engine oil in the dark at room temperature for 21 days. In contrast, a study from Nigeria reported that *Pseudomonas* sp showed the best degradation at 1% of used engine oils after 5 days of incubation (Muhammad *et al.*, 2022). By comparing these results, we can see that our isolates have the best degradation ability at higher concentrations and minimum durations. The isolated *Pseudomonas* spp. exhibited higher degradation in 2M salt concentration within 48 hours of incubation. Pasumarthi *et al*. (2013) stated that *Pseudomonas aeruginosa* and *Escherichia fergusonii* could degrade crude oil at a 3% salt concentration. Correspondingly, Thavasi *et al*. (2007) conveyed that *Pseudomonas aeruginosa* degrades crude oil at 35% of salt concentration. On the contrary, the consortia of alkali-salt tolerant microbes were reported to bioremediate the petroleum hydrocarbons at 50% sodium chloride (Zhang *et al.*, 2021). In comparison to these results, our isolates have a low salt tolerance. The isolates ATK-01 and ATK-02 showed maximum degradation in a 1.5% concentration of Tween 80 as a surfactant at 37^0C and 120 rpm in 24 hours of incubation. Ghosh and Mukherji (2016) informed that the *Pseudomonas aeruginosa* RS-1 strain exhibited a higher rate of pyrene degradation at a 0.016% concentration of Tween 80 at 28^0C and 120 rpm in a shaking incubator for 24 hours. Likewise, the strain isolated from crude oil-contaminated soil was reported to degrade the naphthalene and anthracene at 0.02% concentration of Tween 80 at 45^0C for 3 days in the dark (Mesbaiah *et al.*, 2016). Zhou *et al*. (2008) described that *Pseudomonas alcaligenes* PA-10 displayed 79.63±2.53% degradation of fluoranthene by using 0.05% of Tween 80 at room temperature for 28 days in the dark. Our isolates showed the best degradation at a high concentration of Tween 80 in minimum duration. The highest degradation potential of the carbon source was detected in 0.5% maltose at 37^0C and 120 rpm in 96 hours of incubation. Correspondingly, *Pseudomonas* sp. JP1 anaerobically degraded 72.50% of Benzo[a]pyrene in the presence of 0.05% maltose by incubating in a rotary shaker at 150 rpm and 25^0C for 40 days in the dark (Liang *et al.*, 2014). Wang *et al*. (2019) reported that 10% of maltose and lactose individually could enhance the machine oil degradation by *Pseudomonas* sp. at 37^0C for 7 days. These results indicate that the degradation capability in different concentrations of maltose depends upon the source and species of bacterial strains. In this research, the isolates ATK-01 and ATK-02 utilize 0.5% tryptone as a nitrogen source to degrade spent motor oil at 37^0C and 120 rpm in 72 hours of incubation. Research informed that *Pseudomonas aeruginosa* degraded 71.67% phenol in the presence of peptone at 35^0C and 150 rpm in a shaking incubator for 96 hours (Mahgoub *et al.*, 2023). Zhu *et al*. (2016) described that *Pseudomonas* sp. degraded 99.1% of phenanthrene by using beef extract as a nitrogen source within 7 days at 28 °C and 150 rpm. In contrast, *Pseudomonas aeruginosa* degrades diesel oil in 0.1% of yeast extract as a nitrogen source at 37^0C and 120 rpm in a shaking incubator for a week (Mwaura *et al.*, 2018). These results indicate the degradation capability in the presence of different nitrogen sources, and their concentrations depend upon the source and species of bacterial strains. The degradation percentage of the spent motor was observed as 93.96% for isolate ATK-01 and 89.65% for ATK-02 at 37^0C and 120 rpm in 15 days. Stephen *et al*. (2020) reported that *Pseudomonas* spp degraded 74.82% of spent motor oil after 28 days of incubation. Likewise, another study informed that *Pseudomonas aeruginosa* degraded 74.20 to 74.35% of waste engine oil from day 5 to 7 days of incubation at 60°C and 150 rpm (Gaur *et al.*, 2021). Hussein (2018) described that *Pseudomonas* sp degraded 78.5% of used engine oil after 10 days of incubation at 30°C and 150 rpm. The overall degradation percentages in these researches are quite similar, but the degradation duration and temperatures vary from species.

Conclusion

The isolated *Pseudomonas* species designated as ATK-01 and ATK-02 showed significant degradation of spent motor oil that is 93.96% and 89.65% by consuming 5% hydrocarbon, 2M salt concentration, 1.5% Tween 80, and 0.5% maltose and tryptone. Both isolates exhibited great potential for bioremediation of spent motor oil in minimum duration, becoming the best remediation alternative. The limitation of this study is that only the genus *Pseudomonas* was targeted, and only one type of generator was used. Future recommendations include targeting different genera, using various types and sizes of generators or machines, and increasing the sample size.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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