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Introduction

Over the last several decades, large quantity of oily sludge has been removed during drilling and stored in open pits within the vicinity of oil elds. e entry of such waste to the terrestrial or aquatic environment has threat to the agro-environmental ecosystems.

Oily sludge is composed of a wide range of organic compounds such as alkanes saturate, aromatics and aspaltenes [1]. Short chain hydrocarbons such as nC$_{10}$ to nC$_{12}$ are considered to be phytotoxic even at low concentration in the oily sludge. Moreover, such hydrocarbons are degraded rapidly however the rate of degradation primarily depends on the concentration of these hydrocarbons in oily sludge and secondarily on the length of the exposure of contamination to soil with such hydrocarbons [2]. Rahman et al. [3] reported that the addition of inoculation of bacterial strains, biosurfactant and fertilizers enhanced the degradation of n-alkanes. e n-Alkanes in the range of nC$_{8}$-nC$_{11}$ were completely degraded followed by nC$_{12}$-nC$_{21}$, nC$_{22}$-nC$_{31}$ and nC$_{33}$-nC$_{40}$ with % degradation of 100, 83-98, 80-85 and 57-73% respectively in 10 and 20% sludge at 56 days of incubation. Zand et al. [4] reported that 97% of total hydrocarbon was removed by planting Flax plant.

Oily sludge acts as a carbon substrate for diverse variety of microorganisms [5]. Siddiqui et al. [6] reported that bacterial population increased to 100 fold as compared to soil without previous history of hydrocarbon contamination. Fulekar [7] investigated two di erent soils and found that an increase in total microbial count was experienced with oily sludge contaminated soils, but clay soil showed no change a er oil contamination.

Previous literature reported that there is a lag phase prior to utilize oily sludge as a carbon source by indigenous microorganisms [8-10]. ese lag phases varies and depend on the type and concentration of hydrocarbons present in the oily sludge.

Numerous genera of bacteria such as Aeromonas, Alcaligenes, Acinetobacter, Arthobacter, Bacillus, Brevibacterium, Geobacillus, Mycobacterium, Pseudomonas, Rhodococuss, Sphingomonas, ermus and Xainthomonas species has been isolated from oily sludge using PCR and DNA-DNA hyberdization [11,12]. Cerqueira et al. [13] isolated Stenotrophomonasacidaminiphila, Bacillus megaterium and Bacillus cibi, from petrochemical oily sludge and Pseudomonas aeruginosa and B. cereus capable of degradation of hydrocarbons from oily sludge of Brazil. Jiann-Hong et al. [14] isolated B. altitudinis from oily sludge and found that inoculation of such bacteria to soil become contaminated with benzene can accelerate the degradation of benzene.

Rhizoremediation includes both bioremediation and phytoremediation. Bioremediation means inoculation of oily sludge contaminated sites with hydrocarbon degrading bacterial consortium and nutrients more likely to reduce the lag phase required for indigenous bacterial population to degrade hydrocarbons into biomass, and CO_2 and intermediate products. In addition to that phytoremediation means to use hydrocarbon tolerance plants such as alfalfa, soyabean, perennial ryegrass, fescue or kaller grasses or others to degrade hydrocarbon of oily sludge contaminated sites. Plants or rhizosphere will provide a unique environment for hydrocarbon degrading microorganisms to grow and increase in number and because of their combine e ect of release of root exudates and microorganisms the rate of degradation of hydrocarbons is more rapid than in non rhizosphere environment of oily sludge contaminated sites. rough rhizoremediation natural

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rehabilitation process of degradation of oily sludge is more rapid than either through bioremediation or phytoremediation.

Pradhan et al. [15] reported that the degradation of poly aromatic hydrocarbons (PAHs) were greater in soil where switchgrass (Panicumvirgatum) and little bluestem grass (Schizachyriumscoparium) were grown. ey found that around 57 and 47% of PAHs were degraded than uncontaminated soils. Omotayo et al. [16] reported that inoculation of 50 mg of crude oil per g of soil with consortium of hydrocarbon degrading bacteria such as Arthrobacter sp., Bacillus pumilus, Bacillus sphaericus and Serratiamarcescens and perennial rye grass has increased the rate of degradation of hydrocarbons and around 87.7% of total hydrocarbons were degraded from the soil at 45 days of incubation.

e study was aimed to enhance the natural rehabilitation process of oily sludge contaminated soils inoculated with plant growth promoting bacteria isolated from the host oily sludge and to understand the role of alfalfa on the degradation of hydrocarbons in oily sludge contaminated soils.

Materials and Method

Geology and samples description from potwar plateau

e Kohat-Potwar Plaeatu is situated in the south of the Himalaya and Karakorum Mountains. It is located between latitude 32° and 34°N and longitude 70° and 74°E. It is bounded by the Salt Range and Trans-Indus Range in the south, Kala chitta Range in the north. e Plateau opens up towards Jehlum River in the east. e west of the Plateau is bounded with Kurram-Parachinar Range [17]. Potwar plateau is an oil prone area. Up till now 40 structures have been drilled for oil exploration among which 10 structures have a potential of oil production [18]. e oily sludge from three oil elds within Potwar plateau were selected because of the di erence in the reservoir depth,

Crude oil fractionation into saturates (*n*-alkanes and isopreniods), aromatics and naphthlenes (isoprenoids)

Separation of saturates (n-alkanes and isopreniods), aromatics and polar (risens and asphaltenes) from crude oil was carried out by silica gel 60 column chromatorgraphy as described by Asif et al. [23] For column chromatography, a glass column (40 × 0.9 cm i.d.) with cotton wool at bottom was rinsed with 20 ml of dichloromethane. Around 200 g of silica gel was placed in 250 ml beaker and was activated at 120°C for 24 hours prior to be used for column chromatography. About 10 g of activated silica gel 60 with mesh size 35-70 mm (Fluka-Germany) was removed from the oven and was poured over the column with cotton wool at the bottom. e silica gel 60 was saturated with n-hexane (50 ml). Once the column bed is prepared around 50 mg of crude oil was transferred to the column from Pasteur pipette. e saturate fraction of crude oil was eluted with n-hexane (35 mL) and was collected in 50 ml of bottles. Once saturate fraction is obtained fractionation of aromatic compounds was carried out by adding a mixture of n-hexane:dichloromethane (35 mL, 7:3) which was collected in glass bottle (50 mL) prior to be analyzed by GCFID. Polar compounds were fractionated with 35 mL of methanol: dichloromethane (1:1). e solvent in each fraction was evaporated through roto-evaporator at 60°C temperature.

Gas chromatography- ame ionization detector (GC-FID)

GC-FID analysis was performed using a QC 2010 Shimadzu GCFID. A 30 m × 0.25 mm ID capillary column coated with a 0.25 µm 5% phenyl 95% methyl polysiloxane stationary phase (DB-5 MS, J & W scienti c) was used for the analysis. 1 µL of the saturated or aromatic fractions (1 mg/mL in n-hexane) was introduced into the split/splitless injector using the QC 2010 auto-sampler. e injector was operated at 320° in pulsed splitless mode. Helium maintained at a constant ow rate of 1.1 mL/min was used as carrier gas. e GC oven was programmed from 75°C to 320°C at 10°C/min with initial and nal hold times of 28.83 minutes, respectively.

e peak area of each standard on GC trace was converted to mg kg^{-1} of the hydrocarbon. e response factor of each external standard was calculated by taking the mean of response factor of 5 step dilutions calibration curve using following formulas.

e calibration curve is determined by the analysis of 3 calibration levels, i.e. 0.1, 0.25 and 0.5 mg kg^{-1}. e peak area was calculated and calibration curve was plotted for concentration vs response area or peak area. e calibration curves were best tted to a linear curve. e correlation coe cients (R) were 0.9947. e quanti cation was performed from the mean of two calibration curves surrounding the samples.

Statistical analysis of DATA

e data of isolates were analyzed by complete randomized design using LSD and compare means by statistix 8.1.

Results and Discussion

Alignment of 16S rRNA sequence

For the isolate obtained from oily sludge of Missa kasswal the total length of sequence with 1497 nucleotide was obtained. e comparison of the nucleotide sequence with data nucleotide bank showed highest 99% sequence similarity for 1463/1466 nucleotide bases with that of *B. cereus* strain partial sequence (Acc KF859972).

For the isolate obtained from oily sludge of Missal kasswal the total length of sequence with 1535 nucleotide was obtained. e comparison of the nucleotide sequence with data nucleotide bank showed 99% sequence similarity for 1477/1485 nucleotide bases with that Commamonas bacterium clone EK_An354 16S ribosomal RNA gene, partial sequence (Acc KF859971).

For the isolate obtained from oily sludge of Chak Naurang the total length of sequence with 1501 nucleotide was obtained. e comparison of the nucleotide sequence with data nucleotide bank showed highest 99% sequence similarity for 1477/1482 nucleotide bases with that of *S. maltophilia* strain E56 16S ribosomal RNA gene, partial sequence (Acc KF859973)

For the isolate obtained from Dhakni oil eld total length of sequence with 1506 nucleotide was obtained. e comparison of the nucleotide sequence with data nucleotide bank showed highest 99% sequence similarity for 1466/1475 nucleotide bases with that of *B. altitudinis* strain: 41KF2b 16S ribosomal RNA, partial sequence (Acc KF859970).

Several genera of bacteria were isolated from oily sludge such as Pseudomonas, Bacillus, Proteobacteria (with a predominance of Alphaproteo bacteria and Gammaproteo bacteria), Actinobacteria, Cytophaga-Flavobacterium-Bacteroides, Firmicutes. Spirochaetes [24-26] S. maltophilia and B. cereus were isolated from oily sludge (Chak nand

for certain bacterial strains perhaps acting as C/N source eg in B. altitudines however, the addition of fertilizer and growing alfalfa may be bene cial for other strain e.g. B. cereus both for short term and long term incubation. e B. cereus can survive well in the oily sludge and

the degradation rate in B. cereus, Commamonas, S. maltophilia and B. altitudinis (Figure 2c and 2d).

The concentration of nC_{19}-nC_{29} was 84 % higher at 5 d but was 64 %less in 30% oily sludge at 10 d of incubation as compared to nC_{17}-nC_{18}. B. altitudinis followed by B. cereus were most effective and showed 82 to 64% degradation at 5 d over that of untreated sludge (Figures 3 and 4).

Growing alfalfa significantly increased the rate and % degradation such that negligible amount of nC_{19}-nC_{29} were deleted in inoculated sludge. Addition of fertilizer to alfalfa + inoculation had no such marked effect except that in S maltophilia and B. altitudines which showed early degradation. In presence of both alfalfa and fertilizer the S. maltophilia inoculation resulted in maximum degradation over that of single inoculation (Figure 4c and 4d).

The results (Figure 5) revealed that degradation of pristane (isoprenoid) was enhanced by all the PGPR isolates., but the most effective were B. altitudines followed by Commamonas which enhanced the process of degradation, degrading about 50 to 80 % within 5 d of incubation. B. cereus took 10 d; alfalfa plantation was more effective for B. cereus and S. maltophilia than the fertilizer treatment. Whereas, combined treatment of fertilizer and alfalfa were not much effective to promote the effect of inoculation than that of alfalfa +inoculation. Addition of fertilizer significantly suppressed the process of biodegradation of long chain alkane's nC_{30}-nC_{33} both at 5 and 10 d of incubation. Instead growing alfalfa increased the rate of degradation in B. cereus, Commamonas and S. maltophilia but has suppressed the efficiency of B. altitudinis (Figure 6).

The degradation of nC_{13}-nC_{16} hydrocarbon (alkane series) was faster in presence of fertilizer and fertilizer +alfalfa but the rate and %

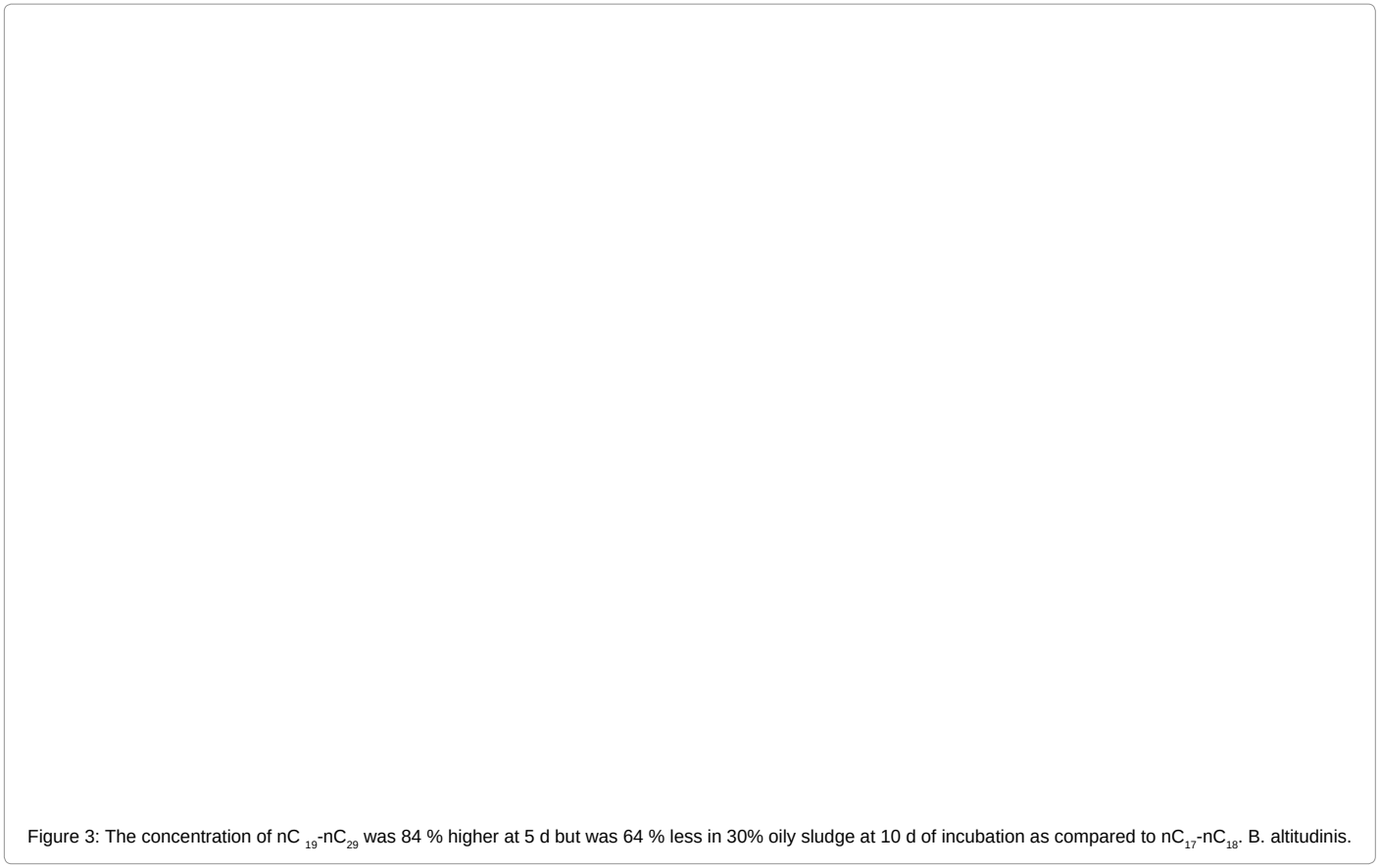

Figure 3: The concentration of nC_{19}-nC_{29} was 84 % higher at 5 d but was 64 % less in 30% oily sludge at 10 d of incubation as compared to nC_{17}-nC_{18}. B. altitudinis.

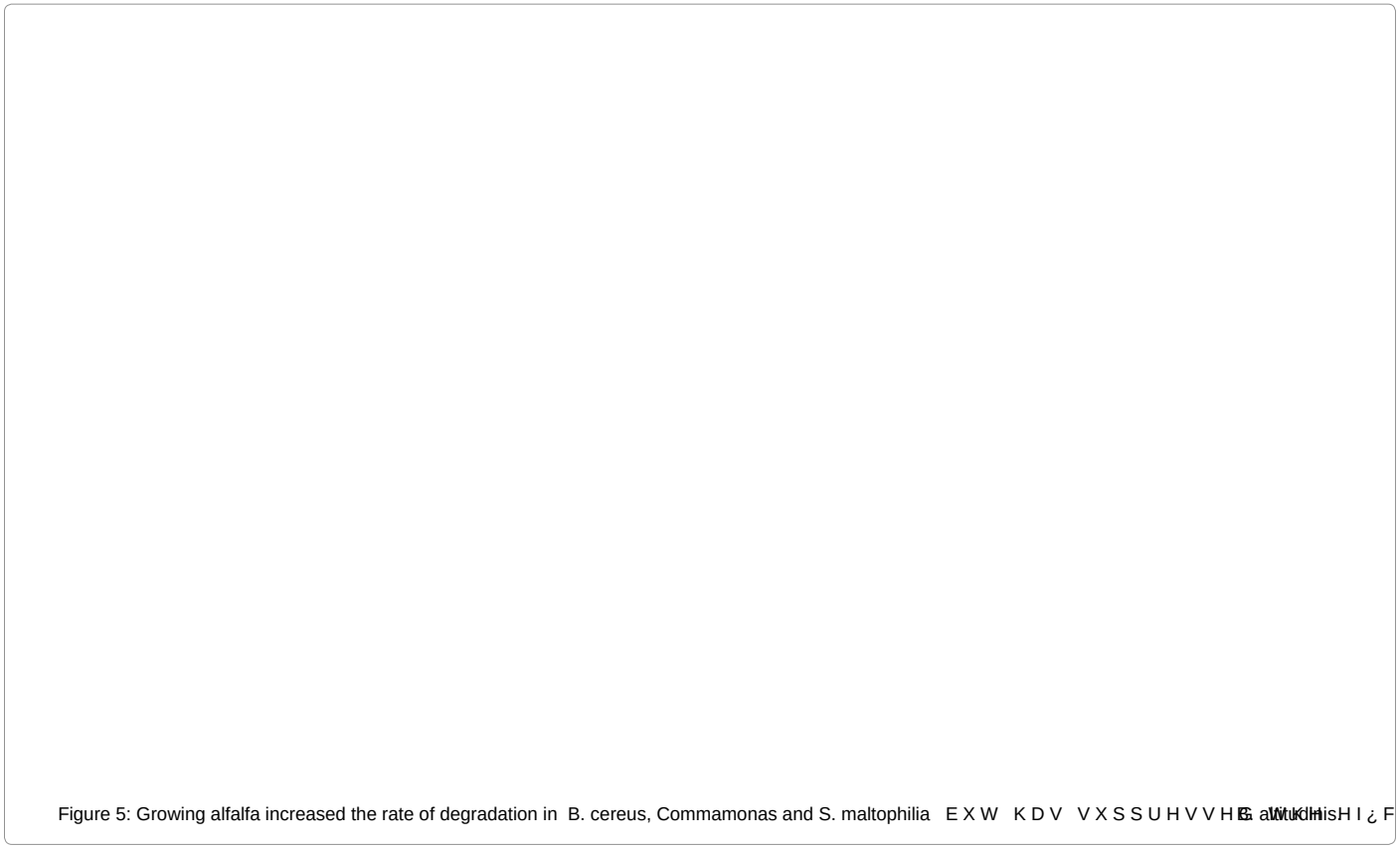

Figure 5: Growing alfalfa increased the rate of degradation in B. cereus, Commamonas and S. maltophilia E X W K D V V X S S U H V V H B a W K H I ¿ F L H Q F

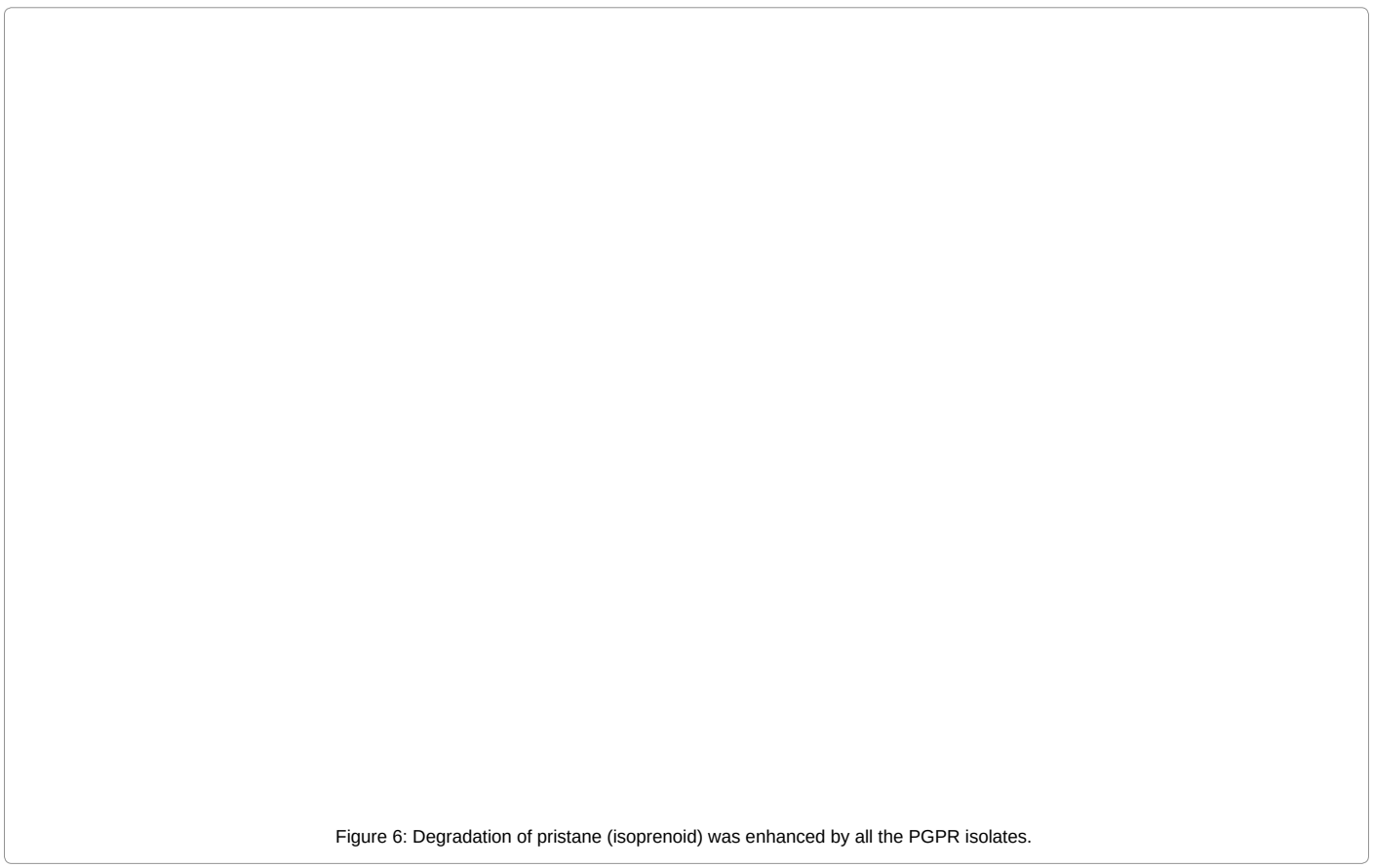

Figure 6: Degradation of pristane (isoprenoid) was enhanced by all the PGPR isolates.

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Commamonadas and S. maltophilia and B. altitudinis respectively, when compared from 5 to 10 d of incubation.

e total hydrocarbon degradation potential of B. altitudinis strains at 5 d was higher from all other strains and present results ndings are comparable to degradation of Nigerian crude-oil by B. subtilis and P. aeruginosa strains isolated from crude oil-polluted soil from Nigeria [41]. Since addition of

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