

A multidimensional study of enzyme activity and hydrocarbon reduction for bioremediation of refinery waste by free living cyanobacteria

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ABSTRACT

The continually increasing demand for fuels with the depleting resources due to expansive production generates tons of waste and effluents in the form of sludge annually across the globe. The inappropriate disposal or inadequate caution for safe elimination practices is the root cause for the deteriorating soil and water quality and the consequent dread toward the marine ecosystem as well as the terrestrial life. Remediation technique that is specific, efficient and that is safe without giving rise to new pollutant sources is the ideal solution. The present available techniques come with drawbacks of inefficiency, insufficiency, laborious, expensive, inadequate scaling, and resource dependent like requiring large span of lands or high-end instruments. The current approach brings along the solution to these shortcomings along with robust evidence. Three cyanobacterial strains cultivated for this procedure were studied morphologically, assessed on growth parameters basis, assayed for their enzyme activities, and monitored to quantify a reduced total petroleum hydrocarbon content in the cultures prior and post-treatment with the tank bottom oil sludge sourced from the Numaligarh Refinery Limited. The obtained outputs of growth parameters indicated the cyanobacteria's survival ability toward the toxic sludge introduced into their cultures. The enzyme assays presented a diverse response of the cultures with esterase as the highest active enzyme in comparison to the rest of the enzymes. On completion of the 28-day course of incubation, the cultures exhibited a slightly reduced lipase and esterase activity and an increased polyphenol oxidase and dehydrogenase activity in comparison with the respective positive control cyanobacterial cultures. The possible reasons for the promotion and reduction of the enzyme activity were discussed in this study. In terms of the gas chromatography flame ionization detection output, the obtained chromatograms reflected significant reductions in the total petroleum hydrocarbon of the sludge post-treatment on day 28. The initial hydrocarbon chain length of C_3-C_{22} was reduced to C_4-C_{12} by the *Nostoc* spp. and the *Anabaena variabilis* cultures, and major peak area reduction, C_3-C_{13} , was observed in the hydrocarbon spectrum of the *Nostoc muscorum* culture post-treatment. The saturated and the unsaturated hydrocarbons, accessory, sulfur compounds, and polycyclic aromatic hydrocarbons (PAHs) were observed to be biodegraded from the gas chromatography-mass spectrometry data of the untreated and the *Nostoc* spp. treated sludge in cultivation. In sum, the obtained data direct toward the high potential of the studied cyanobacterial cultures for the efficient bioremediation of the refinery-acquired sludge.

1. INTRODUCTION

The rapid increase in the global population has provided much impetus to major sources of energy supply, such as coal, petroleum, and natural gas. Due to the inclination toward increase demand for petroleum-derived fuel, a huge amount of petroleum waste in the form

of sludge is generated. The disposal processes employed are very cost bearing involving expensive physico-chemical methods [1]. Hence, biodegradation of the sludge waste using biological organisms is the need of the hour. In this context, cyanobacteria could play a pivotal role in biodegradation as it has marked its presence more than 3 billion years ago and are believed to make their habitat in fresh to marine water bodies, but their dominant presence as primary producers in extremely arid, salt lakes and hot springs, and the hot and cold deserts are significant [2,3]. Cyano in the name cyanobacteria got derived due to the presence of the blue color pigment phycocyanin (PC) combined with the green chlorophyll pigment gives rise to the colloquial label blue-green algae [3]. Because they are photoautotrophs and have the

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ability to fix nitrogen from the atmosphere, cyanobacteria are more resilient in contaminated environments [4]. These organisms have a strong capability for creating copious amounts of effective compounds to guard against oxidative and radical stressors due to their phototrophic lifestyle and frequent exposure to high oxygen and radical stresses [5]. Cyanobacteria by virtue of their vast gene pool and plasticity are stated as potent tools for environmental rehabilitation [6]. Therefore, through either biodegradation or bioaccumulation, cyanobacteria are preferred for the bioremediation of environmental contaminants. Efficient absorption of organic matter facilitates cyanobacteria to be capable of biodegradation, transformation, and biosorption of industrial wastewater pollutants [7]. Products and the petroleum having composition of polyaromatic hydrocarbons (PAHs), mono-, and bi-aromatic compounds, alkanes, asphaltene, resins, are investigated to be bioremediated by cyanobacterial organisms such as *Oscillatoria*, *Spirulina*, *Synechocystis*, to mitigate the toxicological impact on the ecosystem [8,9]. Cultures of blue-green algae, *Synechococcus* spp., axenic and in consortium with *Chlorella vulgaris*, remediate kerosene on cultivation [1].

Along with being the producers of different cellular secondary metabolites and biofuels, cyanobacteria are deemed to be beneficial organisms for waste treatment. These blue-green algae have been as well reported to bear a symbiotic relationship with other aerobic or anaerobic organisms in the natural environment, exhibiting hydrocarbon degradation present in oil. Cyanobacteria have rapid metal removal kinetics and the ability to treat multiple metal-containing molecules [4,7]. Through an elongated recovery duration, cyanobacteria *Nostoc muscorum* and *Anabaena* spp. displayed bioaccumulation to mitigate the deleterious impact of the forms of arsenic, AsV and AsIII [10]. It has been estimated that approximately 28220 tons of sludge are generated in Indian and globally 3 million tons of sludge are generated in China through the exploration by various Chinese refinery companies [11,12]. Beyond that, the composition of these oily sludge is made of compounds and heavy metals as phenol (90–100 mg/kg), chromium (27–80 mg/kg), nickel (17–25 mg/kg), manganese (19–24 mg/kg), zinc (7–80 mg/kg), copper (32–120 mg/kg), cadmium (0.8–2 mg/kg), and lead (0.001–0.12 mg/kg) [7] which are codominantly lethal for disposal in the environment directly before treatment. The hazardous impact of these compounds on lives existing on land or in water bodies (micro-macro organisms) does not remain hidden or unnoticed. Discharge of untreated oil sludge directly to the environment constitutes a peril to the life forms residing in it along with the promotion of unavailability of fresh waters and unpolluted soil. Thus, this demands for holistic understanding and action for characterization, management, and safe and organized disposal of these oil wastes. A treatment before discharge to land or the natural water bodies is a crucial requirement. It shall be an innovative approach for these three specific cyanobacterial organisms to be investigated for the oil refinery-generated sludge through revealing their functional enzyme activities contributing to the bioremediation. The *Nostoc* spp. being yet to be reported for their remediation function toward oil tank sludge, its biodegradation mechanism is quantifiable through the gas chromatography techniques.

Among the plenty of physical and chemical methods and approaches that are expensive, resources dependent, sources of secondary pollutants, laborious, and inefficient for large-scale application, bioremediation comes across as the safest and most suitable solution. The petroleum hydrocarbons are among the most persistent organic pollutants and its removal from the environment is accomplished by microorganism performed degradation [12]. And in that regard,

features like being productive due to the ability of nitrogen fixation, inexpensive, natural resistance acquired against environmental pollutants make these cyanobacteria ideal and advantageous over alternative microorganisms for this purpose.

2. MATERIALS AND METHODS

2.1. Source Location of Treatment Samples

The treatment sample, tank bottom oil sludge, was sourced from the Numaligarh Refinery Limited (NRL) (26.5786° N, 93.7848° E) plant, District Golaghat, Numaligarh, Assam.

2.2. Culture Conditions of Cyanobacterial Samples

Three routinely maintained axenic cyanobacterial cultures of *N. muscorum*, *Anabaena variabilis*, and *Nostoc* spp. in BG11_o medium were selected for the present investigation. Culture conditions include an air-conditioned chamber at 24 ± 2°C with a photon fluence rate of 50 μmol m⁻² s⁻¹ and control illumination of 12 h light and 12 h dark was maintained [13]. The cultures have been purified morphologically and identified through their 16S ribosomal RNA sequence [14] submitted to the gene bank to obtain their accession numbers.

2.3. Microscopical Analysis

The morphology of the treated as well as the untreated cultures was studied using the Radical RXL-4B light microscope with an equipped digital camera under 45×. Features such as the cell shape and size, plane of division, dimensions, color, motility, and sheath were studied in addition to the special feature of heterocyst formation and presence of akinetes, necridiaocytes to resolve a comparative study between the untreated and sludge-treated cyanobacterial cultures.

2.4. Establishment of Sludge Lethal Dose Concentration

Strains of *A. variabilis*, *N. muscorum*, and *Nostoc* spp. were cultivated in BG-11_o medium individually to obtain cells in their exponential growth phase. The sludge sample was prepared for inoculation by dissolving it in n-hexane in a 1:1 ratio. To determine the sludge lethal dose concentration, portions were drawn from the cyanobacterial mother cultures and inoculated with a graded range of concentrations of tank bottom sludge (0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, and 50 mg/mL⁻¹) to culture individually [15]. The culture's optical densities were measured at 663 nm on the 28th day of inoculation to define the sludge lethal dose concentrations.

2.5. Growth Assessment of Cyanobacterial Cultures

2.5.1. Chlorophyll a (Chl a), chlorophyll b (Chl b), and carotenoids

Assessment of the cyanobacterial growth was conducted based on the cultures Chl a, Chl b, and carotenoids content with few modifications [16]. The treated and untreated culture suspensions were prepared for the extraction by centrifuging for 10 min at 10,000 rpm. The pellets obtained were dissolved in methanol in 10 mL, heated in water bath for 30 min, and incubated overnight for the complete extraction of the pigments. The solution was vortexed and centrifuged for 10 min at 10,000 rpm and measured for absorbance by a spectrophotometer. The readings were taken at respective wavelengths specific to chlorophyll a, chlorophyll b, and carotenoids to calculate the pigment content using the following formulae [17],

$$Chl\ a\left(\frac{g}{ml}\right)=(12.47\times A_{665\ nm})-(3.62\times A_{649.1\ nm})$$

$$Chl b \left(\frac{g}{ml} \right) = (25.06 \times A_{649.1nm}) - (6.45 \times A_{665nm})$$

$$Car \left(\frac{g}{ml} \right) = \frac{(1000 \times A_{480nm}) - (1.29 \times Chl a) - (53.78 \times chl b)}{220}$$

Chl a = Chlorophyll a, *Chl b* = Chlorophyll b, *Car* = Carotenoids

2.5.2. Phycobilisomes

The phycobilisome pigment content including phycoerythrin (PE), PC, and allophycocyanin (APC) was prepared by centrifuging the culture suspension at 4000 rpm, thereby washing the pellet with 1M phosphate buffer [18]. The extraction was conducted by giving a 30 min water bath to the pellets dissolved in methanol. The vortexed solution was centrifuged to obtain the supernatant containing the extracted pigment and its absorbance was measured at respective wavelengths using a spectrophotometer and using the following formulae, the pigments were quantified.

$$PC \left(\frac{mg}{ml} \right) = \frac{A_{615nm} - (0.474 \times A_{652nm})}{5.35}$$

$$PE \left(\frac{mg}{ml} \right) = \frac{A_{562nm} - 2.41(PC - 0.849 \times APC)}{9.62}$$

$$APC \left(\frac{mg}{ml} \right) = \frac{A_{652nm} - (0.208 \times A_{615nm})}{5.09}$$

PC = Phycocyanin, *PE* = Phycoerythrin, *APC* = Allophycocyanin

2.6. Hydrocarbon Biodegradation and Degradation Rate

The sample preparation for the determination of total petroleum hydrocarbon (TPH) content of the treated and untreated sludge was conducted [19], sketched protocol including few modifications. Using HPLC-grade hexane in a ratio of 1:1, the samples were extracted with centrifugation at 15000 rpm for 5 min. The obtained pellets were sonicated using a titanium probe of 6 mm diameter of an ultrasonic processor (Rivotek, India) for 90 min with 5 s on and 2 s off intervals. The TPH accumulated solvent was kept resting in a separating funnel to obtain segregated layers with the process being repeated twice to obtain the residual TPH. The complete hydrocarbon was extracted using a rotary evaporator (IKA, Germany). Gas chromatography (GC) was performed to measure the TPH of the prepared samples with an equipped Flame-Ionization Detector (FID) (ThermoFisher TRACE 1600 Series). The used column specifications were TG-WAXMS GC COL capillary column with dimensions of 30 m × 250 mm × 0.25 μm. The analysis was conducted for a total of 50 min run time in a constant flow rate of 1 mL/min of hydrogen carrier gas in a split-less mode. 230°C inlet temperatures, 250°C detector temperatures, and 190°C oven temperature were maintained to run the injected 2 μL samples. Analysis, peak integration, and the required refining procedure were performed in the Chromeleon 7.2/7.3 software for GC SE (single user, single TF instrument license).

The sludge degradation (S) was calculated by the following formula:

$$S = \left(1 - \frac{So}{Sr} \right) \times 100\%$$

Where *So* = Sludge concentration in control and *Sr* = Sludge concentration in treatment culture.

2.7. Sludge Degradation Kinetics

The kinetics of the degradation reactions were measured based on rate laws to determine the order of the reaction.

$$n = 0, [A] = -kt + [A]_0 \quad eq 1$$

$$n = 1, \ln \frac{[A]_0}{[A]} = kt \quad eq 2$$

$$n = 2, \frac{1}{[A]} = kt + \left(\frac{1}{[A]_0} \right) \quad eq 3$$

Where *k* = rate constant, *[A]* = initial sludge concentration, *[A]₀* = sludge concentration relative to time *t* and *n* = order of reaction.

2.8. Enzyme Assays

2.8.1. Preparation of enzyme extracts

The extraction of enzymes from sludge treated as well as positive control cultures of cyanobacteria devoid of sludge was performed with slight modifications [19] by homogenizing in ice-cold 50 mM phosphate buffer and centrifuged at 4°C at 15000 rpm. Freshly prepared cell lysis buffer was added to the obtained pellets and centrifuged to yield a cell-free supernatant. The extract obtained was subjected to 60% ammonium sulfate precipitation and suspended in 50 mM phosphate buffer. Furthermore, purification of the extracts was undertaken for dialysis with phosphate buffer and changed at 3 h intervals for 12 h. This purified and concentrated enzyme extract was then assayed for polyphenol oxidase (PPO), catalase, lipase, esterase, and dehydrogenase enzyme activity.

2.8.2. Measurement of PPO activity

The PPO activity was performed to obtain a standard curve using a concentration range of 0.05 mg/mL to 0.25 mg/mL of a sample [20]. The PPO assay included an enzyme cofactor, a suitable buffer, and PPO enzyme solution. For the determination of the activity of the extracted enzyme, the reaction components were repeated with 100 μL of the enzyme extract instead of the known PPO solution. The reaction contents were incubated at 40°C for 20 min. The obtained absorbance values at 370 nm were plotted on the obtained standard curve and the activity was determined.

2.8.3. Catalase activity

Activity of catalase [EC 1.11.1.6] in the sludge-treated and untreated cyanobacterial cultures was determined by the addition of reaction contents [21] containing definite enzyme extract, 1% Triton X-100, followed by 30% hydrogen peroxide. The activity was supposed to be measured with reference to the height of the effervescence formed in the reaction tube. On the contrary, because the activity was minimal, the presence or absence of the effervescence was used to conclude with the catalase activity as qualitative data.

2.8.4. Lipase activity

Lipase [EC 3.1.1.3] activity was performed by determining the absorbance of the reaction mixture containing lipase solution and Tween 20 in phosphate buffer used as the substrate was taken at 450nm after incubating it for 10 min [22]. The lipase activity of the enzyme extract was determined with the aid of a calibration curve obtained between a concentration range from 0 mg/mL to 4 mg/mL for the mentioned reaction setting with slight increase in the reaction volumes as a modification of the original protocol.

2.8.5. Esterase activity

Esterase [EC 3.1.1.8] activity was determined with slight modifications [23] by adding sodium phosphate buffer and p-nitrophenol acetate in methanol to 1 mL of the enzyme extract and incubated for 10 min at 55°C, followed by measurement of absorbance at 347 nm.

2.8.6. Dehydrogenase activity

Activity of the dehydrogenase [EC 1.1.1.1] enzyme was determined [24] through a reaction mixture containing a buffer substrate solution that is prepared by mixing 4.8 mL of 60% sodium lactate solution (Fisher) and 95.2 mL of 0.1 M Tris buffer (pH 9.0) and diphosphopyridine nucleotide along with the substrate iodonitrotetrazolium chloride dye and gelatine and incubated for 30 min at 37°C. The phenazine methosulfate and the enzyme extract were added to the incubated mixture and kept at 37°C for 15 min. The reaction was stopped by the addition of 0.35M HCl and the absorbance was measured at 540 nm by a spectrophotometer. The activity of dehydrogenase was calculated using a standard curve.

2.9. Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

The sample obtained by processing the oil sludge sample treated by the *Nostoc* spp. culture through centrifuging, followed by sonicating the pellet in an organic solvent and concentrating through rotary evaporation, has been taken for GC-MS analysis. The biodegradation indices were calculated by the following equation,

$$\text{Biodegradation index (\%)} = 100 - \left(\frac{\sum \text{compounds treated}}{\sum \text{compounds untreated}} \times 100 \right)$$

2.10. Statistical Analysis

The experiments generated data were evaluated by one-way analysis of variance with the Tukey-Kramer procedure and the data were considered to be significant with $P < 0.05$.

3. RESULTS

3.1. Morphological Characteristics

The morphological characteristics of the three axenic cultures *N. muscorum*, *A. variabilis*, and *Nostoc* spp. are depicted in Figure 1. These cultures were identified from various habitats and are maintained in the laboratory. The cultures have been identified using molecular phylogeny and accession no have been obtained from the gene bank [14] - *N. muscorum* (KR709122), *A. variabilis* (KR709104), and *Nostoc* spp. (KR709128), respectively.

The sludge treated cultures upon examining with their untreated cultures, *N. muscorum* showed barrel-shaped unbranched filamentous structure indicating to be in the seriate stage, with cell size about $6 \pm 0.38 \mu\text{m}$ in length [Figure 1a, 1b], *A. variabilis* exhibited filamentous vegetative cells in the size range of $5.1 \pm 0.27 \mu\text{m}$ in length, akinetes, and rounded terminal cells and initially devoid of heterocyst's [Figure 1c, 1d] and lastly *Nostoc* spp. exhibited tapered terminal and barrel-shaped filamentous vegetative cells, about $6.3 \pm 0.18 \mu\text{m}$ long [Figure 1e, 1f]. The treated cultures' morphology has not been changes as compared with the untreated cultures [Figure 1a, c, and e] and it confirms that the cell machinery has been functioning in normal way when subjected to sludge stress.

3.2. Establishment of Lethal Dose Concentration

The graph presents the relationship between sludge concentration (mg/mL) and Chl a content in three cyanobacterial species:

N. muscorum, *A. variabilis*, and *Nostoc* spp. [Figure 2]. Chl a is widely used as an important growth parameter in cyanobacteria to determine the threshold concentration at which the sludge treatment could retain the growth parameters of the cell. All cultures showed an initial increase in Chl a concentration with increasing sludge concentration with maximum sludge retention at 7–9 mg/mL. Consequently, beyond 9 mg/mL higher concentrations of the sludge utilization probably decline, reducing their ability to photosynthesize and survive, which is crucial for bioremediation potential. Hence, 9 mg/mL is used as a minimum inhibitory threshold for all experiments that are investigated in the present study.

3.3. Growth Parameters

3.3.1. Chl a, Chl b, and carotenoids

The Chl a content of *N. muscorum*, *A. variabilis*, and *Nostoc* spp. was monitored over 28 days with 9 mg/mL sludge concentration [Figure 3]. Initially, all strains exhibited a higher Chl a concentration, which gradually declined within the first 4 days but subsequently stabilized with a sluggish recovery trend. *Nostoc* spp. exhibited the highest Chl a levels throughout, followed by *A. variabilis*, while *N. muscorum* showed the lowest. After 28 days, the growth was shunted, indicating a gradual adaptation and possible acclimatization to environmental conditions.

Similar trends in context to Chl b were observed for the three cultures under study with sludge concentration of 9 mg/mL incubated for 28 days [Figure 4]. *N. muscorum* exhibits frequent fluctuations with Chl b concentration and in contrast, *A. variabilis* showed consistently low values. Furthermore, the analysis suggests that *Nostoc* spp. may have a higher adaptability or resilience, making it a better candidate for applications requiring sustained chlorophyll retention in cell functionaries.

The carotenoid content in response to the sludge treatment of 9 mg/mL exhibited similar results as Chl a and Chl b growth patterns [Figure 5]. Initially, *Nostoc* spp. unveiled the highest carotenoid concentration of $4.5 \mu\text{g/mL}$, followed by a sharp decrease within the first 4 days and then consistent carotenoid content at lower levels. In contrast, *N. muscorum* and *A. variabilis* displayed lower carotenoid levels of $\mu\text{g/mL}$ and also showed a similar decreasing trend before stabilizing. All the three cultures maintained relatively low carotenoid content, ranging from 0.5 to $1.0 \mu\text{g/mL}$ with minor fluctuations between day 6 and 18, but a gradual increase was observed on day 20 with *Nostoc* spp. cultures exhibiting higher carotenoid concentrations, suggesting a better adaptation upon stress, followed by recovery with increased concentrations.

The overall pattern of the growth characteristics by three cultures suggests that *Nostoc* spp. as a promising candidate for further applications in pigment augmentation and oxidative stress resistance in response to sludge studies.

3.3.2. Phycobilisomes

The phycobilisomes content of cultures under study in the form of PC, PE, and APC in response to sludge treatment showed relatively lower concentrations of phycobiliproteins compared to their respective controls [Figure 6]. Among the cultures, *A. variabilis* demonstrates a moderate increase in all three pigments, followed by *N. muscorum* and *Nostoc* spp., exhibiting similar trends. The results further suggest that the control conditions favored the accumulation of phycobiliproteins, particularly APC, while the experimental cultures show lower but consistent production of these pigments.

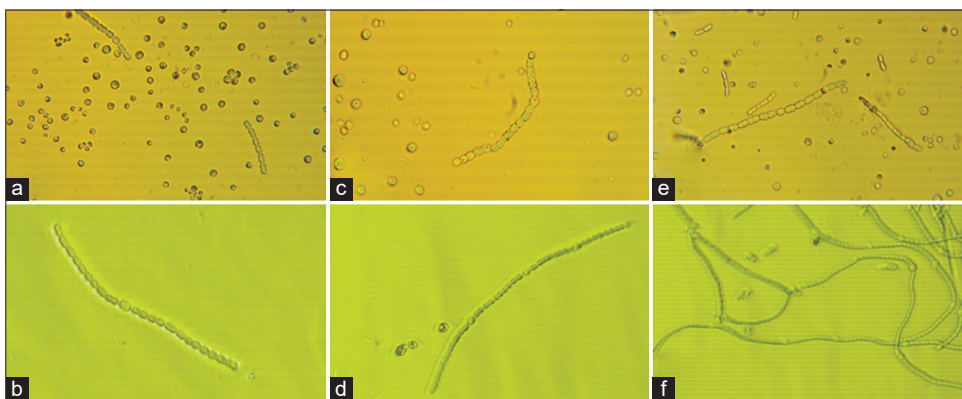


Figure 1: Micrograph of pure cultures and tank bottom sludge treating cultures. (a) pure *Nostocmuscorum*, (b) *Nostoc muscorum* treating tank bottom sludge, (c) pure *Anabaenavariabilis*, (d) *Anabaena variabilis* treating tank bottom sludge, (e) pure *Nostoc* spp., and (f) *Nostoc* spp. cultures treating tank bottom sludge.

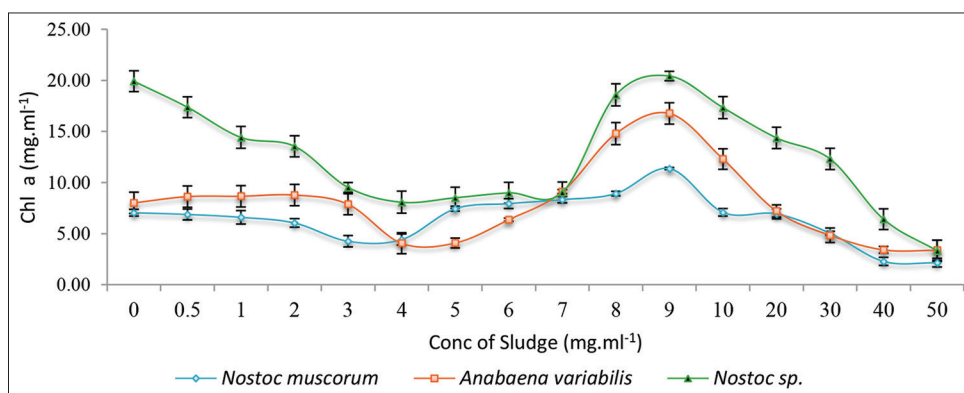


Figure 2: A graphical representation of the lethal dose concentration of the tank bottom sludge on the three treated cyanobacterial cultures on the 28th day of incubation. Individual cultures were duplicated with the respective $\pm$ standard deviation values and $P < 0.05$.

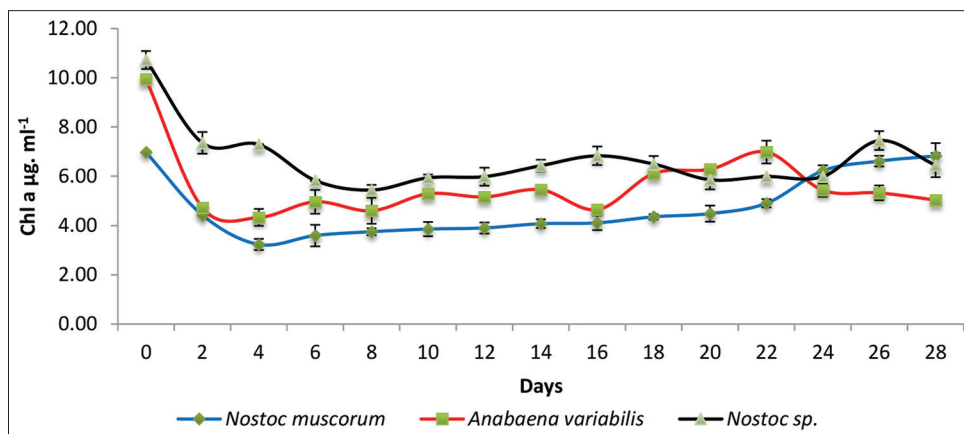


Figure 3: A graphical representation of the chlorophyll a concentration of the three cyanobacterial cultures treated with tank bottom sludge on the 28th day of incubation. Individual cultures were duplicated with the respective $\pm$ standard deviation values and $P < 0.05$.

The differences observed could be attributed to variations in the metabolic activity, growth conditions, or genetic potential of the respective cyanobacterial strains in the sludge stress environment. These findings also indicate that these cyanobacterial cultures may require a longer period to adapt to the initial toxicity of the sludge, as all the pigments after the initial drop did take up a leap, and that with a longer incubation period, if provided, the cultures may exceed the initial pigment content.

3.4. Biodegradation Potential of the Cyanobacterium by Virtue of the Degradation Rates

The composition of the tank bottom sludge from the GC-FID output was found to be of alkanes, isomeric alkanes, alkenes, cycloalkenes, and aliphatic and aromatic hydrocarbons. The pre-incubation TPH content of the untreated sludge was 495.63 g/kg with a spectrum of hydrocarbons ranging from C₃ to C₂₂ illustrated in Figure 7. The residual TPH content of the sludge on the 28th day after incubation with three

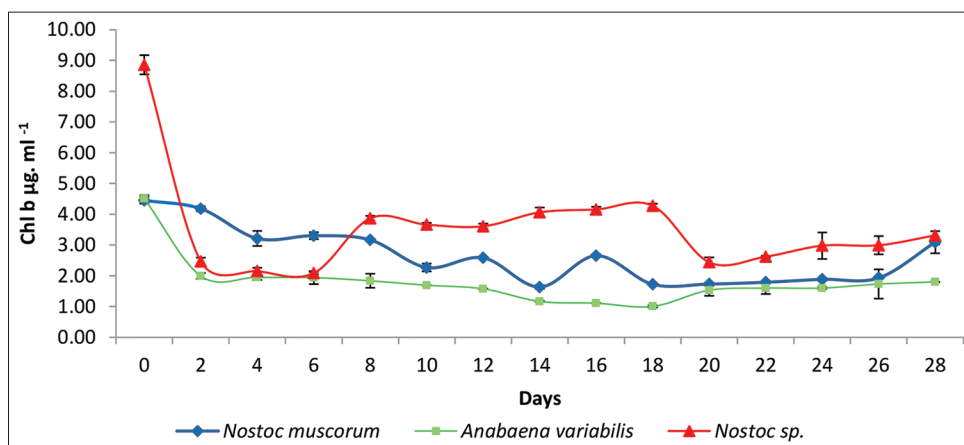


Figure 4: A graphical representation of the chlorophyll b concentration of the three cyanobacterial cultures treated with tank bottom sludge on the 28th day of incubation. Individual cultures were duplicated with the respective $\pm$ standard deviation values and $P < 0.05$.

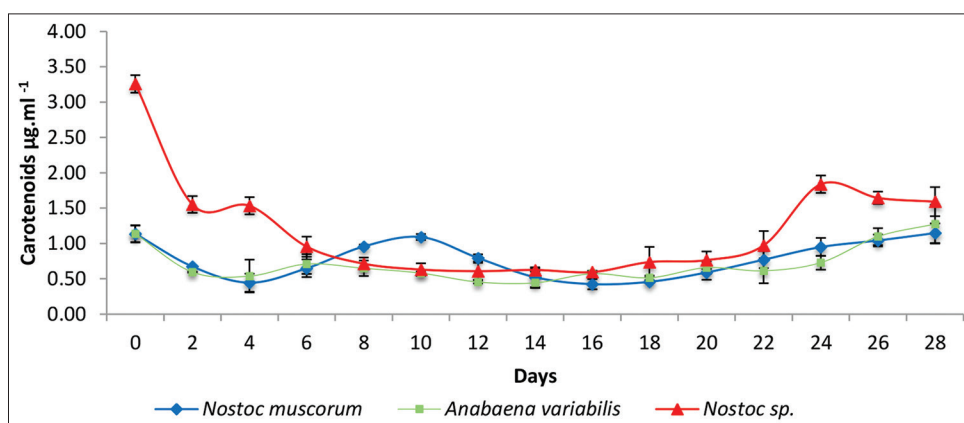


Figure 5: A graphical representation of the carotenoids concentration of the three cyanobacterial cultures treated with tank bottom sludge on the 28th day of incubation. Individual cultures were duplicated with the respective $\pm$ standard deviation values and $P < 0.05$.

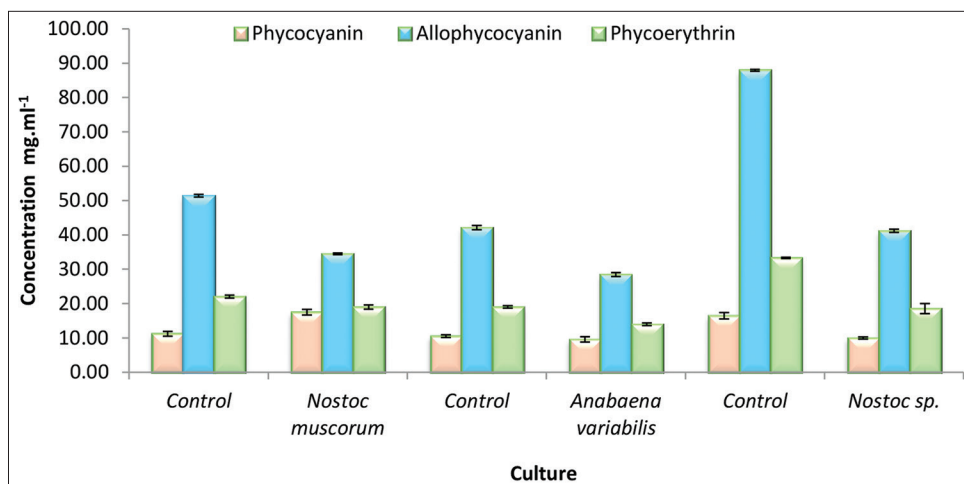


Figure 6: A graphical representation of the phycobilisomes protein concentration of the three cyanobacterial cultures treated with tank bottom sludge on the 28th day of incubation. Individual cultures were duplicated with the respective $\pm$ standard deviation values and $P < 0.05$.

cyanobacterial cultures was obtained to be 29.01 g/kg chla.mg/mL for *N. muscorum*, 0.57 g/kg chla.mg/mL for *A. variabilis*, and 0.62 g/kg chla.mg/mL for *Nostoc* spp., respectively. Subsequently, *N. muscorum* exhibited a TPH reduction of 94.14% followed by *A. variabilis* with reduction of 99.88%, and *Nostoc* spp. with 99.87% TPH reduction

[Table 1]. Evidently, the strain of *Nostoc* spp. performed most efficiently with a reduced hydrocarbon spectrum of C_3-C_{12} , followed by *A. variabilis* with C_3-C_{12} but a slightly higher total peak area than the former and *N. muscorum* with C_3-C_{22} with a highly reduced total peak area from that of the untreated sludge sample. Thus, in addition

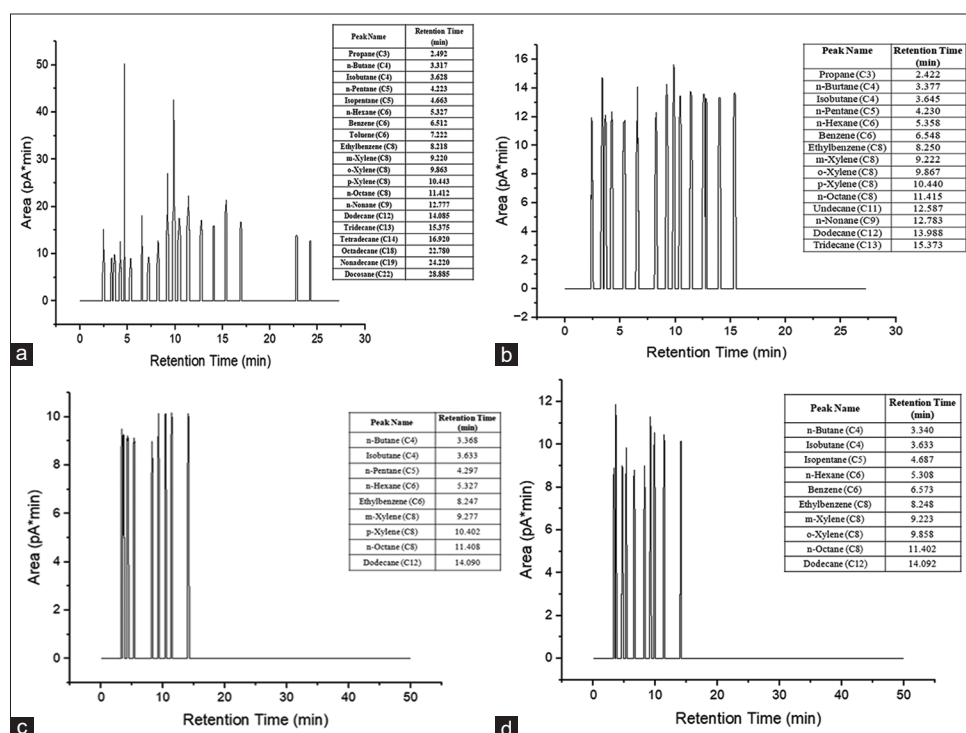


Figure 7: Gas chromatography flame ionization detection chromatogram representing the total petroleum hydrocarbon of (a) Sludge; (b) sludge treated *Nostoc muscorum*; (c) sludge treated *Anabaena variabilis*; (d) sludge-treated *Nostoc* spp. on the 28th day of incubation

Table 1: The reaction rate per cent on the 28th day, reaction kinetic equation, and their respective determination coefficient values by the three treatment cultures, having “y” as the total petroleum hydrocarbon concentration.

Culture	Sludge degradation rate %	Kinetic equation	R ²
<i>Nostoc muscorum</i>	94.14	$y = -2368.x + 94486$	0.99
<i>Anabaena variabilis</i>	99.88	$y = -3094.x + 91226$	0.993
<i>Nostoc</i> spp.	99.87	$y = -3134.x + 92520$	0.99

to these numerical figures, the reduced peak area in the obtained GC-FID chromatogram further contributes to the inference that these cyanobacterial strains are significantly capable of biodegrading the long-chain hydrocarbons present in the tank bottom sludge waste. This describes the potential of these cyanobacterial strains to bioremediate these refinery-generated tank oil sludge, as exhibited in the mentioned frame of incubation, on upscaling. The TPH reduction was further investigated for biodegradation kinetics for all three sludge-treated cyanobacterial cultures [Figure 8], which demonstrated a first-order degradation kinetic model, where the TPH concentration decreases exponentially over time. This suggests that the rate of hydrocarbon degradation is proportional to the remaining TPH concentration as depicted from the eq 1, 2, and 3 which also confirms that the steeper degradation curve results in the higher rate constant (k).

Further analysis of the kinetic model also revealed that *A. variabilis* and *Nostoc* spp. followed a faster first-order biodegradation process compared to *N. muscorum*, making them more efficient candidates for sludge bioremediation.

3.5. Enzyme Assays

The biodegradation ability of the cyanobacterium *N. muscorum*, *A. variabilis*, and *Nostoc* spp. was further experimented with the

activity of some of the important enzymes as an indication of a functional biochemical and metabolic ability of microorganism that has managed to possible hydrocarbon utilization [Figure 9].

3.5.1. PPO

Oxidation of aromatic compounds in nature is an enzyme-dependent process, performed by a group of copper enzymes, PPO [25] with two major varieties: tyrosinases (EC 1.14.18.1) and laccases (EC 1.10.3.2) [26]. The activity of PPO enzyme in the present study exhibited accelerated growth upon treatment with sludge, confirming the better hydrocarbon utilization. On the 28th incubation day, an increase of 22.71%, 15.07%, and 5.48% was observed in the PPO activity by *N. muscorum*, *A. variabilis*, and *Nostoc* spp., respectively. Thus, it corroborates the fact that the ability of cyanobacteria to tolerate as well as remove phenol from different systems within days [27].

3.5.2. Catalase

The confrontation ability of filamentous cyanobacteria *Nostoc punctiforme* ATCC 29133 (*Nostoc* 29133) and *Anabaena* spp. PCC 7120 (*Anabaena* 7120) toward reactive oxygen species (ROS), by virtue of intrinsic constitutive catalase activity [28]. Catalase was identified as a biochemical marker of oxidative stress against hydrogen peroxide with a reported high activity in the marine cyanobacterium *Synechococcus aeruginosus* [29]. The catalase activity in the present study was recorded by either the presence or absence of effervescence, thus taken to be a qualitative physical parameter to mark the functionality of the enzyme in the cultures. This was due to the lack of a scalable amount of effervescence evolved in the reaction assay. On day 28th, the cultures, both treated and positive controls of the three cyanobacterial strains, exhibited a functional catalase enzyme metabolism, thus adding up to the inference of these cyanobacteria being capable of withstanding an oxidative stress that the tank bottom sludge could possibly pose.

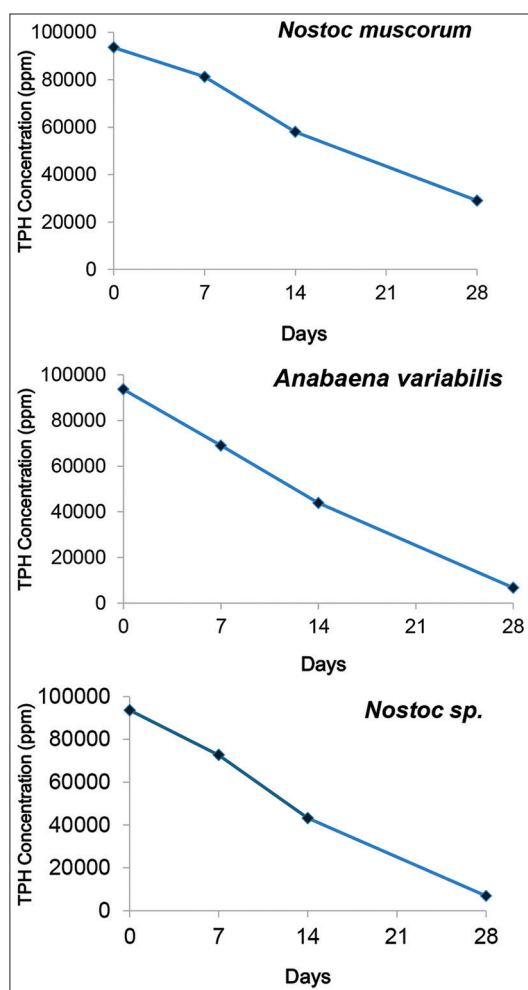


Figure 8: Graphical representation of the biodegradation reaction kinetics by the three cyanobacterial cultures.

3.5.3. Lipase activity

The screening of strains with a nature of degrading oil is determined by an important reference of lipase activity. Its role has been found to be indispensable for biodegradation analysis of oily wastes such as cooking oil [30,31]. The presence of ester bond-cleaving factor (or factors) in cultures composed of *Acinetobacter lwoffii* and *Pseudomonas chlororaphis* PA23 strains, supplied with substrates of p-nitrophenyl-fatty acid and various subunit compositions of PHA polymers [32]. In the present study, a positive, although decreased, activity of lipase was encountered by the three cyanobacterial cultures treated with tank bottom sludge waste. By the completion of the incubation period, the lipase activity by sludge-treated *N. muscorum*, *A. variabilis*, and *Nostoc* spp., decreased was by 10.26, 15.49, and 17.00 percent, respectively, as compared to their positive control cultures. It could therefore be inferred that an active lipase enzyme system present in these cultures could be facilitating the degradation of the sludge added to the cultures and that its activity decreases with the decrease in the concentration of sludge due to the degradation.

3.5.4. Esterase activity

An esterase activity depicts constant increase during the process of degradation of the dimethyl phthalate (DMP) [33], a kind of phthalic acid esters that are classified as a refractory organic plasticizer compound [34-36]. Although the esterase enzyme activity like that of PPO and lipase, in comparison to the dehydrogenase enzymes,

Table 2: A cluster of hydrocarbons and accessory compounds and their biodegradation indices detected through gas chromatography-mass spectrometry of the untreated and the treated oil sludge samples on the incubation for 28th days.

Class	Compound type	ΣCompounds untreated (mg/mL)	ΣCompounds treated (mg/mL)	Biodegradation index (%)
Saturated	Aliphatic hydrocarbon	24	10	58.33
	Cyclic hydrocarbon	2	0	100.00
Unsaturated	Cyclic hydrocarbon	6	3	50.00
	Amide	1	0	100.00
Other functional groups	Ester	5	0	100.00
	Anhydride	1	0	100.00
	Alcohol	2	0	100.00
	Terpenoid	1	0	100.00
	Acidic vitamin	1	0	100.00
Sulfurous compounds	-	15.48	1.48	90.43
Polycyclic aromatic hydrocarbons	Naphthalene	2	0	100.00
	Phenanthrene	5	0	100.00
	Pyrene	1	0	100.00

was observed to accelerate greatly, the recorded activity of the treated cultures was slightly lesser than that of the positive control on completing the incubation. The variation in the activities could be due to the amount of specific enzymes, esterase being produced in the maximum amount, required to be produced by the cells in accustoming to the sludge samples it has been exposed to. The decrease observed was 2.52%, 19.31%, and 7.55% by *N. muscorum*, *A. variabilis*, and *Nostoc* spp., respectively. A similar observation was reported on microalgal cells of *Pseudokirchneriella subcapitata*, with an optimal intracellular esterase activity, met a 20 times higher toxic effect on exposure to oil sands process-affected water than to oil sands exposed to water, and a simultaneous stimulating effect on its photosynthetic function parameters [37]. From this output, it can be inferred that although a decrease, a highly functional enzymatic metabolism is performed by the three cultures, thereby supporting the possibility of efficient biodegradation of tank bottom sludge.

3.5.5. Dehydrogenase enzyme

The dehydrogenase enzyme assay is a prominent index for assessing microbial activity. In this study, the activity was observed to augment in all three treated cyanobacterial strains in comparison to the positive controls, by the 28th day of incubation. *A. variabilis* turned to have the highest activity of $0.69 \pm 0.03 \mu\text{mol/L}$ with an increase of 29%, followed by *N. muscorum* with $0.63 \pm 0.02 \mu\text{mol/L}$ with an increase of 15% and *Nostoc* spp. with $0.28 \pm 0.00 \mu\text{mol/L}$ with an increase

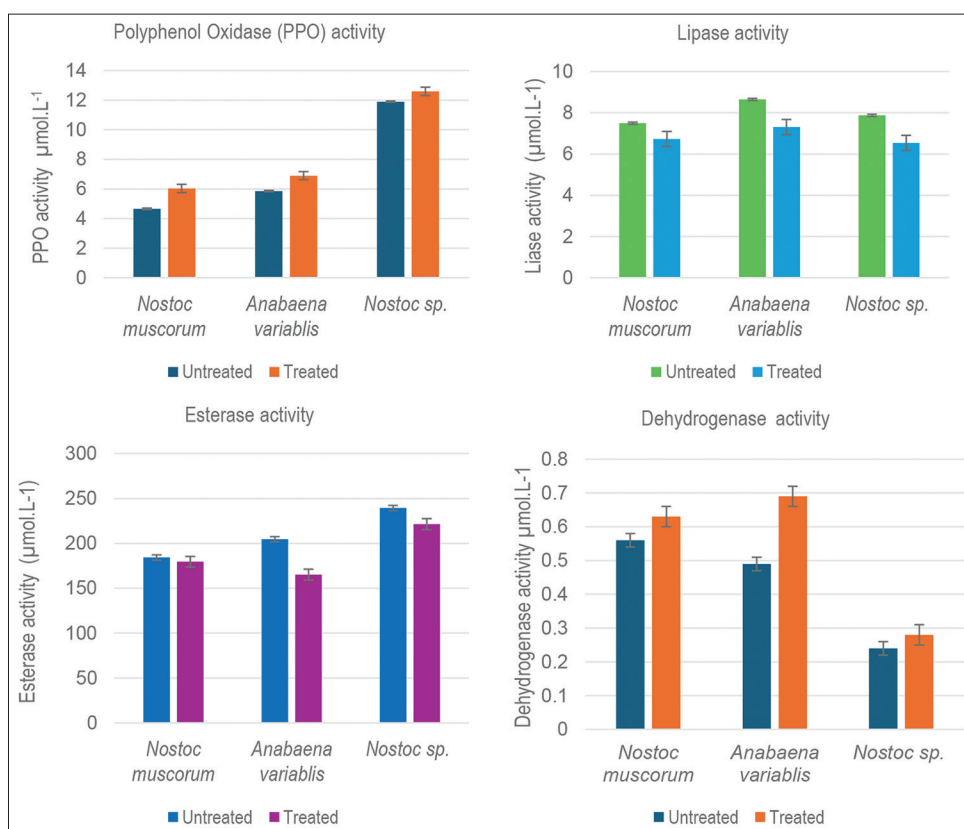


Figure 9: The enzyme activities of the three sludge-treated cultures on the 28th day of incubation. The obtained values in a sample size $n=6$, in duplicates, are represented with their respective $\pm$ standard deviation values.

of 12% from its respective positive control. Thus, in concurrence with an increased dehydrogenase activity by bacterial strains for the biodegradation of weathered hydrocarbons in engine oil-treated soil, it can be concluded that the studied cyanobacterial strains in this study do have the potential to degrade the tank bottom sludge introduced in the culture [38].

3.6. GC-MS Analysis

Based on the holistic inference, there exists no second thought that the biodegradation potential of *Nostoc* spp. is highly efficient in response to sludge hydrocarbon removal in comparison to the other cyanobacterium *N. muscorum* and *A. variabilis*. Thus, the processed sample from the untreated and the treated oil sludge by the culture of *Nostoc* spp. was investigated further through GC-MS. The samples were prepared with the same protocol depicted for GC-FID analysis and were sent to Zibsar Biotech, Guwahati, Assam, for GC-MS analysis. The analysis suggests that the biodegradation efficiency of various organic compounds, including hydrocarbons, sulfurous compounds, and polycyclic aromatic hydrocarbons (PAHs), described as before and after treatment in Table 2, is up scalable.

The biodegradation efficiency of different classes of compounds was assessed by comparing their concentrations in untreated and treated samples with aliphatic hydrocarbons exhibited moderate degradation (58.33%), while cyclic hydrocarbons were completely degraded (100%). The difference suggests that straight-chain alkanes may require more time or optimized conditions for complete breakdown. Cyclic hydrocarbons showed partial degradation (50%), indicating possible resistance to cyanobacterial breakdown and esters, anhydrides, alcohols, terpenoids, and acidic vitamins were fully degraded (100%),

demonstrating strong microbial enzymatic activity. Furthermore, the sulfurous compounds exhibited high degradation efficiency (90.43%), suggesting the presence of effective sulfur-metabolizing microbes and polycyclic aromatic hydrocarbons (PAHs) demonstrated complete degradation of naphthalene. Thus, from the obtained analysis, the culture's ability is determined in regard to the objective of biodegradation of the sludge on incubation in the suitable culture medium, implying its reliability in being replicated on a larger scale.

4. DISCUSSION

Cyanobacteria by virtue of their stress management ability were reported for enhanced carotenogenic properties, facilitating the storage of highly cited carotenoids in the cells [39]. In addition, the phycobiliproteins were specifically reported for their exclusive occurrence in these blue-green algae [40]. The photosynthesis-aiding pigments, chlorophyll, present in the cyanobacterial cells are routinely employed for the growth monitoring and assessment of the cultures. Thus, in addition to the enzyme assays and GC-FID, this pigment profile was regularly monitored for 28 days to make a holistic approach for assessing the biodegradation potential of these cultures. In the course of incubation, the three cultures exhibited a specific pattern of initial decrease, followed by a stable increase in the Chl a and Chl b content, with *Nostoc* spp. with the best chlorophyll profile. In terms of the carotenoids, the three strains maintained a coinciding pigment profile, whereas the treated *N. muscorum* revealed a higher PC content than its positive control culture unlike *Nostoc* spp. and *A. variabilis* cultures with a comparatively lower yet decent phycobilisomes protein content than their respective positive controls. Although carotenoids and phycobilins are known to confer stress protection, their decline

post-sludge exposure may signal their consumption during ROS detoxification or repression due to photodamage of these light-harvesting pigments under sludge-darkened or oil-coated reducing photosynthetic pigment pools due to light shading and resource reallocation to conserve energy or redirect metabolic flux toward stress-mitigating enzymatic pathways (e.g., catalase, dehydrogenases) during hydrocarbon bioremediation.

Cyanobacteria being one of the oldest lineages on the biosphere of the earth with having undergone all types of natural and anthropogenic stresses for billions of years have developed an advanced sensory mechanism and highly systematic metabolism [41]. The cellular organizations of these unicellular or multicellular, filamentous microbes enable them to combat the adverse living conditions in the terrestrial or marine environment. This study aimed to enforce the adaptive nature of these blue-green algae to quantify the transformation and degradation of the hydrocarbon composition of tank bottom oil sludge by monitoring their growth and enzymatic action on exposure to this highly toxic waste generated in tons across the globe by oil refineries.

As mentioned earlier, the sludge is composed of both aliphatic and aromatic hydrocarbons, [41] provided an evidence of an “enzymatic apparatus” in cyanobacterial cells suitable for biosynthesis of precursor compounds of aromatic ring-containing substances, thereby suggesting an ability to transform aromatic compounds such as secondary metabolite with the aid of this enzyme system, investigated through GC-FID and GC-MS. Thus, studying these enzyme systems becomes crucial as it serves as an index for quantifying the biotransformation and biodegradation of the tank bottom sludge exposed to the cultures, adding to the innovation aspect of this investigation by having the *A. variabilis* and *N. muscorum* along the *Nostoc* spp. to be reported for the bioremediation of tank sludge from oil refinery sites in particular. It remains an uncommon approach to identify the enzymatic mechanistic in detail because esterase and lipase enzymes are well-characterized in bacteria and fungi, and investigation in cyanobacterial cultures is commonly regarding their biomass or physiological phenomena, lacking intrinsic data on their enzymatic mechanism. The activity of esterase and lipase uncovers the specific catalytic action conducted by the cyanobacteria to remediate the long-chain hydrocarbons, revealing the enzyme systems that are upregulated, thus providing the mechanistic evidence for metabolic capacities, establishing them as biomarkers [42]. Selecting the optimized cells having superiority in the desired cellular functions by determining the enzymatic activity trends could boost the remediation. Cyanobacteria lack a conventional β -oxidation pathway [43] that is common in other microbes. Thus, the identification and quantification of such similar enzymes shall be aiding in illustrating the unique hydrocarbon remediation mechanism displayed by these cultures.

Intracellular enzymes such as PPO are inducible in nature and thus various species of microalgae have been investigated over the years for an induced degradation of phenolic compounds [33]. In fact, the first marine organism to extract PPO from was cyanobacterium *Phormidium valderianum* [26]. Tyrosinase and laccase are the two major varieties of PPO enzyme [26]. It was unveiled that laccase extracted from *Chlamydomonas moewusii* was the key enzyme responsible. Thus, this finding could be correlated with the present study, indicating a 22.71% increase of polyphenol activity by the culture *N. muscorum* on the completion of the incubation period. The observed qualitative and quantitative upsurge in PPO activity upon sludge exposure a strong correlation can be inferred between PAH breakdown efficiency and PPO-mediated aromatic hydroxylation

mimicking laccase/tyrosinase function. The presence of a tyrosinase-like activity capable of catalyzing ring hydroxylation – a preliminary step in PAH degradation is suggested by the upregulated PPO activity.

About all anthropogenic (herbicides, heavy metals, etc.) and natural (desiccation, high-intensity light, salinity, etc.) strains that cyanobacteria have encountered throughout the period of evolution have culminated in oxidative stress in many cases [44]. Numerous enzymes, of which peroxidases and catalases, are the two main families and detoxify the H_2O_2 produced within cells. It is interesting to note that catalases and peroxidases are widely represented in cyanobacteria [45], like glutathione peroxidases and ascorbate peroxidases, which are important in breaking down H_2O_2 in mammals and plants, respectively, as they are broadly present [46]. In agreement with these reports, the treated along with the positive control cultures in this study exhibited a functional catalase enzyme system on the 28th day of incubation, indicating the combating ability of the cultures toward the stress posed by the tank bottom oil sludge.

Lipases are categorized as ester hydrolases capable of cleaving ester bonds along with having a conserved lipase box and the catalytic triad of histidine, serine, and aspartate residues [32]. Microorganisms that produce lipase have a great deal of scientific and practical significance since they may both directly break down waste oil and encourage the breakdown of oily waste. This suggests that the role of lipase is highly significant in the biodegradation of oily wastes like those from kitchens and that it is necessary for the strains of bacteria that can break down waste oil [47]. The function of the lipase in this study for the breakdown of the oily sludge was assessed and on the 28th day, an observed decreased activity was inferred to be proportional with the decrease in the concentration of the sludge broken down during the course of incubation.

A specific group of enzymes, esterase (EC 3.1.1.2), with an ester hydrolysing nature, are essential for a primary attack on xenobiotics such as phthalates and reports indicated three freshwater unicellular cyanobacteria *Cyanothece* spp. PCC7822, *Synechococcus* spp. PCC7942, and *Synechocystis* spp. PCC6803 performing enhanced esterase activity during biodegradation of DMP a precursor of a more toxic metabolic intermediate mono-methyl phthalate causing endocrine disruption and interference in the development and reproductive system of animals as well as humans [33]. These reports justify the significance to study the esterase in the sludge-treated cultures. The observation from the performed assays indicated a reduction in the activity with *N. muscorum*, showing the lowest decrease in comparison to their respective controls. Although a reduction was encountered, it is noteworthy that in comparison to the dehydrogenase, PPO, and lipase enzymes, esterase was contained in a high amount by all the three cultures. In concurrence with the findings [37], it could be inferred that toxicity constituted by the oily sludge was responsible for the decreased activity and that the cultures are composed of a functional esterase enzyme system contributing to the biodegradation of the sludge.

A unique intracellular enzyme in most soil microbes, dehydrogenase, is often used as the key to measure microbial activity as its functionality serves as the estimate of the complete oxidation potential of these microbes [48-50]. This defines the necessity of the dehydrogenase assay in this study, where a rise of 29%, 15%, and 12% was observed by *A. variabilis*, *N. muscorum*, and *Nostoc* spp., respectively, treated with tank bottom oil sludge. The dehydrogenase activity is associated with oxidative transformations of aromatic hydrocarbons by facilitating the hydroxylation and activation before ring cleavage

by the PPOs. Therefore, with the support of these values, inference could be made that the enzyme facilitated the degradation of the sludge positively.

Collectively, the observations continue to support the aim of this study with substantial findings. Significant reductions in the TPH content with the evidence of reduced peak area of the hydrocarbon spectrum of the treated oil sludge on exposure to the three filamentous cyanobacterial cultures, *Nostoc* spp. being the highest efficient from its additional GC-MS inferences suggest that these strains are immensely capable of remediating bulk tank bottom oil sludge for its safe disposal into the environment without giving rise to secondary pollutants or toxins or threat to surrounding living organisms or deteriorating the soil or water quality. The TPH reduction efficiency for the cyanobacterial cultures displayed as high as >99% by *A. variabilis* and *Nostoc* spp., having *N. muscorum* lagging slightly at ~94%. The reasons like ineffective enzyme isoforms or affinities for the spectrum of the hydrocarbons in the tank sludge, or inadequate expression of the co-factors and transporters, or physiological attributes such as cell wall permeability, formation of microcolonies, or intracellular storage limiting efficient uptake, processing, or excretion of degradation products could be causing the lag in the bioremediation of the sludge by the *N. muscorum* culture. The total degradation of cyclic hydrocarbons (100%) compared to the limited degradation of aliphatic hydrocarbons (58.3%) specifies the substrate-specific enzymatic affinity in the cyanobacterial cultures. This significant disparity in degradation due to the substrate-specific limitations in the cyanobacterial enzymatic repertoire aids in oxidoreductive cleavage of ring structures over linear alkanes, which typically demand monooxygenase systems absent in cyanobacteria. This is because these are more susceptible to enzymatic attack by PPOs and dehydrogenases, which were shown to be significantly upregulated in this study, especially in *A. variabilis* and *Nostoc* spp. The limited beta-oxidation pathway in cyanobacteria [43] affects the efficient breakdown of long-chain aliphatic hydrocarbons. Hence, the partial degradation of saturated aliphatics – despite otherwise high TPH removal rates (>99%) – can be attributed to this biochemical bottleneck in enzymatic compatibility with long-chain alkanes, or suitability of the linear hydrocarbons for their suitability as nutritional sources, intensifying the cellular growth [51-53]. The high degradation rates by *Nostoc* spp. and *A. variabilis* highlight the potential that these cyanobacterial cultures surpass microbial consortia. A 85–92% TPH reduction was asserted by Hamouda *et al.* [1] using an algae consortium – indicating that these particular cyanobacteria strains perform better or at least comparably well individually than typical multi-algal systems documented in literature.

It is a holistic study designs that lead to the unveiling of the strengths and the lag of the catalytic machinery through enzymatic, cellular physiological adaptation, and regulating parameters. Thus, it is a substantial step forward in depicting the potential of these cyanobacterial cultures in the objective of bioremediation that is up scalable for a practical appliance.

5. CONCLUSIONS

The present study underscores the potential of bioremediation as a sustainable approach in combating refinery waste using cyanobacteria as a cost-effective methodology. In addition, the present study emphasizes on the optimizing environmental conditions to enhance the degradation rates and exploring metagenomics and enzyme profiling for deeper insights into biodegradation pathways.

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7. AUTHOR'S CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

10. DATA AVAILABILITY

All data generated and analyzed are included within this research article

11. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors confirm that there was no use of artificial intelligence (AI)-assisted technology for assisting in the writing or editing of the manuscript and no images were manipulated using AI.

12. PUBLISHER'S NOTE

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