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Impact of Effluent Treatment Plant Sludge on Growth, Physiology, and Biodegradation Potential of Cyanobacteria: *Anabaena variabilis*, *Nostoc muscorum*, and *Nostoc* sp.

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ABSTRACT

Organic compounds and pollutants from industries like oil refineries, such as total petroleum hydrocarbon (TPH) and polycyclic aromatic hydrocarbons (PAHs) are known to pose toxic effects to the environment by degrading the soil and water quality along with the deposition of heavy metals thereby causing a threat to the life forms existing in them. This pose of imbalance in the ecosystem calls for an exigent notice and effort regarding controlling it. Hydrocarbon sludge from Effluent Treatment Plant (ETP) is one such toxic effluent eluted from oil refineries, and that is yet to be reported for a biodegradation study. Among a huge number of physical and chemical techniques, bioremediation has been a much talked about measure but is not in the required scale of practice yet. The reason could be a lack of efficient standardization for environmental applications. Cyanobacteria being well-known for their ability to survive difficult environmental conditions efficiently and to adapt to a mixotrophic nature, makes them ideal for performing bioremediation at a large scale in the open environment. The study aimed to quantify this ability of cyanobacteria by assessing the TPH content of the treatment sample, Effluent Treatment plant (ETP) hydrocarbon sludge pre- and post-treatment using GC-FID. The study was designed to treat the sludge by cyanobacterial strains in a pre-determined lethal dose concentration and monitor the activity of enzymes vital for a basic degradation metabolism, in addition to the growth rates of the cultures. The figures obtained from the enzyme assays and the growth rates appeared to be collateral in the direction of it being a highly promising bioremediation approach that could be efficiently performed by increasing the scale of application. The chromatograms obtained from the GC-FID depicted significant reductions in the TPH content of the treated samples that strongly indicated the potential of the cyanobacterial cultures to bioremediate an oil-contaminated site or treat toxic effluents from oil refineries.

Key words: ETP, TPH, enzyme assays, GC – FID, Cyanobacterium, biodegradation.

INTRODUCTION

Petroleum industries generate a massive amount of sludge, approximately more than 28,220 t/yr, in India. This sludge eluted from the Effluent Treatment Plant (ETP), consists of both aliphatic and aromatic hydrocarbons, Total Petroleum Hydrocarbon (TPH), and heavy metals like nickel, chromium, zinc, manganese, cadmium, copper, and lead (An and Huang 2012, Bhattacharya and Shekdar 2003). The United States Environmental Protection Agency (EPA) has listed many of these components as priority pollutants due to their toxic, mutagenic, and carcinogenic properties (Janbandhu and Fulekar 2011). Due to the complex mixture of hydrocarbons and heavy metals, which adversely impact the environment, degradation in natural ecosystems is still complicated. As a result, various approaches, including chemical, mechanical, and biological methods, have been employed as remediation strategies for the cleanup process. Research activities in biodegradation exploiting microorganisms have shown advantageous performance with relatively low capital and maintenance costs, simple design, and operation (Ouyang et al. 2005). However, the microbial strains used in this process are normally not screened and domesticated, which limits the degradation efficiency of oily sludge.

To enhance process efficiency, bioremediation technology has been examined. Based on the composting method, screened and optimized bacteria with high-efficiency degradation are employed to enhance the treatment effect while effectively shortening the treatment time. A variety of microorganisms have been reported capable of using petroleum hydrocarbons as the sole carbon and energy source, such as *Pseudomonas*, *Achromobacter*, *Candida digboiensis*, *Micromonospora*, *Bacillus subtilis*, cyanobacteria, and *Leuconostoc mesenteroides* (Cerqueira et al. 2012, Joo et al. 2008, Ozyurek and Bilkay 2020, Sood et al. 2010).

Cyanobacteria are oxygen-evolving photosynthetic prokaryotes that are known to grow in extreme environments (Thajuddin and Subramanian 2005). Several reports have well documented the ability of cyanobacteria to oxidize oil components. Studies on *Oscillatoria* sp. and *Agmenellum quaduplicatum* demonstrated their ability to oxidize naphthalene to 1-naphthol (Cerniglia et al. 1979). Other studies showed that *Oscillatoria* sp., strain JCM can oxidize biphenyl to 4-hydroxybiphenyl and *Agmenellum quaduplicatum* metabolizes phenanthrene into trans-9,10-dihydroxy-9,10-dihydroxyphenanthrene and 1-methoxy-phenanthrene (Narro et al. 1992). Several other strains were reported to degrade other complex organic compounds, such as surfactants and herbicides. Radwan and Al-Hasan (2000), Raghukumar et al. (2001), Mansy and El-Bestawy (2002), Hoffmann (1996), Hopner et al. (1996), and Grotzschel et al. (2002) demonstrated that cyanobacterial mats were rich in oil-degrading bacterial strains and were capable of degrading petroleum compounds. There is no doubt that cyanobacteria play an important role in the microbial mat by establishing oxygen gradients and supplying nutrients for heterotrophic bacteria. Factors like ubiquity, small size, high rate of reproduction, and large surface-to-volume cell ratio make these microbes an ideal depolluting agent (Cepoi et al. 2016).

Despite many reports showing the ability of cyanobacteria to degrade various organic compounds, including certain constituents of ETP hydrocarbon sludge, convincing proof and detailed investigation are still lacking. The current state of disposal measures of sludge and waste products from industries, particularly oil refineries, is a significant concern for the environment. The need for proper treatment and disposal of these pollutants is of utmost importance due to the hazardous and toxic composition of these effluents.

The organisms employed for this study are cyanobacterial strains, namely *Anabaena* and *Nostoc* species, because they are the most extensively studied cyanobacteria from this perspective. They have been reported to remediate areas contaminated with heavy metals, dyes, and PAHs significantly (Gupta et al. 2012). These bacteria are well-known for their nitrogen fixation capacity and were chosen for their ability to grow in difficult and adverse environments. By their mixotrophic nature, cyanobacteria are capable of metabolizing various organic substrates, including persistent organic pollutants, breaking them down into less toxic or non-toxic substances and using them as nutrients (Cepoi et al. 2016). Thus, it adds to the advantage of using screened cyanobacterial strains to remove these organic pollutants from the environment without causing secondary pollution, while also providing an additional source of nutrition.

MATERIAL AND METHODS

Study area

The ETP hydrocarbon sludge was collected from the Numaligarh Refinery Limited (NRL) (26.5786° N, 93.7848° E) plant, District Golaghat, Numaligarh, Assam.

Cyanobacterial samples

The axenic cyanobacterium *Anabaena variabilis*, *Nostoc muscorum* and *Nostoc* sp. cultures which are routinely maintained in the laboratory, were employed for the present study. The cultures were maintained in the standard $BG11_0$ medium in an air-conditioned chamber with a photon fluence rate of 50 µmol m^{-2} s^{-1} at 24 ± 2°C and control illumination of 12 hrs light and 12 hrs dark (Rippka et al. 1979). Two purified cyanobacterial cultures were identified morphologically as *A. variabilis* and *N. muscorum* and molecularly identified by obtaining their 16S rRNA identification sequence (Jungai and Adhikari 2015). The partial sequence was submitted to the gene bank and accession numbers were obtained to be KR709140 and KR709143, respectively.

Microscopy study

The cultures were viewed under the Radical RXL-4B light microscope with a digital camera under 45X and were observed based on their morphological features such as cell size and shape, dimensions, division planes, colour, shrath and motility and cell specialization features such as heterocyst formation, akinetes, baecytes and necridia formation of the control and the treated ETP-cyanobacteria cultures.

Determination of ETP hydrocarbon sludge lethal dose concentration

Cells in their exponential growth phase were inoculated in $BG11_0$ medium supplemented with graded concentrations of ETP hydrocarbon sludge (0.5, 1, 2, 3, 4, 5 to 50 mg/L) for *A. variabilis*, *N. muscorum* and *Nostoc* sp., respectively. The ETP sludge, due to its sticky nature, was dissolved in n-hexane in the ratio of 1:1. Lethal dose concentration values were subsequently determined in terms of optical density (at 663 nm) on the 28th day (Negi et al. 2019).

Assessment of cyanobacterial growth

The Chlorophyll a, Chlorophyll b and Carotenoids content was measured as per methods developed by Sengar (1970) with slight modifications. The absorbance of the extracted pigments was measured by Spectrophotometer at variant wavelengths and the total chlorophyll a, b and carotenoids were calculated based on the method depicted by Li et al. (2022).The phycobillisomes (phycoerythrin (PE), phycocyanin (PC) and allophycocyanin (APC) pigment) of each of the cultures (treatment and control cultures) were determined as per Bennett and Bogorad (1973) and Bryant et al. (1979). The culture suspension was centrifuged at 4000 rpm followed by washing of pellet with 1M phosphate buffer. The pellets were mixed with methanol and heated in a water bath for 30 min. The Phycobillisomes were quantified spectrophotometrically at the appropriate wavelengths.

Enzyme assays

The enzyme extracts were obtained following the protocol outlined by Suganthi et al. (2018), with minor adjustments. For the purpose of purification, the extracts were subjected to a 60% ammonium sulfate precipitation to then resuspend in 50 mM phosphate buffer. It underwent further purification through a 12 hr dialysis process against a phosphate buffer, with buffer changes performed at 3-hour intervals. Following this, concentration was achieved using barium chloride. The resultant enzyme extract was subsequently subjected to assays for lipase, catalase, esterase, dehydrogenase, and polyphenol oxidase activity.

Dehydrogenase *[EC1.1.1.1]* activity was detected by the method described by Marvin et al. (1960). The activity of dehydrogenases in the enzyme extracts obtained from the three cyanobacterial-ETP hydrocarbon sludge treatment cultures and the positive controls was determined with the help of a standard curve. The activity of Lipase *[EC 3.1.1.3]* was determined with reference to the protocol developed by Válek (2021). The assay was performed having slight modification with an increase in the reaction volumes. The measurement of the unknown polyphenol oxidase (PPO) *[EC 1.10.3.1]* activity was determined using a calibration curve following the protocol by Mayer and Harel (1980). Catalase activity *[EC 1.11.1.6]* of the cultures was performed using the methods as per Iwase et al. (2013). The calibration curve for esterase *[EC 3.1.1.8]* was obtained by following the protocol as described by Peng et al. (2016). The extracellular esterase activity was determined with slight modifications in the protocol.

TPH biodegradation ability of cyanobacterial strains

The determination of TPH content was performed using the protocol developed by Suganthi et al. (2018) with slight modifications. The samples of the untreated and treated ETP sludge were extracted using HPLC grade hexane solvent (1:1 ratio) followed by centrifugation at 15000 rpm for 5 min. The pellet was sonicated for 90 min with 5 sec on and 2 sec off intervals, using a 6 mm diameter titanium probe of an Ultrasonic processor (Rivotek, India). The solvent layer containing the TPH portion was separated using the separating funnel. In order to remove any TPH present in the flask, this procedure was repeated twice. A rotary evaporator (IKA, Germany) was used to extract petroleum hydrocarbons in hexane.

The TPH levels in both the cyanobacteria treated and untreated ETP sludge were determined by a Flame - Ionization Detector equipped Gas Chromatograph (GC-FID, Thermofisher TRACE 1600 Series) using an TG-WAXMS GC COL capillary column with dimensions of 30 m × 250 mm × 0.25 µm. Hydrogen was used as a carrier gas with a constant flow rate of 30 mL min^{-1}. The analysis was carried out at 230ºC inlet temperature, 250ºC detector temperature and 190ºC oven temperature run in a spitless mode for 50 min. The amount of sample injected was 2 µL. The analysis was carried out using the Chromeleon 7.2/7.3 software for GC SE (single user, single TF instrument license).

Statistical analysis

The data generated from the experiments were analysed by one-way analysis of variance (ANOVA) through the Tukey-Kramer procedure. Readings were considered significant when the *p*-value was <0.05.

RESULTS AND DISCUSSION

Morphological study

The purified cyanobacterium cultures were identified morphologically as *Nostoc* sp., while the other two cultures were identified using 16S rRNA sequencing as *Anabaena variabilis* and *Nostoc muscorum*. The microscopic images of Cyanobacteria-ETP sludge treatment culture and positive control culture cells are presented in Figure 1. A notable difference was the increased chain length in the treated culture, although the size of the vegetative cells remained within a narrow range. The vegetative cell width of untreated and treated culture of *A. variabilis* was approximately 6.12 ± 0.30 µm and 6.51 ± 1.27 µm, respectively. While for *N. muscorum* it was about 6.15 ± 0.21 µm and 6.21 ± 0.33 µm, respectively, for *Nostoc* sp. it was 6.18 ± 0.30 µm and 6.33 ± 0.18 µm, respectively. The vegetative cell length of the untreated and treated culture of *A. variabilis* was about 7.29 ± 0.27 µm and 6.99 ± 0.51 µm, respectively. While for *N. muscorum* it was about 7.63 ± 0.21 µm and 6.96 ± 0.33 µm, respectively, for *Nostoc* sp. it was 7.18 ± 0.30 µm and 7.27 ± 0.60 µm, respectively. The number of heterocyst cells increased, indicating a direct role in nitrogen fixation and assimilation (Singh et al. 2008). The filaments were straight, with slightly rounder terminal cells, and the mucilage sheath was present in both treatment and control culture cells.

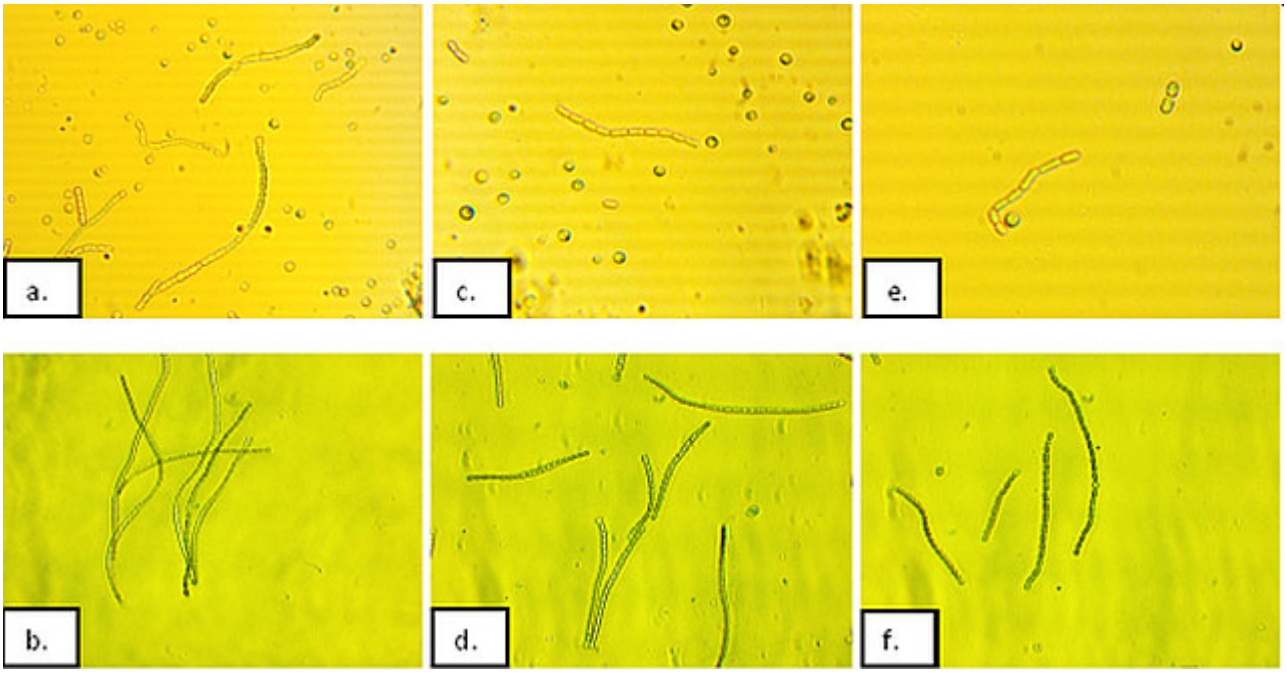


Figure 1. Micrographs of (a.) untreated *Anabaena variabilis*, (b.) treated *Anabaena variabilis*, (c.) untreated *Nostoc muscorum*, (d.) treated *Nostoc muscorum*, (e.) untreated *Nostoc* sp. and (f.) *Nostoc* sp. cultures

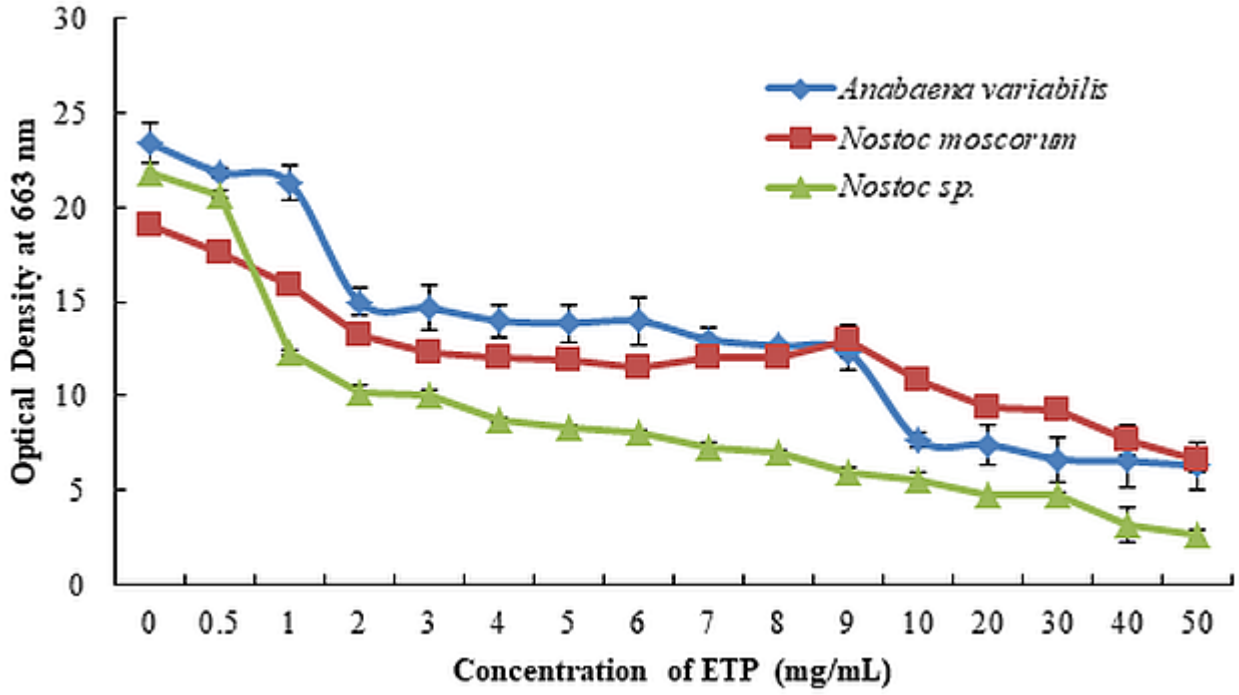


Figure 2. Effect of different concentrations of ETP hydrocarbon sludge on the growth behavior of *Anabaena variabilis*, *Nostoc muscorum* and *Nostoc* sp. cultures taken after 14 days of incubation. The values represent mean ± standard deviation (SD) from two independent experiments with two replicates each, with a *p*-value <0.05

Determination of ETP hydrocarbon sludge lethal dose concentration

The response pattern of the cultures towards the lethal dose concentration of ETP hydrocarbon sludge is depicted in Figure 2. Optimal concentration of 9 mg/mL was found to be sufficiently high for the biodegradation assay without impeding culture growth.

Assessment of cyanobacterial growth

The cyanobacterium growth assessment study in terms of chlorophyll a, chlorophyll b, carotenoids, and the phycobillisome proteins was conducted at a concentration of 9 mg/mL ETP sludge with a total incubation period of 28 days. This is due to the results obtained from the lethal dose concentration determination indicating an optimal growth at 9 mg/mL of ETP sludge concentration in the culture medium. The three ETP sludge treatment cultures demonstrated a positive parallel growth pattern with minimal growth from day 2 to day 9. Distinctly after 10 days, there was a significant increase in the chlorophyll a (Fig. 3a), chlorophyll b (Fig. 3b), and carotenoids (Fig. 3c) content. The phycocyanin content showed a marginal increase in the ETP sludge treatment cyanobacterial cultures, whereas an increase of 25-45% in allophycocyanin content was observed, followed by a slight increase in the phycoerythrin content in comparison to the positive control cultures on the 28th day of incubation (Fig. 4).

All three different cultures employed in the present investigation showed similar behaviour of initial gradual decline, then a slight inclination followed by a sharp descend which indicates the pattern of the cultures of taking the time to adapt to the stress in the beginning, indulged in the form of ETP hydrocarbon sludge and then starting to grow better than the positive control cultures. This behavior leads to the possibility that the cultures start to break down the ETP sludge and utilize the hydrocarbon as an additional source of nutrition. The capacity of cyanobacteria to degrade hydrocarbon were reported by several researchers (Cernglia et al. 1980, Narro et al. 1992, Raghu Kumar et al. 2001, Al-Hasan et al. 1998).

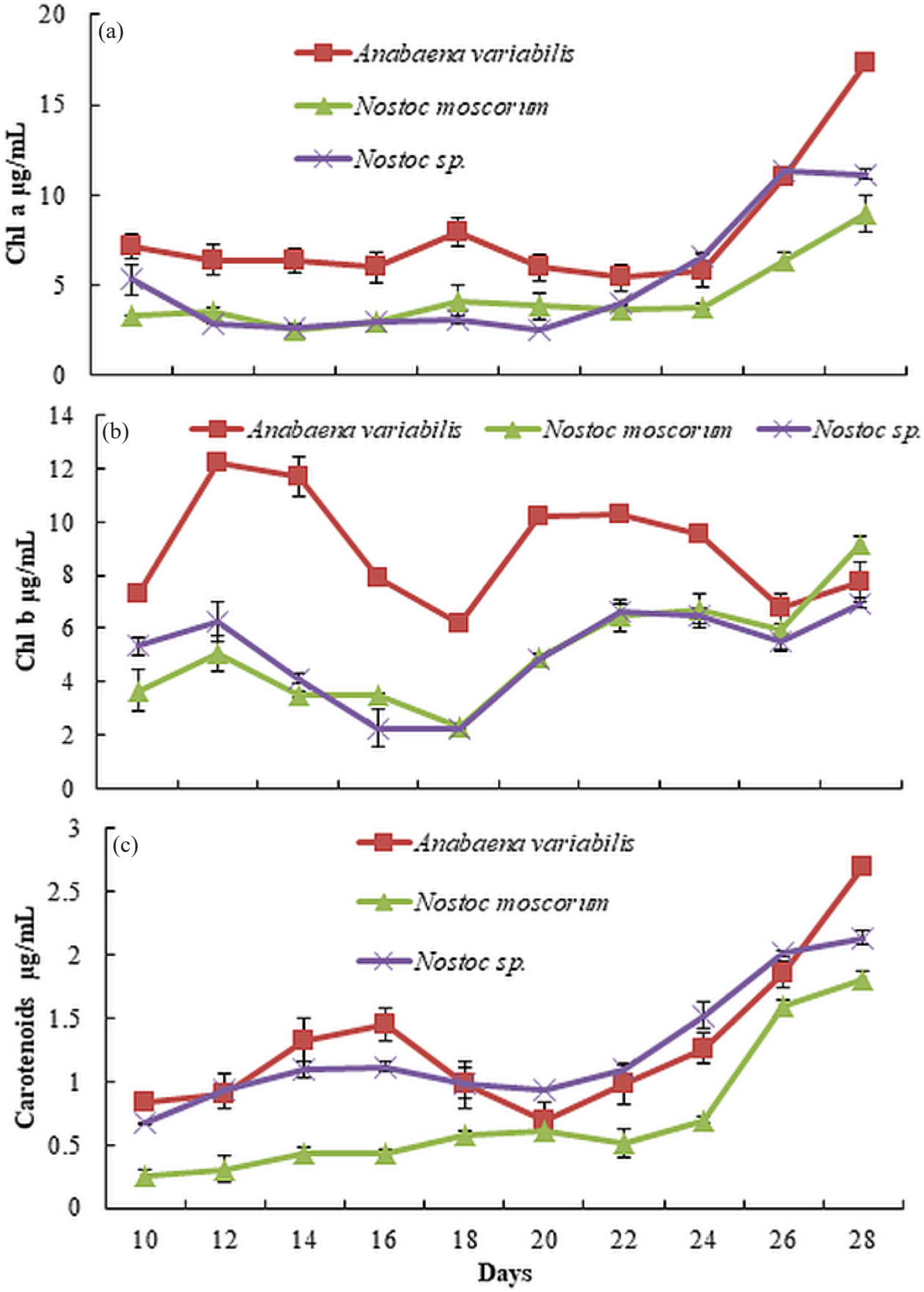
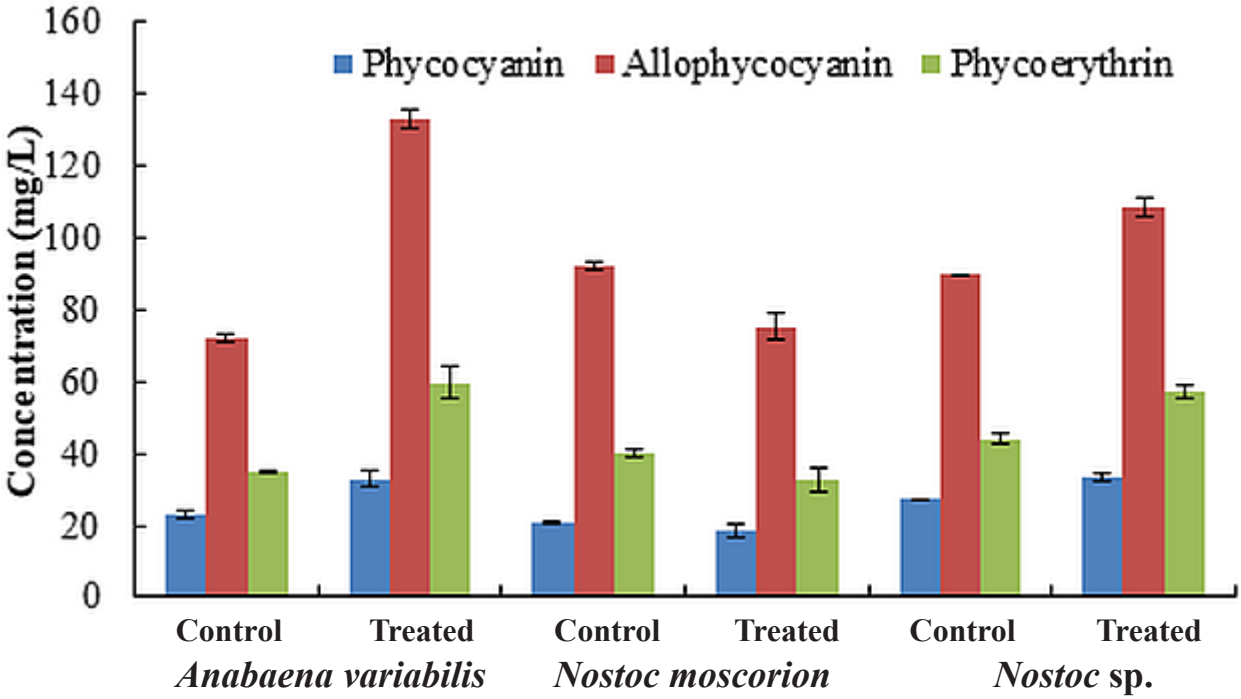


Figure 3. Concentration of (a) chlorophyll a, (b) chlorophyll b, and (c) carotenoids of an *Anabaena variabilis*, *Nostoc muscorum* and *Nostoc* sp. culture treated with ETP hydrocarbon for 28 days.



Enzyme assays

Enzymatic behaviour was analyzed to establish the obtained findings based on the chlorophyll, carotenoids, and phycobillisomes content of the ETP sludge treatment cyanobacterial cultures through assays of dehydrogenase, lipase, esterase, polyphenol oxidase, and catalase (Table 1). In the present study, dehydrogenase activity showed almost 100% increase in all the three cyanobacterium-ETP sludge treatment cultures, in degrading hydrocarbons in comparison to the positive control cultures. Dehydrogenase enzymes, found in cyanobacteria and various other organisms, play a crucial role in various metabolic processes, particularly aiding in hydrocarbon degradation for energy and carbon acquisition. It enhances redox reactions by maintaining electron balance within the cell and facilitating electron transfer between molecules during hydrocarbon degradation. This adaptability allows microorganisms to thrive in challenging environments and promotes the breakdown and utilization of hydrocarbons as a carbon source, which is essential for environmental remediation efforts (Kallberg et al. 2010).

In the present study a decrease in lipase activity can be observed for all the three treatment cultures (Table 1). A slight decrease of 21% is observed in *A. variabilis*, followed by a 22% in *N. muscorum* and a 24% in the *Nostoc* sp. This may probably be due to the limitation of lipase enzymes in degrading complex hydrocarbons and requiring a deeper knowledge and characterization of cyanobacterial lipases. Lipases are enzymes that give rise to glycerol and fatty acids by catalyzing the hydrolysis of triglycerides. They are essential for lipid metabolism and play a crucial role in the degradation of hydrophobic organic compounds like hydrocarbons (Jaeger et al. 1994).

In context to the present study, ETP sludge treating *A. variabilis* and *Nostoc* sp. exhibited an accelerated increase in the PPO activity as compared to *N. muscorum* (Table1). The catalase activity could be attributed as a qualitative physical parameter by the presence or absence of effervescence in the assay due to the low catalase content in the enzyme extract from the cultures. On the 28th day, the catalase activity was observed to be present in all the treatment and control cultures except for the *Nostoc* sp. culture of ETP sludge treatment.

Esterase enzyme activity in *N. muscorum* and *Nostoc* sp. showed a vigorous trend, indicating their cell machinery having undergone an enhanced enzyme activity in transesterification, resulting in the breakdown of complex hydrocarbons. However, in *A. variabilis*, the activity was low on 28th day of incubation with the ETP sludge samples (Table 1).

Table 1. The enzyme activity of *Anabaena variabilis*, *Nostoc muscorum* and *Nostoc* sp. culture treating ETP hydrocarbon sludge, taken on the 28th day. The values represent mean '±' standard deviation (SD) from two independent experiments with two replicates each. '+' and '-' denote to presence and absence of effervescence respectively to mark the catalase activity

Enzyme assay	*Anabaena variabilis*		*Nostoc muscorum*		*Nostoc* sp.	
	Control	Treated	Control	Treated	Control	Treated
Dehydrogenase activity (µmol/L)	0.2 ± 0.01	0.55 ± 0.00	0.27 ± 0.00	0.55 ± 0.01	0.27 ± 0.00	0.69 ± 0.01
Lipase activity (µmol/L)	10.19 ± 0.03	8.07 ± 0.04	8.07 ± 0.02	6.34 ± 0.12	9.80 ± 0.08	7.5 ± 0.01
Esterase activity (µmol/L)	76.66 ± 6.89	28.80 ± 2.43	59.16 ± 0.09	147.5 ± 9.06	20.65 ± 0.49	84.28 ± 3.84
Polyphenol oxidase activity (µmol/L)	0.17 ± 0.02	2.24 ± 0.01	1.55± 0.00	0.34 ± 0.21	0.17 ± 0.06	2.58 ± 0.12
Catalase activity	+	+	+	+	+	-

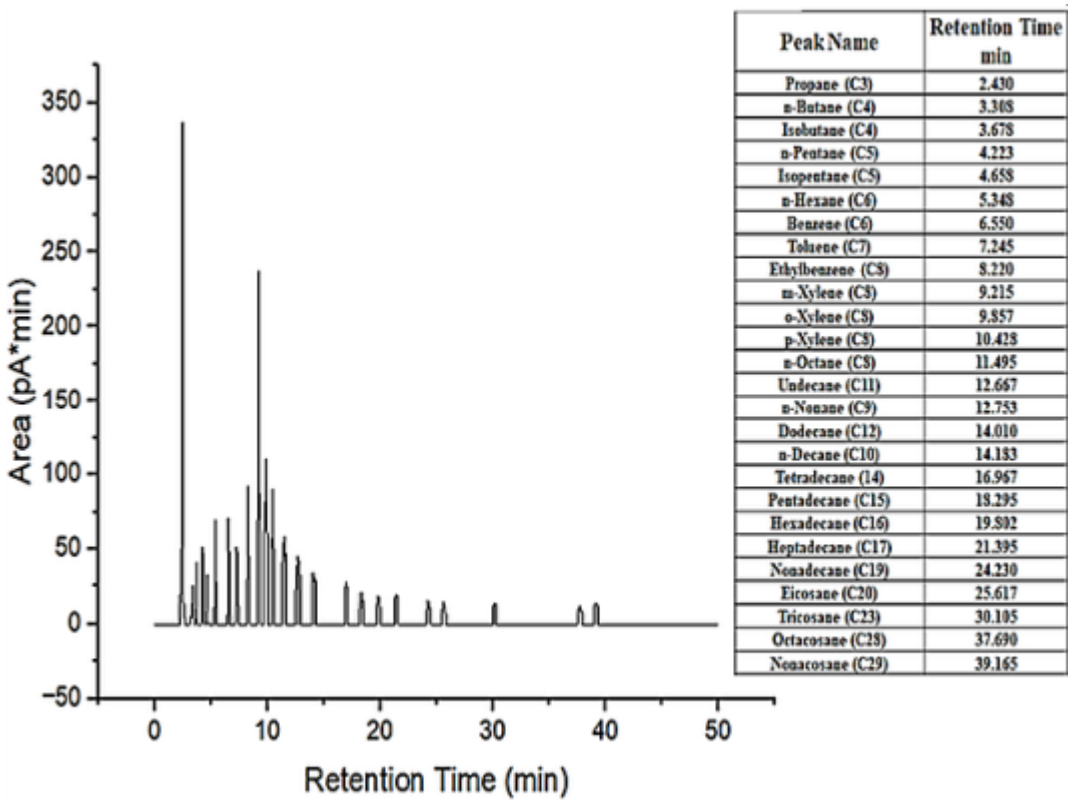
TPH Biodegradation potential of the cyanobacterium species

ETP hydrocarbon sludge characterized by GC-FID showed a chromatogram having numerous peaks indicating the presence of alkanes, alkenes, aliphatic hydrocarbons, cycloalkenes, isomeric alkanes, and polycyclic aromatic hydrocarbons ranging from C_3 to C_{29}. These peaks were gradually reduced by the 28th day when treated with three different cyanobacteria. The initial TPH content in the ETP sludge was detected to be 250.20 g kg^{-1} with hydrocarbon range from C_3 to C_{29} (Fig. 5). The extent of TPH degradation by cyanobacteria in the three treatment cultures is represented in Figures 6, 7 and 8. The TPH content of *A. variabilis* treated ETP sludge was 1.13 g kg^{-1} chl a mg ml^{-1} followed by *N. muscorum* treated sludge being 0.92 g kg^{-1} chl a mg ml^{-1} and *Nostoc* sp. treated sludge being 3.65 g kg^{-1} chl a mg ml^{-1}. The TPH biodegradation capacity of *N. muscorum* (99.62 %) was the highest, followed by *A. variabilis* (99.54%) and *Nostoc* sp. (98.66%). Consequently, the decrease in the TPH content was related to the decrease in the peak area for hydrocarbons on treatment with the three cyanobacteria. The hydrocarbons with higher length carbon chains were degraded, thereby suggesting that the cyanobacteria used can remove higher hydrocarbons and it could be inferred that increasing the incubation further could degrade the residual TPH completely adding to the potential bioremediation activity of these cyanobacteria. This agrees with the report of other researchers using other hydrocarbon sources, although this is the first report on the ETP hydrocarbons removal. Raghukumar et al. (2001) reported the removal of approximately 55% of total fractions of crude oil comprising 50% aliphatics, 31% waxes and bitumen, 14% aromatics and 5% polar compounds by mixed cultures of three cyanobacterial species. These hydrocarbons disappeared within 10 days when measured using gravimetric and gas chromatographic methods.

Species of *Oscillatoria salina, Plectonema terebans, Aphanocapsa* sp. and *Synechococcus* sp. grew as mats in aquatic environments and has been used successfully in bioremediation of oil spills (Raghukumar et al. 2001, Radwan and Al-Hasan 2001, Cohen 2002, Kumari et al. 2012, Ghoreishi et al. 2017). Oil-contaminated soil was also successfully remediated using naturally occurring cyanobacterial association (Sorkhoh et al. 1995). El-Bestawy et al. (2007) tested biodegradation of a well-known chemically and biologically recalcitrant and slow-degrading Lindane using some isolated cyanobacterial species with high proof of efficiency in degrading the pesticide at a very fast rate as well as demonstrating a high level of resistance against its toxicity. Ichor et al. (2014) and Latha and Kalaivani (2012) reported a correlation between increased oil degradation to an increase in the cell number of bacteria indicating that the isolates were responsible for the degradation which corroborates the results of our present findings.

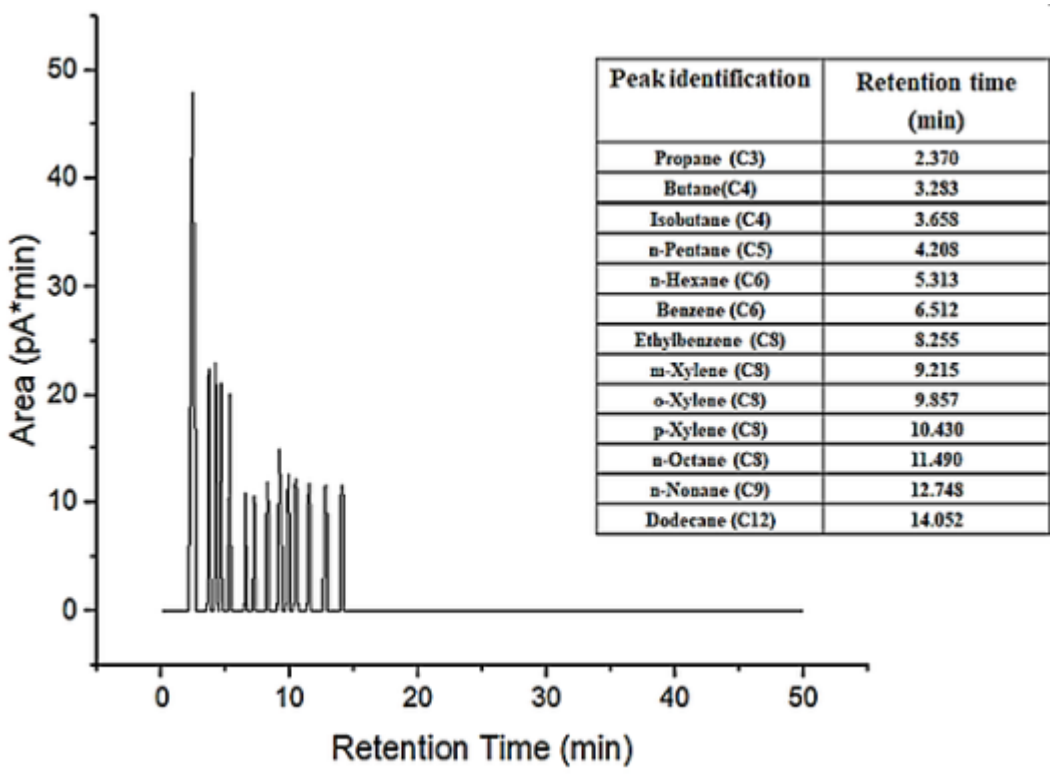
CONCLUSION

Cyanobacteria, due to their ubiquitous occurrence, exhibit excellent models in petroleum byproduct degradation. The present study investigated an effective TPH removal from ETP hydrocarbon sludge by cyanobacterial cultures. After obtaining the



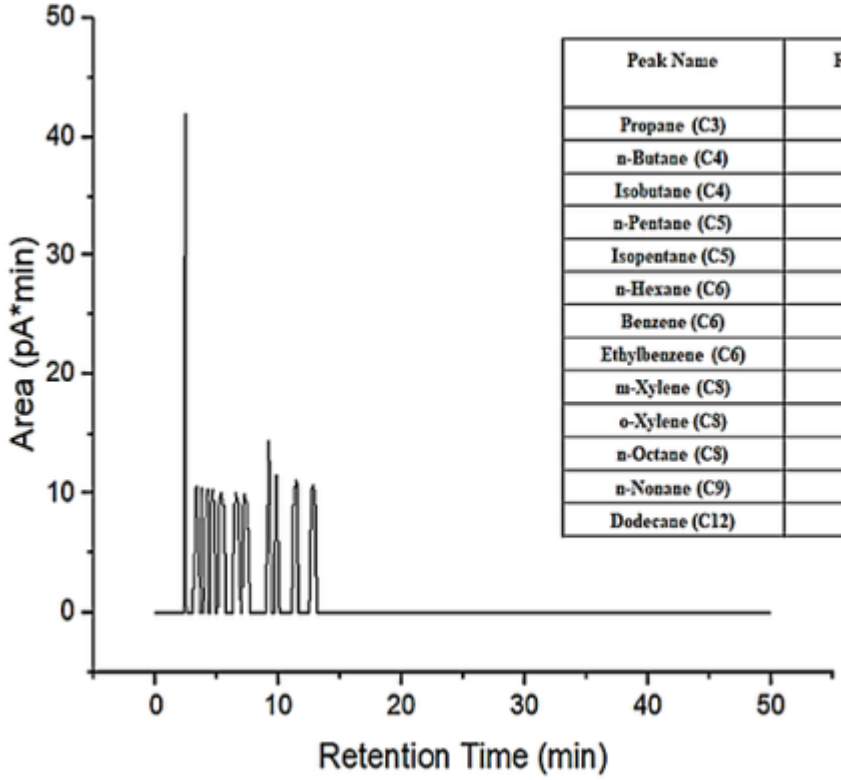
Peak Name	Retention Time min
Propane (C3)	2.430
n-Butane (C4)	3.308
Isobutane (C4)	3.678
n-Pentane (C5)	4.223
Isopentane (C5)	4.658
n-Hexane (C6)	5.348
Benzene (C6)	6.550
Toluene (C7)	7.245
Ethylbenzene (C8)	8.220
m-Xylene (C8)	9.215
o-Xylene (C8)	9.857
p-Xylene (C8)	10.428
n-Octane (C8)	11.495
Undecane (C11)	12.667
n-Nonane (C9)	12.753
Dodecane (C12)	14.010
n-Decane (C10)	14.183
Tetradecane (14)	16.967
Pentadecane (C15)	18.295
Hexadecane (C16)	19.502
Heptadecane (C17)	21.395
Nonadecane (C19)	24.230
Eicosane (C20)	25.617
Tricosane (C23)	30.105
Octacosane (C28)	37.690
Nonacosane (C29)	39.165

Figure 5. Total petroleum hydrocarbon of the ETP hydrocarbon sludge (Standard/Untreated)



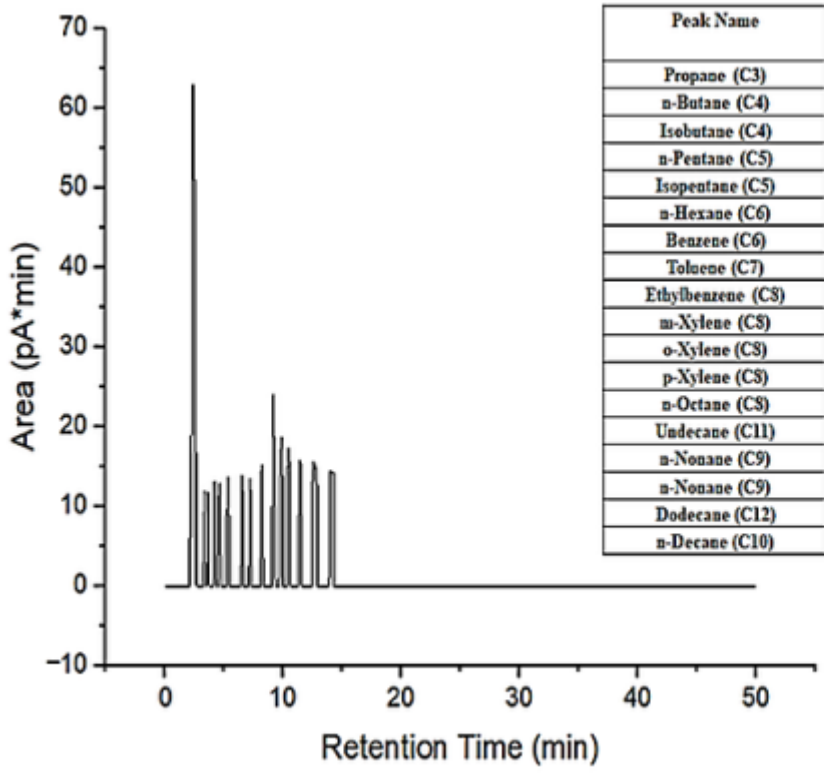
Peak identification	Retention time (min)
Propane (C3)	2.370
Butane(C4)	3.283
Isobutane (C4)	3.658
n-Pentane (C5)	4.208
n-Hexane (C6)	5.313
Benzene (C6)	6.512
Ethylbenzene (C8)	8.255
m-Xylene (C8)	9.215
o-Xylene (C8)	9.857
p-Xylene (C8)	10.430
n-Octane (C8)	11.490
n-Nonane (C9)	12.748
Dodecane (C12)	14.052

Figure 6. Total petroleum hydrocarbon of the ETP hydrocarbon sludge on the 28th day of incubation treated by an *Anabaena variabilis* culture



Peak Name	Retention Time min
Propane (C3)	2.368
n-Butane (C4)	3.302
Isobutane (C4)	3.675
n-Pentane (C5)	4.227
Isopentane (C5)	4.602
n-Hexane (C6)	5.300
Benzene (C6)	6.523
Ethylbenzene (C8)	8.263
m-Xylene (C8)	9.222
o-Xylene (C8)	9.813
n-Octane (C8)	11.405
n-Nonane (C9)	12.777
Dodecane (C12)	14.002

Figure 7. Total petroleum hydrocarbon of the ETP hydrocarbon sludge on the 28th day of incubation treated by a *Nostoc muscorum* culture



Peak Name	Retention Time min
Propane (C3)	2.388
n-Butane (C4)	3.302
Isobutane (C4)	3.633
n-Pentane (C5)	4.227
Isopentane (C5)	4.600
n-Hexane (C6)	5.332
Benzene (C6)	6.522
Toluene (C7)	7.220
Ethylbenzene (C8)	8.200
m-Xylene (C8)	9.220
o-Xylene (C8)	9.863
p-Xylene (C8)	10.438
n-Octane (C8)	11.405
Undecane (C11)	12.590
n-Nonane (C9)	12.702
n-Nonane (C9)	12.762
Dodecane (C12)	14.002
n-Decane (C10)	14.193

Figure 8. Total petroleum hydrocarbon of the ETP hydrocarbon sludge on the 28th day of incubation treated by a *Nostoc* sp. culture

minimum concentration of ETP sludge that a cyanobacterium can resist, their growth study in terms of chlorophyll a, chlorophyll b, carotenoids and phycobillisomes content was designed and executed. The cyanobacterial growth was also correlated with the enzyme activities that were measured independently for the extracts and test substrates. Degradative enzymes such as dehydrogenase, lipase, esterase, polyphenol oxidase and catalase showed great metabolic diversity in the present study and, hence, it verifies their endless competent biodegradation ability in degrading hydrocarbons present in the ETP sludge.

These interesting findings further elaborated the need to study the TPH removal efficiency in the cyanobacterium treating with ETP hydrocarbon sludge. The TPH degradation in the present study with three different cyanobacteria possessing an inherent capability was attempted to quantify using GC-FID. The rates of TPH reductions performed by the three cyanobacterial cultures are highly evident from the obtained trimmed spectrum of hydrocarbons after the treatment. The reduction from initial hydrocarbon content of C_3 to C_{29} to a residual hydrocarbon content of C_3 to C_{12} establishes the biodegrading capacity of the cyanobacterial cultures in the 28 days of treatment period.

The cyanobacteria when treated with ETP hydrocarbon sludge exhibited photosynthetic activity which can utilize hydrocarbons as a source of carbon and energy. The cyanobacterial species investigated for biodegradation in this present study are highly recommended for beneficial bioremediation applications for in-situ and ex-situ removal of petroleum hydrocarbons in the ETP hydrocarbon sludge. Further research on biostimulation and bioaugmentation may be carried out to establish the best and most efficient methods of optimizing the degradation potentials of these isolates.

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