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Analysis of aromatic hydrocarbon degrading capacity by thermophilic bacteria isolated from oil contaminated soil

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Abstract: Thermophilic bacteria was isolated from oil-contaminated soil to analyze their aromatic hydro carbon degrading capacity. The bacteria was characterized by 16S rRNA sequencing and was found to contain *Bacillus lichenoformis* A10, which showed lipase activity of 28 U/ml, and was used for biosurfactant production. On the other hand, diesel oil was characterized by silica gel fractionation and separated into resins, aromatic and aliphatic compounds. The aromatic fractions obtained were analyzed using gas chromatography-mass spectrometry. *Bacillus licheniformis* A10, isolated from oil-contaminated soil has the potential of degrading diesel oil and aromatic hydrocarbon to about 83.52% and 80.72% respectively in 30 days.
Keywords: Thermophilic bacteria; Contamination; Hydrocarbon; Biodegradation; biosurfactant; chromate-graphy;

1. Introduction

Diesel oil, which is one of the major products of crude oil, constitutes a major source of pollution in our environment. Diesel oil spills generally causes harm to flora and fauna[1]. Besides, diesel oil spills on agricultural land generally reduce plant growth. Suggested reasons for the reduced plant growth in diesel oil contaminated soils range from direct toxic effect on plants and reduced germination to unsatisfactory soil condition due to insufficient aeration of the soil because of the displacement of air from the space between the soil particles by diesel oil[2]. One biological strategy that can enhance contact between bacteria and water-insoluble hydrocarbons is emulsification of the hydrocarbon[3]. Therefore, it is not surprising that bacteria growing on petroleum usually produce potent emulsifiers. These surfactants help to disperse the oil, increase the surface area for growth, and help detach the bacteria from the oil droplets after the utilizable hydrocarbon has been depleted. The majority of known biosurfactants are synthesized by microorganisms grown on water immiscible hydrocarbons, but some have been produced on such water- soluble substrates as glucose, glycerol and ethanol[4]. Chemically-synthesized surfactants have been used in the oil industry to aid clean up of oil spills, as well as to enhance oil recovery from oil reservoirs. These compounds are not biodegradable and can be toxic to environment[5]. Biosurfactant have special advantage over their commercially manufactured counterparts because of their lower toxicity, biodegradable nature, and effectiveness at extreme temperature, pH, salinity and ease of synthesis. They are potential candidate for much commercial application in the pharmaceutical, food processing and oil recovery industries[6].

Microbial degradation is an environment friendly and cost-competitive alternative to chemical decomposition process. Many bacteria belonging to different taxonomic groups have been reported for their ability to degrade hydrocarbons[7]. Pure bacterial cultures have been used to develop bioprocesses for the mineralization of hydrocarbons. However, the long growth cycle and moderate degradation rate limit the performance of bacterial degradation system. In contrast, bacterial consortiums are normally faster, but it may require a highly resistant mixed community to mineralize hydrocarbons. The main aim of this study was to investigate hydrocarbon degradation potential of a thermophilic bacterium isolated from oil contaminated site of an industrial scale and provide biologically favourable environment required to accomplish hydrocarbon degradation in an effective manner.

2. Materials and Methods

2.1 Isolation of bacterial consortium

Microorganisms capable of degrading diesel oil were isolated from an oil refinery. One gram of diesel oil contaminated soil was serially diluted up to 10^{-8} dilution. An aliquot (0.1 ml) of the 10^{-8} dilution of the contaminated diesel oil was plated unto modified diesel medium using the pour plate method. The plates were incubated at 55°C for 48 h. After incubation, each bacterial colony type was subcultured repeatedly into nutrient agar plate to obtain a pure culture. The isolates were characterized based on cultural characteristics, cell morphology and biochemical characteristics.

2.2 16S rRNA sequencing and phylogenetic analysis of isolates

The DNA isolated was amplified using 16S rRNA universal primers and sequenced for the identification of bacterial strain at molecular level. **Forward Primer:** 8f (5'-GAGTTTGATCATGGCTCAG-3'); **Reverse primer:** 1495r (5'-CTACGGCTACCTTGTTACG-3'). Amplification of the PCR products of expected size was confirmed by electrophoresis. The sequence of the 16S rRNA was determined with a Dye terminator sequencing kit (Applied Biosystems). The gene sequences of each isolate obtained in this study were compared with known 16S rRNA gene sequences in the GenBank database.

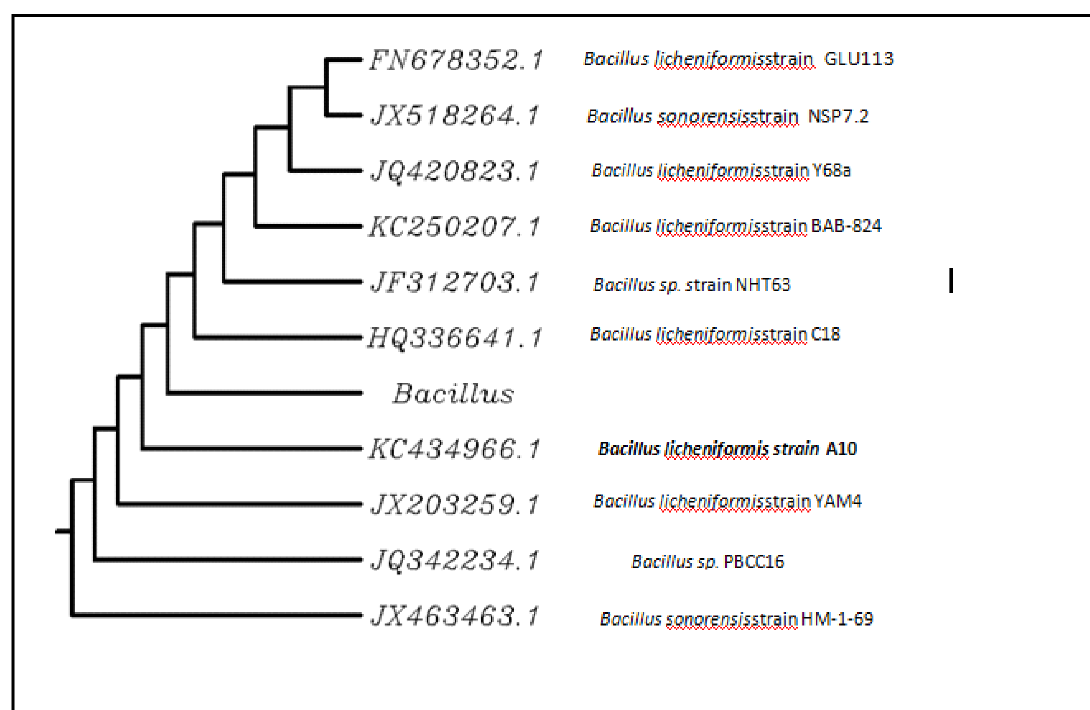

Figure 1: Phylogenetic tree of the consortium

2.3 Hydrocarbon utility test

The modified diesel medium was used in this study to identify the hydrocarbon degrading micro organism. The medium composition comprised of 1.4 g K_2HPO_4, 0.2 g $(NH_4)_2SO_4$, 0.6 g KH_2PO_4 and 0.6 g $MgSO_4.7H_2O$. The mineral components of the medium were dissolved in 100 ml of distilled water and mixed

with 4 g agar-agar and 2 ml diesel oil. The medium was autoclaved at 121°C for 15 min. The isolated bacteria were each tested for their ability to utilize diesel oil as the sole carbon and energy source. Each isolate was streaked onto modified mineral salt agar medium which contains a filter paper soaked in diesel oil. The plates were incubated for 7 days at room temperature. Using a sterile pipette, 5 ml of nutrient broth was transferred into a conical flask, aseptically inoculated with a loop full of pure stock culture of bacterial isolate and incubated at 55^0C. Five percent (v/w) of the inoculum samples were taken in a cuvette at 6 h intervals beginning from zero and their corresponding absorbance is measured at 540 nm using uv-visible spectrophotometer. This procedure was continued until 36h. Microbial inoculum (0.5 ml) was used to inoculate the polluted and control soil samples for degradation studies.

2.4 Diesel oil degradation studies

The ability of bacterium to degrade diesel oil was demonstrated in terms of reduction in the quantity of diesel oil introduced to pollute the soil samples. The rate of utilization was monitored on the first day of the study and subsequently at 3-day interval for 30 days. Carbon tetrachloride was employed as the extractant. On each day, one sample per group was analyzed for the quantity of residual diesel oil. Each of the samples was mixed with 40 ml of carbon tetrachloride, placed in a separating flask, shaken vigorously for 3 min and allowed to settle for 5 min. The liquid phase was separated by allowing (diesel oil – carbon tetrachloride) to pass gradually through a funnel fitted with Whatman No. 1 filter paper. Anhydrous sodium sulphate spread on the filter paper was employed to remove any moisture in the mixture. The beaker containing the extract was placed in an oven and the extractant, allowed to evaporate at 50^0C. The beaker with the residual diesel oil was allowed to cool to room temperature and weighed to determine the quantity of residual diesel oil. The percentage of diesel oil degraded at three days interval was determined from the equation:

$$\%\ diesel\ oil\ degraded = \frac{Weight\ of\ diesel\ oil\ degraded}{Original\ weight\ of\ diesel\ oil\ introduced}\ X\ 100 - - - - (1)$$

Where, the weight of diesel oil degraded was determined as,

$$Weight\ of\ diesel\ oil$$
$$= original\ weight\ of\ diesel\ oil$$
$$- weight\ of\ residual\ diesel\ oil\ obtained\ after\ evoparating\ the\ extractant - - (2)$$

The rate of degradation was determined as,

$$Rate\ of\ degradation = \frac{weight\ of\ diesel\ oil\ degraded\ (g)}{time\ taken\ (h)} - - - - - (3)$$

2.5 Silica gel fractionation

Elution liquid chromatography: elution liquid chromatography for the fractionation of crude oils and petroleum products into aliphatics, aromatics and resins was carried out in a standard burette. After cleaning and drying, the column was carefully packed with silica gel and moistened with 30ml of n-pentane. 5ml of diesel oil dissolved in 10ml of n-pentane was then introduced into the column. The aliphatic component was obtained by adding n-pentane (25-40ml) to the column to flush it. Thereafter, resin component was eluted by using chloroform and methanol (1:1). The elution of the aromatic component was done using benzene to flush the column. These fractions were then analyzed using gas chromatography to observe the presence of aromatic compounds.

2.6 Gas chromatography analysis

The GC MS - 5975 C Agilent gas chromatograph equipped with a flame ionization detector was used for the analysis. The column was packed with polyethylene glycol (EG 200) on chromosorb P(80-100mesh) solid support. The GC conditions include initial and final column temperatures of 65°C and 245°C respectively. The detector temperature was 260°C and nitrogen was used as the carrier gas. In a typical analysis, 1µl of the sample was injected into the injection port of the GC using a microsyringe. The sample was immediately vaporized and swept down the column by the carrier gas. Following separation in the column, the components were identified and quantitatively analyzed by the flame ionization detector whose output was transmitted to a recorder which produced a chromatogram. The output of the recorder interfaced with a computer with a suitable chromatographic program also includes retention time, peak area, peak height, area percent and height percent.

2.7 Lipase assay

Lipase activity was assayed by spectrometric measurement of the release of p-nitro phenol at 405 nm from p-nitro phenol palmitate (PNPP). The reaction mixture consists of 100 micro litre of 10 mM PNPP in water, 800 micro litre of acetate buffer (0.2M pH 3.6) and 100 micro litre of approximately diluted enzyme. After incubation at 40ºC for 15 minutes, the reaction was stopped by addition of 3 ml of 0.2M sodium carbonate. One unit of enzyme activity is defined as the amount of enzyme releasing 1 micromole of p-nitro phenol per minute under assay conditions**.**

2.8 Extraction of biosurfactants

Culture was inoculated in 50ml of R2B broth with 1ml of diesel. Then it was incubated at 55°c for 7 days under shaking condition. After incubation bacterial cells were removed by centrifugation at 5000rpm for 20 minutes. Equal volume of chloroform and methanol (2:1) was added to the supernatant. This mixture was shaken well for mixing and left overnight for evaporation. The biosurfactants settled as white coloured sediment.

2.9.1 Oil spreading technique*:*

30ml of distilled water was taken in the pertiplates. 1 ml of diesel oil was added to the centre of the plates containing distilled water. Now add 20µl of the supernatant of the cultures isolated from the soil to the centre. The biosurfactant producing organism can displace the oil and spread in the water.

Results and Discussion

Microbial Degradation of hydrocarbons in the environment[8] is a well studied concept. One of the first known in this field is *Cunninghamella elegans* oxidising naphthalene and an array of other aromatic molecules[9]. In this study, thermophilic bacteria was isolated from diesel contaminated soil sample and identification was carried out by 16s rRNA sequencing. The sequence of the 16S rRNA was determined with a dye terminator sequencing kit (Applied Biosystems). The gene sequences of the isolate obtained in this study were compared with known 16S rRNA gene sequences in the GenBank database. The gene sequence of that isolate were used for phylogenetic tree construction and it can inferred from that phylogenetic tree that the bacterium isolated from oil contaminated soil contains *Bacillus licheniformis* A10. The DNA which was isolated from the culture was amplified using 16S rDNA universal primers and sequenced for the identification of bacterial strain at molecular level.

Lipase (E.C.3.1.1.3) is the enzyme which degrades hydrocarbons. It is mainly used because of its high positional specificity[10], and staying active even in organic solvents[11]. Many bacterial strains produces lipases; *Bacillus* sp *Staphylococcus* sp, *Micrococcus* sp, *Pseudomonas* sp, *Psychrobacter* sp, and *Alcaligens* faecalis; and have been reported to degrade diesel oil[7]. In this study, the isolated thermophillic bacteria *Bacillus licheniformis* A10 was grown on diesel oil and it has the ability to produce 28 U/ml of lipase activity. It can be inferered that growth of bacteria can be directly related to the degradation of diesel oil.

Several types of biosurfactant have been isolated and characterized, including glycolipids, phospholipids, lipopeptides, natural lipids, fatty acids and lipopolysacharides . In oil spreading technique, no zone of displacement was observed in the control. Whereas, there was a zone of displacement observed in the test sample which indicates biosurfactant production. Further confirmation test confirms that thermophillic bacteria *Bacillus lichenoformis* A10 has the ability produce lipopeptide biosurfactant.

Polycyclic-Aromatic Hydrocarbon (PAH), is an immunotoxic and carcinogenic compound[12] and poses serious threats when present in the environment. In this current investigation, n-pentane, benzene and Chloroform : methanol(1:1) were used for separation of aliphatic compounds, aromatic compounds and resins .GC-MS was performed for the aromatic sample and was found to contain: p-Xylene; Benzene, 1,3-dimethyl-; o-Xylene; acetophenone; Benzene, 1,2,4,5-tetramethyl-; Benzene, 1,2,3,4-tetramethyl-; Benzene, 1-ethyl-2,3-dimethyl-; Naphthalene, 1,2,3,4-tetrahydro-5- methyl-;Naphthalene, 1,2,3,4-tetrahydro-6-methyl-; Carbonic acid, neopentyl 2-ethylhexyl ester; 2'-Ethylpropiophenone 4'-Ethylpropiophenone; Benzene, 2,4-dimethyl-1-(1-methylpropyl)-; 1H-Azepine, 1-acetylhexahydro-; Naphthalene, 1,2,3,4-tetrahydro-1,4-dimethyl-; Naphthalene, 1,2,3,4-tetrahydro 2,7-dimethyl-; 1H-Inden-1-one, 2,3-dihydro-3,3-dimethyl-; Benzene, 4-(2-butenyl)-1,2-dimethy l-, (E)-; Benzene, 1,3,5-trimethyl-2-(1-methylethenyl)-; Naphthalene, 2,7-dimethyl-; Naphthalene, 1,4-dimethyl-; Naphthalene, 1,2,3,4-tetrahydro-1,6,8 trimethyl-; Naphthalene, 1,2,3,4-tetrahydro-1,5,7-trimethyl-; Naphthalene, 1,2,3,4-tetrahydro-1,5,8-trimethyl-; Naphthalene, 1,2,3,4-tetrahydro-1,5,7-trimethyl-; Naphtha lene, 1,2,3,4-tetrahydro-2,5,8-trimethyl; trans-calamenene; 3-Acetoxy-2-methyl-hex-4-enoic acid, methyl ester and Oxirane (Figure 2)

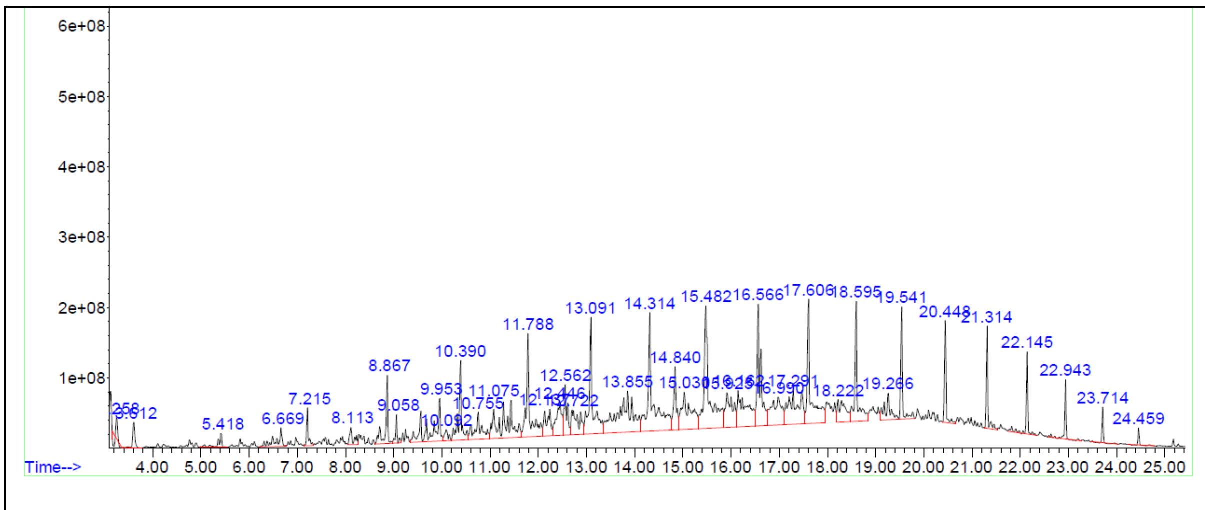

Figure 2: GC-MS of aromatic compounds

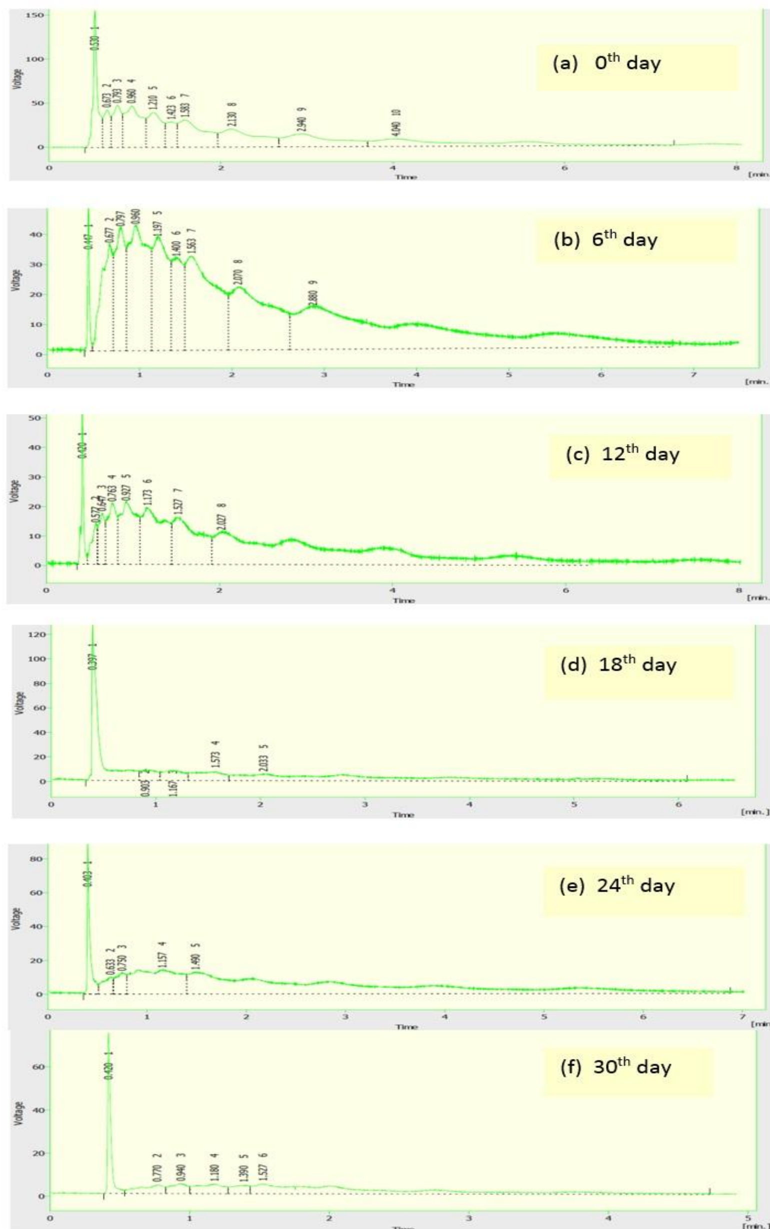

Figure 3: Gas chromatography analysis of degradation studies.

- 0th day ;Height(nV): 298.782; Area(mV.s): 4869.157

- 6th day ;Height(nV): 298.782; Area(mV.s): 4869.15
- 12th day ;Height(nV): 167.175 Area(mV.s): 2533.321
- 18th day ;Height(nV): 154.720; Area(mV.s): 2218.933
- 24th day ;Height(nV): 137.244; Area(mV.s): 1568.287
- 30th day ;Height(nV): 95.387; Area(mV.s): 584.172

PAH's like phenanthrene are effectively degraded by microbial consortium[13]. A co-culture of bacterial and fungal bacterium can degrade high molecular PAH's[14]. *Bacilli* and *Pseudomonas* are the two major genus of bacteria which can successfully degrade PHA's. In this present study, *Bacilluslicheniformis* A10, isolated from oil contaminated soil produced lipase with activity of 28U/ml and was able to degrade 80.72% of aromatic hydrocarbon in 30 days (Figure 3). But, *Pseudomonasaeruginosa* IU-5 was able to degrade at the rate of $8.33x10^{-3}$g/hr,[15] when compared to $1.43x10^{-3}$g/hr for the bacillus strain. This proves the already established fact that *Pseudomonas* is the most degradative microorganism present in any oil contaminated soil sample[15]. Other *Pseudomonas* strains like *P. putida* PB4 degraded aromatic hydrocarbons like benzene, toluene and ethyl-benzene at room temperature[16]. Among others , the mixed culture of *Micrococcus* sp. GS2-22, *Coryne bacterium* sp. GS5-66, *Flavobacterium* sp. DS5-73, *Bacillus* sp. DS6-86 and *Pseudomonas* sp. DS10-129 were only able to degrade Bombay high crude oil upto 78% in 20 days[17] in 30□C. At that low temperature, the viscosity of the oil is high, which ultimately delays the biodegradability[17]. The temperature of 30□C is optimum for PAH degradation[17] and microbial growth. From Table 1, it can be inferred that the maximum % of diesel oil degradation was found to be 83.52 in 30th day. The rate of degradation of diesel oil was found for 30 days with an interval of 3 days and maximum rate was found to be during the 15th day. From Table 2, it can be inferred that the maximum % of aromatic hydrocarbon degradation was found to be 80.72 in 30th day. From Table 3, the rate of degradation of aromatic hydrocarbon was found for 30 days, with a 3day interval and the maximum rate was found to be at the 21st day. *Bacillus licheniformis* A10 is a thermophilic bacteria it degrades them at 55□C. Though some bacterium are able to degrade 93.75% of the total aromatics[7], to have a thermophilic bacteria which can naturally degrade upto 80% of the aromatics in the diesel is promising. This amount can be increased by using biosurfactants[16]. Since there are very few cases of a thermophilic bacteria able to degrade hydrocarbons [18] , this is an area where further research is required.

Table 1: Percentage degradation and rate of degradation of diesel oil by thermophillic bacteria *Bacillus licheniformis* A10 at 55°C.

Day	Wt. of diesel oil extracted(g)	% degradation	Wt. of diesel oil degraded(g)	Rate of degradation (g/hr)
0	0.85	0	0	0
1	0.83	2.35	0.02	8.33×10^{-4}
3	0.78	8.23	0.07	9.72×10^{-4}
6	0.65	23.52	0.20	1.38×10^{-3}
9	0.54	36.47	0.31	1.43×10^{-3}
12	0.48	43.52	0.37	1.28×10^{-3}
15	0.32	62.35	0.53	1.47×10^{-3}
18	0.24	71.76	0.61	1.41×10^{-3}
21	0.19	77.64	0.66	1.30×10^{-3}
24	0.16	81.17	0.69	1.19×10^{-3}
27	0.14	83.52	0.71	1.09×10^{-3}
30	0.14	83.52	0.71	9.86×10^{-4}

Table 2: % degradation and rate of degradation of aromatic hydrocarbons by thermophillic bacteria *Bacillus licheniformis* A10 at 55°C.

Day	Wt. of diesel oil degraded(g)	Rate of degradation (g/hr)	Wt. of aromatic compounds extracted(g)	% degradation
0	0	0	0.83	0
1	0.02	8.33×10^{-4}	0.83	0
3	0.07	9.72×10^{-4}	0.76	8.43
6	0.20	1.38×10^{-3}	0.69	16.86

9	0.31	1.43×10^{-3}	0.62	25.30
12	0.37	1.28×10^{-3}	0.55	33.73
15	0.53	1.47×10^{-3}	0.48	42.16
18	0.61	1.41×10^{-3}	0.37	55.42
21	0.66	1.30×10^{-3}	0.24	71.08
24	0.69	1.19×10^{-3}	0.17	79.51
27	0.71	1.09×10^{-3}	0.16	80.72
30	0.71	9.86×10^{-4}	0.16	80.72

Table 3: Rate of degradation of aromatic hydrocarbons in diesel oil by thermophillic bacteria *Bacillus licheniformis* A10 at 55°C.

Day	Wt. of aromatic compounds degraded(g)	Rate of degradation (g/hr)
0	0	0
1	0	0
3	0.07	9.72×10^{-4}
6	0.14	9.72×10^{-4}
9	0.21	9.72×10^{-4}
12	0.28	9.72×10^{-4}
15	0.35	9.72×10^{-4}
18	0.46	1.06×10^{-3}
21	0.59	1.17×10^{-3}
24	0.66	1.14×10^{-3}
27	0.67	1.03×10^{-3}
30	0.67	9.30×10^{-4}

Conclusion

Thermophillic bacteria *Bacillus licheniformis A10* isolated from oil-contaminated soil was found to produce lipopeptide biosurfactant and also produce lipase activity of about 28 U/ml. It has the potential of degrading diesel oil and aromatic hydrocarbon to about 83.52% and 80.72% in 30 days. Rate of degradation of diesel oil was highest to about 1.47×10^{-3} g/hr in 15th day and for aromatic hydrocarbon it was 1.17×10^{-3} g/hrin 21st day. From the gas chromatography analysis of degradation studies, it can be inferred that the *Bacillus lichenoformis A10* has the potential of degrading aromatic hydrocarbon present in diesel oil because sum (height & area) of the peak was decreasing gradually.

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