ISOLATION AND OPTIMIZATION OF BACTERIAL CONSORTIUM FOR THE POTENTIAL BIOREMEDIATION IN CRUDE OIL CONTAMINATED SITES

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ABSTRACT

The bacterium designated strain, able to degrade crude oil was isolated from petroleum contaminated soil at automobile workshops and petrol pumps in Chennai, Tamil Nadu, India. Bacterial analysis of the samples revealed the presence of crude oil degrading bacteria belonging to the genera Pseudomonas, Enterobacteriaceae, Moraxella, Bacillus and Micrococcus sp. The microbial consortium consists of (*Pseudomonas aeruginosa* and *Bacillus pumilus*) is having higher degradation. The biodegradation rate of crude oil concentration in Bushnell Haas (BH) broth, but addition of carbon and nitrogen source had a substantial impact on the biodegradation of crude oil, which suggested that carbon might provide a factor that was necessary for its crude oil biodegradation. Influence of various effect of pH, temperature, carbon sources, nitrogen sources and on crude oil degradation from BH broth also studied. The results showed a rapid and efficient process of crude oil degradation (90%) in BH broth supplemented with 1% cellulose and 1% peptone inoculated by bacterial consortium at incubation temperature of 35 °C at pH 7. This study suggests that isolated *Pseudomonas aeruginosa* and *Bacillus pumilus* may play an important role for biodegradation of crude oil in the contaminated soil.

Key words:

Crude oil, Biodegrdation, Bacterial consortium, *Pseudomonas aeruginosa* and *Bacillus pumilus*.

1. INTRODUCTION

In recent years, petroleum and its byproducts pollution has been increasing concern both nationally and internationally. These pollutants are removed by biotechnological process using microorganisms (Agarry *et al*. 2012). Bioremediation process is used to remove environmental pollutants such as organic and inorganic substances from soils, water and sediments. It has countless advantages when compared to other processes employed to remove pollution such as extraction with solvents addition of chemical oxidizers (Nano *et al*., 2003). The presence of hydrocarbon contamination in the environment has influenced the biodiversity of the region. The petroleum hydrocarbon degradation from environment is limited by large number of factors. An important factors in the biodegradation of contaminated soils is low bioavailability and solubility of the hydrocarbon (Latha and Kalaivani 2012). The degradation of petroleum hydrocarbons and its byproducts using environmental microorganisms have been recognized as an efficient, versatile, economic and environmental friendly treatment. The research for effective and efficient methods of petroleum hydrocarbons from contaminated sites has intensified in recent years, because biodegradation is responsible for cleaning of the environment pollutants (Grangemard *et al*., 2001).

Bioremediation is also environmentally friendly, it does not produce waste products and is cost effective. Microorganisms with potentials for oil degradation are widespread in nature. It can be combined with other technologies and naturally occurring process when the conditions are suitable for the growth of microorganisms (Roy *et al*., 2014).In general, bioremediation process optimization may be incomplete by the lack of studies showing the simultaneous effect of different environmental factors). Hence, our study focused was to set up the optimum values of five abiotic factors: pH, temperature, carbon source, nitrogen source and salt concentration for the biodegradation of crude oil. In order to achieve the efficient biodegradation of the above factors on the microbial growth and the biotic degradation was studied (Amass *et al*., 1998). Generally, microbial communities present in crude oil contaminated soils are enriched by microorganisms able to use as a carbon and nitrogen source (Gallego *et al*., 2007). The present study focused on to isolate, identify and screen the efficient oil degrading bacterial strains, bacterial consortium and optimization of bacteria consortium with different carbon source, pH and temperature oil under different concentration.

2. MATERIALS METHODS

2.1. Sample collection

Petroleum hydrocarbon contaminated soil samples were collected from motor vehicle workshops, water service stations and vehicle parking areas located in and around Chennai, Tamil Nadu. Soil samples were collected randomly 5-10 cm beneath the surface using spatula and packed in sterile container. The samples were transported to the laboratory in an ice box and stored at 4^{o}C for analysis. The collected soil samples were serially diluted from 10^{-1} to 10^{-6}, spreaded on nutrient agar plates and incubated at 37^{o}C for 24 hours. The obtained cultures were purified by quadrant streaking on sterile nutrient agar plates (Khan and Rizvi, 2011).

2.3. Primary screening of crude oil degrading bacteria

Bushnell Hass media (BHM) along with redox indicator 2, 6-dichlorophenol indophenol was prepared, 1% of crude oil was added. The isolated strains were inoculated into broth and incubated at 37°C for 7 days (Ibrahim *et al*., 2013). Ten ml of broth was taken, centrifuged and the supernatant was used to measure the optical density at 640nm for degradation ability of the isolates. About 0.1ml of each 7 days old BH broth culture was spread over to the nutrient agar plates to count bacterial population.

2.3. Secondary screening by gravimetric analysis

The bacterial strains (15 nos) showed more efficiency on crude oil degradation and increased growth in primary screening was selected for secondary screening. About 100ml of Bushnell Hass broth media was prepared with this one gram of crude oil was added in the broth. Oil degrading isolates were aseptically added as an inoculum. The flask was incubated at 37°C for 7 days in a rotary shaker at 120rpm. After incubation, the flask was added with diethyl ether solvent and transferred to the separating flask. The estimation of residual oil left after degradation was made by the amount of oil in a preweighed plate (Anupama *et al*., 2009).

2.4. Degradation by Bacterial Consortium

Bacteria were grouped for consortium such as A + B, A + C, A + D, B + C, C + D, A + B + C, A + B + D, A + C + D, B + C + D and A + B + C + D. Ten milliliter of the above isolates were taken and mixed. The suspension was taken as seed inoculums and 1 ml of inoculum of above consortium was inoculated in BH medium with 100 ppm of crude oil and kept in a shaker at 37 °C for 7 days.

2.5. Effect of pH on crude oil degradation

The BH broth was prepared with various pH (3, 5, 7, 9 and 11). 100 ppm of crude oil was prepared and one ml of one OD bacterial consortium (*Pseudomonas aeruginosa* and *Bacillus pumilus*) was inoculated to the broth and kept in a shaker (120 rpm) at 37^{o}C. The samples were drawn aseptically at regular intervals. The bacterial growth was checked by standed plate count method and concentration of crude oil in the medium was determined by using UV visible spectrophotometer (Singh and Chandra, 2014).

2.6. Effect of temperature on crude oil degradation

The sterile BH Medium at pH 7 with 100 ppm of crude oil was prepared and one ml of one OD bacterial consortium (*Pseudomonas aeruginosa* and *Bacillus pumilus*) was inoculated to the medium and kept in a shaker (120 rpm) at different temperature (15, 30, 35, 40 and 45^{o}C). The bacterial growth was checked by standed plate count method and crude oil concentration in the medium was determined using UV visible spectrophotometer method (Sarkar *et al*., 2017).

2.7. Effect of carbon sources on crude oil degradation

The sterile BH medium at pH 7 with 100 ppm of crude oil and 100 mg/l various carbon substrates such as dextrose, sucrose, cellouse, starch and glucose was prepared and one ml of 1 OD bacterial consortium *(Pseudomonas aeruginosa* and *Bacillus pumilus)* was inoculated and kept in a shaker (120 rpm) at 35^{o}C. The samples were drawn aseptically at regular intervals, analyzed the bacterial growth was checked by stranded plate count method and concentration of crude oil using UV-Vis spectrophotometer (Teng *et al*., 2010).

2.8. Effect of nitrogen sources on crude oil degradation

The BH broth at pH 7 with 100 ppm of crude oil and 100 mg/l various nitrogen sources peptone, beef extract, yeast extract, soya bean and casein acid hydrolysate was prepared and one ml of one OD bacterial consortium (*Pseudomonas aeruginosa* and *Bacillus pumilus*) was inoculated to the broth with added 1% cellouse and kept in a shaker (120 rpm) at 35^{o}C. The samples were drawn aseptically at regular intervals. The bacterial growth was checked by stranded plate count method and concentration of crude oil in broth was determined by using UV visible spectrophotometer (Teng, *et al*., 2010). The best nitrogen sources was selected and used for further the crude oil degradation studies.

3. RESULTS

3.1. Sample collection and isolation of bacteria from soil sample

There are 14 crude oil contaminated soil samples were collected for obtaining efficient crude oil degrading bacteria isolates from in and around Chennai. Totally 135 bacterial strains were isolated from crude oil contaminated samples and identified by various biochemical tests according to the Bergey's manual of determinative bacteriology. Among the 135 bacterial strains the major genera present in petroleum contaminated sites were *Micrococcus*, *Pseudomonas*, *Bacillus*, *Moraella* and *Enterobacteriaceae* sp. (Fig 1).

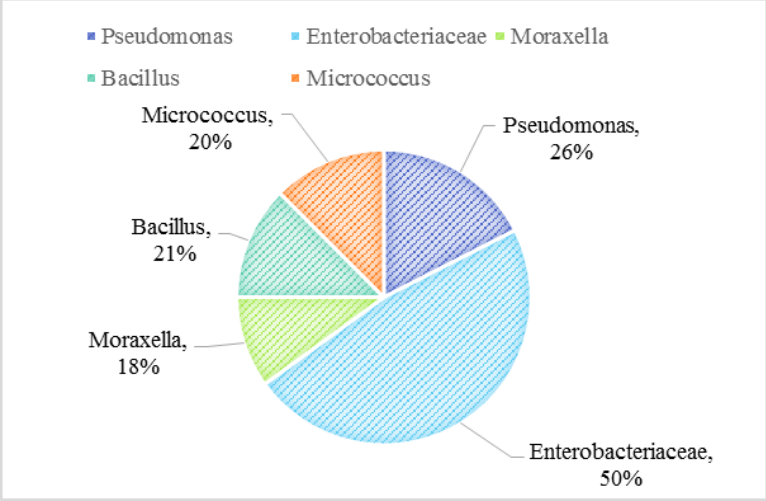

Figure 1. Major microbial genera in oil contaminated soil

3.2. Primary screening of by DCPIP test

Among the 135 bacterial isolates, 15 isolates were screened for degradation of petroleum hydrocarbons by primary screening method using DCPIP test. Ability of the isolates to degrade the hydrocarbon was confirmed by the color change from blue to colourless.

3.3. Secondary screening by gravimetric analysis

The rate of degradation was confirmed by gravimetric method. Among the 15 bacterial isolates only two bacterial genera namely *Bacillus* sp. 18 and *Pseudomaonas* sp. 35 showed higher degradation ability was confirmed in secondary screening. The bacterium *Bacillus* sp 18 degraded 37% and *Pseudomaonas* sp 35 degraded 38% within7 days (Fig 2).

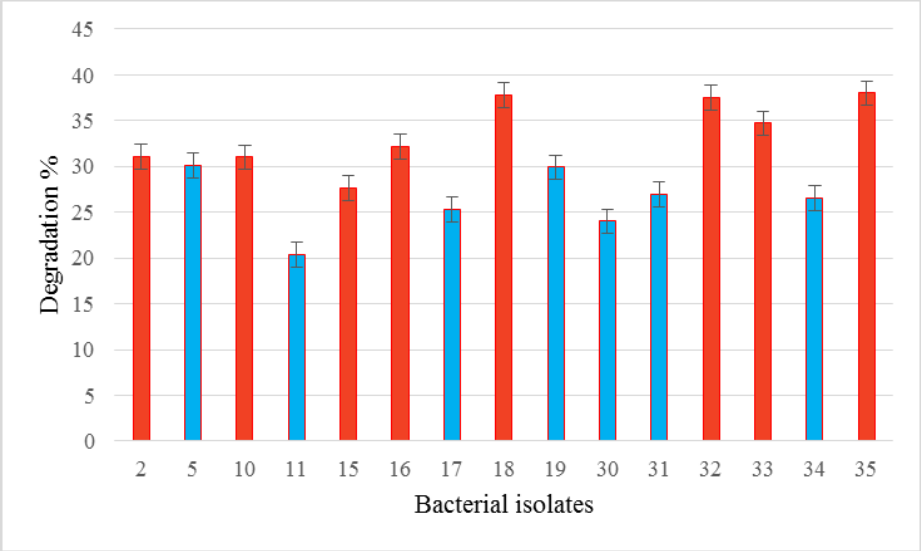

Figure 2. Secondary screening of bacteria in crude oil degradation

3.4. Efficient microbial consortium

In this study, the two combination of bacterial consortium was used to check the efficient microbial consortium (Fig.3). The results showed that consortium B+C was more efficient in crude oil degradation. Hence, this consortium was used for further degradation study. The selected two bacterial consortium were subjected to morphological and biochemical character studies and identified as *Pseudomonas aeruginosa* and *Bacillus pumilus*.

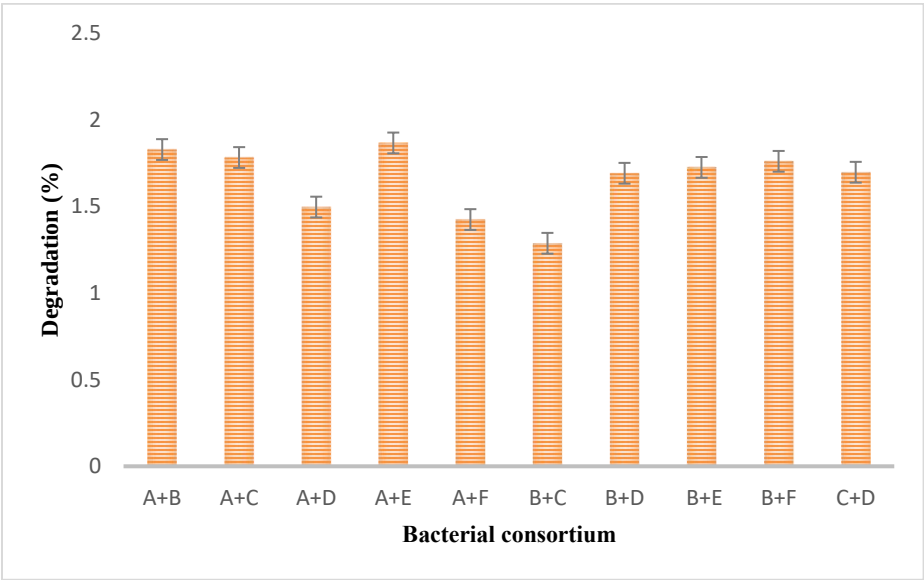

Figure 3. Efficient bacterial consortium

3.5. Effect of pH on crude oil degradation

The effect of pH on the bacterial consortium (*Pseudomonas aeruginosa* and *Bacillus pumilus*) with various pH like 3, 5, 7, 9 and 11. The pH in the broth is directly proportional to the degradation of crude oil. Periodically the bacterial growth was determined by standard plate count. The maximum growth of (85×10^6 CFU mL^{-1}) was observed in pH 7, at 35°C in 7 days followed by pH 3 (75×10^6 CFU mL^{-1}). In pH 5, pH 9 and pH 11 growth showed about 69×10^6 CFU mL^{-1}, 66×10^6 CFU mL^{-1} and 60×10^6 CFU mL^{-1} respectively (Fig.4). The result showed that pH 7 was ideal for the growth of the bacterial consortium.

The maximum crude oil degradation 95% (100 to 5 ppm) was observed in BH broth at pH 7 during 7 days. At pH 3, 5, 7, 9 and 11 the degradation of crude oil was 70, 85, 95, 88 and 78% respectively. The complete degradation was obtained in pH 7 indicated that the neutral pH 7 was the suitable for degradation of crude oil (Fig. 5).

3.6. Effect of temperatures on crude oil degradation

Crude oil degradation was studied at different temperatures like 15, 30, 35, 40 and 45°C the broth was containing bacterial consortium (*Pseudomonas aeruginosa* and *Bacillus pumilus*) with 100ppm of crude oil concentration at pH 7. The maximum growth was observed in 35°C (75×10^6 CFU mL^{-1}) in 7 days followed by 15, 30, 45 and 40°C (71×10^6 CFU mL^{-1}, 68×10^6 CFU mL^{-1}, 64×10^6 CFU mL^{-1} and 60×10^6 CFU mL^{-1}) respectively (Fig.6). The result showed that 35°C was ideal for the growth of the bacterial consortium.

The maximum crude oil degradation 95% was observed in pH 7, at 35°C in 7 days by the bacterial consortium (*Pseudomonas aeruginosa* and *Bacillus pumilus*) with 1% cellouse in pH 7 at 35°C in 7 days incubation. At the temperatures 15, 30, 40 and 45°C degradation was observed 49, 75, 95, 90 and 88% respectively (Fig .7). At the 35°C for the higher of degradation (95%) was observed 7 days incubation. This indicate that 35°C was finest temperature for crude oil degradation study.

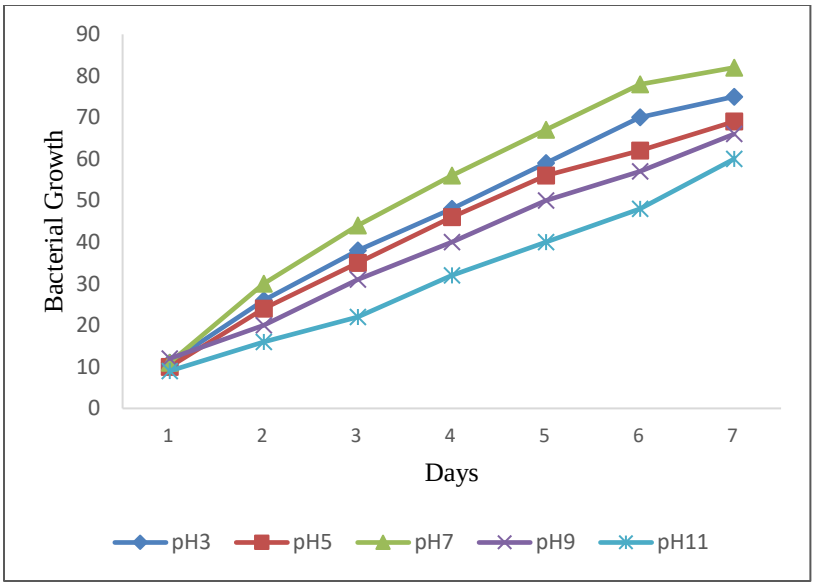

Figure 4. Effect of various pH on the growth of bacterial consortium

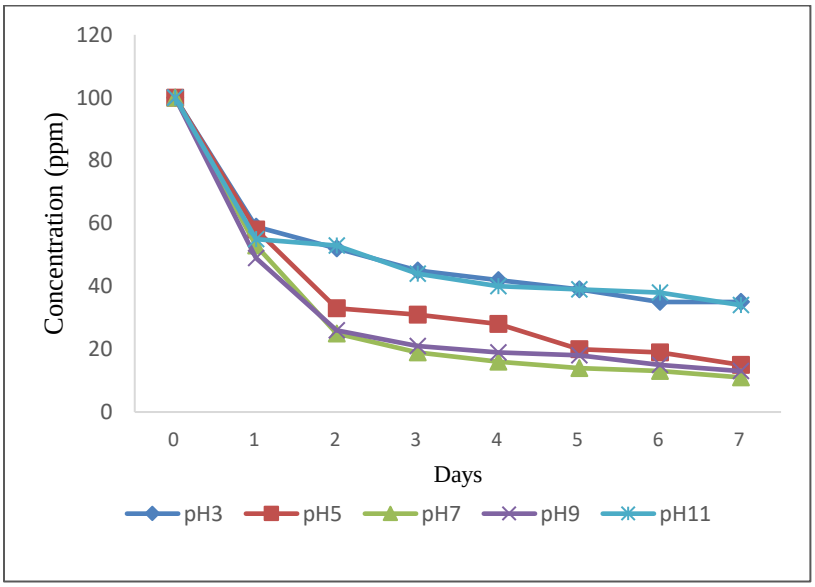

Figure 5. Effect of various pH during crude oil degradation

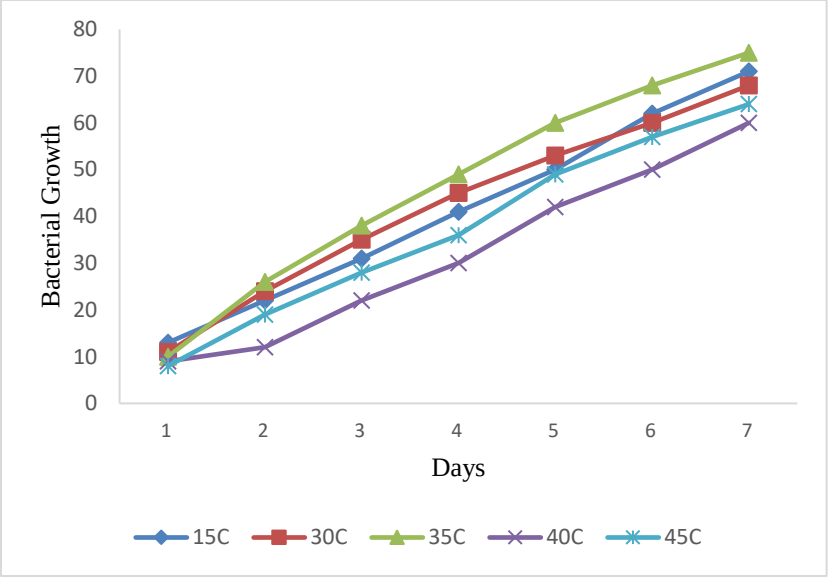

Figure 6. Effect of various temperature on the growth of bacterial consortium

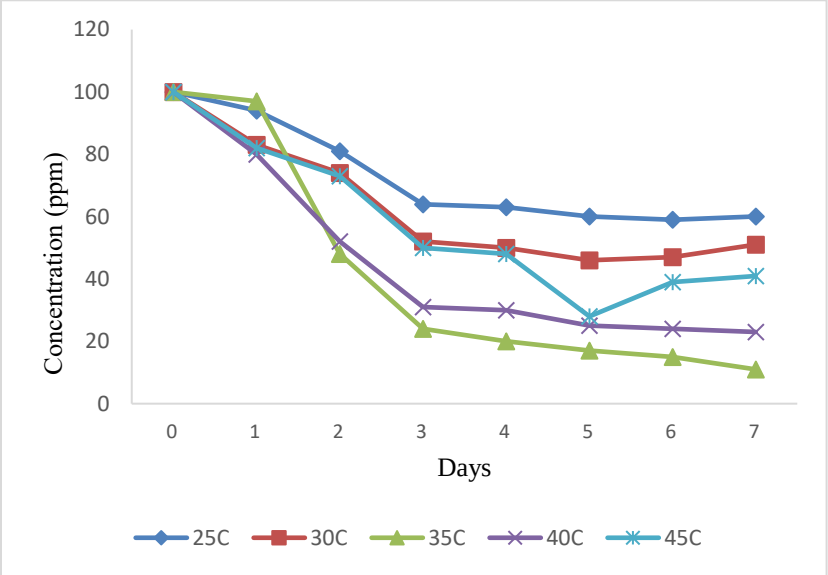

Figure 7. Effect of various temperature during crude oil degradation

3.7. Effect of carbon sources on crude oil degradation

BH medium with various carbon sources and bacterial consortium (*Pseudomonas aeruginosa* and *Bacillus pumilus*) was studied. Totally five different analytical grade carbon sources namely dextrose, sucrose, glucose, starch and cellulose were used. The maximum growth was observed cellulose (80×10^6 CFU mL^{-1}) in pH 7, at 35°C in 7 days followed by dextrose (75×10^6 CFU mL^{-1}), starch (66×10^6 CFU mL^{-1}), sucrose (60×10^6 CFU mL^{-1}) and glucose (58×10^6 CFU mL^{-1})

respectively (Fig.8). The result showed that cellulose was ideal carbon growth of the bacterial consortium. The maximum 96% (100 to 4ppm) degradation of crude oil was observed in BH broth with supplemented cellulose as a carbon source in pH 7 at 35°C in 7 days. In order to other carbon sources degradation was observed in starch 95% (100 to 5ppm), glucose 92% (100 to 8ppm), sucrose 90% (100 to 10ppm) and dextrose 85% (100 to 15ppm) respectively (Fig. 9). The cellouse showed best degradation and selected for the further studies. Cellouse used as co substrate to enhance the metabolic activity of bacterial consortium (*Pseudomonas aeruginosa* and *Bacillus pumilus*).

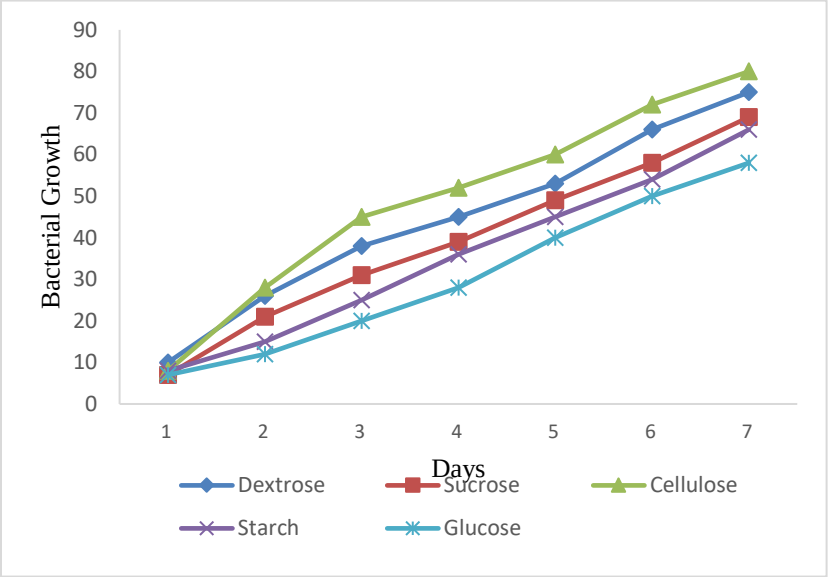

Figure 8. Effect of various carbon source on the growth of bacterial consortium

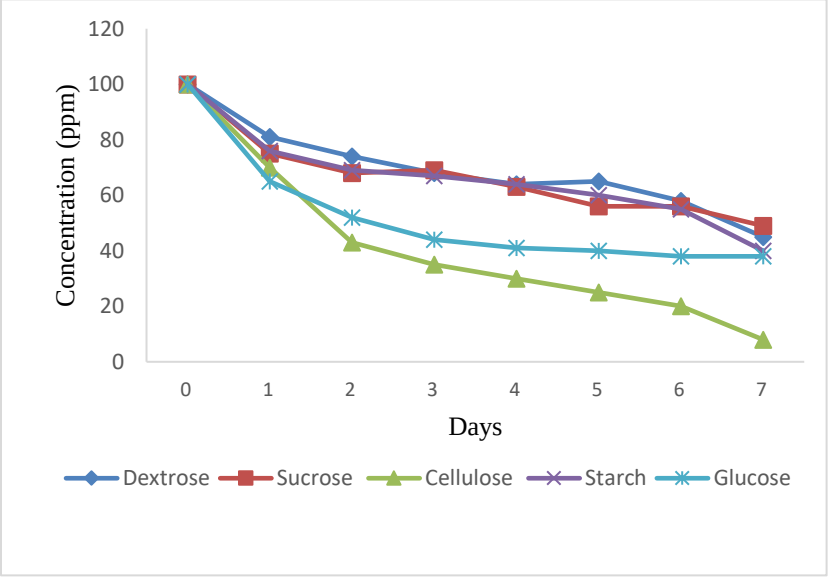

Figure 9. Effect of various carbon source during crude oil degradation

3.8. Effect of nitrogen sources on crude oil degradation

BH medium with various nitrogen sources and bacterial consortium (*Pseudomonas aeruginosa* and *Bacillus pumilus*) was studied. Nitrogen sources, namely peptone, beef extract, yeast extract, soya bean powder and casein acid hydrolysate were used. The maximum growth was observed in peptone (85×10^6 CFU mL^{-1}) in the pH 7 with 1% cellouse, at 35ºC for 7 days followed by soya bean powder (80×10^6 CFU mL^{-1}), yeast extract (76×10^6 CFU mL^{-1}), casein acid hydrolysate (70×10^6 CFU mL^{-1} and beef extract (62×10^6 CFU mL^{-1}) respectively (Fig.10).

The result showed that peptone was ideal for the growth of the bacterial consortium. In this study, the maximum crude oil degradation was observed in nitrogen sources peptone amended with 1% cellouse in pH 7 at 35ºC during the experimental days. The degradation was observed 93% (100 to 7ppm) in beef extract, 92% (100 to 8ppm) soya bean powder, 91% (100 to 9ppm) yeast extract and 89% (100 to 12ppm) casein acid hydrolysate respectively (Fig. 11).

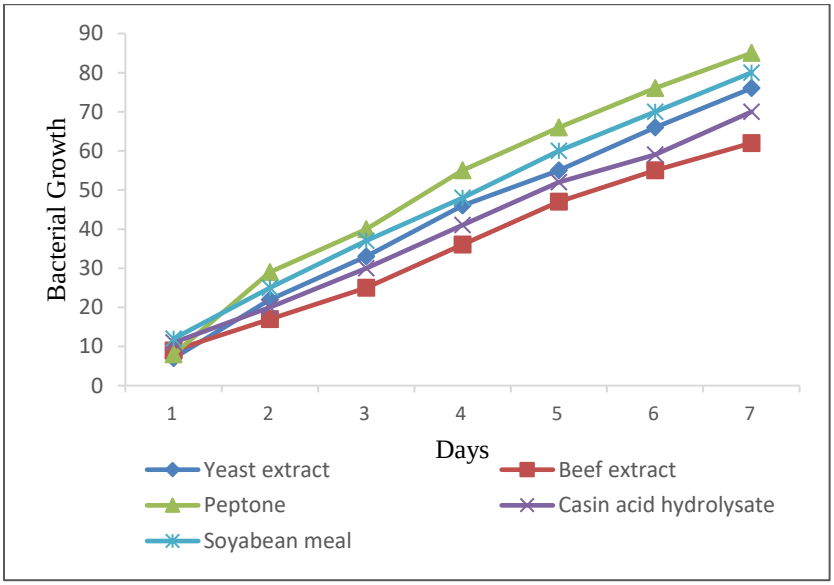

Figure 10. Effect of various nitrogen source on growth of bacterial consortium

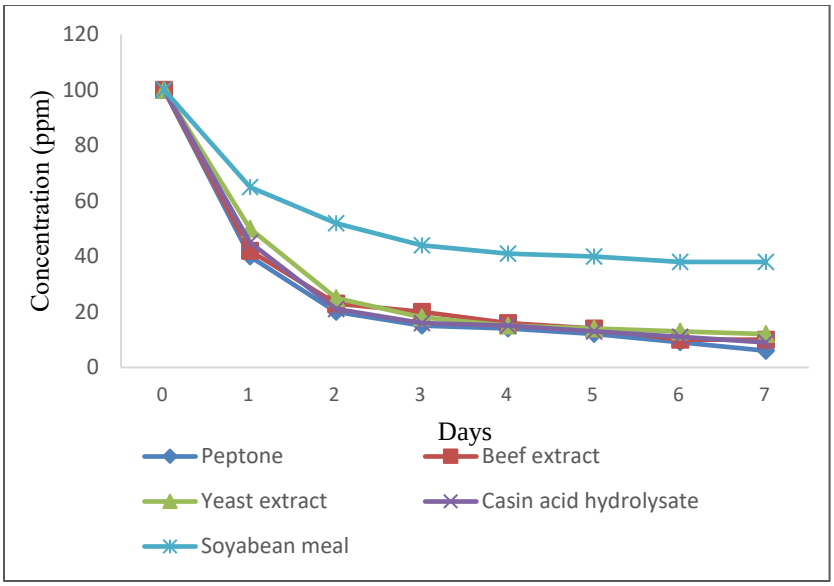

Figure 11. Effect of various nitrogen source during crude oil degradation

4. DISCUSSION

Presently the toxic, mutagenic and carcinogenic properties of polycyclic aromatic hydrocarbons (PAHs) have been concerned by the Unites States Environmental Protection. (Luning Prak and Pritchard, 2002) Microbial bioremediation removes or immobilizes the pollutants reducing toxicity with a very low environmental impact. Environmental pollution acts on the indigenous biota of the ecosystem to eliminating or selecting microorganisms in accordance sensitivity in the presence of the toxic agent. There are several reports on bioremediation of pollutants by the action of different bacterial strains have been capable of degrading hydrocarbons (Rahman *et al.*, 2002; Varjani *et al.*, 2015) have been reported on the roles of *Bacillus* sp. and *Pseudomonas* sp.in hydrocarbon bioremediation.

Rapid primary screening procedure was performed to assess the indicator dye (2, 6-DCPIP) decolourization efficiency of selected strains for confirmation of crude oil biodegradation. To ascertain microbial ability to utilize hydrocarbon substrate by simple observing the color change of DCPIP in which the quickest decolourization time represents the best oil biodegradation is a major breakthrough in biodegradation studies. Total bacterial count was determined by standard plate count method (Selvakumar *et al.*, 2014).

Secondary screening of purified culture is also done by recovering oil from the flask and the estimated amount of oil is left after degradation. Increase in oil degradation is directly proportional

to an increase in cell count indicating that bacterial isolates were capable for oil degradation (Rahman *et al*., 2002). *Pseudomonas* sp. is an outstanding and natural crude oil degrader reported in the literature which is wide spread in nature and can degrade wide range of xenobiotics. It has been postulated that *Bacillus* sp. are predominant and more tolerant to high level of crude oil contaminated soil due to their ability of the resistant endospore which may protect them from the toxic effect of the hydrocarbon (Usman *et al*., 2012).

The present study was undertaken to determine the optimum condition and efficient bacterial isolate to attain the maximum degradation possible in soil contaminated samples. Among different combinations of bacterial consortium (*Pseudomonas aeruginosa* and *Bacillus pumilus*) used a maximum of growth was recorded in the combination B+ C at 7 days. Similarly, Rajakumar *et al*. (2008) reported that bacterial consortium of *Pseudomonas* sp. KW1 and Bacillus sp. YW4 from nitrate contaminated soil were found to be the most efficient in terms of nitrate reduction was recorded in the two bacterial consortium combination. Prabhakaran *et al*. (2014) studied crude oil degradation and showed that mixed bacterial consortia degraded maximum level of 93.85% of crude oil.

The effect of various pH on bacterial growth was studied in BH broth containing 100ppm of crude oil. The maximum growth (85×10^6 CFU mL^{-1}) was observed in pH 7, at 35^{o}C in 7 days followed by pH 3 (75×10^6 CFU mL^{-1}), pH 5 (69×10^6 CFU mL^{-1}), pH 9 (66×10^6 CFU mL^{-}) and pH (60×10^6 CFU mL^{-1}) respectively. In the current study, bacterial growth was maximum at pH 7, though the process was active from pH 7 to 9. Similarly, Hambrick *et al*. (1980) found increased growth rates of microbial mineralization of octadecane when pH was increased from 6.5 to 8.0.

The effect of various pH on bacterial growth was studied in BH broth containing 100ppm of crude oil. The maximum level of bacterial growth was observed in pH 7 at 7 days. The maximum 95% degradation was observed. Pawar (2015) suggests that pH 7.5 was most suitable for the degradation of all PAHs as 50% degradation was also observed for all in soil pH 7.5 within the first seven days. The effect of various temperature on bacterial growth was studied in BH broth containing 100ppm of crude oil pH 7. The maximum growth was observed in 35^{o}C (75×10^6 CFU mL^{-1}) followed by 15, 30, 45 and 40^{o}C (71×10^6 CFU mL^{-1}, 68×10^6 CFU mL^{-1}, 64×10^6 CFU mL^{-1} and 60×10^6 CFU mL^{-1}) respectively. The bacterial consortium (*Pseudomonas aeruginosa* and *Bacillus pumilus*) degraded crude oil from 100 to 5ppm (95%) at the temperature 35 ^{o}C. At the

temperatures of 15, 25, 45 and 55ºC, the crude oil degradation was 49, 75, 90 and 88% respectively. Most of the reports revealed that, the best biodegradation efficiency of crude oil was achieved at the highest temperatures (35-40°C). Husain and Ahmad, (2013) also reported 35ºC temperature showed higher efficiency of oil degradation.

The effect of various carbon source on bacterial growth was studied in BH broth containing 100ppm of crude oil. The maximum growth was observed in cellulose (80×10^6 CFU mL^{-1}) in pH 7, at 35ºC in 7 days followed by dextrose (75×10^6 CFU mL^{-1}), starch (66×10^6 CFU mL^{-1}), sucrose (60×10^6 CFU mL^{-1}) and glucose (58×10^6 CFU mL^{-1}) respectively. The bacterial consortium (*Pseudomonas aeruginosa* and *Bacillus pumilus*) degraded crude oil from 100 to 4ppm (96%) in the broth amended with 1% cellulose. The carbon sources of dextrose, sucrose, lactose and starch the crude oil degradation was 95, 92, 90 and 85% respectively. The above results showed that 1% of cellulose as carbon source was found to be optimum for crude oil degradation by the bacterial consortium (*Pseudomonas aeruginosa* and *Bacillus pumilus*). Rajakumar *et al*., (2008) also reported that carbon sources enhance the nitrate degradation percentage.

The effect of various nitrogen source on bacterial growth was studied in BH broth containing 100ppm of crude oil. The maximum growth was observed peptone (85×10^6 CFU mL^{-1}) was observed in pH 7 with 1% cellouse, at 35ºC in 7 days followed by soya bean powder (80×10^6 CFU mL^{-1}), yeast extract (76×10^6 CFU mL^{-1}), casein acid hydrolysate (70×10^6 CFU mL^{-1} and beef extract (62×10^6 CFU mL^{-1}) respectively. The bacterial consortium (*Pseudomonas aeruginosa* and *Bacillus pumilus*) degraded crude oil degraded from 100 to 5ppm (95%) in BH broth containing peptone as a nitrogen source. The nitrogen sources of beef extract, yeast extract, soya bean powder and casein acid hydrolysate the crude oil degradation was 93, 92, 91, and 89 % respectively. The above results showed that 1% of peptone, as nitrogen source was found to be optimum for crude oil degradation by the bacterial consortium (*Pseudomonas aeruginosa* and *Bacillus pumilus*). Ashok *et al*., (2016) also reported that nitrogen sources enhance the ethylbenzene degradation. In the nutrient enhancement, the effect of the nitrogen form on biodegradation is well documented in the literature (Shewfelt *et al*., 2005). The microbes found in soil, groundwater and superficial waters can break down hydrocarbons which will be utilized as carbon and energy source, thereby eliminating them from polluted environments (Mazzeo *et al*., 2010).

5. CONCLUSION

In this study, hydrocarbon utilizing Pseudomaonas sp. and Bacillus sp. bacteria was isolated from contaminated soil. Screening of crude oil degrading bacteria was performed by DCPIP redox indicator spectrophotometric technique. *Pseudomonas aeruginosa* and *Bacillus pumilus*. both are predominant bacterial strains have more ability to degrade the petroleum oil contamination was proved. Bacterial consortium used for crude oil degradation process and obtained better results for oil degradation. It was further investigated for degradation of hydrocarbon by gravimetric analysis revealed 78% of degradation. Using microbial consortium process is successful and safe way to enhance environment health in particular with low cost, technique and high public acceptance to cleaning up aquatic ecosystems from oil spills.

In this optimization study, *Pseudomonas aeruginosa* and *Bacillus pumilus* were found to be the most efficient in terms of crude oil degradation. The above results revealed that crude oil degradation by *Pseudomonas aeruginosa* and *Bacillus pumilus* were influenced by various pH, temperature carbon sources and nitrogen sources. The rate of bacterial growth and crude oil degradation was high with 1% cellulose and 1% peptone as the sole carbon source and nitrogen source under optimum conditions at an optimum temperature of 35^{o}C and pH 7. The cellulose and peptone is a successive nutrient source for the bacterial growth and could be useful to remediate containing crude oil. The maximum degradation was recorded up to 90% for a period of 7 days in optimization study. In the study carried out with bacterial consortium as inoculum. The bacterial consortia showed a superior crude oil degrading ability.

Many factors were studied such as pH, temperature, carbon and nitrogen sources were optimized and enhanced the bacterial consortium (*Pseudomonas aeruginosa* and *Bacillus pumilus*) to use crude oil as a substrate, degrade and detoxify. The bacterial growth with biocarrier materials was high with 1% cellulose and 1% peptone as the sole carbon source and nitrogen source under optimum conditions at an optimum temperature of 35^{o}C and pH 7 at 7 days. Therefore, information derived from metabolites during the process was effective and defined important factors interference on efficient clean-up of pollutants. Based on the observation, it could be concluded that the bacterial consortium (*Pseudomonas aeruginosa* and *Bacillus pumilus*) by bacterium is a potential biological resource and can be used for remediation of oil contaminated environment sites.

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