Discussion

We are lacking knowledge on the impacts of oil pollution and its elimination from our ecosystem (land or water). As well as its economic and geopolitical contributions, pipeline transportation, waste disposal has a great risk in terms of oil contamination by spillage, leakage, accidents and dumping etc., on the natural sources. Improving our knowledge on the effects and remediation of oil-related pollution has therefore a critical significance in the respect of sustainable use of the environment.

In bioremediation process, microbes metabolize the target contaminants to derive energy through enzyme driven oxidation-reduction reactions. The end products of bioremediation processes are non-toxic are relatively less toxic as compared to the parent contaminants. Prevailing environmental conditions are among the most important limiting factors for optimum bioremediation. The factors affecting the success rate of microbial bioremediation are nutrient availability, reaction (pH), temperature, C/N ratio, etc. The present study was carried out to access the viability of water. These qualities determine the capability of bacterial isolates to biodegrade petroleum compounds and their wastes like oily sludge, a hazardous hydrocarbon waste generated by the petroleum industry. The bacterial communities are believed to adapt the local environment. These environmental factors play a vital role in the bioremediation (Erdogan and Karaca, 2011).
The present study aimed to access the feasibility of using a bacterial consortium to degrade highly toxic oily waste water under optimized conditions during the course of treatment. The bacterial consortium, consisting of six bacterial strains, was applied as a carrier based inoculant to the contaminated water. The site of sampling was a part of Mathura Refinery situated in the state of Uttar Pradesh in Northern India. Bioremediation employs microorganisms capable of degrading toxic contaminants for reclamation of polluted sites. It is practically not possible to degrade it by single microbial species so a mixed microbial population is required to perform this task that has broad substrate specificity.

For the bioremediation studies, the oil degrading bacterial strains were obtained from the waste water itself. Different strains were obtained from the waste water samples at different times of sampling. Moreover, using an indigenous bacterial consortium ensures that the organisms has a higher tolerance to the toxicity of hydrocarbons and are resistant to variations in the environment (Dibble and Bartha, 1979; Ericksson et al., 1995). So it can be said that any waste environment contains its remediates in itself as shown in the studies prior the present work also.

The bioremediating bacterial strains isolated from the waste water samples were *Acinetobacter baumanii, Alcaligenes faecalis, Bacillus licheniformis, Bacillus subtilis, Corynebacterium propinquum* and *Pseudomonas aeruginosa.*
Few workers have reported the above mentioned bacteria helpful in bioremediating the crude oil waste water earlier. Aerobically degrading bacteria in organo-polluted site belongs to *Pseudomonas* sp., *Acinetobacter* sp., *Alcaligenes* sp., *Xanthomonas* sp., *Nocardia* sp., *Corynebacterium* sp., *Mycobacterium* sp., *Arthrobacter* sp. and *Bacillus* sp. (Ishak *et al.*, 2012; Shokrollahzadeh *et al.*, 2008).

The principal of the aerobic biodegradation is that: oxygen is needed by degradable organisms in their degradation at two metabolic sites, at the initial attack of the substrate and at the end of respiratory chain (Pedro and Walter, 2006). Bacteria could produce oxygenases and peroxidases they could help with the pollutant oxidization and get benefits from observing the energy, carbon and nutrient elements released during this process. A huge number of bacteria generally possess the capability to release non-specific oxidases and degrade organic pollutants. There are generally two types of relationships between the microorganism and organic pollutants: one is that the microorganisms use organic pollutant as sole source of carbon and energy, the other is that the microorganisms use a growth substrate as carbon and energy source, while another organic compound in the organic substrate which could not provide carbon and energy resource is also degraded, namely co-metabolism (Li *et al.*, 2013; Chandra *et al.*, 2012).
Discussion

These potential strains were selected due to their resistance on higher concentrations of crude oil belonging to aforementioned species identified by biochemical tests and later confirmed by BD-BBL Crystal Mind Autoreader™. The screening results showed that these species were predominated hydrocarbon degraders in the waste water. These findings are in agreement with many reports which indicate wide spread existence of *Acinetobacter*, *Alcaligenes*, *Bacillus*, *Corynebacterium*, and *Pseudomonas* species at the hydrocarbon contaminated areas (Jamijan et al., 1997; Khan et al., 2006).

In the experiments done for the study, pH 7 and 35°C temperature were found to be most suitable for the microbial growth. This data indicated that bacterial consortium can retain their degradation ability in a wide range of pH with optimum degradation at 35°C temperature where the pH of the water sample was done at neutral (pH7). This finding is in support with the reports of Ashok and Seth (1989) and Jilani and Khan (2013). The characteristics of many hydrocarbon degrading bacteria are being examined by the scientists for their possible use in bioremediation. Deeb and Alvarez-Cohen (1999) studied the temperature effects on the consortium of bacteria. They found their consortium grew best at 35°C temperature.

Verstraete *et al.* (1975) reported that a doubling rate of biodegradation of gasoline in acidic (pH 4.5) by adjusting the pH to 7.5. Rates dropped significantly, however, when the pH further rose to 8.5 which go with the
findings of present study. In present study, crude oil degrading bacteria grew well at the range of pH 7-8 but at pH less or more than this range showed a significant decrease in the bacterial population. Similarly, Dibble and Bartha (1976) observed an optimal pH of 7.8, in the range from 5.0 to 7.8 for the mineralization of oily sludge (Jain et al., 2011).

The presence of sulphide in the waste water samples could be due to the presence of trace concentration of sulphide compounds (i.e., mercaptanes and hydrogen sulphides) in wastewater. Hydrogen sulphides, mercaptanes and other sulphide compounds were reported to be present in petroleum refinery waste water (Skrtic, 2006; Hamza et al., 2012). It was found that the sulphide was present in the water samples ranged from 25.2-46.0 mgL⁻¹. After bioremediation with the bacterial consortium, the waste water samples showed reduction upto (53.27%) in the sulphide concentration present.

The observations showed upto 82.57% and 66.84% reduction in nitrogen and nitrate concentrations found in the waste water samples. Following data was observed that the bacterial strains incorporated in the consortium can lead to bioremediation. This study is supported by Focht and Verstraete (1973), Amin et al. (2013), they stated that unlike the limited species diversity of bacteria mediating nitrification, other genera of bacteria can also reduce nitrogen and nitrates. Among these, Pseudomonas, Bacillus and Alcaligenes are the most prominent.
The levels of heavy metals, namely cadmium, chromium, lead and zinc analyzed in the Mathura Refinery waste water were generally, above W.H.O. standards recommended for surface and sea waters. This is an indication of pollution. The oxidation state, solubility and association of metals with other organic and inorganic molecules can vary, however the microorganisms as well as higher organisms can play an important role in the bioremediation of the concentration of metals, so that they become less available and less hazardous (Bonaventura and Johnson (1997).

In the present study, the reduction of heavy metals is analyzed upto 96% which indicates that the isolated bacterial strains have good ability to bioremediate the heavy metals. These findings correlate with the earlier studies of some scientists that the isolated bacterial strains lead to bioaccumulation of heavy metals hence bioremediate the environment and reduces heavy metal concentration (Clausen, 2000; Rajbanshi, 2008; Kumar et al., 2013).

Bioremediation is a potential method for solving the problem of heavy metal pollution (Samal and Kotiyal, 2013). Among bacteria, Bacillus (Kim et al., 2007) and Pseudomonas (Jayashree et al., 2012) acts as a potent metal biosorbent. There are several researches carried out on the removal of heavy metals from wastes. Pseudomonas aeruginosa strains were found to be resistant to Pb, Cd, Zn, Cr and Cu (Soltan, 2001; Shakibaie et al., 2008, Bestawy et al., 2013). Prior studies shows that Bacillus subtilis is able to accumulate lead ions in its cell wall.
and has been shown to have a high absorptive capacity for lead, copper, zinc (Hookoom and Puchooa, 2013).

The organic matter present in the petroleum or crude oil provides food in form of nutrients to the microbes, hence the production of many organic and inorganic acids increases their population which results in hastening of the bioremediation process. The oxidation and reduction reactions carried out through bacteria detoxify the heavy metals by making them either more water-soluble and less-toxic or less water-soluble and precipitated or volatilized. Since most of the metals exist in the combined form as oxides, hydroxyl carbonates, chlorides, sulphides, silicates, their interactions with the gases being produced, converts them to respective oxides, sulphides, carbonates, hydroxides, sulphates or other compounds. Thus, the properties of heavy metals are changed. Alkali metals and alkaline earth metals are oxidized at a quicker rate at low temperature and at high temperature all the metals are oxidized.

Microbial remediation of a hydrocarbon site is accomplished with the help of a diverse group of microbes. These indigenous bacteria can degrade a wide range of target constituents present in oil contamination. According to the present study, *Acinetobacter*, *Alcaligenes*, *Bacillus*, *Corynebacterium* and *Pseudomonas* species were isolated from refinery waste water which degraded oil contents of water as well as hydrocarbons present in the waste water alongwith the heavy metals and other chemical components. Present
observations are in support with the prior findings of Kiyohara et al., (1992), Johnson et al. (1996), Pathak et al. (2008). *Bacillus licheniformis* has the potential to be used in bioremediation of petroleum crude oil. Thus, the bacterium was considered as good candidate for application in bioremediation process of petroleum contaminated sites. This bacteria is being reported only once in the previous studies as the potential bacterium in oil degradation by El-Sheshtawy et al., 2013.

In the present study, the total hydrocarbon reduction using bacterial consortium was recorded 22.61% minimum and 67% maximum. Some prior findings supports the research results of the study as reported efficiency of biodegradation ranges from 0.003% (Hollaway et al., 1980) to 100% (Phillips and Stewart, 1974) for marine bacteria. Many scientists reported that mixed populations with overall broad enzymatic capacities are required to degrade complex mixtures of hydrocarbons such as crude oil in fresh water (Cooney, 1984) and marine environments (Atlas, 1985; Floodgate, 1984; Das and Chandran, 2011). Several studies have indicated that extent of oil and total petroleum hydrocarbon (TPH) biodegradation is linked to oil type and its molecular composition (Sarma et al., 2004; Mandal et al., 2011; Mandal et al., 2012; Mohanty and Mukherji, 2013). According to Lal and Khanna (1996), 58% degradation in 15 days period was recorded in crude oil samples by *Acinetobacter* sp. and *Alcaligenes* sp. in combination. (Bhatnagar and Kumari, 2013).
The biodegradative pathways of petroleum hydrocarbons have been reviewed by many researchers as Higgins and Gilbert (1978), Chapman (1979), Horowitz (1989), Murugan *et al.* (2013), Chikere (2013). The biodegradation of hydrocarbon substrate occurs by inductive synthesis of enzymes when microorganisms are offered with the broad substrate range, then they do not attack the substrates simultaneously, but rather in definite sequence controlled by metabolic regulation called as diauxine and sequential biodegradation as described by Monod (1942).

The present data reported is in strong agreement with the earlier study of Ajao *et al.*, (2013) reported percentage decrease in COD (97%), BOD (94%), phenol (98%), TPH (79%) and oil & grease (90%) of refinery waste water after 20 days of treatment with bacterial consortium. He also showed a remarkable reduction in nitrate and sulphate.

The specificity of metabolic transformation of hydrocarbons is related to the genetic potential of particular microorganism to introduce molecular oxygen into the hydrocarbon with a few reactions to generate intermediates that subsequently enter general energy yielding catabolic reactions of the cell. The specific genetic capacity is expressed in the substrate specificity of enzymes for its substrate(s) and in ability of carbon source to induce various enzymatic activities necessary for its biodegradation. The final products of hydrocarbon oxidation are carbon dioxide, water, cell material and products of incomplete
oxidation and co-transformation reactions (Shailubhai, 1986). The investigation suggests that total biodegradation if hydrocarbon is a dynamic process including carbon transformation in carbon dioxide (mineralization), water, biomass, accumulation of extra cellular products and the persistence of traces of undegradable hydrocarbons.

Petroleum is in one sense, a natural product, resulting from the anaerobic conversion of biomass under high temperature and pressure. Most of its components are subjected to biodegradation, but at relatively slow rates (Cohen, 2002). Degradation of the hydrocarbons in the presence of synthetic surfactants is a delicate issue. Generally, the toxicity of surfactants increases with their hydrophobicity. The use of surfactants of biological origin solves the toxicity problem. Barkley et al. (1999) tested the effect of polymeric biosurfactant, Alasan, on the solubilization and biodegradation of polyaromatic hydrocarbons (PAHs), which resulted in solubilized PAHs by a physical interaction, probably hydrophilic. The reintroduction of indigenous microorganisms isolated from a contaminated site after culturing seems to be a highly effective bioremediation method, especially when microbial growth is supplemented by oxygen and fertilizers (added nutrients).