1. INTRODUCTION

Oil hydrocarbons are the most common soil contaminants. It has been estimated that the natural crude-oil seepage amounts to $6 \times 10^5$ metric tons per year, with a range of uncertainty of $2 \times 10^5$ to $2 \times 10^6$ metric tons per year (Kvenvolden and Cooper, 2003).

Petroleum hydrocarbons (crude oil and intermediate products) are the most prevalent environmental pollutants to all biological systems. Particularly, oil spills release crude oil into environments during exploitation, transportation and refining and cause critical concerns in environments, ecological systems and human health problems. Oil spills often result in both immediate and long-term environmental damage (Chandankere et al., 2014).

Soil is one of the earth’s most important natural resources to sustain human life. One of the main threats to soils, compromising their ability to perform the functions we expect, is pollution (Mikkonen et al., 2011). The contamination of soil and groundwater by hazardous chemicals has become a major concern due to the associated risks to human health and the environment. One hundred years ago oil exploitation began, first as a source of energy and later to include oil as a source of raw material. As a source of energy, crude oil went through an uninterrupted progression of its extraction during more than one century, driven by development of transport and industry.

Crude oil serves as the mainstay and the bedrock of economy of the Organization of Petroleum Exporting Countries (OPEC) and some other countries exporting crude oil. Petroleum-based products are the major source of energy for industry and daily life. Peoples have used petroleum since ancient times. The ancient Chinese and Egyptians burned oil to light their homes.
Before 1850s, Americans used whale oil to light their homes. When whale oil became scarce, people skimmed the oil that seeped to the surface ponds and streams. The demand for oil grew and in 1859, Edwin Drake drilled the first oil well near Titusville, Pennsylvania. At first, the crude oil was refined or made into kerosene for lighting. Gasoline and other products made during refining were thrown away because people had no use for them. This all changed when Hendry Ford began mass producing automobiles in 1913. Everyone wanted an automobile and they all ran on gasoline.

### 1.1. National and International status of crude oil demand

Annual worldwide consumption of petroleum hydrocarbons was estimated to be of the order of $10^{12}$ US gallon (Prince, 1993). India is both a major energy producer and consumer. India currently ranks as the world’s eleventh greatest energy producer, accounting for about 2.4% of the world’s total annual energy production and as the world’s sixth greatest energy consumer, accounting for about 3.3% of the world’s total annual energy consumption (Kandasamy, 2002). Oil is both the principle source of energy for man and an important environmental pollutant (Ferrari et al., 1996; Vasudevan and Rajaram, 2001). Crude oil is a complex mixture of many petroleum hydrocarbons, like alkanes, aromatics, resins and asphaltenes associated with other organic compounds containing sulfur, nitrogen and oxygen. Among petroleum hydrocarbons, aromatic compounds constitute a major fraction containing 30 poly aromatic hydrocarbons (Kumar et al., 2011).

### 1.2. Petroleum hydrocarbons

Petroleum was first obtained by the Chinese in pre-Christian times and has been known for several years to occur in surface seepage (Okoh, 2006). The modern petroleum industry had its beginning in Romania and in a Pennsylvania well sunk by Colonel E.A. Drake in 1859 (Alloway and Ayres, 1993). Petroleum is a naturally occurring, oily, flammable liquid that is
present beneath the surface of the earth. It is a mixture of hydrocarbons and elements like sulphur, oxygen and nitrogen. In the refining industry, petroleum refers only to crude oil (Van, 1997). Crude oil originates in naturally occurring geological deposits formed from the organic decomposition of ancient plants and animals under high temperatures and pressure. Crude oil is principally composed of liquid hydrocarbons having four or more carbon atoms (Manning and Thompson, 1991; Van, 1997). Alkanes, cyclo alkanes and aromatics are the three principle classes of hydrocarbons present in petroleum (Strausz and Lown, 2003). The major types of hydrocarbons are alkanes, alkenes, alkynes and aromatic compounds.

1.2.1. Alkanes

Alkanes, also called paraffin or aliphatic hydrocarbons, are hydrocarbons in which the C atoms are joined by single covalent bonds (sigma bonds) consisting of 2 shared electrons. As with other organic compounds, the carbon atoms in alkanes may form straight chains, branched chains or rings. These three kinds of alkanes are straight-chain alkanes, branched-chain alkanes and cycloalkanes. Related to environmental or aquatic toxicology, (unsubstituted) alkanes are generally not of much toxicological concern. Other than fire or explosion hazard, the main concern seems to be potential effects on the CNS or as general asphyxiants or irritants. The same cannot be said for the more hazardous halogenated alkanes such as carbon tetrachloride (Maple et al., 2004).

1.2.2. Alkanes and Alkyl Groups

Most organic compounds can be derived from alkanes. In addition, many important parts of organic molecules contain one or more alkane groups minus a hydrogen atom bonded as substituents onto the basic organic molecule. As a consequence of these factors, the names of many organic compounds are based upon alkanes and it is useful to know the
names of some of the more common alkanes and substituent groups derived from them (Maple et al., 2004).

1.2.3. Alkenes and Alkynes

Alkenes or olefins are hydrocarbons that have double bonds consisting of 4 shared electrons. The simplest and most widely manufactured alkene is ethylene, used for the production of polyethylene polymer. Another example of an important alkene is 1,3-butadiene, widely used in the manufacture of polymers, particularly synthetic rubber. The lighter alkenes, including ethylene and 1,3-butadiene, are highly flammable. Like other gaseous hydrocarbons, they form explosive mixtures with air. Acetylene is an alkyne, a class of hydrocarbons characterized by carbon-carbon triple bonds consisting of 6 shared electrons. Acetylene is a highly flammable gas that forms dangerously explosive mixtures with air. It is used in large quantities as a chemical raw material. Acetylene is the fuel in oxyacetylene torches used for cutting steel and for various kinds of welding applications. Some types of organochlorides have significant toxicity to plants or animals, including humans. Dioxins, produced when organic matter is burned in the presence of chlorine and some insecticides, such as DDT, are persistent organic pollutants which pose dangers when they are released into the environment. For example, DDT, which was widely used to control insects in the mid 20th century, also accumulates in food chains and causes reproductive problems (i.e., eggshell thinning) in certain bird species (Muller et al., 2008).

1.2.4. Aromatic Hydrocarbons

The ary1 compounds have substituent groups containing atoms of elements other than hydrogen and carbon and are called aromatic compounds or aryl compounds. Aromatic compounds have ring structures and are held together in part by particularly stable bonds that contain
delocalized clouds of so-called p (pi, pronounced “pie”) electrons. These compounds are formed by the incomplete combustion of other hydrocarbons, a process that consumes hydrogen in preference to carbon. The carbon residue is left in the thermodynamically favoured condensed aromatic ring system of the PAH compounds. Because there are so many partial combustion and pyrolysis processes that favour production of PAHs, these compounds are encountered abundant in the atmosphere, soil and elsewhere in the environment from sources that include engine exhausts, wood stove smoke, cigarette smoke and charbroiled food. Coal tars and petroleum residues such as road and roofing asphalt have high levels of PAHs. Some PAH compounds, of which the most widely investigated has been the 5-ringed compound benzo (a) pyrene, are of toxicological concern because they are precursors to cancer-causing metabolites. The majority of hydrocarbons found on earth naturally occur in crude oil, where decomposed organic matter provides an abundance of carbon and hydrogen which, when bonded, can catenate to form seemingly limitless chains. Oil contamination with petroleum and petroleum-based hydrocarbons in accidental spills has caused critical concerns in environment, ecological systems, human health, tourism and recreation activities (Tahir et al., 2014).

1.3. Impacts of crude oil pollution

As petroleum exploration and commercialization continues to increase, routine and accidental spills are causing greater damage to the environment (Plate 1.1 and 1.2). The persistence of these contaminants can cause irreversible damage to the soil, air, rivers, seas and groundwater (Vieira et al., 2009). Leaks and accidental spills occur regularly during the exploration, production, refining, transport and storage of petroleum and petroleum products. The amount of natural crude oil seepage was
estimated to be 600,000 metric tons per year with a range of uncertainty of 200,000 metric tons per year.

The oil industry unavoidably generates large quantities of oily and viscous residue of oily sludge, which is formed during various production, transportation and refining. Petroleum hydrocarbons are among the most common and widespread environmental contaminants, adversely affecting human health and posing environmental problems (Boulding, 1996; Kingston, 2002). The exploitation of petroleum has generated various sources of pollution in soil, air, water. Oil spills destroy ecosystems and kill wildlife, but people's health is directly affected too. Oil is semi-volatile, which means that it can evaporate into the air and create a heavy vapor that stays near the ground - in the human breathing zone. When winds whip up oily sea water, the spray contains tiny droplets - basically a fume of oil, which are small enough to be inhaled deep into the lungs. Oil contains petroleum hydrocarbons, which are toxic and irritating to the skin and airways. It also contains volatile chemicals, called VOCs, which can cause acute health effects such as headaches, dizziness and nausea. Over the long term, many of these chemicals have been linked to cancer, so there are lots of reasons to worry about inhaling them. In the case of plants oil effect the growth by messing up the nutrients by going into the soil and erupting the root so it stops its growth.

In order to resolve this problem, several techniques have been developed, including Physical, chemical and bioremediation, which is of great interest because of the possibility soil reuse. Physical methods, such as stripping or sorption, are not as effective as biological methods for treating hazardous organic compounds (Knox et al., 19860). The various conventional cleaning methods such as physical methods like the first response selection, rarely achieve complete cleanup of oil spills. According to
the Office of Technology Assessment it’s considered to the current mechanical methods typically recover no more than 10-15% of the oil after a major spill. Several types of physical method were used for crude oil removal that is booms, skimmers, manual removal, mechanical removal, washing, sediment relocation, tilling and in-situ burning (U.S. Congress, 1991).

The chemical structure and then the behavior of pollutants are changed by means of chemical reactions to produce less toxic or better separable compounds from the matrix. Thermal processes use heat to increase the volatility, to burn, decompose, destroy or melt the contaminants. Chemical constituents are burned and chemically oxidized by applying a high heat input. The most important problem in incinerating hazardous wastes and soil is the generation of by-products such as polychlorinated dibenzofurans (PCDF), chlorinated benzenes, chlorinated phenols and nitrogen oxides (Song et al., 1992; Nito et al., 1997).

Bioremediation is based on the capacity of microorganisms to degrade organic pollutant compounds, such as hydrocarbons. These compounds are important soil pollutants because of the high toxicity of the polycyclic aromatic hydrocarbon (PAH) fraction. According to the environmental protection agency (EPA), 16 PAHs have been reported as carcinogenic and mutagenic compounds (Verdin et al., 2004). So it is necessary to remove them from contaminated sites. A studies have reported several bacteria and filamentous fungal species with the capacity to mineralize or to degrade PHAs (Boonchan et al., 2000).

1.4. Remediation strategies for crude oil pollution

Recovery of spilled crude oil by physical and chemical methods over the marine water is possible only to 10-15% of the oil. As compared to physical and chemical methods such as booms, skimmers, adsorbents, chemical surfactants, oxidants, etc. bioremediation are thought to be a self-
driven, economical and ecofriendly method using suitable microorganisms, highly hazardous oily material can be easily mineralized to harmless end products at a very low cost (Rahman et al., 2002).

In recent years, researchers are working hard to find an effective and efficient way to remove the oil contaminants from the environment (Wei et al., 2004). Biodegradation can be described as the conversion of chemical compounds by living microorganisms, into energy, cell mass, carbon dioxide and biological waste products. Conventional activated sludge treatment has some drawbacks, such as low resistant to loading rate, sludge expansion, sensitivity to low temperatures and toxic compounds, loss of activity, biomass and more equipment needed for accumulating sludge, instability due to loading shock and fluctuation and further treatment of excess sludge (Chen et al., 2008). Hence, the biological methods are environmental friendly and retain the quality of environments (soil or water) during the remediation process. Besides, these methods are cheaper than physical and chemical techniques used for remediation.

1.5. Bioremediation of crude oil contaminated soil

The hydrocarbon degrading microorganisms occur in most environments, where hydrocarbons may serve as organic carbon sources. Bioremediation is based on the use of microorganisms or microbial processes to degrade environmental contaminants and offers several advantages over the conventional chemical and physical technologies. It can be a cost effective and environmental friendly technology. Biodegradation is defined as the biologically catalyzed reduction in complexity of chemical compounds (Alexander, 1994; Pepper et al., 1996).

It would be interesting to develop the bioremediation process further using fungi, because of their capacity to incorporate rapidly into the soil matrix. Furthermore, they have the ability to grow in environments with low
nutrient concentrations, low humidity and acidic pH (Potin et al., 2004; Mollea et al., 2005). Several different bioremediation techniques have been developed, but biostimulation is the most often used (Head, 1998). This consists of the activation of native soil microorganisms through the addition of nutrients (Providenti et al., 1993) reported that an efficient removal of contaminants requires $1 \times 10^3$ CFU g$^{-1}$ of soil, although other factors to be considered are the molecular structure and bioavailability of the contaminants (Juhasz and Naidu, 2000). The microbial culture must have the ability to withstand different soil environmental condition and to survive in the presence of other microorganisms (Roberts, 1998). Biosurfactants act by emulsifying hydrocarbons, increasing the solubilization of crude oil and subsequent availability for microbial degradation (Menezes et al., 2005; Zhang et al., 2005).

1.6. Bioremediation of crude oil contaminated water

Diverse components of crude oil and petroleum, such as polycyclic aromatic hydrocarbons (PAHs) have been found in waterways as a result of pollution from industrial effluents and petrochemical products (Beckles, et al., 1998). Poor miscibility of crude oil accounts for accumulation of free oil on the surface of groundwater and this may migrate laterally over a wide distance to pollute other zones very far away from the point of pollution. Industrial and municipal discharges as well as urban run-offs, atmospheric deposition and natural seeps also account for petroleum hydrocarbon pollution of the environment. It is worthy of note that groundwater is one of the many media by which human beings, plants and animals come into contact with petroleum hydrocarbon pollution.

1.7. Surfactants

The term surfactant was derived from the phrase ‘surface active agents’ and describes the activity of these amphiphilic molecules at the
interfaces between different phases, gaseous, liquid and solid. Nowadays, the huge demand of surfactants is currently provided by chemical surfactants derived from petroleum, but these compounds have the problem of being toxic to the environment and non-biodegradable. Surfactants are acts as detergents, wetting agents, emulsifiers, dispersants and foaming agents and form major ingredients of many product formulations ranging from household detergents, shampoos, personal care products and pharmaceuticals to paints. The worldwide use of surfactants is enormous, estimated in 2008 to be 13 million tonnes per annum (p.a.) with a predicted increase in use by approximately 2% per annum (Reznik et al., 2010).

1.8. Biosurfactants

Biosurfactants are a leading group of valuable microbial natural products with unique biochemical properties. From a biotechnology prospective, the production of biosurfactants is important owing to their vast applications in food, cosmetics, pharmaceuticals, agricultural and the petrochemical industries (Pruthi and cameotra, 2003; Nguyen et al., 2008; Abouseoud et al., 2008). They are interesting amphiphilic biomacromolecules with various biological functions/properties such as enhancer of polycyclic aromatic hydrocarbons bioavailability to microorganisms and accordingly their degradation, reducer of the interfacial tension by partitioning preferentially at the interfaces, effective activity on surfaces, oil recovering and so on (Desai and Banat, 1997; Pruthi and cameotra, 2003; Nguyen et al., 2008; Joshi et al., 2008).

Biosurfactant is a surfactant produced extra cellularly or as part of the cell membrane by bacteria, yeasts and fungi from various substrates including sugars, oils, alkanes among others (Mulligan, 2005). They are amphiphilic compounds with considerable potential in commercial applications within various industries, such as health care and food processing industries,
enhancing oil recovery, crude oil drilling lubricants, bioremediation of water-insoluble pollutants (Peypoux et al., 1999; Fox and Bala, 2000).

Microbial biosurfactants exert some influence on interfaces in both aqueous solutions and hydrocarbon mixtures. These properties cause micro-emulsions in which micelle formation occurs where hydrocarbons can solubilize in water, or water in hydrocarbons. Generally, biosurfactants are classified into five major groups, viz. glycolipids, phospholipids and fatty acids, lipopeptides (lipoprotein), polymeric and particulate biosurfactant.

They are potential alternatives of chemically synthesized surfactant in a variety of applications because of their advantages such as lower toxicity, higher biodegradability, better environmental compatibility, lower critical micelle concentration, each of production, ability to be synthesized from renewable resources, higher foaming, higher selectivity, specific activity at extreme temperature, pH and salinity (Desai and Banat, 1997; Mukherjee et al., 2006). In this recent year, the biosurfactants have been placed on the environmental impacts of chemical surfactants and new surfactants for use in any field (Surachai et al., 2007).

1.8.1. Microbial enhanced oil recovery: biosurfactants and other applications

The largest possible market for biosurfactant is the oil industry, both for petroleum production and for incorporation into oil formulations (Dyke et al., 1991). Other applications related to the oil industries include oil spill bioremediation/dispersion, both inland and at sea, removal/mobilization of oil sludge from storage tanks and enhanced oil recovery (Georgiou et al., 1992; Khire and Khan, 1994a,b). Emulsification and de-emulsification, dispersion, foaming, wetting and coating are some of the numerous surface activities that biosurfactants can achieve when applied within systems such as immiscible liquid/liquid (e.g., oil/water), solid/liquid (e.g., rock/oil and
rock/water) and gas/liquid. Therefore, the possibilities of exploiting these bioproducts in oil related sciences are vast and made petroleum industry their largest possible market at present. The role of biosurfactants in enhancing oil recovery from reservoirs is certainly the best known; however they can be effectively applied in many other fields from transportation of crude oil in pipeline to the cleanup of oil storage tanks and even manufacturing of fine petrochemicals. When properly used, biosurfactants are comparable to traditional chemical analogues in terms of performances and offer advantages with regard to environment protection/conservation.

The second largest market for biosurfactants is emulsion polymerization for paints, paper coatings and industrial coatings. Layman (1985) described other uses of surfactants including asphalt, cement, textile and fiber manufacturing, in addition to metal treatment, mining, water treatment, coal slurry defoamers and as wood preservatives. Surfactants are also used in food and cosmetic industries, industrial cleaning of products and in agricultural chemicals to dilute and disperse fertilizers and pesticides and to enhance penetration of active compounds into plants (Kosaric et al., 1987). A comprehensive detail of various potential applications of biosurfactants as fine specialty chemicals are shown in Table 1.1. Ishigami (1997) has speculated on potential applications for biosurfactants in bioengineering including their use as cryopreservatives, protein solubilizers and enzyme stabilizers, DNA isolating agents, preservatives for cut flowers, growth enhancers for plants, recovery enhancers for wounds and swelling and for the control of biomembranous functions.

1.9. Bacterial consortium on remediation of pollutants

Bacterial consortia are used as black boxes without analyzing the constituent microbial population for environmental remediation. The complexity of bacterial consortium enables them to act on a variety of
pollutants (Watanabe and Baker, 2000). Although, many microorganisms are capable of degrading the crude oil present in the soil, it has been found beneficial to employ mix culture opposed to pure cultures in bioremediation as it shows the synergistic interactions. Bacteria for hydrocarbon decomposition are commercially available as freeze dried bacteria, which can be used for bioremediation after propagation to a minimum of $2 \times 10^8$ CFU/ml. Bacteria can degrade petroleum products are *Pseudomonas*, *Aeromonas*, *Moraxella*, *Beijerinckia*, *Flavobacteria*, *Chrobacteria*, *Corynebacteria*, *Atinetobacter*, *Mycobactena*, *Modococi*, *Bacilli*, *Arthrobacter*, *Aeromonas*, *Cyanobacteria* etc (Emami et al., 2012).

### 1.10. Fungi on remediation of crude oil contamination

In natural ecosystems, fungi plays an important role during their ability in removing hazardous compounds from the water, whereas sediment particles contaminated with crude oil from oil spills is one of the desired ecological niche to fungi which inhabits such substrate and use carbon source from hydrocarbons in polluted sediment particles to biodegrade crude oil from the sediments in the beaches. Fungi have been found to be better degraders of petroleum than traditional bioremediation techniques including bacteria. Although hydrocarbon degraders may be expected to be readily isolated from a petroleum oil- associated environment, the same degree of expectation may be anticipated for microorganisms isolated from a totally unrelated environment (Batelle, 2000; Ojo, 2005).

Many researchers studied the role of fungi on biodegradation of petroleum contaminants and the most common fungi which have been recorded as a biodegrades belongs to following genera: *Alternaria*, *Aspergillus*, *Candida*, *Cephalosporium*, *Cladosporium*, *Fusarium*, *Geotrichum*, *Gliocladium*, *Mucor*, *Paecilomyces*, *Penicillium*, *Pleurotus*, *Polyporus*, *Rhizopus*, *Rhodotolura*, *Saccharomyces*, *Talaromyces and Torulopsis* (Saraswathy et al.,
1.11. **Insitu and exsitu remediation of crude oil**

Several authors studied removal of crude oil through application of chemical surfactants, emulsification, absorption using natural resources and etc. for the wastewater system. However, removal of crude oil in soil and sediments through biodegradation and emulsification using microorganisms and bioactive agents has not yet been conducted. Therefore, in this study an integrated approach was attempted using biological methods for a more efficient removal of crude oil from contaminated soil (Carberry and Wik, 2001).

Because of the above significance the present study was framed for bioremediation of crude oil by efficient bacterial consortium isolated from crude oil contaminated soil and water samples. In the absence of effective measures to remove crude oil from the contaminants, it was felt that it would be more appropriate to use aerobic microorganisms and microbial biosurfactant to tackle the problems of crude oil pollution. Hence, this present study was made with the following objectives.
Objectives of this study

1. To enumerate and identify the bacterial populations present in the crude oil contaminated soil and water.
2. To analyse the crude oil resistance and degrading ability by primary and secondary screening.
3. To optimize the degrading ability by various factors (carbon source, nitrogen source, pH and temperature).
4. To remediate the crude oil contaminated water using bacterial consortium through an aerobic bioreactor.
5. To determine the biosurfactant production by various assays and evaluate the mass production, extraction, preliminary characterization, composition, stability studies (Effect of pH, Temp and NaCl concentration) and antibacterial activity of biosurfactant.
6. To remediate the crude oil contaminated soil using individual and bacterial consortium through soil column.
7. To evaluate the application of biosurfactant and synthetic surfactant on emulsification of crude oil contaminated soil through soil column.
8. To evaluate the toxicity of the soil column extracts on the germination and genetic characteristics of onion (var: Allium oschaninii).