Land and water are precious natural resources on which rely the sustainability of agriculture and the civilization of mankind. Unfortunately, they have been subjected to maximum exploitation and severely degraded or polluted due to anthropogenic activities Water pollution is a serious problem in India as almost 70 per cent of its surface water resources are contaminated by biological, toxic, organic, and inorganic pollutants [1]. The water bodies get polluted by vehicle exhaust and metals from smelting and mining, soluble salts (natural and artificial), PCO spills, use of insecticides/pesticides and fertilizers from agriculture, disposal of industrial and municipal wastes etc[2]. Among these pollutants, contamination by heavy metals and petroleum polyaromatic hydrocarbons are most serious because of their persistence in the environment which leads to bioaccumulation, toxicity and carcinogenicity in human beings and aquatic life[3].

1.1 HEAVY METALS

Heavy metals are commonly defined as those having a specific density of more than 5 g/cm^3.Heavy metals pollution is an environmental problem of worldwide concern because most of them can be toxic even at low concentrations. Industrialized societies are responsible for increasing environmental contamination by trace metals produced as wastes from industrial and agricultural processes and household activities[4]. Some of these metals include cadmium, lead, mercury, arsenic, silver, cobalt, etc.

1.1.1 Possible sources and adverse effects of heavy metal pollution

The main threats to human health from heavy metals are associated with exposure to lead, cadmium, lithium and mercury. However other heavy metals such as copper, manganese etc also have adverse effects on living organisms.

A. Lead

Batteries remain the main contributors of lead pollution and metals processing is the major source of lead emissions to the air today. Paints are also source of exposure to lead[5]. During the last century, lead emissions to ambient air have

caused considerable pollution, mainly due to lead emissions from petrol[6]. While natural levels of lead in PCO ranges between 50 and 400 ppm, mining, smelting, and refining activities have resulted in substantial increases in lead levels in the environment, especially near mining and smelting sites. When lead is released to the air from industrial sources or vehicles, it may travel long distances before settling to the ground, where it usually sticks to soil particles. Lead may move from soil into ground water depending on the type of lead compound and the characteristics of the soil[7].

Lead has a high tendency to interact with proteins and enzymes of the living systems[9].Lead is also observed to create cell toxicity by reacting with anionic group like OH^- in the cell to precipitate as hydroxides. Children are particularly susceptible to lead exposure due to high gastrointestinal uptake and the permeable blood–brain barrier. Blood levels in children should be reduced below the levels so far considered acceptable, recent data indicating that there may be neurotoxic effects of lead at lower levels of exposure than previously anticipated[6].

B. Cadmium

Cadmium compounds are used in the metal plating and battery industry, and as stabilizing agents in many polyvinyl chloride (PVC) products. Cadmium is a component of petrol, diesel fuel and lubricating PCOs. Metallic cadmium has mostly been used as an anticorrosion agent (cadmiation). Cadmium is also present as a pollutant in phosphate fertilizers[10]. Natural as well as anthropogenic sources of cadmium, including industrial emissions and the application of fertilizer and sewage sludge to farm land, may lead to contamination of soils, and increased cadmium uptake by crops and vegetables, grown for human consumption[11]. Weathering and erosion result in the transport of large quantities of cadmium by rivers to the world's oceans. An annual gross input of 15,000 tonnes of cadmium has been estimated. Moreover, between about 900 and 3,600 tonnes of cadmium are estimated to be deposited to aquatic environments throughout the world through atmospheric deposition of emissions originating from anthropogenic and natural sources[6].

The most significant route of exposure to cadmium and or cadmium compounds for most members of the general public is through food, since food

materials tend to take up and retain cadmium. Cadmium, especially cadmium oxide is a 'probable carcinogen'. There is evidence of it causing prostate and kidney cancer in humans, it has been shown to cause lung and testicle cancer in animals[11]. Animal experiments have suggested that cadmium may be a risk factor for cardiovascular disease. However, a Japanese study showed an excess risk of cardiovascular mortality in cadmium-exposed persons with signs of tubular kidney damage compared to individuals without kidney damage [12].

C. Lithium

Lithium is a widely used and is an effective treatment for mood disorders. Lithium batteries are also used extensively these days in electronic gadgets like laptops which also lead to accumulation of lithium and toxicity after its improper disposal. Lithium can be found throughout the world, but brine waters contain up to 0.050 to 2000 ppm, seawater up to 0.170 ppm, and freshwater typically less than 0.001 to 0.003 ppm and daily lithium content from food has been estimated at 2 milligrams per day with the primary source being grains and vegetables[13].

Lithium toxicity presented a neurological syndrome suggesting a diagnosis of Creutzfeldt-Jakob disease[14]. Exposure to lithium via drinking water and other environmental sources may affect thyroid function. This stresses the need to screen for lithium in all drinking water sources[15].

D. Mercury

Metallic mercury is used in thermometers, barometers and instruments for measuring blood pressure. A major use of mercury is in the chloralkali industry, in the electrochemical process of manufacturing chlorine, where mercury is used as an electrode[16]. The general population is primarily exposed to mercury *via* food, fish being a major source of methyl mercury exposure, and dental amalgam. Inorganic mercury is converted to organic compounds, such as methyl mercury, which is very stable and accumulates in the food chain[17].

Methyl mercury poisoning has a latency of 1 month or longer after acute exposure, and the main symptoms relate to nervous system damage. High doses may lead to death, usually 2–4 weeks after onset of symptoms. The Minamata

catastrophe in Japan in the 1950s was caused by methyl mercury poisoning from fish contaminated by mercury discharges to the surrounding sea[18].

E. Copper

Copper is used widely in electronic and electrical applications, in heat exchangers, in motors, for plumbing fittings, in building construction and roofing, in chemical and marine equipment, coal-fired power stations, metal production, waste incinerators, sewage treatment processes and from the application of agricultural chemicals. Smaller amounts are also released naturally from the earth's crust[19]. The concentration of copper in lakes and rivers ranges from 0.5 to 1,000 ppb with an average concentration of 10 ppb. The average copper concentration in groundwater (5 ppb) is similar to that in lakes and rivers; however, monitoring data indicate that some groundwater contains levels of copper (up to 2,783 ppb) that are well above the standard of 1,300 ppm for drinking water.

Some species accumulate copper. Toxic effects on fish and other aquatic organisms have also been observed. No significant effects on the global environment are expected. High level exposure (following an accident or in an occupational setting) might cause chest pains, vomiting and irritation of the eyes and nose.

F. Manganese

Manganese is released to the environment from industrial emissions, fossil fuel combustion, and erosion of manganese-containing soils. Almost 80% of industrial emissions of manganese are attributable to iron and steel production facilities[20]. Manganese occurs naturally in surface water and groundwater. A median dissolved manganese concentration of 24 µg/L in samples from 286 U.S. rivers and streams was reported.

Cholestatic disease and nervous system disorders have been associated with toxic concentrations of manganese[21]. An epidemiological study in Japan described adverse effects in humans consuming manganese dissolved in drinking-water, probably at a concentration close to 28 mg/l[22]. The manganese was derived from

400 dry-cell batteries buried near a drinking-water well. Fifteen cases of poisoning were reported among 25 persons examined, with symptoms including lethargy, increased muscle tone, tremor and mental disturbances[23].

1.2 PETROLEUM POLY AROMATIC HYDROCARBONS

Poly Aromatic Hydrocarbons (PAH) are a class of fused-ring aromatic compounds which are of great concern because of their toxicity, carcinogenicity, potential for toxic biomagnifications and resistance to biodegradation. The demand for processed petroleum products and agricultural produce has exposed our environment to PAH contamination. PAH sticks to solid sediments and are ubiquitous including soil, water and air. PCO which is the source for fractional distillation of PCO comprises of carbon and hydrogen molecules in complex chains[24]. In addition, impurities like sulphur, nitrogen and metals are also present in the crude. This PCO consists of hydrocarbons which are classified as paraffinic, naphthenic, or aromatic crudes depending on their composition[25].

1.1.1 Possible sources and adverse effects of petroleum PAH pollution

India's oil and gas sectors meet around 42% of the country's primary energy demand and contribute over 15% to the gross domestic product. The major source of oil pollution is the oil spills. Oil spilling accidents are very common in the areas near the seashore because of oil tankers shipping crude oil and bursting of oil supply pipelines[26]. The oil spill which began in the Gulf of Mexico in the year April 2010 was not fully contained until late July 2010 and throughout the duration of the spill it was estimated that 53,000 barrels of PCO per day were leaked into the Gulf of Mexico. In total almost 5 million barrels of PCO were released. In the year 1979, an amount of 140 million gallons of PCO was spilled in the Gulf of Mexico. An PCO spill of the amount of 400-800 tonnes occurred in Mumbai in the month of August 2011 which caused a wide spread mortality of sea animals[27].

Pollution caused by petroleum hydrocarbons in terrestrial and aquatic environment is a common phenomenon that causes significant ecological and social problems[28]. PCO when spilled on land affects the physicochemical properties of the soil such as temperature, structure, nutrient status and pH. Toxicity of PCO includes

liver necrosis, congestion of the liver, fatty degeneration, and dissociation of hepatocytes. Birds and animals in PCO-contaminated area are found to have black emulsion in the digestive tract with a petroleum odor. This leads to decrease in the absorption of nutrients and finally leads to death of these birds and animals[29]. Adverse effects such as genetic damage, liver disease, and cancer can occur within the wildlife among other aquatic life. Reproductive and developmental defects, as well as immune impairments have occurred and are still being documented as of 2012. When the aquatic environment becomes toxic this affects humans too[30].

The removal of these harmful pollutants from the environment is being done by many physical and chemical methods. These methods have many disadvantages like high cost, non-specificity and incapability to cause complete mineralization and therefore they are used in a small scale. Hence, the present day challenge is to develop a novel cost effective environmental friendly method to remove these pollutants from the environment.

1.3 BIOREMEDIATION

According to the Environmental Protection Agency (EPA), Bioremediation is a "treatment that uses naturally occurring organisms to break down hazardous substances into less toxic or non toxic substances". Technologies can be generally classified as *in situ* or *ex situ*. *In situ* bioremediation involves treating the contaminated material at the site, while *ex situ* involves the removal of the contaminated material to be treated elsewhere. Some examples of bioremediation related technologies are phytoremediation, bioventing, bioleaching, bioreactor, composting, bioaugmentation, rhizofiltration and biostimulation. The bioremediation method is mindful of the environment and secondly, no dead-end products are produced to initiate further contamination of the environment.

Microbes play direct or indirect roles in several biological activities. Metals and nonmetals present on earth are in constant association with biological components. This interaction is natural and continuous since the inception of life and provides the exciting areas of research. Hence, bioremediation also involves the use

of these micro organisms which degrade the hazardous substances by mineralizing the compound to harmless inorganic molecules such as carbon dioxide and salts or converting compound to some other compound, which may also be toxic and recalcitrant to further degradation or producing enzymes which degrade compounds through exploitation of the organism's energy need [31].

The ecology of hydrocarbons and metal degradation by microbial populations in the natural environment has been reviewed, emphasizing the physical, chemical, and biological factors that contribute to the biodegradation of petroleum, hydrocarbons and heavy metals[32].

1.3.1 Mechanism of bioremediation of heavy metals

Micro organisms can change the oxidation state of metals and concomitantly deposit metal oxides and zerovalent metals on or into their cells. Because of their specific characteristics, such as high specific surface areas and high catalytic reactivity, biogenic metals offer promising perspectives for the sorption and (bio) degradation of contaminants[33].

Another method by which micro organisms remediate the metals is by nanoparticle synthesis. Micro organisms grab the target ions from the environment and convert them into elements after the release of enzymes. This process may be intracellular or extracellular. In the intracellular method, the ions are transported into the cell after which the enzymes act and nanoparticle synthesis occurs inside the cell. In extracellular synthesis, the ions are trapped on the surface of the cells after which the enzymes act resulting in the synthesis of nanoparticles[34].

1.3.2 Mechanism of biodegradation of PCOs

A. Chemical Degradation

Determination of PAH degradation in the environment is quite an onerous task. The occurrence of PAH in anaerobic conditions depends on certain factors which include substrate interaction, pH and redox conditions[35]. PAH in soil could be degraded through biotic processes. Oxidation reactions are viewed as the most effective in this regard, although some authors have suggested that photochemical

reactions may also aid oxidative reaction processes[36].It must be noted that most oxidation reactions in the environment are initiated by oxidants such as peroxides (H_2O_2), ozone (O_3) and hydroxyl radicals generated by photochemical processes. When PAH undergo chemical reactions, they are transformed into other polyaromatic hydrocarbons (they do not lose their aromatic character). Their aromaticity is conserved since considerable amounts of energy are required to change an aromatic compound into a non aromatic compound. The efficiency of PAH chemical degradation is limited by their low aqueous solubility and vapour pressure [37].

B. Biodegradation

Biodegradation is one of the forms of bioremediation applied to treat soils, water or sediments contaminated with PAH. It is the use of microorganisms to degrade or detoxify environmental pollutants[38].Biodegradation is also a clean-up method that presents the possibility to eliminate organic contaminants with the aid of natural biological activity available in the substrate[39]. The microorganisms used for biodegradation should be indigenous to the contaminated area or site[40].It is expected that the bacteria will be able to degrade the contaminant by multiplying in population and then decline when the contaminant is finally degraded[41].The products of complete mineralization of the pollutant by biodegradation process include; CO_2, H_2O and cell biomass [42].

The ultimate goal of any remediation process should not be limited to removing contaminants from polluted substrates but should also include restoring the ability of the soil to function according to its potential[43].In this regard, the bioavailability of PAH becomes a very important factor to consider. Bioavailability of PAH in biodegradation is determined by certain complex interactions between biotic and abiotic factors. Biotic factors affecting bioavailability include; the metabolic ability of microorganisms to degrade PAH as well as the ways in which the bacteria encourage PAH accessibility. One way by which microbes encourage PAH accessibility is by the production of biosurfactants. Biosurfactants are produced to increase the solubility of insoluble substrates or solubilised (emulsify) insoluble

substrates in order to enhance their direct contact between microorganisms and contaminants [44].

1.4 BIOSURFACTANTS

Hydrocarbon degrading microorganisms produce biosurfactants of different chemical nature and molecular size which are surface active compounds which increases the surface tension of the hydrophobic water-insoluble substrates and thereby enhances their bioavailability and the rate of bioremediation[45]. Almost all surfactants currently produced are chemically derived from petroleum. These synthetic surfactants are usually toxic themselves and are hardly degraded by microorganisms. They are, therefore, a potential source of pollution and damage to the environment. These hazards associated with synthetic emulsifiers have, in recent years, drawn much attention to the microbial production of surfactants or biosurfactants [46].

Biosurfactants derived from living organisms, mainly microorganisms have attracted much attention because of advantageous characteristics such as structural diversity, low toxicity, higher biodegradability, better environmental compatibility, higher substrate selectivity, and lower Critical Micelle Concentration (CMC). These properties have led to several biosurfactant applications in the food, cosmetic and pharmaceutical industries[47,48].Thus, based on their chemical composition, the biosurfactants produced by microorganisms are of many types such as glycolipids, lipopolysaccharides, oligosaccharides, and lipopeptides [49].

1.4.1 Role of Biosurfactants in uptake of metals

All biosurfactants are amphiphiles, they consist of two parts—a polar (hydrophilic) moiety and non polar (hydrophobic) group. A hydrophilic group consists of mono-, oligo- or polysaccharides, peptides or proteins and a hydrophobic moiety usually contains saturated, unsaturated and hydroxylated fatty acids or fatty alcohols[50]. A characteristic feature of biosurfactants is a hydrophilic-lipophilic balance (HLB) which specifies the portion of hydrophilic and hydrophobic constituents in surface-active substances. Due to their amphiphilic structure, biosurfactants increase the surface area of hydrophobic water-insoluble substances,

increase the water bioavailability of such substances and change the properties of the bacterial cell surface. Surface activity makes surfactants excellent emulsifiers, foaming and dispersing agents[51].

Extracellular polysaccharides or exopolysaccharides (EPSs) of bacterial, algal and fungal origin are recommended as surface active agents for heavy metal removal [52].The adsorption of heavy metal by EPS is a metabolism-independent process, and it is attributed to interaction between metal cations and negative charges of acidic functional groups of EPS [53].

Lipo Poly Saccharides (LPS) confers a considerable negative charge upon the cell surface[54] which favors electrostatic interactions with cations, removal of LPS may reduce the magnitude of the negativity of the cell surface charge, thus reducing interactions with cations, such as cadmium. The mechanism by which metal-chelating agents reduce toxicity clearly warrants further exploration.

1.4.2 Role of biosurfactants in the biodegradation of PCOs

The low solubility and high hydrophobicity of many hydrocarbon compounds make them highly unavailable to microorganisms[55].Biosurfactant production helps the hydrocarbon degrading bacterium to gain better access to their hydrophobic substrates as it brings about changes like reduction of surface tension of the environment around the bacterium, reduction of interfacial tension between bacterial cell wall and hydrocarbon molecules, membrane modifications like increasing the hydrophobicity of cell wall by reducing the lipopolysaccharide content of cell wall, enhancing the dispersion of hydrocarbon by encapsulation of the hydrocarbon into micelles, etc [56,57].

1.5 *BREVIBACTERIUM*

Brevibacterium is a genus of bacteria of the order Actinomycetales. They are soil organisms. Although understood primarily as soil bacteria, they might be more abundant in freshwaters. Brevibacteria are short, nonbranched, asporogenous, obligately aerobic gram positive rods which may exhibit a marked rod-coccus cycle when cells grow older. Some of the species belonging to this genus are *B.*

acetyliticum, B. albidum, and B.antiquum. The genus is best known for its important role in the ripening of certain cheeses (*B.linens*) and for its supposed over-production of L-amino acids. Other interesting industrial applications, including the production of ectoine, have recently been proposed[58].*Brevibacterium* sp. has been known to have extra chromosomal, circular DNA which acts as a vector and commonly called as a plasmid[59].

1.6 AIM OF THE STUDY

Environmental pollution is a serious problem in India most of its environment has been contaminated by biological, toxic, organic, and inorganic pollutants. The current physico-chemical processes for treatment involve high energy and large capital investment. The bacterium under study i.e. *Brevibacterium* sp. MTCC10313 that was previously isolated by Sneha et al, 2000[59] is reported to have a plasmid with caffeine degradation ability. Hence, it is presumed that bacterium may have the ability to degrade possible polyaromatic hydrocarbons and also may aid in bioremediation of heavy metals. Hence, the aim of our study is to utilise this bacterium for the effective treatment of heavy metal and PCO contaminated environment. This bacterium could be grown in large scale and a novel cost effective method could be exploited for treatment of polluted environment. On the basis of this the following objectives were formulated.

1.7 OBJECTIVES OF THE WORK

The objectives of the project work were

1. To study the ability of *Brevibacterium* sp. MTCC10313 to biosorb heavy metals and to biodegrade petroleum crude oil (PCO)
2. To extract and characterize the biosurfactants secreted by the bacterium in response to PCO and heavy metal stress.