5.1 Physico-chemical Attributes of the Soil Samples

Soil is a highly diverse environment for the microbial population that dwells in it. The different components like sand, silt, clay and organic matter of the soil provide different kind of habitats (Van Elsas and Trevors, 1997). The type of soil can influence the population of the microbial biomass. Soil with high clay contents has a higher microbial biomass generally as they retain more water and often contain more organic carbon (Carson, 2011).

The organisms present in soil are exposed to various abiotic factors as well as nutritional conditions that may vary in concentration around micrometers. As per the difference in pH, particle size distribution, cation exchange capacity or organic matter content of the soil, it can determine the microbial community structure. Microorganisms in soil are important because they affect the structure and fertility of soils. The diversity of bacteria also controls the quality of soil by detoxification of contaminants present in the soil to their less toxic forms. Bacteria can affect physicochemical properties of soil. Hence, soil biology plays an important role in determining many characteristics of soil.

In the present study arsenite tolerant bacteria were isolated from soil collected near textile industry (Sanganer area, Jaipur) at the depth of 0-40 cm; at the distance of 100-200 m from textile industry. This soil is used in field for cultivating seasonal vegetables and is usually irrigated with wastewater of dyeing industry. Thus, it has been considered to be constantly subjected to dyes and mordants used in the industry. The physicochemical characterization of soil seems to indicate the possible reason for the development of arsenic resistance in bacteria.

The pH of all the samples ranged from neutral to alkaline i.e. 7.27-8.61. The pH of the soil samples was found to be slightly higher than that of neutral values i.e. 6.6-7.3 (Bruce
and Rayment, 1982; Table 5). Soil pH has an important role in determining the type of microorganisms that inhabits in different soils (Lynch and Hobbie, 1988; Matthies et al., 1997). The pH condition of natural soil is an important criteria as the growth of microbes declines under too acidic or too alkaline conditions. According to Carson (2011) soil pH around 7.0 is most suitable for growth of microbes. The pH of the soil is an independent and important factor that determines soil bacterial diversity because the intracellular pH of most microorganisms is usually within 1 pH unit of neutral (Madigan et al., 1997).

Electrical conductivity, which is a measure of soil salinity, ranged from 0.356-2.58 mS in the soil samples. Soil salinity can influence physical, chemical as well as biological processes in soils (Carson, 2011). The high values of electrical conductivity in our result might be due to the presence of high concentration of ions and dyes as contributed by the dyeing industries.

Range of organic carbon and organic matter observed was 0.396 ± 0.019% to 1.924 ± 0.086% (low-medium range) and 0.683 ± 0.181% to 3.317 ± 0.087% (low-high range) respectively. The microbial biomass is also affected by change in carbon content of soil. The amount of labile carbon (easily broken down by microbes and largely made up of crop residues and particulate organic matter) present in soil is of particular importance for determining the microbial biomass as labile carbon provides a readily available energy source for microbial decomposition. Soils with more labile carbon usually tend to have higher microbial biomass. The amount of organic matter present in the soil affects soil fertility, soil structure and water holding capacity and has a large impact on the surrounding biodiversity (Carson, 2011).

The minimum Ca\(^{2+}\) present in the soil samples determined was 16.832 ppm of soil, while maximum was 217.232 ppm of soil. The minimum Mg\(^{2+}\) present in the soil samples was
11.24 ppm of soil, while maximum was 101.644 ppm of soil. According to the Ca/Mg ratio as depicted in our study, low level of calcium in the soil samples was observed (Eckert, 1987; Table 9).

Calcium occurs in soil as a component of residual minerals, inorganic compounds and organic matter as an exchangeable cation, which is available for plant uptake (Bruce et al., 1988). Exchangeable magnesium is the most important form present in soil for plant growth. In neutral and slightly acidic soils, exchangeable magnesium is usually second to exchangeable calcium as the dominant cation on the exchange capacity. Problems associated with high magnesium in soil may include deficiency of potassium, poor structure of soil; slow water infiltration as well as cracking clays. Even though soil with high magnesium concentration has sufficient level of potassium but it appears that excess magnesium interferes with potassium uptake (Hailes et al., 1997).

Saline soils have high salt concentrations, which are being dominated by calcium and magnesium salts. Acidic soil samples have less calcium and magnesium and high soil pH increases availability of calcium and magnesium. Soil with high cation exchange capacity holds more of calcium and magnesium (Bruce et al., 1988). The supply of magnesium to plants is highly governed by the exchangeable magnesium concentration (Sinclair, 1981) Magnesium deficiencies attributable to ion imbalances in the soil have led to use of exchangeable cation ratios (Ca/Mg) as estimates of magnesium availability (Metson, 1974).

Water holding capacity indicates the moisture content of soil, which in turn affects the growth of bacteria. The water holding capacity ranged from 43.8-71.0%. The WHC observed in our study was suitable for microbial survival and growth in the soil.
High concentration of arsenic contamination was observed in the samples. The arsenic content in samples ranged from 68-464 mg/kg of the soil. Iron, magnesium, lead and zinc content were also determined in the soil samples and were in variable ranges. Very low level of zinc was determined in the samples. The estimation of metals like As, Fe, Mg and Pb in of the four soil samples revealed that the values were higher than permissible limits prescribed by ISI, 1991. This presence of metal ions in the soil suggested to have been introduced in soil in considerable amount by use of materials used in the dyeing process, or from metal complex dyes (Correia et al., 1994 and Heinfling et al., 1997).

Metals are considered to have an important role in the metabolic processes of the microorganisms. Presence of heavy metals in any environmental condition generally influence the population of microbes by affecting their growth, morphology as well as biochemical activities that results in decrease in biomass and diversity (Roane and Pepper, 2000). Heavy metal stress in soils causes the change in structure, quantity, distribution and metabolic functioning of bacterial community (Frostegard et al., 1996). Under the condition of the long-term heavy metal exposure, the biomass of microorganisms in soil decreases significantly (Hiroki, 1993; Bardgett et al., 1994). Arsenic contamination can cause a steep decline in the amount of microbial biomass carbon (MBC) and microbial biomass nitrogen (MBN) in soil (Yang et al., 2012).

The results revealed that soil samples exhibited high arsenic and lead concentrations, while iron and magnesium were present at lower concentration with zinc at the lowest concentration. As the pH of the contaminated soil was near neutral, it is possible that arsenic is in unionized form. Thus, it can passively move across the bilayer membrane or can be transported by a carrier protein similar to those that transport unionized organic compounds (Salam et al., 2009).
The fluoride content of the samples ranged from 3.405-4.43 mg/g of soil. Fluoride is immobile in soil so have a negative effect on the microbial community as it decreases microbial biomass (Tscherko and Kandeler, 1997).

The present investigation was in agreement and differed in some aspects like pH, electrical conductivity, organic carbon and matter as well as zinc and iron content in the soil samples with the results of the experiments conducted by Joshi and Kumar, 2011 and Mathur and Kumar, 2013 who studied physico-chemical parameters of the contaminated soil samples of Sanganer area.

Similar soil characterization was done by Jaroenmit et al. 1987, and the soil from which they isolated arsenite resistant bacteria exhibited pH range from 5.8-7.9 and organic matter percentage ranged between 0.23 and 2.90%. The soil samples exhibited varied level of arsenic (124-711 mg/kg of soil), lead (2-66 mg/kg of soil) and zinc (28-101 mg/kg of soil).

Aksornchu et al.,(2008) isolated arsenite resistant bacteria from arsenic contaminated soil samples. The pH and arsenic concentrations of soils samples were found in the range of 5.39-8.19 and 42.14-1010.96 mg/kg of soil respectively.

Yoon et al.,2009 isolated arsenite resistant bacteria from two abandoned mines. The total arsenic concentrations from Duckum mine soils were 67.9 ± 0.7, 78.8 ± 11.0, 58.0 ± 0.8, and 37.0 ±1.1 mg/kg at the sampling sites, while the total arsenic concentration from Myoungbong mine soil was 143.0 ± 17.8 mg/kg. The level of arsenic contamination in these studies is in the range of arsenic as observed in our study.

A wide variation in the physico-chemical properties of soil was observed in the soil samples from Sanganer area were found in the present study. The physico-chemical characteristics of soil varied with one another, which may be due to relatively wide
spectrum of dyes used in textile industry. Long-term irrigation of agricultural lands with such effluents obtained from industries could be the reason of increased electrical conductivity, organic carbon and other heavy metals content (Narwal et al., 1993; Brar and Arora, 1997; Olaniya, 1998).

5.2 Resistance in the Isolated Bacteria

In the present study, four arsenite resistant bacterial strains were isolated from soil sample number 3 and 4 (two isolates from each soil sample) collected at the depth 10-40 cm and distance of 200 meters from textile effluent. These soil samples showed neutral pH and medium range of electrical conductivity, exchangeable calcium and water holding capacity. The arsenic concentration in soil ranged between 68-84 mg/kg of soil and was also found contaminated with iron, zinc, lead and magnesium.

The possible reason for the occurrence of arsenite tolerant bacteria from soil (200 meters distance from textile effluent and at depth of 0-40 cm from top soil) might be because of favourable conditions of growth in medium range of pH, calcium and electrical conductivity leading to gradual development of arsenic tolerance in presence of arsenic. All the isolated strains were studied for their colony morphology and Gram’s reaction. Bacterial colonies were yellow or cream coloured having circular shape and all were Gram’s positive.

Arsenite resistant bacteria have been isolated from various sources. Few studies have reported isolation of arsenite resistant bacteria belonging to genera of Alcaligenes, Acinetobacter, Flavobacterium, Pseudomonas, Sinorhizobium, Sphingomonas, etc. from soil (Quastel and Scholefield, 1953; Osborne and Erhlich, 1976; Jaroenmit et al., 1987, Kinegam et al., 2008).
Garcia-Dominguez et al.,(2008) isolated a gram-negative *Thiobacillus* species from soil at the depth of 20 cm that was able to oxidize arsenite to less toxic arsenate and was able to tolerate 10 mM of arsenite.

Many species of *Bacillus*, *Achromobacter* and *Pseudomonas* have been reported from arsenic contaminated ecosystems (Jackson et al.,2003). Huysmans and Frankenberger (1990) have reported the isolation of arsenic resistant bacteria from agricultural drainage water and evaporation pond sediments. *Pseudomonas putida* and *Alcaligenes eutrophus* were isolated from gold arsenic deposits of Kazakhstan (Jackson et al.,2003). Conversely, some reports are available regarding the isolation of arsenic-resistant bacteria found in arsenic-free soils (Jackson et al.,2005).

### 5.3 Minimum Inhibitory Concentration

The MIC of the arsenite resistant bacteria was determined and the isolated arsenite resistant strains exhibited MIC in range of 3-9 g/L (23.09-69.2 mM). The strain IR-1 exhibited highest MIC of 9 g/L.

The growth for the strain IR-1 ceased at concentration 9 g/L of sodium arsenite in nutrient broth. The strain reached to the exponential phase of growth within 24 hours of incubation at 1-3 g/L concentration of sodium arsenite. With further increase in arsenite level decline in the growth of the isolate IR-1 was observed. No significant difference in growth was detected, when the isolate was grown in the absence of sodium arsenite (control) and presence of 1 g/L of sodium arsenite, this suggests that arsenite at this dose pose no negative effect on the isolate. But at higher doses of sodium arsenite (2-9 g/L), statistically significant difference at p<0.05 was observed for 2-5 g/L sodium arsenite.
and highly significant difference at p<0.001 for 6-8 g/L respectively as compared to control (no arsenic in medium) (Table 20).

Microorganisms present in soil exhibit wide variation in resistance to arsenic forms. Arsenic is one of the most extensively studied metals, which induce oxidative stress that generates intracellular reactive oxygen species (ROS) and free radicals and alters levels of antioxidants (Suntres, 2002; Liu et al., 2004). ROS may also be formed during the oxidation of arsenite to arsenate (Aposhian and Aposhian, 2006; Flora et al., 2008). Shi et al., 2004 have explained that arsenic generates free radicals, which results cellular injury as well as death by the activation of oxidative sensitive signalling pathways. Hence, with the increase in sodium arsenite concentration in nutrient medium, growth of the bacteria declines.

In literature, high level of tolerance in bacteria to arsenite have been reported as 100 mM by Green 1918 and 60 mM by Turner 1949. Bacteria have been observed to tolerate varying levels of arsenite as upto 1.0 g/L (Jaroenmit et al., 1987; Huysmans and Frankenberger, 1990), 2-80 mM (Chen and Shao, 2008), 10 mM (Joshi et al., 2009), 100 ppm (Salam et al., 2009), 40 mM (Chitpirom et al., 2009), 120 mM (Dave et al., 2010). Santini et al., 2000 studied arsenite oxidizing bacterium from gold mine with MIC for arsenite as 5 mM. Cai et al., (2009a) isolated arsenite resistant bacteria from soil contaminated with arsenic and reported MIC greater than 20 mM.

Since arsenate is less toxic than arsenite, various workers have reported very high bacterial tolerance to arsenate as compared to arsenite like 1500 mg/L for arsenate and 1000 mg/L arsenite (Jaroenmit et al., 1987), 66.7 mM for arsenate and 26 mM for arsenite (Chang et al., 2007b), 50 mM for arsenate and 0.2 mM for arsenite (Patel et al., 2007; Joshi et al., 2008), 200-500 mM for arsenate and 5-20 mM for arsenite (Joshi et al., 2009).
Bacteria are capable to tolerate or resist the toxic effects of arsenic. Despite of arsenic toxicity, microorganisms are able to use oxidized or reduced form of arsenic in their metabolism (Silver and Phung, 2005). Numerous bacteria have been reported, which are involved in arsenic transformation by using reduction, oxidation and methylation mechanisms (Weeger et al., 1999; Ellis et al., 2001; Oremland and Stolz, 2003).

Genetically, the resistance to both arsenite and arsenate is found among both Gram-positive and Gram-negative bacteria. Operons encoding analogous arsenic resistant genes have been found on the chromosome and on transmissible plasmids in a wide variety of both Gram-positive and Gram-negative microorganism (Silver and Misra, 1988; Silver and Walderhaug, 1992; Cervantes, 1995). The major modes of arsenic resistance in bacteria are sequestration, exclusion of metal ion or transformation of inorganic arsenite into less toxic form arsenate through oxidation. Thus, it can be interpreted that the arsenite resistant bacteria isolated in the study exhibit medium to high level of resistance in terms of MIC, which might have gradually developed because of continuous exposure of arsenic in the soil. This resistance can be attributed by genetic defense mechanism for arsenic.

5.4 Identification and Characterization of the Isolated Strain

Based on nucleotide homology and phylogenetic analysis of the 16S rDNA gene sequence, the bacterial strain, IR-1 with highest MIC of 9 g/L was identified as Microbacterium paraoxydans (GenBank Accession Number: NR_025548.1). Phylogenetically the species is closely related to Microbacterium sp. CQ0110Y, Microbacterium paraoxydans strain 3109, Microbacterium sp. GE1017 and Microbacterium paraoxydans strain 3200.
According to Bergey’s Manual of Systematic Bacteriology, 2012 the isolated bacteria fall under class Actinobacteria, order Actinomycetales and family Microbacteriaceae and genus *Microbacterium* and species *paroxydans* (Laffineur *et al.*, 2003).

As per earlier reports other bacteria of class Actinobacteria and order actinomycetales have been isolated from arsenic rich ecosystem (Delavat *et al.*, 2012).

Bachate *et al.* (2009) isolated and identified aerobic arsenic-resistant bacteria (heterotrophic) from soil that belonged to different genera in the Gram-positive and Proteobacteria groups. Out of the twenty–one isolates mostly belonged to Actinobacteria and Firmicutes division and only a small number of isolates belonged to Proteobacteria and six isolates belonged to *Microbacterium* genus that had the similar arsenic resistance pattern as all of them were able to resisted upto 30 mmol/L arsenite.

In a study by Chen and Shao, 2009, fifty-four isolates belonged to γ-proteobacteria (25 isolates) Actinobacteria (14 isolates), α-proteobacteria (11 isolates), and Cytophaga-Flavobacterium (CFB) group (4 isolates). Actinobacteria as represented by *Microbacterium* exhibited high arsenic resistance

*Microbacterium paroxydans* IR-1 was further studied for its colony morphology and biochemical characterization according to Bergey’s Manual of Determinative Bacteriology. Discrete, small, cream colored colonies of *Microbacterium paroxydans* IR-1 were seen on nutrient agar plate, when grown at 37°C for 48 hours. The strain was found to be gram positive bacilli, negative for acid fast and spore staining. It was aerobic with positive results for catalase and motility test.

Physiological and biochemical traits of *Microbacterium paroxydans* IR-1 were in accordance to amended description of *Microbacterium paroxydans* by Laffineur *et

According to the study of Schumann et al., (1999) and Aksornchu et al., (2008) on *Microbacterium oxydans*, it is considered as an aerobic bacterium, which produces acid from sucrose but cannot utilize Simmons’ citrate agar for its growth. It also exhibited catalase-positive, oxidase and methyl red-negative. These results of *Microbacterium* species were similar to those obtained in the present study for *Microbacterium paraoxydans* IR-1.

Many workers have isolated *Microbacterium* from various environmental conditions like sludge, air, soil and deep sea sediments. Many workers have reported their study on *Microbacterium* species that have shown the ability to resist many heavy metals like chromium, uranium and gold (Humphries et al., 2005; Nedelkova et al., 2007; Inomata et al., 2007).

In 2002, Mokashi and Paknikar isolated *Microbacterium lacticum* with its minimum inhibitory concentration for arsenite of 50 mM and it was identified as arsenite oxidizing bacteria. *Microbacterium* was detected as an arsenic resistant bacterium from an unsaturated soil by Macur et al. (2004). Jackson et al., (2005) isolated a *Microbacterium* species from woodland leaf litter that can grow in the presence of 15 mM arsenite. Achour et al., (2007) reported that *Microbacterium* sp. A33 obtained from forest soils can grow in the presence of 56 mM arsenite. Aksornchu et al., (2008) isolated *Microbacterium oxydans* isolated from arsenic contaminated site that was able to tolerate arsenite upto 40 mM. An arsenic-resistant *Microbacterium esteraromaticum* was detected in deep-sea sediments from the Southwest Indian Ridge. It was found to tolerate arsenite upto 80 mM (Chen and Shao, 2009). Bachate et al., (2009) isolated
Microbacterium hydrocarbonoxydans that was resistant to arsenite at a concentration of 30 mM. Microbacterium paraoxydans had been reported by Laffineur et al., (2003).

Thus, so far many strains of Microbacterium genera have been reported to show resistance to arsenite but the MIC varies widely and few of them have also shown arsenic oxidizing ability.

5.5 Metal and Antibiotic Sensitivity of Microbacterium paraoxydans IR-1

Microbacterium paraoxydans IR-1 isolated from soil contaminated with arsenic, lead, zinc and iron exhibited resistance not only to arsenite but also to heavy metals like cobalt, cadmium, mercury, lead, nickel, zinc, chromium, selenium, stannous at the concentration of 100 µg/ml of each. The MIC was determined for the selected heavy metals of the isolate. It was found to be highest for chromium (1.6 g/L) than the other metals employed under study namely cadmium (0.7 g/L), lead (1.2 g/L), mercury (0.7 g/L) and nickel (1.4 g/L).

Metals are involved in all aspects of microbial life. Some metals are essential, while others are non-essential for microorganisms. Heavy metals are present in the surroundings of microbes due to their release through several natural and anthropogenic processes. Microbes have developed various mechanisms to tolerate the presence of heavy metals. In general, microbes are able to grow at high metal concentration due to a variety of specific environmental factors and mechanisms of resistance. The mechanisms reported so far include metal sorption, mineralization, uptake and accumulation, enzymatic oxidation or reduction to its less toxic form followed by its efflux from the
cell (Mergeay, 1991; Hughes and Poole, 1991; Nies, 1992; Urrutia and Beveridge, 1993; Joshi-Tope and Francis, 1995).

The other mechanisms of resistance by microorganism include exclusion of metal ions, binding of metal ions to the outer surface of bacteria extra cellular precipitation, or its intracellular sequestration (Satchanska et al., 2005). The environmental factors such as the surrounding pH and redox potential, metal speciation, soil particulates and soluble organic matters also influence the resistance mechanisms in microbes (Srinath et al., 2002; Zoubilis et al., 2004, Aksornchu et al., 2008).

Till date, tolerance mechanisms for various metals like arsenic, cadmium, copper, zinc, chromium and nickel have been identified and described in detail. The major modes of such tolerance or resistance involve the efflux of metal ions outside the cell. The genes responsible for this resistance may be present on both chromosomes as well as plasmids. These metal resistant bacteria are can to grow on metals and thus have an important role in the biogeochemical cycling of metal ions. As the intake and subsequent efflux of heavy metal ions by microbes many a times involves a metal redox reaction. Some of the bacteria can also utilize this redox reaction for production of energy and growth. Moreover, the oxidation state of a heavy metals is also related to the solubility and toxicity of the metal itself. Therefore, many scientists have been trying to isolate and use microbes, which can oxidize or reduce heavy metals and in turn reduce its toxicity. These bacteria can be further used to remediate metal-contaminated sites (Spain, 2003).

Mergeay et al.,(1985) have worked on metal resistance of Escherichia coli on agar medium and determined the minimal inhibitory concentrations of various metals. As per their results the mercury was most toxic metal whereas the least toxic metal was manganese.
So, the multi-metal resistance observed in the bacterium can be due to adaptation to the polluted soils. The isolated bacteria might have developed certain metal resistance system to protect its sensitive cellular components. The dissimilarity between the levels of resistance exhibited by the isolated strain to various metals may have resulted from the variations in the levels of metal contamination, in the period of time over which metal exposure and the source of contamination experienced by the strain, the characteristics of soil and in the variations of metal bioavailability.

### 5.5.1 Correlation of Metal Tolerance and Antibiotic Resistance

In the Antibiotic sensitivity tests the bacterium was found to be resistant to antibiotics cephalothin (30 µg), amoxyclav (30 µg) and ampicillin (10 µg) and sensitive to all others.

Another inference that can be drawn from heavy metal tolerance by bacteria is that it may contribute to the stability of antibiotic resistance genes by increasing the selective pressure of the environment in favor of metal resistant bacteria Many workers have speculated and have shown that there is a correlation between metal tolerance and antibiotic resistance in bacteria as that genes resistant to both antibiotics and heavy metals may be located closely on the same plasmid in bacteria and are thus more likely to be transferred together in them (Baquero et al., 1998; Spain, 2003).

This resistance to antibiotics can be achieved gained by a alteration in the genetic makeup of a bacterium, which can be due to either a random mutation or by transfer of antibiotic resistance genes between bacteria in the same environment (American Academy of Microbiology, 1999).
According to Lawrence's (2000) discussion on the Selfish Operon Theory, clustering of genes on a plasmid is beneficial because those genes are more likely to be transferred together during conjugation. Thus, in an environment with antibiotics and heavy metals stress, it would be more favorable for bacteria to acquire resistance to both the stress for its survival (Spain, 2003).

In most of the studies, metal resistance has been reported that are associated with antibiotic resistance (Verma et al., 2001). In an metal stress environment the metal and antibiotic resistance in bacteria helps them to acquire adaptation by the spread of resistant factors rather than by mutation and natural selection (Silver and Misra, 1988).

In reported literature by Huysmans and Frankenberger (1990), 14 bacterial isolates were tested for their tolerance to metals. Cadmium in high concentrations was found to be toxic to Rhizobium fredii USDA 201 (Angle and Chaney, 1989). Other elements that inhibited the bacterial growth were Te, Zn and Hg. All the As-resistant bacteria were also tolerant to other metals like Cu, Pb, Co, Ni, Mo, Se (IV), Se (VI), Sb, Cr, Sn, and Ag at 50 µg/mL.

In a study by Dave et al. (2010), the MIC cobalt, chromium, nickel, molybdenum, copper, selenate and selenite was studied for the four isolates in the presence of 20 mM of arsenite. Cobalt and nickel were most toxic metals as per the results of this study. All the four isolates were able to grow in presence of 30 mM of these metals and in presence of 40 and 100 mM of zinc and antimony respectively. Selenite was found to be least toxic among the metals studied and all the isolates showed growth even in the presence of 300 mM of selenite.

As reported in literature by Rehman et al., (2010), P. lubricans was found to tolerate arsenite at the concentration of 3 mg/ml. This strain also exhibited resistance against
Ni$^{2+}$, Cu$^{2+}$, Cd$^{2+}$, Hg$^{2+}$ and Cr$^{6+}$ at a concentration of 300, 700, 100, 400 and 500 µg/ml, respectively. In a study by Butt and Rehman (2011), *K. pneumoniae* and *K. variicola* were found to tolerate arsenite at a concentration of 26.6 and 24 mM, respectively. These bacterial strains were also found to resist other metals like Ni$^{2+}$, Cu$^{2+}$, Cd$^{2+}$, Hg$^{2+}$ and Cr$^{6+}$.

In reported literature by Huysmans and Frankenberger (1990), 14 bacterial isolates were tested for their susceptibility to antibiotics and all the isolates were found to be susceptible to, streptomycin, novobiocin, chloramphenicol, rifampin and tetracycline. The antibiotics like kanamycin and gentamicin were found to be intermediate in the inhibition of growth.

In a study by Nagvenkar and Ramaiah, 2010, many arsenic tolerant bacteria were found which exhibited sensitivity to most of antibiotics except chloramphenical, penicillin and oxytetracycline.

In ecological terms the bacteria, which are resistant to metals play an important role in the biogeochemical cycling of those metals (Gadd, 1990). Bioremediation of heavy metals using microorganisms has received a great deal of attention in recent years because of scientific novelty and its potential application in industries. Therefore, the application of heavy metal-resistant bacteria in bioremediating the metal contaminated site is a promising approach for human beings.

### 5.6 Standardization of Growth Conditions

For standardization of optimum growth conditions of *Microbacterium paroxydans* IR-1, the parameters studied were temperature, pH and media:
5.6.1 Temperature

The maximum growth in term of optical density was observed at 37°C as significant difference in growth was observed at 37°C as compared to growth at 30°C and 45°C at significance level of p<0.01 and p<0.001 respectively. Hence it can be concluded that isolate is mesophilic in nature.

Temperature is an important factor that affects bacterial growth (Herbert and Bhakoo, 1979). The optimum temperature (37°C) for the growth of the strain (Microbacterium or other bacteria) in the present study supports the results as observed by Bouchard et al., 1996; Christiansen and Ahring, 1996; Niggemeyer et al., 2001 and Salam et al., 2009.

According to amended description of Microbacterium paraoxydans IR-1 by Laffineur et al., (2003) and Buczolits et al., (2008) the strain was able to grow well at temperature 37°C and 42°C but growth was absent at 4°C. Similarly, we have also observed that the strain Microbacterium paraoxydans IR-1 isolated by us grew well at 37°C but was unable to grow at 4°C.

According to a study by Mokashi and Paknikar, 2002, optimum temperature required for oxidation of arsenite by Microbacterium lacticum was found to be 30°C. The most suitable temperature for the isolated arsenic resistant was 30°C in the studies conducted by Rehman et al. (2010) and Butt and Rehman (2011).

Arsenite oxidizing bacteria have also been isolated from hot springs like Thermus aquaticus and Thermus thermophiles, which grew well at 40-79°C and were able to oxidize As (III) to As (V) efficiently at 70°C (Gihring et al., 2001).

A bacterial strain, which was able to oxidize As (III) to As (V) efficiently at 50-60°C was isolated from hot spring water. The oxidation rate of As (III) decreased with decrease in
temperature. Oxidation of arsenite occurred in the pH range of 7.0-8.7 (Hiroshi and Yasou, 2003).

5.6.2 pH

The optimum pH condition of the medium for growth of *Microbacterium paraoxydans* IR-1 was found to be neutral to alkaline. Although non-significant difference in growth pattern was observed at pH 7, 8 and 9. The pH of soil from which the bacteria was isolated was 7.55.

Toxicity of heavy metal and their binding depends on pH (Wood, 1983) and the bacterial strains require environmentally relevant pH for their growth. Bouchard *et al.*, 1996; Christiansen and Ahring, 1996 and Niggemeyer *et al.*, 2001 reported optimum pH 7.5 for arsenic resistant strains *Desulfitobacterium frappieri*, *Desulfitobacterium hafniense* and *Desulfitobacterium* strain GBFH, respectively. According to a study by Mokashi and Paknikar, 2002, optimum pH required for oxidation of arsenite by *Microbacterium lacticum* was found to be 7.5.

Salam *et al.* (2009) reported 8.5 as the most appropriate pH for the growth of arsenic resistant bacteria. The most suitable pH for arsenic resistant bacteria isolates was observed to be pH 7 in the studies conducted by Rehman *et al.* (2010) and Butt and Rehman (2011).

Acidophilic bacterium like *Acidiphilium multivorum* isolated from acid mine drainage in Japan was found resistant to arsenite at concentrations upto 1125 mg/L of arsenite (Suzuki *et al.* 1997).

*Microbacterium paraoxydans* IR-1 grew well in nutrient broth, minimal media with carbon source and chemically defined media, when supplemented with sodium arsenite.
Microbacterium paraoxydans IR-1 grows best in nutrient broth as compared to minimal media and chemically defined media. No significant difference was obtained, when strain was grown in minimal media (with carbon source) and chemically defined media, when both were supplemented with arsenite.

No significant difference was observed in the growth, when culture was grown in minimal media supplemented with varying concentration (10, 15 and 20 g/L) of carbon source (lactose monohydrate). No growth was observed in control, which was minimal media without carbon source. There was a decline in growth (p<0.001) of the isolate in minimal media, when supplemented with 1g/L of sodium arsenite. On the basis of the nutritional requirement, this bacterium was considered as chemoheterotrophic.

Arsenic oxidation is mediated by both heterotrophic (grows in the presence of organic matter) and chemoautotrophic (aerobes or anaerobes, using arsenite as the electron donor and $\text{CO}_2/\text{HCO}_3^-$ as the sole carbon source) microorganisms. Some heterotrophic and chemoautotrophic microorganisms have been reported that were able to oxidize arsenite to arsenate under both aerobic and anaerobic conditions (Oremland and Stolz 2003).

These kind of microorganisms have been isolated from arsenic contaminated environments, such as soils, lake water or sediment (Oremland et al., 2002a; Campos et al., 2009; Escalante et al., 2009; Valenzuela et al., 2009), gold mine wastewater (Ilyaletdinov and Abdrashitova, 1981; Santini et al., 2002) and geothermal environments (Gihring et al., 2001).

Some microbes (chemolithotrophic bacteria) gain energy from oxidizing arsenite (Turner and Legge, 1954; Nriagu, 2002; Ramirez-Solis et al., 2004; Silver and Phung, 2005, Inskeep et al., 2007). Heterotrophic bacteria have not been shown to derive major energy from arsenite in growth experiments. In case of heterotrophic bacteria, it is usually
considered to be a detoxification mechanism. The oxidation arsenite oxidation is considered as a thermodynamically exergonic reaction under standard conditions which provide sufficient energy to support chemoautotrophic growth of microbes (Ehrlich, 1996).

Turner, 1949 and 1954 have isolated arsenite-oxidizing bacterial strains by supplementing organic matter in the medium and all the strains were found to be heterotrophic arsenite oxidizers. Similarly, heterotrophic arsenite-oxidizing bacteria have been isolated from raw sewage and soil and most of the isolates have been identified as strains of *A. faecalis*, (Phillips and Taylor, 1976; Osborne and Ehrlich, 1976).

Many bacteria have been reported that are capable of arsenite oxidation and are heterotrophic in nature (Anderson *et al.*,1992; Santini *et al.*,2000; Martin and Pederson, 2004). Anderson *et al.*,1992 reported that the two strains namely, *Alcaligenes* sp. and *Agrobacterium albertimagni* were able to grow heterotrophically but can even oxidize arsenite rapidly using arsenic detoxification mechanism rather than energy generation mechanism. Other heterotrophic bacteria like *Haemophilus, Micrococcus* and *Bacillus* were reported to be arsenite oxidizers (Ike *et al.*,2008).

A chemoautotrophic arsenite oxidizing strain *Thiomonas arsenivorans* was isolated from disused mine that utilized arsenite, sulphur and thiosulphate. This bacterium could grow using arsenite as sole energy source and can also grow heterotrophically on yeast extracts and a variety of different organic compounds (Battaglia-Brunet *et al.*,2006).

A study by Santini *et al.*,2000 showed that NT-26 strain grew well in minimal media containing lactate as carbon and energy source. A strain *Microbacterium lacticum* studied by Mokashi and Paknikar in 2002 was able to utilize a variety of carbon sources such as lactate, methanol and citrate supplemented in the medium.
Hal arsenatibacter silvermanii, an anaerobic extremophile isolated from the sediments of salt-saturated, arsenic-rich Searles Lake could grow either as a heterotroph using lactate as its electron donor or as a chemoautotroph using sulfide as electron donor (Oremland et al., 2005; Switzer Blum et al., 2009).

Thiomonas sp. (strain 3 AsT), a facultative chemoautotroph was shown to grow in minimal media supplemented with lactate at 5 mM concentration (Slyemi et al., 2011).

Most chemolithotrophic arsenite oxidizers isolated to date are members of the α-Proteobacteria, while the heterotrophic oxidizers are members of the β-Proteobacteria (Lugtu et al., 2009).

5.7 Arsenite Detoxification by Microbacterium paraoxydans IR-1

The ability of arsenite detoxification by oxidizing arsenite to less toxic arsenate by Microbacterium paraoxydans IR-1 was studied qualitatively and quantitatively as well as at genetic level.

Some bacteria could oxidize arsenite to arsenate and are termed as arsenite-oxidizing bacteria or arsenite oxidizers. The oxidation of arsenite to arsenate by bacteria represents a partial detoxification mechanism that is employed in bioremediation of arsenic contaminated sites (Simeonova et al., 2005) as it generates 100 times less toxic and less mobile form of arsenic that is arsenate. Thus, arsenite-oxidizing bacteria have been used in bioremediation of arsenic contaminated water (Lievremont et al., 2003).

As per the literature the arsenite oxidizers have been isolated majorly from arsenic contaminated site but there are some isolates, which are from uncontaminated site also.

Two isolates namely, Achromobacter SPB-31 and Bordetella SPB-24 were isolated from arsenic uncontaminated garden soil from Pune by Bachate et al. (2012). Prasad et al.
(2009) identified *Arthrobacter* sp. 15b from Sewage treatment plant site (non-contaminated with arsenic).

The first arsenite oxidizing bacterium, *Bacillus arsenoxydans* was isolated by H. H. Green in 1918. Thereafter, various researchers have reported numerous oxidizing strains. For example: *Agrobacterium/Rhizobium* isolated from gold mines in Australia that can grow chemolithoautotrophically (Santini *et al.*, 2000). *Alcaligenes* sp. and *Agrobacterium albertimagni* strains grow heterotrophically and can rapidly oxidize arsenite (Anderson *et al.*, 1992; Salmassi *et al.*, 2002). *Microbacterium lacticum* oxidizing up to 50 mM of arsenite was isolated from municipal sewage by an enrichment culture technique (Mokashi and Paknikar, 2002). Numerous arsenic-oxidizing microorganisms, especially belonging to *Proteobacteria*, are able to oxidise arsenite in order to detoxify their surroundings (Oremland and Stolz, 2003). Similar isolates have also been found in soils and sewage (Osborne and Ehrlich, 1976; Philips and Taylor, 1976).

Oxidizing ability of the strain *Microbacterium paraoxydans* IR-1 was determined qualitatively by silver nitrate assay in agar plate, microplate screening assay and paper chromatogram.

For screening of the arsenite-oxidizing bacteria the AgNO$_3$ method described by Lett *et al.*, 2001 has been used by many workers in which agar plates with sodium meta-arsenite were inoculated with arsenite resistant bacterial strains and later flooded with a solution of 0.1 M AgNO$_3$. A brownish precipitate revealed the presence of arsenate in the medium (Krumova *et al.*, 2008; Rehman *et al.*, 2010; Butt and Rehman, 2011).

The transforming abilities of different isolates were confirmed by “Microplate screening assay for the detection of arsenite oxidizing and arsenate reducing bacteria” by many workers (Mokashi and Paknikar, 2002; Simeonova *et al.*, 2004; Krumova *et al.*, 2008).
The paper chromatographic separation of 24 hours nutrient broth supplemented only with arsenite indicated the presence of arsenite as well as arsenate (oxidized product) in the media; this was also confirmed by comparing the $R_f$ values of separated spots with standard arsenite and arsenate solution.

The quantitative determination of the arsenite oxidation to arsenate was done by molybdene blue method (Cai et al., 2009a) that confirmed the oxidizing ability of the strain, *Microbacterium paraoxydans* IR-1. Highly significant ($p<0.001$) increase was observed in the quantity of oxidized product (arsenate) during the first 24 hours of incubation of cell pellet with 800 µM arsenite solution. Statistically significant ($p<0.05$) rise in oxidation was observed from 24 to 48 hours. There was no change in arsenite concentration in the control medium over time.

The amount of arsenate observed after 24, 48 and 72 hours of oxidation by the bacterial cell pellet was found to be 556.6 µM (69.57%), 763.06 µM (95.39%) and 755.43 µM (94.43%) respectively, when initially supplemented with 800 µM (100%) of arsenite solution. The oxidized product that is arsenate was obtained in maximum concentration after 48 hours. *Microbacterium paraoxydans* was able to oxidize 95% of arsenite after 48 hours only (Table 33).

The results of molybdene blue method were further supported by separation of arsenic species (arsenite and arsenate) by anion exchange chromatography and quantitative estimation by Inductively Coupled Plasma Mass Spectrometry. Maximum arsenate concentration after oxidation was observed at 48 hours.

According to Salmassi et al. (2002) the strain AOL15 grew exponentially for 15 hours and then reached to stationary phase in which the decline in the arsenite concentration indicated that the oxidation occurred during the late exponential phase (the culture
reached to high cell density). Oxidation of 585 µM arsenite (i.e., 290 µmole) was completed within 24 hours (Salmassi et al., 2002).

In a study, Cai et al. (2009b) isolated SY8 and TS44 (identified as Achromobacter and Pseudomonas) from soils with intermediate and high levels of arsenic contamination, respectively. Arsenite oxidation was monitored by spectrophotometry. The average oxidizing velocity of SY8 (52.9 µM/h) and TS44 (59.1 µM/h) was found to be almost equivalent.

The isolate, Pseudomonas stutzeri strain DY-BDan2 (EF429003) exhibited the complete oxidation of 1 mM arsenite to arsenate within 30 hours, which was also confirmed by of arsenic speciation analysis in the culture medium as well as the existence of aox genes (Chang et al., 2010).

Rehman et al. (2010) reported that P. lubricans exhibited high resistance against arsenite up to 40 mM and could oxidize 42% 78% and 95% of arsenite from the medium after 24, 48 and 72 hours, respectively.

According to Butt and Rehman (2011), arsenite oxidizing activity of the bacterial isolates was determined in medium containing 1.3 mM arsenite. K. pneumoniae could oxidize arsenite 36% (24 hours), 64% (48 hours) and 87% (72 hours). K. variicola was also able to oxidize arsenite 33%, 59% and 83% after 24, 48 and 72 hours, respectively. No change in arsenite concentration was found in the control medium over time.

Pseudomonas lubricans was able to oxidize 95% of arsenite after 72 hours as studied by Rehman et al. in 2010 and in a study by Butt and Rehman (2011) after 72 hours K. pneumonia and K. variicola was able to oxidize only 87% and 83% of arsenite respectively. In comparison, Microbacterium paraoxydans IR-1 isolated by us was able to oxidize 95% of arsenite after 48 hours only. According to Salmassi et al. (2002), the
isolated strain AOL15 completely oxidized 585 µM arsenite (i.e., 290 µmole) within 24 hours. *Pseudomonas stutzeri* strain DY-BDan2 (EF429003) exhibited arsenite detoxification potential through the complete oxidation of 1 mM As (III) to As (V) within 30 hours (Chang *et al.*, 2010).

The genetic basis of this oxidation process was also studied and confirmed by PCR amplification of *aoxB and aoxC* genes (Figure 30). These *aox* genes may code for the arsenite oxidase enzyme responsible for the oxidation. *aoxB* codes for the large molybdopterin subunit of enzyme that is arsenite oxidase and *aoxC* encodes for a putative oxyanion reductase required in oxidation (Muller *et al.*, 2003; Silver and Phung, 2005).

The detoxification potential of the bacterial strains is carried out with the arsenite oxidation gene (*aox* genotype that shows the activity of arsenite oxidation to arsenate) (Chang *et al.*, 2009). Number of bacteria has been isolated, which showed the arsenite oxidase enzyme activity as well as presence of genes encoding arsenite oxidase in various groups of bacteria and archaea (Muller *et al.*, 2003, Anderson *et al.*, 1992; vanden-Hoven and Santini, 2004; Cai *et al.*, 2009a; Branco *et al.*, 2009). The gene conferring arsenite oxidation has been variously described by a number of workers but in the present study *aox* operon as given by Chang *et al.*, 2010 is considered. The *aox* operons usually contain additional genes, like *cytC*, which encodes a cytochrome c, and *moeA*, which encodes an enzyme involved in molybdenum cofactor biosynthesis (Silver and Phung, 2005).

A cluster of four contiguous genes, which encoded arsenite oxidase, was also identified (Muller *et al.*, 2003) from *Centibacterium arsenoxidans* (member of the β- Proteobacteria) isolated from industrial wastewater (Weeger *et al.*, 1999). The four identified genes were named *aoxA, aoxB, aoxC, aoxD*. An additional, partial gene
upstream of \(aotA\) was also observed. Moreover, the inactivation of the \(aotA\) or \(aotB\) gene caused loss of the arsenite oxidase activity (Muller et al., 2003).

Branco et al. (2009) isolated a heterotrophic bacterium, \(Ochrobactrum tritici\) SCI24, which was able to oxidize arsenite to arsenate. Sequence analysis of arsenite oxidase genes in the strain revealed structural genes for arsenite oxidase (\(aotAB\)), a \(c\)-type cytochrome (\(cytC\)), and molybdopterin biosynthesis (\(moeA\)).

Three gene clusters involved in arsenic redox transformation of two arsenite oxidizers \(Achromobacter\) sp. SY8 and \(Pseudomonas\) sp. TS44 was identified. A 17.5 kb sequence containing arsenite oxidase \(aot\) gene clusters (\(aotX-aotS-aotR\) and \(aotA-aotB-aotC-aotD\)) was isolated from \(Achromobacter\). Similarly, a 14.6 kb sequence including \(aot\) clusters (\(arsD-arsA-aotA-aotB\)) and arsenic resistance (\(ars\)) gene cluster (\(arsC1-arsR-arsC2-ACR3-arsH\)) was obtained from \(Pseudomonas\) sp. TS44 (Cai et al., 2009b). Chang et al., 2010 also identified \(aotB\) and \(aotR\) genes in \(Pseudomonas strutzeri\).

Several genes encoding arsenite oxidase (\(aot\)) have been cloned and characterized. The crystal structures of arsenite oxidases have also been studied (Silver and Phung, 2005). The enzyme catalysing arsenite oxidation (arsenite oxidase) has been well characterized in several bacterial strains (Ellis et al., 2001). Arsenite oxidase enzyme contains two subunits which is encoded by the genes \(aotA\) (small Fe-S Rieske subunit) and \(aotB\) (large Mo-pterin subunit) respectively (Mukhopadhyay et al., 2002; Silver and Phung, 2005). The \(aotB\) gene, which encodes the large subunit of arsenite oxidase, has been found in different soil and water systems containing arsenic (Inskeep et al., 2007).

Arsenite oxidase enzyme has been found widely in various groups of bacteria and has been studied in detail for the arsenite oxidizing bacteria namely \(Alcaligenes faecalis\) (Anderson et al., 1992), NT-26 (Santini and vanden Hoven, 2004), and NT-14 (vanden
Hoven and Santini, 2004). The arsenite oxidases of these strains were purified and characterized.

The \textit{aoxB} genes act as a functional marker of aerobic arsenite oxidisers (responsible for oxidation) that result in the formation of arsenite oxidase, which facilitates biotransformation of arsenite to arsenate (Quemeneur \textit{et al.} 2008). Several novel arsenite oxidase genes (\textit{aoxBs}) were identified from the upper layers of the sediments of land surface (0-7 m) and were found to be specific for arsenite-oxidizing bacteria (Fan \textit{et al.}, 2008).

Quemeneur \textit{et al.}, 2008 isolated 25 genera of arsenite oxidizing bacterial strains. The \textit{aoxB} gene was found to be a valuable molecular marker for investigating potential of arsenite oxidation. Primers targeting the first quarter of the \textit{aoxB} gene were used to detect its presence and expression in the environment. Some bacteria like \textit{Herminiimonas arsenicoxydans}, \textit{Thiomonas arsenvorans}, \textit{Agrobacterium tumefaciens} and \textit{Rhizobium} species strain NT 26 were protein sequenced for \textit{aoxB} protein.

Arsenite oxidizing property has also been reported in other members of \textit{Actinobacteria}. In 2002, Mokashi and Paknikar isolated \textit{Microbacterium lacticum} with its minimum inhibitory concentration for arsenite of 50 mM and it was identified as arsenite oxidizing bacteria.

In our knowledge, no other member of \textit{Actinobacteria}, order \textit{Actinomycetales} and family \textit{Microbacteriaceae} and genus \textit{Microbacterium} has been reported that possess arsenite oxidizing ability. Although various arsenic resistant bacteria member of \textit{Actinobacteria} have been reported.

To understand the evolutionary relationship of arsenite oxidizing genes present in diverse bacteria, some studies on evolutionary trees of gene and protein sequence relationship
are considered. Silver and Phung (2005), drew two conclusions relevant to environmental microbiology: *asoBA* or *aoxAB* genes, which are found widely in a wide range of prokaryotes. However, there is great diversity in sequences, which precludes the use of gene-specific universal probes or primers for identifying these genes in the recently explored isolates.

The results obtained in the present study provide the evidence of the presence of diverse arsenite-resistant bacteria in agricultural soil that receives effluent from textile dyeing industry. The resistance level of arsenic by these bacteria can be due to the arsenic contamination observed in the sampling site. Although direct role of the bacterium *Microbacterium paraoxydans* IR-1 in mobilization of arsenic was not studied, the ability of the isolate to oxidize arsenite suggests that the isolate can be used to remediate arsenic transformations in the contaminated soils. The role of the isolate in the arsenic cycling in the soil may become relevant with changing environmental conditions. Our results indicate that *Microbacterium paraoxydans* IR-1 is an important arsenite oxidizing bacteria in the arsenic contaminated environments. This bacterium can grow heterotrophically and work as a natural catalyst for oxidizing the most toxic form of arsenic to less toxic arsenate. Although, there is no direct comparison between laboratory experiments and field conditions, but it may play a vital role in the biogeo cycling of the arsenic element and have potential in bioremediation of arsenic-contaminated environments.