Declaration

Certified that the work embodied in this thesis entitled, "**IMPACT OF HEAVY METALS IONS ON BOD EXERTION**" which is being submitted by Mrs. Siloni Goel, in the fulfillment of requirements for the award of the degree of Doctor of Philosophy in the Department of Biotechnology and Environmental Science of Thapar University, Patiala is a record of candidate's own work carried out by her under my supervision and guidance. The matter presented in this thesis has not been submitted in part or full for the award of any degree in any other University.

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Chapter-1

INTRODUCTION

Chemically, wastewater is composed of organic and inorganic compounds as well as various gases. Organic components may consist of carbohydrates, proteins, fats and greases, surfactants, oils, pesticides, phenols, etc. Inorganic components may consist of heavy metals, nitrogen, phosphorus, pH, sulfur, chlorides, alkalinity, toxic compounds, etc. In domestic wastewater, the organic and inorganic portion is approximately 50% respectively. Since wastewater contains a higher portion of dissolved solids than suspended solids, about 85 to 90% of the total inorganic component is dissolved and about 55 to 60% of the total organic component is dissolved. Gases commonly dissolved in wastewater are hydrogen sulfide, methane, ammonia, oxygen, carbon dioxide and nitrogen. The first three gases result from the decomposition of organic matter present in the wastewater. Over the years, a number of different tests have been developed to determine the organic content of wastewaters. Laboratory methods commonly used today are biochemical oxygen demand (BOD), chemical oxygen demand (COD), and total organic carbon (TOC).

Waste Water and Its Sources

Wastewater is the flow of used water from a community. Characteristics of the wastewater discharges will vary from location to location depending upon the population and industrial sector served, land uses, groundwater levels, and degree of separation between storm water and sanitary wastes. Domestic wastewater includes typical wastes from the kitchen, bathroom, and laundry, as well as any other wastes that people may accidentally or intentionally pour down the drain. Sanitary wastewater consists of domestic wastewater as well as those discharged from commercial, institutional, and

similar facilities. Wastewater composition refers to the actual amounts of physical, chemical and biological constituents present in it.

Waste Water Quality Parameters

Physically, a grey colour, musty odour, a solid content of about 0.1%, and 99.9% water content usually characterize wastewater. The solids can be suspended (about 30%) as well as dissolved (about 70%). Chemical and biological processes can precipitate dissolved solids. From a physical point of view the suspended solids can lead to the development of sludge deposits and anaerobic conditions when discharged into the receiving environment. The most important physical characteristic of wastewaters is total solids content, which is composed of floating matter, matter in suspension and, matter. Temperature is a very important parameter because of its effect on the aquatic life, chemical reactions and reaction rates, and the suitability of the water for beneficial uses. In addition, oxygen is less soluble in warm water than in cold water.

Toxins found in wastewater pass through wastewater treatment facilities that have not been designed to remove them and can interfere with their operation. In a biological treatment process toxic materials can upset a treatment process or even kill the biological community and make the process ineffective. Removal of toxic pollutants at the treatment facility can be very costly since they can very be numerous and varied in a larger community and therefore it is generally advantages to remove them at the source. Source control can be achieved by the use of municipal by laws limiting pollutant discharges to the sewerage system. The removal of toxic pollutants at the source can be achieved by requiring treatment prior to discharge, recycling of waste by products, manufacturing process changes, and the substitution of raw materials.

Table 1.1: Typical composition of untreated domestic wastewater

Constituent	Concentration, mg/L		
	Strong	**Medium**	**Weak**
Solids, Total:	1200	720	350
Dissolved, Total	850	500	250
Fixed	525	300	145
Volatile	325	200	105
Suspended, total	350	220	100
Fixed	75	55	20
Volatile	275	165	80
Settle able Solids, mg/L	20	10	5
BOD mg/L	400	220	110
Total organic carbon (TOC)	290	160	80
COD	1000	500	250
Nitrogen (total as N):	85	40	20
Organic	35	15	8
Free Ammonia	50	25	12
Nitrites	0	0	0
Nitrates	0	0	0
Phosphorous (total as P):	15	8	4
Organic	5	3	1
Inorganic	10	5	3
Chlorides	100	50	30
Alkalinity	200	100	50
Grease	150	100	50

Role of Heavy Metal Ions in Waste Water Environment

Biosphere pollution by chemicals and heavy metals such as cadmium, nickel, zinc, lead, copper, etc., has accelerated dramatically during the last few decades due to mining, smelting, manufacturing, use of agricultural fertilizers, pesticides, municipal wastes, traffic emissions, industrial effluents and industrial chemicals, etc. Some of these metal ions, e.g. Na^+, K^+, Mg^{2+}, and Ca^{2+} are most essential for life and behave more like major elements such as P with respect to their cycling rates and accumulation patterns (Lobersli and Steinnes, 1988). The metal ions of transition metals essential for life include vanadium, manganese, iron, cobalt, nickel, copper, zinc, and molybdenum. These metal ions play a variety of functions in biological systems as important structural components

in proteins. But higher levels of these metal ions are highly toxic to animals including humans as well as plants, and their solubility in water is considered to be one of the major environmental issues. As vegetation is usually the first interceptor of heavy metals (HMs) in the soils and water (Rauser, 1990; Chaudhry et al., 1998), it is very important to clean up soils and groundwater, which is a challenging proposition for a range of technical and economical reasons. Movement of HMs through soils is largely dependent on the organic matter (OM) contents of the soil, i.e. the greater the OM contents of the upper horizon of soil, the greater the affinity of that horizon for HMs (Schnitzer, 1978). There are OM/HM interactions (metallo-organic complexes) in the soils with most of the OM in soils being comprised of humic substances. Many terms such as chelation, complexation, absorption, etc. are used to describe them. Higher concentrations of HMs in soils have been reported to inhibit plant growth, nutrient uptake, and physiological and metabolic processes. This also results in chlorosis, damage to root tips, reduced water and nutrient uptake and damage to enzymes (Baisberg-Påhlsson, 1989; Sanità di Toppi and Gabbrielli, 1999). Heavy metals, like other environmental stressors, also induce increased antioxidant enzyme activities in plants (Iannelli et al., 2002). HMs are found to induce tumor and mutations in animals at higher concentrations (Degraeve, 1981). They are capable of causing genetic damage to germ cells of male and female animals including humans (Hayes, 1984; Groten and Vanbladeren, 1994; Wagner,1993). They are regarded as cumulative toxins which through biomagnification in plants affect human health (Groten and Vanbladeren, 1994). Heavy metals persist in soils and can be adsorbed in soil particles or leached into ground water. Human exposure to these metals through ingestion of contaminated food or uptake of drinking water can lead to their accumulation in humans, animals and plants (Khan, 2006a).

There is large variability in the quality of industrial effluents, which varies with industrial processes. The effluents discharged by different industries contain a high range of physico-chemical parameters like temperature, pH, conductivity, hardness, alkalinity, COD, TSS, nitrates, nitrites, cations (Na^+, K^+, Ca^{2+} and Mg^{2+}) and anions (Cl^-, CO_3^{2-}, HCO_3^-, SO_4^{2-}). These effluents from different industries including the tannery industry (Tandon et al. 2001) also contain heavy metals and trace metals including chromium,

cadmium, copper, lead, nickel, zinc, cobalt, magnesium, iron and arsenic. Generally, the growers are unaware of ion toxicity, which could be introduced into the food chain by vegetables and crops irrigated with effluents. These toxic ions may retain in soil or leach out through the soil and may contaminate ground water along with the soil itself and finally enter the food chain and cause health hazard in animals and plants. So there is dire need to quantify these toxic elements in the effluents prior to their use for irrigation, drinking and other purposes.

Biochemical oxygen demand (BOD)

BOD-biochemical (biological) oxygen demand is a test used to measure the concentration of biodegradable organic matter present in a sample of water. It can be used to infer the general quality of the water and its degree of pollution by biodegradable organic matter. It is used in water quality management and assessment, Ecology and environmental science. BOD is not an accurate quantitative test and should be considered as providing an indicator of the quality of a water body.

It measures the molecular oxygen utilized during a specified incubation period for the biochemical degradation of organic material (carbonaceous demand) and the oxygen used to oxidize inorganic material such as sulphides and ferrous ions. The BOD test is one of the simplest biotests of water analysis. This test is widely applied to define organic water pollution and to control the performance of wastewater treatment plant. Generally BOD is standardized by the measurement of oxygen consumption in five days (BOD_5) at 20° or three days (BOD_3) at 27° and in the dark (Standard Methods, 20^{th} addition). Utilization of BOD_3 as an evaluation method of the biodegradability of domestic wastewater treatment plants is widely accepted. It is, however, noted that standard domestic wastewater has generally constant composition and rarely contains compounds or factors inhibiting biodegradation. In contrast, in the case of industrial effluents, the presence of many different inhibitory substances, the infinite number of possible mixtures and high variability of chemical conditions necessitate an evaluation of the BOD_3 in order to test the suitability of this approach for the predictive biodegradability characterization of

industrial wastewaters. Many municipal activated sludge plants receive combined wastewater containing heavy metals. These metals typically include copper, zinc, nickel and cadmium and originate predominantly from industrial discharges.

BOD_3 values were calculated using the following equation and results are used to find change in BOD (increase/ inhibition)

$$BOD_5(mgL^{-1}) = \left[(D_1 - D_2) - (B_1 - B_2)\left(1 - \frac{1}{D_f}\right) \right] D_f$$

Where,
D_1 = DO of diluted sample immediately after preparation, mgL^{-1}
D_2 = DO of diluted sample after 3 day incubation at 27°C, mgL^{-1}
B_1 = DO of seed control before incubation, mgL^{-1}
B_2 = DO of seed control after incubation, mgL^{-1}.
Here, D_f=100, (1% of substrate).

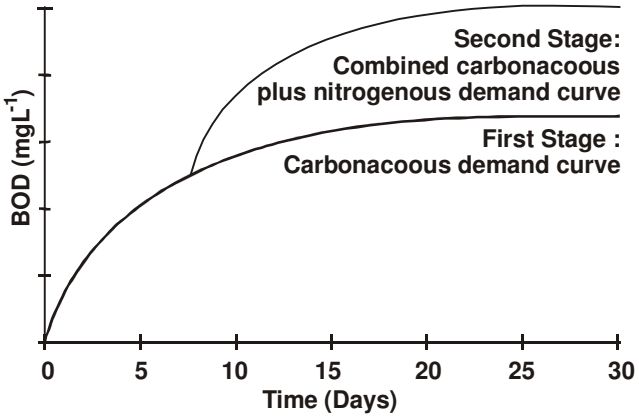

Figure 1.1: BOD curve showing the superimposed nitrogen oxidation curve

The BOD test is carried out by diluting the sample with de-ionised water saturated with oxygen, inoculating it with a fixed aliquot of seed, measuring the dissolved oxygen and sealing the sample (to prevent further oxygen dissolving in). The sample is kept at 20° in the dark (to prevent photosynthesis and thereby the addition of oxygen) for five days and the dissolved oxygen is measured again. The difference between the final D.O and initial D.O is the B.O.D. The apparent BOD for the control is subtracted from the control result to provide the corrected value. The loss of dissolved oxygen in the sample, once

corrections have been made for the degree of dilution, is called the BOD5. In the UK allylthiourea is also added at the start of the test to prevent oxidation of ammonia. Results from such tests are represented as BOD_5 (ATU) and referred to as Carbonaceous BOD (CBOD) in the U.S.. Less frequently used is the Ultimate BOD (UBOD) test, in which DO is repeatedly measured by DO meter in the same specialized bottles until it has reached equilibrium.

BOD is similar in function to chemical oxygen demand (COD), in that both measure the amount of organic compounds in water. However, COD is less specific since it measures everything that can be chemically oxidised rather than just levels of biology active organic matter. BOD is used as a gauge of the effectiveness of wastewater treatment plants. Various commercial devices are available for its determination. BOD can be calculated by: Undiluted; Initial D.O - Final D.O = BOD Diluted; ((Initial D.O - Final D.O)- BOD of Seed) x Dilution Factor

Factors affecting BOD

The main factors affecting BOD are as follows:

- pH
- Temperature
- Heavy Metal Ions
- Seed

Effect of pH on BOD

pH: The pH of a liquid is the negative logarithm of the concentration of hydrogen ions in the solution. The pH of natural water depends on several factors: the carbonate system, types of rock, types of soil, and nature of discharged pollutants. The concentration of carbonates (CO_3^{2-}, HCO_3^-) and carbon dioxide ($CO_2(aq)$) has the main influence on the pH of clean water. High concentrations produce alkaline waters (high pH), while low concentrations usually produce acidic waters (low pH). The types of soil in the drainage area also affect the pH. Drainage water from forests and marshes is often slightly acidic due to the presence of acids produced by decaying vegetation. Nitrogen oxides (NO,

NO_2) and sulfur dioxide (SO_2) from automobile and power plant emissions are converted into nitric acid (HNO_3) and sulfuric acid (H_2SO_4) in the atmosphere. The pH values of natural surface waters usually range from 5.5 to 8.5. Extremely high (9.6) and low (4.5) values are unsuitable for most aquatic organisms. Changes in pH can also affect aquatic life indirectly by altering other aspects of water chemistry. Low pH levels accelerate the release of heavy metals from sediments on the stream bottom.

Effect of Temperature on BOD

The effect of temperature on toxicity is a complex phenomenon. Both natural processes and human activities influence the temperature of stream water. Water temperature affects the amount of dissolved oxygen and other gases that water can hold at specific atmospheric pressure. A rise in temperature decreases the ability of water to hold oxygen molecules. There are two main sources of dissolved oxygen in stream water: the atmosphere and photosynthesis. Waves and tumbling water mix air into the water where oxygen readily dissolves until saturation occurs. Aquatic plants and algae also introduce oxygen as a byproduct of photosynthesis. The amount of dissolved oxygen is limited by physical conditions, such as water temperature and atmospheric pressure.

Lower temperature ---> higher potential dissolved oxygen level

Higher temperature ---> lower potential dissolved oxygen level

Activity of living organisms increases in warmer water, requiring more oxygen to support their metabolism and magnifying the temperature effect on dissolved oxygen. Low dissolved oxygen indicates a demand on the oxygen of the system. Build up of organic material from human activities is one source of oxygen depletion. Microorganisms in the stream consume oxygen as they decompose inadequately treated sewage, urban and agricultural runoff, and discharge from food-processing plants, meat-packaging plants, and dairies that has entered the stream. Natural organic materials, such as leaves, also accumulate in the stream and create an oxygen demand as they decompose. Some pollutants, such as acid mine drainage, produce direct chemical demands on oxygen in

the water. Dissolved oxygen is consumed in the oxidation-reduction reactions of introduced chemical compounds, such as nitrate (NO_3^-) and ammonia (NH_4^+), sulfate (SO_4^{2-}) and sulfite (SO_3^{2-}), and ferrous (Fe^{2+}) and ferric (Fe^{3+}) ions. One measure of dissolved oxygen in water is parts per million (ppm), which is the number of oxygen molecules (O_2) per million total molecules in a sample. Calculating the percent saturation is another way to analyze dissolved oxygen levels. Percent saturation is the measured dissolved oxygen level divided by the greatest amount of oxygen that the water can hold under various temperature and atmospheric pressure conditions multiplied by 100.

The temperature at which the microorganisms are living and growing markedly influences the process of oxygen utilization in the degradation of the organic constituents present in wastewater, or deoxygenation. The rate of oxygen usage, as does the rate of all metabolic activities, increases with an increase in temperature where, in the range of 30 - 40°, the maximum value is achieved. Increases in temperature beyond this point result in a very rapid decrease in the rate, reportedly due to the denaturing of certain heat labile proteins, such as enzymes, and the actual destruction of some of the microorganisms. At lower temperature the deoxygenation rate is generally reduced to where, near 0°C, it practically ceases.

Effect of Heavy Metal Ions on BOD

Microorganisms require metals in trace quantities for metabolism and growth, but higher concentrations can be toxic (Ford, et al., 1995). The toxicity of metals is caused primarily by their ability to denature proteins. This effect can be caused by the blocking of functional groups, displacing an essential metal, or modifying the active conformation of the molecule (Gadd and Griffiths, 1978). Microbial cells require mechanisms for the acquisition of the essential metals to metabolize, grow, and reproduce. However, for survival in environments containing high concentrations of available metals, mechanisms to counter the inherent toxicity of the metal ions are required (Gadd, 1993). The important role-played by fungi in biogeochemical cycles make them candidates for studies of metal-microbe interactions.

Heavy metals are metals with density above 5g/cm^3. Thus the transition elements from V (but not Sc and Ti) to the half-metal As, from Zr (but not Y) to Sb, from La to Po, the lanthanides and actinides can be referred to as heavy metals (Weast, 1984). Of the 90 naturally occurring elements, 21 are non-metals, 16 are light metals and the remaining 53 (with As included) are heavy metals (Weast 1984). Most heavy metals are transition elements with unfilled d orbitals. These d orbitals provide heavy metal cation with the ability to form complex compounds that may or may not redox-active. Thus, heavy metal cation play an important role as trace elements in sophisticated biochemical reactions. At higher concentration, however, heavy metal ions form unspecific complex compounds in the cell, which leads to toxic effects. Some heavy metal cations, e.g. Hg^{2+} Cd^{2+} and Ag^{+}, form strong toxic complexes, which makes them too dangerous for any physiological function. Even highly reputable trace elements like Zn^{2+} or Ni^{2+} and especially Cu^{2+} are toxic at higher concentration. Thus, the intracellular concentration of heavy metal ions has to be controlled, and heavy metal ion resistance is just a specific case of the general demand of every living cell for some heavy –metal homoeostasis system.

To have any physiological or toxic effect, most heavy metal ions have to enter the cell. At first glance, divalent heavy metal cations are structurally very similar; the divalent cations Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+} have ionic diameters between 138pm and 160pm (Weast, 1984), a difference of 14 % and all, of course, carry a double positive charge. Oxyanions like chromate, with four tetrahedrally arranged oxygen atoms and two negative charges, differ mostly in the size of the central ion, so the structure of chromate resembles that of sulphate. The same is true for arsenate and phosphate. Thus, uptake systems for heavy metal ions have to bind those ions tightly if they want to differentiate between a couple of structurally very similar ions. However, tight binding costs both time and energy.

When a cell faces a high concentration of any heavy metal that is accumulated by such an unspecific system, the specific heavy metal ion is transported into the cytoplasm in spite of its high concentration, because these unspecific transporters are constitutively

expressed. Thus gate cannot be closed. This open gate is the first reason why the heavy metal ions are toxic (Nies and Silver, 1995). Once inside the cell, heavy metal cations, especially those with high atomic numbers, tend to bind SH groups, e.g. Hg^{2+}, Cd^{2+} and Ag^{+}. The minimal inhibitory concentration of this metal ion is a function of the complex constants of the respective sulphides. By binding to SH groups, the metals may inhibit the activity of sensitive enzymes. Other heavy metal cations may interact with physiological ions, Cd^{2+} with Zn^{2+} or Ca^{2+}, Ni^{2+} and Co^{2+} with Fe^{2+}, Zn^{2+} with Mg^{2+} thereby inhibiting the function of the respective physiological cations.

Trace quantities of heavy metals such as nickel, manganese, lead, chromium, cadmium, zinc, copper, ferrous and mercury are essential constituents of most wastewaters, for example, some of these metals are necessary for growth of biological life and absence of sufficient quantities of them could limit growth of algae. Since both the biological treatment process and BOD measuring technique are based on same principle of biochemical degradation of organic matter by microorganisms in presence of oxygen, the presence of toxic metals like copper, zinc, lead and other heavy metals will have an influence on both the processes. Because of the limited solubility of oxygen in the aquatic medium contained in the BOD bottle, the toxic effect of heavy metal will be sufficiently large as compared to that in the treatment plant producing effluent being tested for BOD. This difference may be due to the reason that on continuous aeration, the effluent of the treatment plant gets the regular supply of oxygen.

Concentration of heavy metal ions in the effluent of different industries has been cited to a greater extent (Tandon et al, 2001) but effect on BOD exertion under different conditions like pH, temperature, quality of seed, concentration of different metal ions like cobalt, copper, zinc, nickel, cadmium, silver, mercury etc. and its kinetics in presence of above said metal ions are limited. When a raw wastewater or wastewater treatment plant effluent is discharged into a receiving body of water, the organic matter present is rapidly degraded and assimilated by micro-organisms.

Ammonium chloride is fairly effective in deactivating the free Hg^{2+} and Ag^{+} to below the inhibiton level. Among the other complexing agent used to reduce the concentration of

free heavy metal ions are ethylenediamine tetraccetic acid (EDTA), sodium di- ethyl dithio-carbamate (DDTC), ate-hydroxy-quinoline (oxine) and di-phenyle thiocarbazone (dithizone). EDTA and DDTC are most successful complexing agent probably because of their better solubility in an aqueous medium. Complete removal of effect of the heavy metal ion requires the addition of a large amount of complexing agent the complete removal of their inhibitory effect of the metal ions cannot be achieved. In addition, such compounds when added in large quantities will effect interfere in the normal BOD measurement process.

Effect of Seed on BOD Exertion Process

Different types of seed affect BOD exertion process. BOD values were observed to be more in presence of indigenous seed than with the seed from sewage. The results of ultimate BOD and rate constants for the system were also more with indigenous seed (Shrivastava et al, 2000).

Presence of Metal –Microbe Interactions in Nature

Metal ions are known to interact with microbes in nature through their immobilization. It may be cellular sequestration and accumulation or extracellular precipitation. Bioleaching of metals from ores is a practical example.

Microorganisms present several mechanisms of metal tolerance. Metal-microbe interactions can be conveniently divided into three main mechanisms:1) extracellular interactions, involving extracellular polymers, proteins, acid metabolites and changes in the localized environment due to biochemical processes; 2) cell surface interactions, in which metals bind to microbial cell surfaces as a result of specific functional groups, and 3) intracellular interactions, where metals accumulate in microbial cells due to specific active transport processes. Detoxification occurs through transformation to insoluble or more volatile forms, or incorporation of specific metals into proteins (Ford and Mitchell, 1992).

The effect of seed on BOD exertion, which would generally encounter during its biological treatment, has not been studied in detail. In the past, different parameters of

BOD kinetics for an industrial waste have been estimated using a sewage seed. This in turn has probably been a major reason for malfunctioning of various effluent treatment plants and hence there is a need to study the effect of seed on BOD parameters. Streeter and Phelps (1925) proposed the first order formulation of BOD exertion. After this Young and Clark (1965) proposed a second order equation for BOD exertion kinetics. Hewitt et al. (1979) tried to view BOD exertion kinetics in multiple perspective. Eldrige and Ekenfeldeer reported that seed may affect 5 days value, but ultimate BOD value is not affected significantly. At very low seed concentration (0.1%), there is the existence of lag phase.

Trace amounts of heavy metal ions such as Cu^{2+}, Fe^{2+}, Fe^{3+}, Mn^{2+} and Zn^{2+} are essential minerals for life sustenance and healthy growth of a microorganism and also as co-factors for the enzymic reactions in the cell (Lehninger, 1982; Hughes and Poole, 1989). However, there is generally a threshold concentration for each of the heavy metal ions above which it would become toxic to the microbial system. The toxicity of the metal ions varies from adversely affecting the growth and characteristics of the microbial system to its ultimate death (Webb. 1966: Macgregor and Clarkson, 1974; Collins and Stotzky, 1989: Hughes and Poole, 1989). Although the mechanism by which these heavy metal ions affect a microbial system is still not well understood (Wakatsuki, 1995), it is however generally described by their displacement of the native metal ions from the normal binding sites or their attachment to the proteins and nucleic acids in the microbial cells (Hughes and Poole, 1989). Their adsorption on the enzyme could deactivate or poison the enzyme when the adsorbed amounts exceed their useful function limits as co-factors for the enzymic reactions. Most microorganisms do however develop resistance to some heavy metal ions during their culture and growth. High tolerance of some microbial systems to metal ion toxicity is often attributed to their ability to convert them to less toxic or volatile compounds. For example, microbial resistance to mercuric ions is often attributed to its reduction to volatile Hg^{o} in the presence of mercuric reductase found in microorganisms such as E. coli and S. aureus (Hamdy and Noyes, 1975: Hughes and Poole, 1989; Wakatsuki, 1995). BOD biosensors generally use living microorganisms as the biosensing component and are thereof sensitive to the presence of heavy metal ions. Ignorance of the effect of heavy metal ions could result in significant

error in the BOD measurement (Karube et. al. 1977; Riedel and Lange, 1990; Li and Chu, 1991; Li and Tan, 1994) Wastewater contains varying amounts of different heavy metal ions. Particularly those from the metal-finishing, electronic paint, mineral and metallurgical processing industries. Pretreatment of the test sample containing excessive amount of heavy metal ions and other toxic compounds is an integral part of the standard procedure for the conventional measurement of the 5-day BOD values.

Heavy metal ions in the substrate have been known to affect the biochemical activities and metabolism of the microorganism and hence its growth, health conditions and assimilability of the substrate (Collins and Stotzky 1989, Hughes and Poole 1989). Apart from the ions of alkaline and alkaline earth metals, trace concentration in the order of 10 ppm of some heavy metal ions such as manganese, iron, cobalt, copper, zinc and molybdenum are essential for growth of the microorganism. These metals are known to be involved in the stabilization of the biological structure ranging from cell walls to protein confirmation. They are often highly effective catalysts for the essential biochemical processes involved in the well-being and growth of the microbes. However at high concentration, even with the essential metals, the metal ions are toxic to the microorganism. It is possible nevertheless for the microorganism to develop resistance to toxic metals. Hence the effect of heavy metal ion depends both on the type and concentration of the heavy metal ion and the type and growth history (conditioning) of the microbial system. This would directly affect the consumption of oxygen by the microorganism during its assimilation of the substrate and hence the BOD measurement. The effect of heavy metal ions is therefore another important and necessary parameter apart from temperature, pH and dissolved solutes in the substrate that should be considered in the evaluation of the efficacy of a BOD biosensor.

The way microbes interact with metals depends in part on whether the organisms are prokaryotic or eukaryotic. Both types of microbes have the ability to bind metal ions present in the external environment at the cell surface or to transport them into the cell for various intracellular functions.

There are primarily three types of interactions: Metabolic-enzymatic, metabolic-non-enzymatic and natural occurrence of metal/microbe interactions. Uptake of trace metals and their subsequent incorporation into metalloenzymes occurs in all microbes. A number of microbes are able to use some metals or metalloids as electron donors or acceptors in energy metabolism. Enzymatic microbial detoxification of harmful metals or metalloids is a third type of interaction. In this process, a toxic metal species may be converted into a less toxic or non-toxic species by enzymatic oxidation or reduction, e.g. bacterial oxidation of AsO_2 to AsO¾ by a strain of Alcaligens faecalis (Ehrlich 1996).

As it is established that microbes drive the processes that cycle and transform metals in sediments, it is important to examine closely the interactions between metals and microbes. These interactions play a particularly important role in understanding the fate of metals in the environment, yet little is known about metal-microbe interactions such as the microbial control of metal speciation. The literature suggests that microbial populations may play important roles in metal speciation in a variety of environments. Copper complexing ligands have been found to form in sediments (Skrabal et. al., 1997) and certain bacteria produce extracellular copper binding ligands (Schreiber et. al., 1990; Croot et. al., 1999). It is thought that some of these ligands may actually control the speciation of copper in the ocean and point to an active biological cycling of copper (Gordon, 1998). In addition, bacteria can also cause the precipitation of metal minerals either inside the cell or at the cell membrane (Beveridge and Doyle, 1989).

It is well documented that microbes interact with metals in a variety of ways (Mills, 1997). First, many metals are essential elements for life, as they serve critical functions in many proteins and biochemical reactions, e.g., Cu, Zn, Fe, Ni, Mn, and Co (Theil and Raymond, 1994). For example, zinc is crucial for the structural integrity of “zinc finger” proteins responsible for DNA transcription (Mills, 1989). Manganese is responsible for the redox chemistry at the active site of phytosynthetic reaction centers as well as in some superoxide dismutases in bacteria (Madigan et. al., 1997). Copper and iron also participate in a similar manner in a host of biological functions, such as the binding of dioxygen in hemoglobin, electron transfer in cytochromes, and redox centers in superoxide dismutases (Theil and Raymond, 1994). Cobalt is found in vitamin B12

(Sennett et. al., 1981) and nickel is a key metal in various hydrogenases and S-methyl CoM reductase, which is the catalyst of methane production in all methanogenic bacteria (Theil and Raymond, 1994). These transition metal nutrients, normally available at nanomolar concentrations in the environment, may also be toxic at elevated concentrations (Lippard and J.M., 1994). Second, other transition metals, which serve no known biological function (e.g., Hg, and Pb), are toxic, in particular when they bioaccumulate.

Third, some metals serve a role either as electron donors or electron acceptors (e.g. Fe^{2+} or Fe^{3+} respectively) in energy generating reactions. Dissimilatory metal reduction has been recognized as a significant process (Myers and Nealson, 1988; Lovley, 1991; Nelson and Myers, 1992; Davison, 1993; Nealson and Saffarini, 1994) in sediments and may play an important indirect role in heavy metal speciation. For instance, the reduction of iron or manganese oxides may serve to remobilize co-precipitated or sorbed metals (Francis and Dodge, 1990). Finally, many metal mineral phases, such as carbonates, oxides, phosphates, and sulfides, can nucleate at the surface of bacterial cell walls (Doyle, 1989; Ferris, 1989; Ferris et. al., 1989). As a consequence of the integral relationship with metals, microbes have developed a variety of both uptake and resistance mechanisms. Some of these mechanisms may alter metal speciation or prevent metals from reaching toxic concentrations inside the cell.

The existing wastewater contains various kinds of organic and/or inorganic pollutants and some of them may inhibit the microbial activities. Refractory organic pollutants and small amounts of heavy metal ions in the effluent water and it also contained a rather a high concentration of inorganic salts formed by controlling the pH value of the water. Chemical additives like caustic soda, lime, hypochlorite alum, and clay, starch are used in the paper making industry. Many other chemicals are also used for drying, tinting, cleaning and quality improvement. The effluent from a paper mill can contain thousands of different chemical species, which, if discharged directly into the environment, could cause untold damage.

The seed culture that is used to prepare the dilution water for the BOD test is a mixed culture. Such cultures contain large numbers of saprophytic bacteria and other organisms that oxidize the organic matter. In addition, they contain certain autotrophic bacteria that oxidize the noncarbonaceous matter. In the dairy wetland wastewater effluent, the bacterial community composition was dominated by bacteria from phylogenetic clusters related to Bacillus, Clostridium, *Mycoplasma*, *Eubacterium*, and *Proteobacteria* originally retrieved from the gastrointestinal tracts of mammals. The population of ammonia-oxidizing bacteria showed a higher percentage of *Nitrosospira*-like sequences from the wetland samples, while a higher percentage of *Nitrosomonas*-like sequences from manure and facultative pond was found (Ibekwe et al, 2003).

An inorganic toxicant may be cationic such as metallic ions of mercury, cadmium, chromium, lead nickel and uranium, etc. Toxic inorganics may also be alkylated or aromatized forms of metal ions such as methyl-mercury and phenyl mercury. Liquid wastes containing toxic heavy metals may be generated in various industrial processes, e.g., in chemical manufacturing, electric power generating, coal and ore mining, melting and metal and metal refining, distillery industries, pulp and paper wastes, etc. There is wide variety of microorganisms, including bacteria, fungi, yeast, and algae that can interact with metals through several mechanisms to transform them (Poole and Gadd, 1989). Berkun (1974) investigated the suitability of the first- and second-order models using BOD data obtained from extensive experiments using a respirometer and conventional dilution technique. In some studies effects of settleable BOD were also taken into account (Tyagi et al., 1999), but metal related deoxygenation data are limited. Some inhibiting effects of metals on deoxygenating rates in rivers were reported by Baity and Bell (1929), and Felegy et al. (1948) on various stream reaches receiving various industrial pollutions. Baker (1971) showed that even very small concentrations of HgCI, can effect the BOD values using a standard dilution technique. Research committee (1954) showed the effects of $HgCl_2$, $Cr_2(SO_4)_3$ and Na_2CrO_4 using a standard dilution technique. Research carried out to investigate the metal effects on BOD, indicated that the reliability of related Sag curve parameters should be carefully evaluated. Heukelekuan and Gelman (1955) studied the effects of Cu, Ni, Zn, Cd and Cr on BOD testing. Berkum (1982) investigated the effects of Hg, Cr, Cu, Zn and Al on synthetic

wastewater BOD values obtained from a large volume respirometer. Albek et al. (1997) investigated the effects of nickel on respirometric BOD values. Gokcay and Dilek (1991) investigated the effects of nickel and chromium and substrate concentration on the microbial growth of acclimatized microbes of sewage origin in batch cultures. Yetis et al. (1992) investigated the effects of heavy metals on biological activity in BOD bottle-seed biomass concentration.

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Chapter-2

LITERATURE SURVEY

Parameters like Biochemical oxygen demand (BOD), Chemical Oxygen demand (COD) and total and volatile solid (TSS or VSS) are traditionally used to characterize organic matter in wastewater. These parameters provide an indirect determination of the quantity of the organic matter present. COD and VSS are measures of the total concentration of the organic matter and particulate organic matter respectively, while the empirical BOD test provides a partial estimate of biodegradable organic matter only.

In 1912, the Royal Commission on Sewage Disposal took the view that the five-day Biochemical oxygen Demand (BOD) test was the most reliable chemical index of river water quality. The BOD figures recommended by the committee became known as the 'Royal Commission River Classification'. It was perhaps reasonable to note that the BOD test was not introduced to measure the strength of raw wastewater, rather the effect upon the receiving waters. Interestingly, the 5-day duration for BOD determination has no theoretical grounding but is based on historical convention (Tchobanoglous and Schoeder, 1985). In a report prepared by the Royal Commission on Sewage Disposal in the United Kingdom at the beginning of the last century, it was recommended that 5-day, 18.3°, BOD value be used as a reference in Great Britain. These values were selected because British rivers do not flow to the open sea in greater than 5 days and average long-term summer temperatures do not exceed 18.3°. The temperature has been rounded upward to 20°, but 5-day time period has become the universal scientific and legal reference.

Since BOD is measured on the effluent, perhaps it is only natural to see BOD removal as an important process parameter. But should BOD be measured on the influent as well to provide that information even though it is an inappropriate measurement? BOD is seen by many agencies as the most appropriate way of measuring the strength of carbonaceous load, even though BOD is essentially inferior to COD in almost all ways including

accuracy and precision. BOD is further affected by nitrification and although it is often assumed that the 5-day test of raw sewage will not nitrify, absence of nitrification cannot be guaranteed. In any particular BOD analysis even a small amount of nitrification will markedly increase the result. Incorporation of all or some of the ammonia-N in a BOD measurement is a needless complication, as ammonia-N can be measured directly rather than indirectly as an oxygen demand.

The BOD exertion phenomena are a complex biochemical reaction between heterogeneous assemblage of biota and complex organic substrate. The effect of varying environmental conditions such as temperature, pH, presence of toxicants etc. have been studied in details over the years. The phases during the BOD exertion have also been studied. Various models representing BOD kinetics have also been proposed and evaluated for various field applications.

The concentration level of dissolved organic pollutants in wasterwater is normally measured under specified standard conditions in terms of the biochemical oxygen demand (BOD) or the amount of oxygen required for the biochemical oxidation of the organic materials in the water in the presence of a suitable microbial culture such as activated sludge. Since biochemical oxidation is generally a slow process, the results obtained five days after seeding the water with the microorganism or BOD_5 (mg O_2 consumed/L of water) is universally considered and accepted to be a reasonably good indication of the concentration of the organics in the water. The BOD measurement also depends significantly on the temperature, oxygen concentration, toxicants and the type, quality and quantity of the seeding microorganisms used. Acceptability or rejection of the test results is usually decided upon counterchecking the analytical procedure and the microorganism with standard glucose/glutamic acid BOD check solutions. Although BOD_5 is a good index of the organic concentration, the results are only available 5 days after the actual sampling. This implies that the test water has to be held back for five days before any follow-up action can be taken. It is therefore of considerable interest to develop alternative methods which would give the same or better measurement of organic concentration in water or to develop a sensing device which can provide fast BOD_5

measurement. Such a sensing device is usually a microbial sensor which consists of a dissolved oxygen (DO) probe overlaid with a biofilm in which a suitable microorganism is immobilized. The organics in the test solution are biochemcially oxidized by the immobilized microorganisms in the biofilm. The dissolved oxygen measured by the DO probe will decrease with higher concentration of the organics in the test solution. BOD sensors reported with good reproducibility and stability were generally based on biofilms containing one species of microorganism.

A rapid biochemical degradation of carbohydrates-i.e., a high biochemical oxygen demand (BOD) in effluents from, for example, pulp and paper industries, may be a disadvantage in that it leads to a rapid lowering of the oxygen content of the water system near the mill. On the other hand, a rapid degradation may be an advantage from the point of view of efficiency and time of treatment when aerated ponds or activated sludge treatment are used. In addition, dissolved high-molecular carbohydrate material can be recoagulated before the sedimentation tank, whereas low-molecular carbohydrates can not. The rate of biochemical or chemical degradation of carbohydrates seems to be dependent on their degree of polymerization (DP). Monosaccharides are degraded more rapidly and have higher BOD_5 per unit mass than polysaccharides. A comparison of disaccharides and their monosaccharide constituents have shown an apparent lag in the BOD, due the existence of the bond holding the two monosaccharides (Varma and Hall, 1966). Among the most important hemicelluloses dissolved from the wood in, for example, thermomechanical pulping, are xylan and glucomannan. These have an original DP of 50-200 i.e., they consist of 50-200 monosaccharide units in the original wood. Data (Varma and Hall, 1966) indicate that the BOD_5 for these hemicelluloses is only one sixth of the BOD_5 for the corresponding monosaccharides. The chemical oxygen demand (COD) determined by permanganate oxidation, is higher and more rapid for monosaccharides than for polymeric saccharides (Meissner, 1958).

Metals play an integral role in the life processes of living organisms. Some metals (e.g. Ca, Co, Cr, Cu, Fe, K, Mg, Mn, Na, Ni and Zn) are essential, serve as micronutrients and are used for redox-processes, to stabilize molecules through electrostatic interactions; as

components of various enzymes; and regulation of osmotic pressure (Bruins, 2000). Many other metals have no biological role (e.g. Ag, Al, Cd, Au, Pb, and Hg), and are nonessential (Bruins, 2000) and potentially toxic to living organisms, specially microorganisms. Toxicity of nonessential metals occurs through the displacement of essential metals from their native binding sites or through ligand interactions (Bruins, 2000; Nies, 1999). In addition, at high levels, both essential and nonessential metals can damage cell membranes, alter enzyme specificity, disrupt cellular functions, and damage the structure of DNA (Bruins, 2000).

Heavy metals in wastewater come from industries and municipal sewage, and they are one of the main causes of water and soil pollution. Accumulation of these metals in wastewater depends on many local factors such as type of industries in the region, way of life and awareness of the impacts made to the environment by careless disposal of wastes (Chipasa, 2003). Therefore the presence of heavy metals in wastewater is not only of great environmental concern, but also strongly reduces microbial activity and as a result adversely affects biological wastewater treatment processes. Moreover, the toxicity of heavy metals in wastewater was shown to be dependant on factors like metal species and concentration, pH, wastewater pollution load (Dilek, et al 1998) and solubility of the metal ions.

Trace amounts of heavy metal ions such as Cu^{2+}, Fe^{2+}, Fe^{3+}, Mn^{2+} and Zn^{2+} are essential minerals for life sustenance and healthy growth of a microorganism and also as co-factors for the enzymic reactions in the cell (Lehninger, 1982; Hughes and Poole, 1989). However, there is generally a threshold concentration for each of the heavy metal ions above which it would become toxic to the microbial system. The toxicity of the metal ions varies from adversely affecting the growth and characteristics of the microbial system to its ultimate death (Webb,1966; MacGregor and Clarkson, 1974; Collins and Stotzky, 1989; Hughes and Poole, 1989). Although the mechanism by which these heavy metal ions affect a microbial system is still not well understood (Wakatsuki, 1995), it is however, generally described by their displacement of the native metal ions from the normal binding sites or their attachment to the proteins and nucleic acids in the microbial

cells (Hughes and Poole, 1989). Their adsorption on the enzyme could deactivate or poison the enzyme when the adsorbed amounts exceed their useful functional limits as co-factors for the enzymic reactions. Most microorganisms do however develop resistance to some heavy metal ions during their culture and growth. High tolerance of some microbial systems to metal ion toxicity is often attributed to their ability to convert them to less toxic or volatile compounds.

Wastewater contains varying amounts of different heavy metal ions, particularly those from the metal-fnishing, electronic, paint, mineral and metallurgical processing industries. Pretreatment of the test sample containing excessive amounts of heavy metal ions and other toxic compounds is an integral part of the standard procedure for the conventional measurement of the 5-day BOD values. It is therefore logical and essential to evaluate the effects of commonly used heavy metal ions in industry on the sensing efficacy of any newly developed BOD biosensor, particularly those using new bioactive materials. The reports by Karube et al. (1977), Riedel (1998), Li et al (2000) and Li and Tan (1994) showed conclusively that the resistance of BOD biosensors to heavy metal ions such as silver, cadmium, cobalt, chromium, copper, ferrous, ferric, mercuric, manganese, nickel, lead and zinc ions depends significantly on the heavy metal ion concentration, the microbial system and the operating conditions. Mitigation methods have also been proposed. Li and Tan (1994) reported the effectiveness of mitigation by the phenotypic modification of the microbial cells, the isolation of the microorganisms from the metal ions by overlaying the biofilm with a poly (4-vinylpyridine) membrane and the complexation of the heavy metal ions with ethylene diamine tetraacetate (EDTA) to reduce the free metal ion concentration to below its threshold toxic level. Besides heavy metal ions, common electrolytes including the ammonium, alkaline and alkaline earth metals chlorides, sulphates and nitrates are also generally present in the wastewater. These salts could affect the oxygen solubility in the solution and change the baseline current and biosensor response (Karube et al., 1977; Hyun et al., 1993; Li and Tan, 1994).

The impact of heavy metals on the environment and their accretion through the food chain have promoted research aimed at developing alternative, efficient and low cost wastewater purification systems (Wilhelmi and Duncan, 1995). Conventional methods for removing dissolved heavy metals include chemical precipitation and sludge separation, chemical oxidation or reduction, ion exchange, reverse osmosis, filtration, adsorption using activated charcoal, electrochemical treatment and evaporative recovery (Volesky, 1999). However, these techniques can be expensive, as they may not always be feasible and their metal-binding properties are non-specific (Price, Classen and Payne, 2001). These are reasons why alternative processing methods, such as those using microbial biomass are now being considered more seriously (Volesky, 1999, Slaveykova, Wilkinson, Ceresa and Pretsch, 2003).

Bioremediation of industrial wastes containing heavy metals has been demonstrated by several biotechnology companies employing bioaccumulation (Dilek and Yetis,1992; Lovley, Philips, Gorby and Landa, 1991 and Lovley and Coates, 1997). Biosorption, biopreciptation, and uptake by purified biopolymers derived from microbial cells provide alternative and/or additive processes for conventional physical and chemical methods (Wagner, et al 1999 and Lu et al 2000). Intact microbial cells, live or dead, and their products can be highly efficient bioaccumulators of both soluble and particulate forms of metals (Wagner et al 2001; Niu, Xu and Wang, 1993; Norberg and Persson,1984; Silver and Phung, 1986). The cell surfaces of all microorganisms are negatively charged owing to the presence of various anionic structures. This gives bacteria the ability to bind metal cations. Various microbial species, mainly Pseudomonas, have been shown to be relatively efficient in bioaccumulation of the different heavy metals from polluted effluents (Hussein et al 2004).

Heavy metals are commonly found in effluents from electroplating and other metal-processing industries. Conventional methods of heavy metal removal from aqueous solutions usually involve physico-chemical treatments such as precipitation, filtration, ionic exchange, adsorption, electron-deposition, reverse osmosis etc. (Dean, Bosqui and Lanoettle, 1972; Sag and Kutsal, 1995). Although the mechanisms by which heavy

metals affect biological treatment processes are not well defined, the general response of these processes to varying concentrations of metals is well documented (Bagby and Sherrard, 1981). It was reported that activated sludge microorganisms and process efficiency were inhibited by cadmium, chromium and nickel at concentration above 10 mg/L (Zarnovsky, Derco, Kuffa and Drtil, 1994). However, trace amounts of heavy metals are still required by microorganisms for optimum growth (Wood and Tchobanoglous, 1975). The deleterious effects of toxic compounds on biological processes are complex and are generally related to the species, and the solubility of the metal concentration of the toxicant (Dilek, Gokcay and U. Yetis, 1998).

In fact, a single metal species exists very seldom in wastewater. The presence of more than one metal often gives rise to interactive effects. Although the interactive effects of a mixture of heavy metals are extremely complex, it has been shown that the final expression of heavy metal toxicity in a biological treatment process depends on types and concentration levels of metals, order of metal addition, types of microorganisms present in the medium, mean cell residence time, type and strength of the influent wastewater and the pH of the medium (Nurdan, Tulay and Ozbelge, 1997).

Isao Karube (1990) reported a new biochemical oxygen demand (BOD) sensing method employing a double-mediator (DM) system coupled with ferricyanide and a lipophilic mediator, menadione and the eukaryote Saccharomyces cerevisiae. In this study, a stirred micro-batch-type microbial sensor with a 560 μL volume and a two-electrode system was used. The chronamperometric response of this sensor had a linear response between 1 μM and 10 mM hexacyanoferrate(II). Next, the optimum conditions for BOD estimation by the DM system (BODDM) were investigated and the findings revealed that the concentration of ethanol, used to dissolve menadione, influenced the sensor response and a relationship between the sensor output and glucose glutamic acid concentration was obtained over a range of 6.6–220 mg O_2 L^{-1} when using a reaction mixture incubated for 15 min. The sensor responses to 14 pure organic substances were compared with the conventional BOD_5 method and other biosensor methods. Similar results with the BOD biosensor system using Trichosporon cutaneum were obtained. In addition, the influence

of chloride ion, artificial seawater and heavy metal ions on the sensor response was investigated. In addition, no influence of the heavy metal ions (1.0 mg L^{-1} Fe^{3+}, Cu^{2+}, Mn^{2+}, Cr^{3+} and Zn^{2+}) was observed. Real sample measurements using both river water and seawater were performed and compared with those obtained from the BOD_5 method. Finally, stable responses were obtained for 14 days when the yeast suspension was stored at 4° (response reduction, 93%; R.S.D. for 6 testing days, 9.1%).

Milton Mori (2004) reported that water quality measurements and process information are used to develop models to predict biochemical oxygen demand (BOD) at the inlet and outlet of an aerated lagoon of a pulp and paper mill. The results show that linear steady-state and dynamic models are able to predict inlet and outlet BOD even for a complex process that has operational data limitations. Jianbo Jia (2003) reported that a novel type of biochemical oxygen demand (BOD) biosensor was developed for water monitoring, based on co-immobilizing of Trichosporon cutaneum and Bacillus subtilis in the sol–gel derived composite material which is composed of silica and the grafting copolymer of poly (vinyl alcohol) and 4-vinylpyridine (PVA-g-P(4-VP)). Factors that influence the performance of the resulting biosensor were examined.

Tan (1999) studied that the measurement of BOD of an aqueous solution using a thermally-killed Bacillus subtilis BOD biosensor was unaffected by the presence of 5 mM of Al^{3+}, Cd^{2+}, Co^{2+}, Cr^{3+}, Fe^{2+}, Fe^{3+}, Ni^{2+} and Pb^{2+} in the sample. In the presence of 5 mM of Zn^{2+}, Mn^{2+} and Sn^{2+}, the sensor over-estimated the BOD_5 of the test samples by 12–68% compared with 13% under-estimation at the same concentration of Cu^{2+}. The sensor completely and irreversibly lost its BOD sensing ability in the presence of 0.5 mM of Ag^{+} or Hg^{2+}. Similar effect was observed with a living B. subtilis BOD biosensor. The interference of copper, manganese, stannous and zinc ions was effectively eliminated by dosing the substrate with 6.2 mM of ethylene diamine tetra-acetate (EDTA) or 5.8 mM of sodium diethyl dithiocarbamate (DDTC).

Li and Tan (1994) reported that heavy metal ions such as cadmium, mercuric, lead, chromic, stannous, ferrous, ferric and aluminium at a concentration of 4 mM have negligible or no effect on the BOD sensing of a mixed Bacillus subtilis and Bacillus

licheniformis 7B microbial BOD sensor. In the presence of cobalt, zinc and nickel ions, the BOD measured by the sensor was higher than the true value of the test solution. This was attributed to the increase in the microbial activity by the metal ions present. Cupric and in particular silver ions significantly suppressed the microbial assimilation of the substrate resulting in a lower measured BOD value of the substrate. The interference of silver and cupric ions in the BOD measurement were successfully eliminated by chelating these ions with EDTA or by phenotypic modification of the microbial system by pre-conditioning the sensor in a BOD solution containing the interfering ions.

Ademoroti (1996) reported that the trans-isomer of sodium ethylenediamine-tetraacetic acid (EDTA) was used to chelate metal ions known to inhibit accurate determination of BOD of wastewater. The results showed that as much as 5 mg Cu(II) and 6 mg Zn present in a litre of poultry wastewater were effectively chelated by 1 ml of 0·1 EDTA so that the metals had no suppressive effect on the BOD values.

Williamson (1981) reported that that a bioassay using freeze-dried Nitrobacter as the test organism has been shown to successfully detect various toxicants in municipal and industrial wastewaters. The test is simple, sensitive, rapid and inexpensive; as a result, this test shows potential as a quantitative measurement technique for wastewater toxicity.

Mohammad Ajmal (1984) studied the effect of water hardness at different concentrations (viz. 0, 80, 120, 160, 240, 320, 400 and 480 mg/L as $CaCO_3$) on the toxicity of cadmium metal (5 mg/L) as sulphate to saprophytic and nitrifying bacteria, with respect to the rate constant (K) and ultimate biochemical oxygen demand (L) which were calculated from BOD data (15 days) using the Thomas Graphical Method. Glucose was used as a source of carbon for micro-organisms. It was observed that the toxicity of cadmium to micro-organisms (both saprophytic and nitrifying) decreased with increasing hardness and reached a maximum at 320 mg/L as $CaCO_3$ for nitrifying and 400 mg/L as $CaCO_3$ for saprophytic bacteria. After these hardness levels, the ultimate BOD (L) and rate constant (K) showed a decrease. Nitrifying bacteria were found to be more sensitive to the metal as well as to its complexation with calcium or with other ions as they retained their normal activity at a lower hardness level as compared to saprophytic bacteria.

Chen (2004) studied the photocatalytic degradation of organic wastes with nanosized titanium dioxide particles has been studied for a long time in order to offer an appropriate method for wastewater treatment, but its practical application is greatly limited by the slow process. In this work, an electrochemically assisted TiO_2 photocatalytic system was set-up by combining a TiO_2 photocatalytic cell with a three-electrode potentiostatic unit. The composite system revealed high photocatalytic activity towards organic wastes mineralization. After continuous treatment for 0.5 h, the maximum absorption of rhodamine 6G (R-6G) was reduced by more than 90%; chemical oxygen demand (COD) and biochemical oxygen demand (BOD_5) of textile dye wastewater (TDW) were decreased by 93.9 and 88.7%, respectively. The biodegradability of TDW was also improved because the COD/BOD_5 ratio decreased from 2.1 to 1.2. All these results indicated that the composite system could be used for effective organic wastes mineralization or as a feasible detoxification and color removal pretreatment stage for biological post treatment.

Barros (2007) carried out the present study for evaluating the retention behavior of sanitary sewage and sand in relation to chromium and nickel ions in upflow reactors. It was found that the sludge presented a greater assimilation of the metals studied when compared to the inert material, probably due to the presence of anionic groups, which favors adsorption and complexation processes. Thermal analyses of the samples showed a shift in the decomposition peaks of the “in natura” sludge, when compared with those of the samples spiked with the metals, confirming the possibility of interactions between the heavy metals and the anionic groups present in the sludge.

Wang (2006) studied that biosorption, using biomaterials such as bacteria, fungi, yeast and algae, is regarded as a cost-effective biotechnology for the treatment of high volume and low concentration complex wastewaters containing heavy metal(s) in the order of 1 to 100 mg/L. Hussein, (2005) reported a group of Pseudomonads, previously isolated from wastewater, was used to study the accumulation of a specific metal in the presence of a second binary metal or a combination of other metal(s). The growth of Cr(VI)-resistant Pseudomonas fluorescens strain was directly inhibited when the Cr(VI)

concentration reached 3 mmol/L. The presence of binary metal ions decreased the amount of accumulated Cr(VI). Furthermore, a *Pseudomonas putida* strain was shown to tolerate Cu(II) up to a concentration of 3 mmol/L, while higher concentrations (>4 mmol/L) showed a greater inhibitory effect. This pattern of inhibition was varied in the presence of other binary metal ions.

Li and Tan (1994) observed that heavy metal ions such as cadmium, mercuric, lead, chromic, stannous, ferrous, ferric and aluminium have negligible or no effect on the BOD sensing of a mixed Bacillus subtilis and Bacillus licheniformis 7B microbial BOD sensor. In the presence of cobalt, zinc and nickel ions, the BOD measured by the sensor was higher than the true value of the test solution. This was attributed to the increase in the microbial activity by the metal ions present. Cupric and in particular silver ions significantly suppressed the microbial assimilation of the substrate resulting in a lower measured BOD value of the substrate. The interference of silver and cupric ions in the BOD measurement were successfully eliminated by chelating these ions with EDTA or by phenotypic modification of the microbial system by pre-conditioning the sensor in a BOD solution containing the interfering ions.

Cimino and Caristi (1990) studied the effects of the acidity and of metal ions on metabolic activity of activated sludge from waste cheese whey during biological treatment process. In a decreasing order of toxicity two groups were found: $Hg^{2+} > Cd^{2+} > CrO_4^{2-} > Cr^{3+} > Cu^{2+}$ and $Pb^{4+} > Zn^{2+}$. The discrepancy in results of other toxicity studies revealed the impossibility of defining on an absolute scale an order of toxicity for these ions. A very strong synergistic effect was noted in the mixed Cu and Zn studies, thus pointing out the difficulty of setting a water-quality standard based on single toxic chemicals.

Factors affecting the fate of heavy metals in the activated sludge process. Factors include the metal uptake and pH. Adsorption isotherms were used to represent equilibrium distributions of metals between solution and bacterial solid phases. At pH 7.0, the affinity of the bacterial solids for metals was zinc>copper>cadmium. Cadmium and zinc adsorption increased steadily from 15 to 20 % at pH 4 to greater than 90% at pH 10.

Copper appeared to reach maximum adsorption in the 7.0 to 8.0 pH range and for zinc it was 4.5 to 6.5. The pH of a solution in which interactions between metal ions and organic matter take place is an important factor in determining the association of metal ions with the organic functional groups (Chna et al, 1981). The Irving-Williams series indicated that the stability complexes of bivalent metal ions, regardless of the nature of complexed ligand or ligand molecules, involved, followed the sequence of zinc > copper > nickel > cobalt > cadmium > iron > manganese.

Ajmal et al (1982) studied the microbial uptake of cadmium and its effects on the BOD at various temperatures (20°, 30°, 40° and 50°) and at pH 7. Toxic effect of cadmium has been studied with respect to rate constant and ultimate BOD was calculated from BOD data using Thomas Graphical method. They concluded that the highest consumption of Cd was at 30° and 40° and the lowest at 50°.

Babaich and Stotzky (1977) tested a variety of different bacteria including actinomycetes and fungi for their growth in presence of metal ions. Various microorganisms were studied in pure culture with addition of various levels of cadmium.

Ong et al, (2005) investigated the effects of Ni^{2+}, Cr^{3+} and Zn^{2+} on the treatment performance of sequencing batch reactor (SBR) system. The kinetics of adsorption study showed that the pseudo second-order reaction model provided the best description of the data obtained. From the Langmuir isotherm, the maximum adsorption capacities of Ni^{2+}, Cr^{3+} and Zn^{2+} were 30 mg/g, 23 mg/g, and 18 mg/g, respectively. Cr^{3+} and Ni^{2+} were found to exert a more pronounced inhibitory effect on the bioactivity of the microorganisms compare to Zn^{2+}. The increase of Cr^{3+} and Ni^{2+} concentration from 5 to 10 mg/L caused significant effect on the suspended solids (SS) and total organic carbon (TOC) removal efficiency in SBR system but vice versa in the case of Zn^{2+}.

Thompson et al (1984) investigated the strength and nature of the binding of heavy metal ions to bacterial extracellular polymers. They determined the conditional stability constants for complexes formed between extracted *Klesbsiella aerogenes* polymer and copper, cadmium, cobalt, and nickel. Adsorption isotherms for metals indicated that

metal uptake occurred after intial complexation capacity had been exceeded, suggesting the presence of more than one binding site. An affinity series for polymer-metal complexes can be identified as: copper > nickel, cobalt > cadmium. Steiner et al (1976) have suggested that the differential adsorption of cations by polymers may be due to the occurrence of two types of binding site, corresponding to carboxyl and hydroxyl groups associated with soluble and solid state forms of the polymer respectively.

Doyle et al (1975) found that *Escherichia coli* and *Bacillus cereus* were able to grow at 40 and 80µg Cd/ml, but *Lactobacillus acidophilus*, *Staphlococcus aureus*, and *Strptococcus faecalis* were inhibited. Various microorganisms have been studied in pure cultures with addition of various levels of cadmium. Babich and Stotzky (1984) tested a variety of different bacteria, including actinomycetes, and fungi for their sensitivity to cadmium. They have also studied the effect of pH levels in the broth cultures and agar plate cultures used for the bacteria and fungi. It was found that the gram-negative eubacteria such as *Enterobacter aerogenes*, were more tolerant of cadmium than were the gram-positive such as *Bacillus megaterium*.

Nies (1999) described the workings of known metal – resistance systems in micro-organisms and compared the transport of 17 most important heavy metal ions on the basis of the basic principles of homoeostasis. Mitra et al. (1975) studied the influence of other metal ions on the toxicity of cadmium. In case of *E. coli*, the addition of zinc shortened the lag phase in the growth of the bacteria when it was treated with cadmium.

Zanoni (1967) conducted a large number of BOD_5 determinations on a raw municipal sewage using the dilution technique and at incubation temperatures ranging from 2° to 40°C. The data were used to evaluate the first stage velocity constant, k and ultimate demand, L of the molecular deoxygenation expression and these in turn were related to temperature. He stated that the deoxygenation kinetics could be more accurately described by a second order reaction rate equation than by the conventional first order or monomolecular equation.

Leong and Lim (2004) employed a quantitative approach to evaluate the relative inhibitory effect of toxic metals, namely Zn, Pb, Cd and Cu, singly and in combination on biochemical oxygen demand (BOD) exertion. Domestic wastewater sample was spiked with the metals singly and in combination in the concentration range of 1 – 50 mg/L for daily BOD determination over a 10-day period. The BOD values with and without metals were fitted to the first-order kinetic model to obtain the rate constants and ultimate BOD values. A toxicity coefficient defined as the fractional reduction in the ultimate BOD values was employed to determine the relative inhibitory effect of the metals and was found to increase in the order: Zn<Pb<Cd<<Cu. The overall inhibitory effect seemed to be determined by the most toxic metal, namely Cu, and no synergistic effect of metals was observed. An empirical relationship between the toxicity coefficient and metal concentration was proposed. BOD values were observed to be more in presence of indigenous seed than with the seed from sewage. The results of ultimate BOD and rate constants for the system were also more with indigenous seed. The ratio of BOD exerted with indigenous seed to sewage seed is greater than unity. It was 1.29 and 1.94 for paper mill and distillery wastewater, respectively and BOD rate constant ratio is also greater than unity and was 1.22 and 1.41, respectively (Shrivastava , 2000).

Pulgarin et al (2003) established that BOD_5 obtained by adding manufactured inocula to the synthetic medium (effluent containing known and easily biodegradable substances) is close to the values obtained with inocula taken from the treated effluent of an urban and a rural purification plant. The use of synthetic media showed that even for effluents of very simple composition, multiple synergetic and antagonist interactions between different parameters values of BOD_5. Even if several parameters like temperature, light, pH, microorganisms, nutritive substances, etc. are controlled, the measured BOD_5 values of such samples have to be considered with the like copper present greatest caution. A toxicant like copper present in the effluent can influence the BOD_5 value in different ways. The toxic effect of a substance can be neutralized by complexation with the growth medium. pH can act on the complexation and bioavailability of the toxicant for microorganisms. In industrial effluents, the organic matter can mask toxicants and BOD_5

can be inhibited by different factors as toxicity, lack of nutrients, absence of adapted microorganism, etc.

Complexation of copper with glutamic acid as a function of pH has been investigated by Methenitis et al (1987). They showed that three types of complexes are successively formed when pH increases. At pH < 5, copper is bound to the carboxylic groups of two glutamic acid molecules. Around pH 6.5, the major species is a complex containing one copper ion for one glutamic acid molecule. At high pH, one copper ion is bound to the nitrogen atoms of two glutamic acid molecules. Bacterial cell walls displayed a strong affinity for a wide variety of aqueous metal cations (Liu et al, 2001).

Tan and Qian (1999) measured the BOD of an aqueous solution using a thermally-killed *Bacillus subtilis* BOD biosensor and was unaffected by the presence of 5mM of aluminium, cadmium, cobalt, chromium, iron, nickel and lead ions in the sample. Presence of 5mM of zinc, manganese and tin ions, BOD_5 was over-estimated by 12-68% compared with 13% under-estimation at the same concentration of copper ions. For the study they limited the concentration of metal ions to 5mM. Li and Tan (1994) measured BOD in presence of cobalt, zinc and nickel ions and was higher than the true value of the test solution. This was attributed to the increase in the microbial activity by the metal ions present. Cupric and silver ions significantly suppressed the microbial assimilation of the susbstrate resulting in lower measured BOD value of the substrate.

Thompson and Prior (1984) studied the problems for a water authority caused due to toxic metals present in trade effluents. Haug and Smidsrod (1970) stated that a high selectivity of polyanions exists for copper, compared to calcium.

Macaskie and Dean (1982) made a survey of accumulation of cadmium by 174 strains of bacteria and 29 moulds, isolated from polluted sites. Hermann et al (1981) studied the inhibitory effect of substances like mercuric chloride, potassium cyanide, nickel chloride, copper sulphate etc., in combination on oxygen utilization. Substances in combination may exert additive, antagonistic or synergistic toxic effects and an additive inhibitory effect on oxygen utilization was observed.

Ion-selective transport systems participate in both the uptake of essential ions and the efflux of toxic metals (Silver et. al., 1993). Many resistances to toxic metals are encoded on plasmids (Ji and Silver, 1995), including resistances to Ag^{+}, AsO_4^{3-}, Cd^{2+}, CrO_4^{2-}, Cu^{2+}, Hg^{2+}, Ni^{2+}, Sb^{3+}, and Zn^{2+}.

Cobalt is found mainly in the cobalt(II) form, cobalt(III) is only stable in complex compounds. Kobayashi et al 1998 described a new class of cobalt-containing enzymes, nitrile hydratases. Co^{2+} is rapidly accumulated by the corA system inmost bacterial cells (Smith and Martell, 1993). Cobalt resistance seems always to be the by-product of resistance to another heavy metal, either nickel (Schmidt and Schlegel 1994) or zinc (Nies 1987). Nickel is an essential nutrient for selected microorganism where it participates in a variety of cellular processes. Many microbes are capable of sensing cellular ion concentration and taking up this nutrient. The temperature at which the microorganism are living and growing, markedly influences the process of oxygen utilization in the degradation of the organic constituents present in wastewater or deoxygenation. The rate of oxygen, as does the rate of all metabolic activities, increase with increase in temperature.

Copper is used by cells in small quantities in cellular enzymes (e.g. cytichrome oxidase). However, copper is so widely used in industry and agriculture, high levels of copper may exist in some environments. Bacteria have evolved several types of mechanisms to resist toxicity due to high copper concentrations. In a study conducted by Lin and Olson (1995), bacteria isolated from a water distribution system experiencing copper corrosion, 62% found to be copper resistant. In E.coli, resistance to copper is based on an efflux mechanism by which coppe was removed from the cell.

Zinc is another essential trace element. It is not biologically redox reactive and thus not used in respiration. However, it is important in forming complexes. Bacterial cells accumulate zinc by a fast unspecific uptake mechanism and it is normally found in higher concentrations but it is less toxic than other heavy metals (Nies, 1999). Silver is isoelectronic to copper; however, while the standard electrochemical potential of the copper (II) / copper (I) pair is –268mV, the potential of the silver (II)/ silver (I) pair is

1.56 V at pH 7. Thus, main ionic forms of the two elements are copper (II) and silver (I). Because of its toxicity, silver is no trace element, but it has been used as an antimicrobial agent in medicine (Slawson et al, 1992). Cadmium uptake is barely understood on the molecular level. The solubility product of CdS is 1.4×10^{-29} but 2.91×10^{-25} for ZnS (Weast, 1984). Thus, cadmium is more toxic (Ragan and Mast, 1990) than zinc. Resistance to cadmium in bacteria is based on cadmium efflux.

Steiner et al (1976) have suggested that the differential adsorption of cations by polymers may be due to the occurrence of two types of binding site, corresponding to carboxyl and hydroxyl groups associated with soluble and solid state forms of the polymer respectively. By contrast, although Ni^{+2} was the lesser affected at 20 mg ml^{-1} Cd^{2+}, the extended cadmium concentration-dependent lags observed at higher concentrations of the metal reduced its potential as a useful tool for cadmium removal by growing micro-organisms. This type of growth response to cadmium is similar to that observed by *Escherichia coli* (Mitra et al, 1975), *Klebsiella aerogenes* (Pickett and Dean, 1976) and *Bacillus subtilis* absp. *Niger* (Pickett and Dean, 1979), in that a period of accommodation was required prior to the onset of growth. The mechanism of accommodation is, in the case of Escherichia coli, associated with the production of cadmium-binding protein (Khazeali and Mitra, 1981) which is presumed to bind with, and thus compartmentalize, the metal intracellularly.

An alternative method of microbial resistance to heavy metals is by conclusion of the metals from the cells, corresponding to an observed low uptake of the metal (Chopra, 1971; Tynecka et al, 1975; Konda et al, 1974; Foster, 1977 and Baldry and Dean, 1980).

A third mechanism of metal resistance may be by the production of an exopolymer forming a protective layer around the cell. This has been observed with Klebsiella aerogens (Bitton and Friehofer, 1978). Bacterial exopolymers are often negatively charged, and are believed to function also in the uptake of metals onto microbial cells (Brown and Lester, 1979).

Liu and Mattiasson (2002) reviewed the field of biosensors for measuring biochemical oxygen demand (BOD) BOD sensors constructed on the biofilm configuration are discussed regarding performance characteristics like linearity, response time, precision, agreement between BOD values obtained from the biosensors and the conventional 5-days test, as well as toxic resistance to various compounds and operational stability. The techniques for improving the agreement between the sensor BOD and BOD_5 are described. Information provided also included BOD biosensors based on respirometery and other measuring principles, the commercial BOD instruments, as well as the current limitations of BOD biosensor development.

Chee, Nomura and Karube (1999) described a highly sensitive sensor for biochemical oxygen demand (BOD) using Pseudomonas putida. Instead of glucose and glutamic acid, artificial wastewater (AWW) was employed for calibration of the BOD sensor. Typical response times of the BOD sensor were 2–15 min, and the service life of the immobilized microorganisms is >10 days. The optimal BOD response was observed at 30°C and pH 7.0. The lower limit of detection was 0.5 mg L^{-1} BOD. The sensor response was not influenced by chloride ion up to 1000 mg L^{-1}, and also was not affected by heavy metal ions. For natural river waters, BOD values estimated by this biosensor correlate well with those determined by the conventional 5-day BOD test.

Praet, Reuter, Gaillard and Vasel (1995) discussed the utilization of biomembranes and immobilized microorganisms for biochemical oxygen demand (BOD) estimation. Brenner and Horner(1992) reported the potential for CMA to deplete dissolved oxygen (DO) in water bodies. In laboratory biochemical oxygen demand (BOD) experiments, 100 mg CMA/1, at the high end of the concentration routinely expected in highway runoff, completely depleted oxygen within 2 days at 20°C. A concentration of 10 mg/1 caused a net depletion of about 4.5 and 7.0 mg O_2/L for reagent-grade and corn-based CMA, respectively. This extra oxygen demand was created by the corn-based CMA even though the two products had equal acetate concentrations. Most of the extra demand can probably be attributed to the presence of butyrate as a contaminant in the corn-based CMA. The rate of DO depletion was strongly dependent on temperature. While the rate

appeared to follow an Arrhenius relationship with temperature, the classic, first-order BOD equation did not represent initial DO depletion well at low temperatures. An alternative model was proposed for this region, where depletion better followed a logistic-type curve, probably in direct proportion to the growth of bacteria. Oxygen depletion after CMA additions was also observed in microcosm ecosystems and field ponds, although reaeration reduced net depletion compared to the BOD experiments. When ice covered one of the ponds, a large DO drop occurred following a relatively small CMA inflow. It is recommended that CMA applications be avoided in situations where receiving waters are close to the road, have a low dilution potential, or support populations of fish sensitive to low oxygen levels. Applications during late spring storms and where there is potential for CMA runoff to get into ice-covered water bodies are also discouraged.

Riedel, Lange, Stein, Kühn, Ott and Scheller (1990) a microbial amperometric sensor for the determination of the biochemical oxygen demand (BOD) using Trichosporon cutaneum cells immobilized in polyvinylalcohol. This sensor allowed BOD measurement with very short response times (< 30 s), an operation stability of 48 days and a serial coefficient of variation of ±3.3% when a sample solution containing 22 mg L^{-1} BOD was employed. A linear range was obtained up to 100 mg L^{-1} BOD using a glucose/glutamic acid standard. The sensitivity and specificity was increased by incubation of the BOD sensor with the respective wastewater. Comparable results were obtained for BOD values estimated by the biosensor and those determined by the 5-day method. Samples of poultry wastewater were analyzed to examine the effect of some metallic toxicants on their BOD values (Admeroti, 1988). Results showed that 1 mg Cu (II) or Zn ions per litre of the wastewater caused as much as 33% and 16·9% BOD suppression, respectively. This was due to the toxic effect of the metals on the bacteria responsible for the biochemical oxidation of the organic wastes in the wastewater. The metals did not show any appreciable effect on the COD measurements of the wastewater.

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Chapter – 3

BOD EXERTION IN PRESENCE OF MICROBIAL SEED FROM DIFFERENT WASTEWATER STREAMS

Measurement of BOD of an aqueous medium is affected by the presence of some heavy metal ions. There is generally a threshold concentration upto which a microorganism can tolerate the metal ion presence, beyond which it becomes toxic to the microbial system. Although the mechanism by which these heavy metal ions affects the microbial growth is not well understood, yet there are some references (Huges and Poole 1989; Fitzmaurice and Gray, 1989; Mittal and Ratra, 2000; Pulgrian et al, 2003) available in literature which have put forward some probable mechanisms to explain the concept of BOD inhibition due to the presence of heavy metal ions.

Microbes use metals for metabolic processes, however, in high concentration, metallic ions are typically toxic to microbes. The interacting microbes must be able to balance this toxicity and utility when interacting with a metal surface. As such, there are three main types of interaction:

Extracellular: The microbes changes the localized environment in some sort of biochemical processes.

Cell-surface: The metal binds to the cellular surface as either an ion or as elemental metal.

Intracellular: Accumulation of metal within the cell, either to detoxify the environment or for metabolic purposes.

The second two interaction types are significant for many practical applications. However, they rarely play an important role in corrosion. Therefore, we will only consider extracellular interactions. In extracellular processes we see many different corrosion related processes. For example, microbes will produce salts that complex with metal ions in solution and cause them to precipitate as insoluble salts.

A specific example of a microbe that interacts extracellularly is the siderophore (iron loving) class of bacteria. Siderophores can also strongly uptake Aluminium, gallium, chromium, molybdedenum and copper producing extermly stable compounds (Hider, 1984).

BOD exertion is affected by factors like nature of heavy metal ion, type of seed used as a source of microbes, temperature, pH, etc. (Zanoni 1967; Reidal and Lange 1990; Li and Chu 1991; Mittal et al, 2004). It is also reported that most of the microorganisms develop resistance towards some heavy metal ions during culture and growth (Reidal and Lange 1990; Li and Chu 1991; Li and Tan 1994a, b). It may be due to the biochemical reactions of cell enzymes with metal ion (Huges and Poole 1989). BOD inhibition by metal ion has been found to be quite reproducible by a number of independent researchers (Hermann 1959; Subcommittee 1956; Mowat, 1976). It is reported that indigenous seed exert more BOD than in case of seed drawn from industrial wastewater. (Shrivastva et al, 2000). Heavy metal ions such as cadmium, mercury, lead, chromium, tin, iron and aluminum at a concentration of 4mM have negligible or no effect on the BOD exertion by mixed Bacillus subtilis and Bacillus licheniformis 7B, whereas in the presence of cobalt, zinc and nickel ions the BOD measured by a BOD sensor was higher than the true value of the test solution (Li and Tan, 1994). The BOD in this case is influenced by the synergic and antagonist interactions between numerous and variable parameters like pH, nature and concentration of nutrients, amount and nature of assimiable substances, presence of toxicants etc. (Pulgarin, 2003). In the present study, the effect of seed quality on BOD in presence of heavy metal ions is studied.

EXPERIMENTAL SET-UP

The standard 3-day BOD (27°C) determination was chosen as test procedure for its known relationship to stabilization of organic wastewater. Dilution technique was used to prepare samples for the BOD measurement according to standard method (APHA, 1998).

The substrate was synthesized from standard BOD dilution water saturated with oxygen at 27°C and supplemented with 300mg/L of glucose and 300mg/L of glutamic acid. The substrate was then seeded with 1.5mL wastewater from industries, namely, pulp & paper, distillery, dairy and the sewage. The wastewaters were collected from aerated tanks and conditioned for aeration at 37°C for 72 hours before using as a seed. Metal ion solutions were prepared in demineralised distilled water, using metal salts of AR grade.

Kinetics of BOD Exertion Process

Biochemical oxidation is a slow process and theoretically takes an infinite time to go to completion. Within a 20-day period, the oxidation is about 95 to 99 per cent complete, and in the 5-day period used for the BOD test, oxidation is from 60 to 70 per cent complete. The 20° temperature used is an average value for slow moving streams in temperate climates and is easily duplicated in an incubator. Different results would be obtained at different temperatures because biochemical reaction rates are temperature-dependent.

The kinetics of the BOD reaction are, for practical purposes, formulated in accordance with first-order reaction kinetics and may be expressed as

$$dL_t/dt = -K'L_t$$

where L_t is the amount of the first-stage BOD remaining in the water at time t. This equation can be integrated and become

$$L_t/L = e^{-K't}$$

Where L is the ultimate first-stage BOD when t = 0. and K' is constant.

y, the amount of BOD that has been exerted at any time t, equals

$$y = L - L_t = L\,(1 - e^{-K,t})$$

therefore, 5-day BOD equals

$$y_5 = L - L_5 = L\,(1 - e^{-5K,})$$

For polluted water and wastewater, typical value of K (base 10, 20°) is 0.10 d^{-1}. The value of K varies significantly, however, with the type of waste. The range may be from 0.05 to 0.3 d^{-1} or more. For the same ultimate BOD, the oxygen uptake will vary with time and with different K values. The effect of different K values is shown in Figure 1.

The temperature at which the BOD of a wastewater sample is determined is usually 20°. It is possible, however, to determine the reaction constant K at a temperature other than 20°. The following approximate equation, which is derived from the van't Hoff-Arrhenius relationship, may be used:

$$K_T = K_{20}\,\theta^{(T-20°)}$$

The value of θ has been found to vary from 1.056 in the temperature range between 20 and 30° to 1.135 in the temperature range between 4 and 20° (Schroeder, 1977). A value of θ often quoted in the literature is 1.047 (Phelps, 1944), but it has been observed that this value does not apply at cold temperatures (Schroeder, 1977).

Noncarbonaceous matter, such as ammonia, is produced during the hydrolysis of proteins. Some of the autotrophic bacteria are capable of using oxygen to oxidize the ammonia to nitrites and nitrates. The nitrogenous oxygen demand caused by the autotrophic bacteria is called the second stage BOD. At 20°, however, the reproductive rate of the nitrifying bacteria is very slow. It normally takes from 6 to 10 days for them to reach significant numbers and to exert a measurable oxygen demand.

The value of K is needed if the BOD_5 is to be used to obtain L, the ultimate or 20-day BOD. There are several ways of determining K and L from a series of BOD measurements, including the least-squares method (Waugh, 1943; Young 1962), the method of moments (Moore et al, 1950), the daily-difference method (Tsivoglou, 1958)

the rapid-ratio method (Sheey, 1960) and Thomas method (Thomas, 1950). The least squares method is illustrated here and followed for calculations.

The least-squares method involves fitting a curve through a set of data points, so that the sum of the squares of the residuals (the difference between the observed value and the value of the fitted curve) must be minimum. Using this method, a variety of different types of curves can be fitted through a set of data points (Waugh, 1943).

The following equations which are derived from least square method have been used for the calculation of K and L values.

$$na + b\Sigma y - \Sigma y' = 0$$

$$a\Sigma y + b\Sigma y^2 - \Sigma yy' = 0$$

RESULTS AND DISCUSSION

BOD Measurements

Inhibition/ increase in BOD in presence of heavy metal ions is an accepted phenomenon (Mittal and Ratra, 2000; Li and Tan, 1994a, b; Collins and Stotzky, 1989). Experiments were conducted to measure BOD_3 at 27°C in presence of 0.2mM of Co^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+} ions. In general, BOD_3 is found to be inhibited in the presence of metal ions. Maximum inhibition is observed from sewage seed in presence of all the identified metal ions while it shows minimum inhibition, rather an increase in BOD when seed from pulp & paper industry is used (Figures 3.1 to 3.4). This is due to the reason that in sewage waste a mixed flora microbes are present while in pulp & paper waste the bacterial community is dominated by clusters like *Bacillus, Clostridium, Mycoplasma, Eubacterium, Proteobacteria, Nitrosospira* and *Nitrosomanas* are present (Ibekwe et al, 2003). The behavior of these microbes is similar to some extent towards all the transition metal ions (Table 3.1).

Table 3.1 Percentage change in BOD for a GGA system in presence of some metal ions (0.2mML^{-1}) using different types of seeds.

Metal ions	Change in BOD (%)			
	Sewage	Distillery	Pulp & Paper	Dairy
Co^{2+}	(-) 22.0	(-) 18.9	(+) 12.5	(-) 16.4
Ni^{2+}	(-) 29.3	(-) 28.3	(-) 2.1	(-) 24.6
Cu^{2+}	(-) 21.1	(-) 18.9	(+) 17.7	(-) 11.5
Zn^{2+}	(-) 30.8	(-) 16.0	(+) 11.5	(-) 3.3

For the sewage seed, the heavy metal ions influence BOD almost to the same extent and an inhibition in the range 21-31% is observed. Presence of Ni^{2+} is found to be most toxic to the microbes of the distillery waste for which the inhibition is 28% while it is 16 % in presence of zinc. Nickel is also the most toxic for bacteria present in dairy wastewater leading to 25% decrease in BOD, whereas zinc ions inhibit the BOD only to 4%.

The presence of zinc has a different effect on BOD in comparison to other transition metal ions like cobalt, nickel and copper. This is true for all the seeds derived from distillery, pulp & paper and dairy wastes. This may be due to the reason that zinc is the last element of the first transition series in the periodic table having electronic configuration of the outermost shell as $3d^{10}$ and there is no vacant d-orbital available for forming complex with protein molecules of the microbial cell. Cobalt and nickel, due to the available vacant d-orbitals have a tendency to form complex with microbial cells (Cotton and Wilkinson, 1976). Metal ions are known to form complexes with proteins (Mitra et al, 1975) of the cell wall of microorganism like E-coli, Bacillus subtilis, etc.

The most interesting feature of the study is an increase in BOD to about 18% in presence of cobalt, copper and zinc when seed from pulp & paper industry is used.

From a small piece of work reported here, it can be safely concluded that a presence of heavy metal ions does affect BOD exertion process. The extent of change in BOD is different when different seeds are taken from different sources as well as when same amount of different metal ion are present during BOD exertion process.

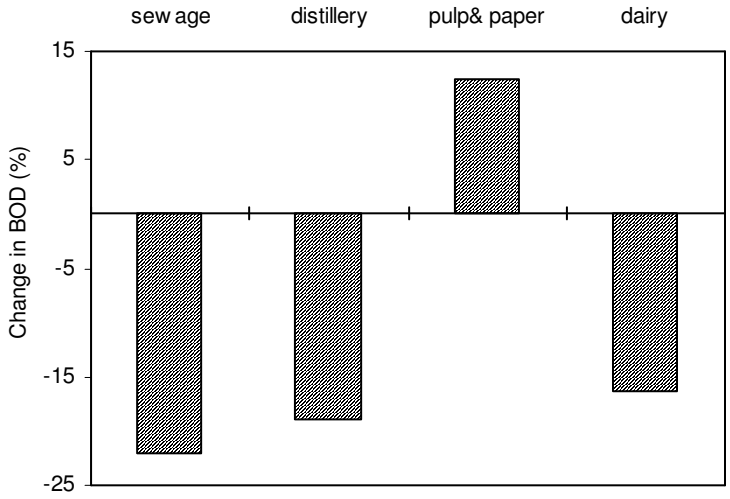

Figure 3.1 Bars of change in BOD in presence of cobalt ions using different types of seed

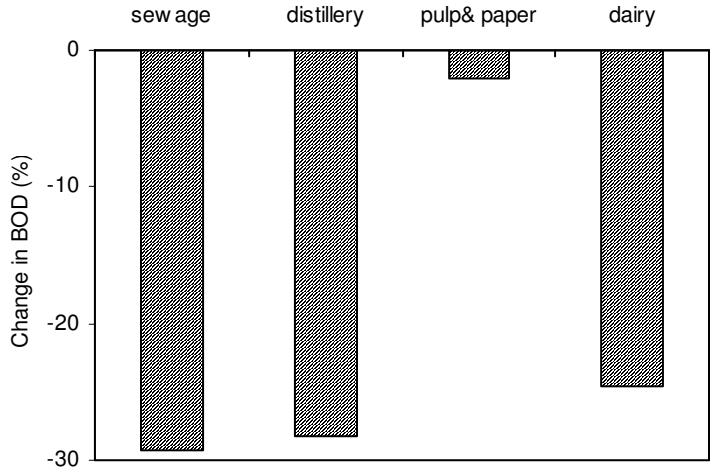

Figure 3.2 Bars of change in BOD in presence of nickel ions using different types of seed

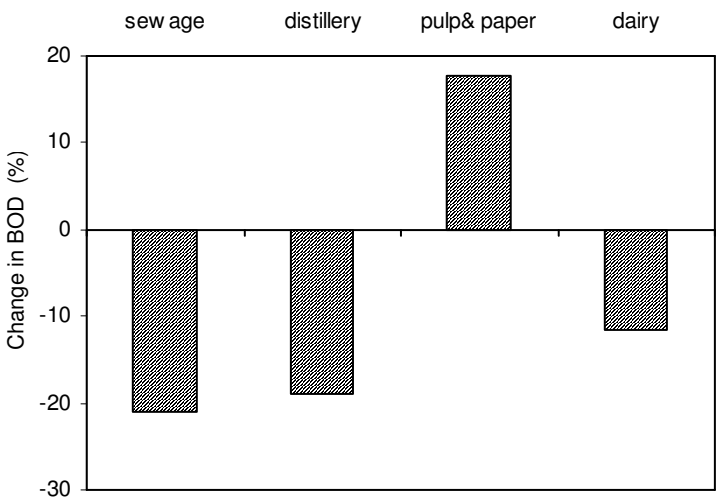

Figure 3.3 Bars of change in BOD in presence of copper ions using different types of seed

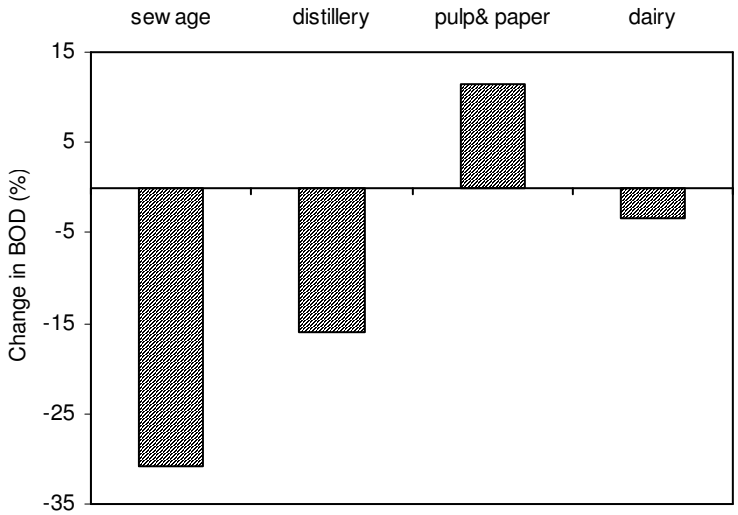

Figure 3.4 Bars of change in BOD in presence of zinc ions using different types of seed

BOD Kinetics

Experiments were conducted to study kinetics of BOD exertion process in the presence of a constant concentration ($0.2mML^{-1}$) of metal ions known to exert inhibition in BOD through metal microbe interaction. Different experiments were designed to use wastewater from dairy industry, pulp & paper industry, distillery industry and sewage as "seed".

BOD exertion was also measured in the absence of metal ions to find out the change in BOD due to the presence of metal ions. Readings of DO were taken using DO meter (model 805A+ using DO probe 080510, ORION) at different time interval 0.0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 days. Results indicate that with change in source of microbes, the magnitude of BOD inhibition changes in the presence of a given metal ion. Thus, the nature of microbes does play an important part in deciding the extent of BOD exertion.

Table 3.2 BOD values for GGA system at different incubation periods using seed from dairy waste at 27° in the absence/presence of Ni^{2+} ions (0.2 mML^{-1})

Time (days)	BOD (mg/L)		BOD inhibition (mg/L)	Change in BOD (%)
	Without metal ion	With metal ion		
0.0	0	0	0	-0.0
0.5	10	0	10	-100.0
1.0	70	25	45	-64.3
1.5	105	45	60	-57.1
2.0	140	65	75	-53.6
2.5	175	75	100	-57.1
3.0	180	85	95	-53.0

With dairy waste, as well as, distillery waste as a seed, the maximum change in BOD is obtained within 0.5 day and the magnitude decreases drastically up to day1 and stabilizes to an inhibition of 55±2%, 60±2 %, thereafter, respectively (Tables 3.2 and 3.3). With seed from pulp & paper wastewater, a similar trend is noticed only with a difference in magnitude in inhibition (79% on day ½) Table 3.4. With mixed flora sewage seed the decrease in BOD is to the extent of 65% on day ½ and decreases by day 2.0 to 18.0% and later on stabilizes to 25% (Table 3.5).

Initial fast inhibition in BOD during first half day of incubation in almost all cases is due to the fast growth of microbes, which is quite obvious from the growth curve of microbes responsible for the BOD exertion process (McCarty & Sawyer).

Table 3.3 BOD_3 values for GGA system at different incubation period using seed from distillery waste at 27° in the absence/presence of Ni^{2+} ions (0.2 mML^{-1})

Time (days)	BOD of GGA system (mg/L)		BOD inhibition (mg/L)	Change in BOD (%)
	Without metal ion	With metal ion		
0.0	-	-	-	-
0.5	98	0	98	-100.0
1.0	200	75	125	-62.5
1.5	335	120	215	-64.5
2.0	405	150	255	-63.0
2.5	440	175	265	-60.2
3.0	450	190	260	-58.0

Table 3.4 BOD values for GGA system at different incubation period using seed from pulp & paper waste at 27° in the absence/presence of Ni^{2+} ions (0.2 mML^{-1})

Time (days)	BOD of GGA system (mg/L)		BOD inhibition (mg/L)	Change in BOD (%)
	Without metal ion	With metal ion		
0.0	0.0	0.0	0.0	-0.0
0.5	95	20	75	-79.0
1.0	150	85	65	-43.3
1.5	200	160	40	-20.0
2.0	240	185	55	-23.0
2.5	285	210	75	-26.0
3.0	315	220	95	-30.0

Table 3.5 BOD values for GGA system at different incubation periods using seed from sewage waste at 27° in the absence/presence of Ni^{2+} ions (0.2 mML^{-1})

Time (days)	BOD of GGA system (mg/L)		BOD inhibition (mg/L)	Change in BOD (%)
	Without metal ion	With metal ion		
0.0	-	-	-	-
0.5	85	30	55	-64.7
1.0	180	90	90	-50.0
1.5	230	155	75	-32.6
2.0	275	225	50	-18.2
2.5	330	240	90	-27.3
3.0	345	260	85	-24.6

BOD curves were drawn as a function of time in days and are shown in Figures 3.5 to 3.8. In all curves the presence of a constant amount of nickel ions ($0.2mML^{-1}$) lowers down the BOD values. The seed from distillery and pulp & paper show similar shapes of curves (Figures 3.5 to 3.6) while seed drawn from dairy and sewage wastewaters (Figures 3.7 to 3.8) show identical effect on BOD exertion. It can be seen from the curves that nickel is more toxic for microbes present in sewage or dairy wastewaters as compared to those present in distillery and pulp & paper wastes.

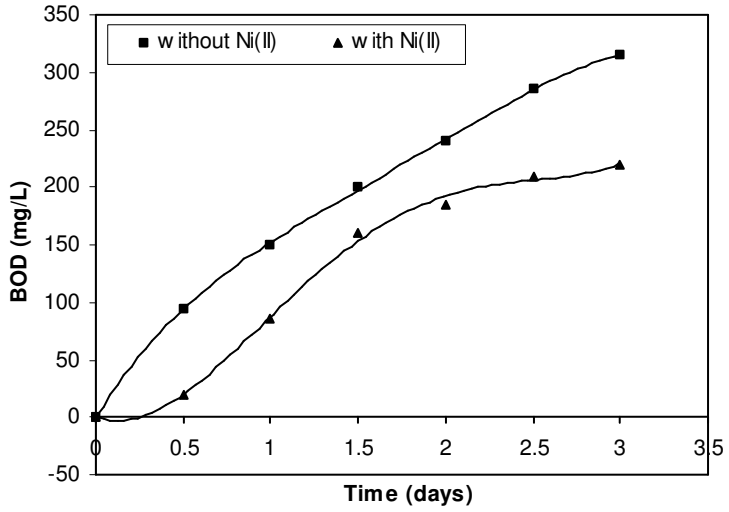

Figure 3.5 BOD curve in presence/ absence of Ni(II) ion (0.2mM/L) using distillery waste as seed

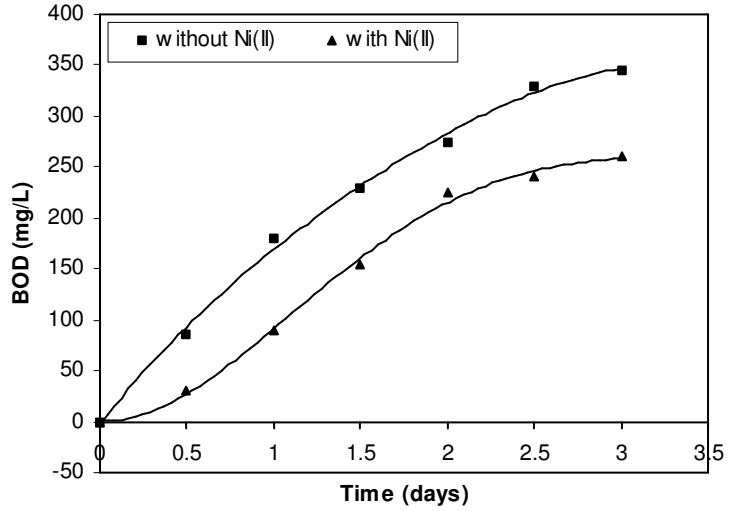

Figure 3.6 BOD curve in absence/ presence of Ni(II) ions using (0.2mM/L) pulp & paper as seed

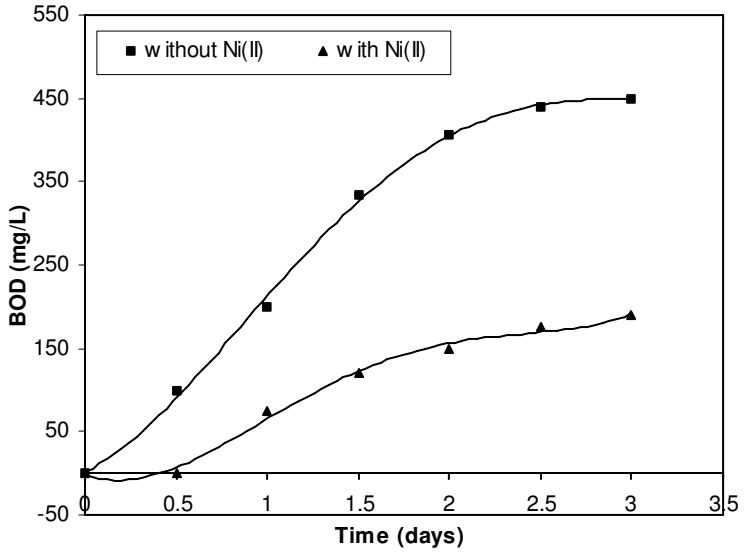

Figure 3.7 BOD curve in absence /presence of Ni(II) ions using (0.2mM/L) dairy waste as seed

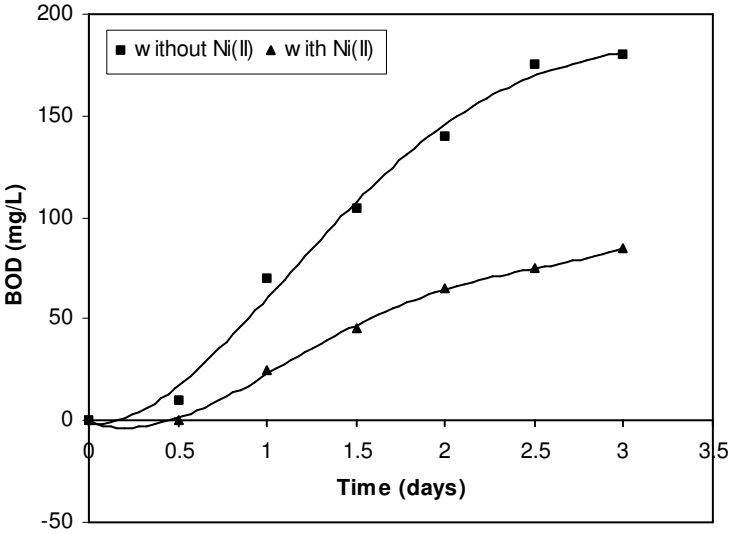

Figure 3.8 BOD curve in presence / absence of Ni(II) ions (0.2mM/L) using sewage waste as seed

Rate constants were calculated by using BOD ultimate values for each setup of experiments (Table 3.6). Rate constants for the system with metal ions are quite less than their respective values without metal ions, which establish that metals do inhibit the process of BOD exertion and makes it slower. The magnitude of decrease in rate constant

is more or less the same for sewage and distillery wastes. There is a very small change in rate constant values for pulp & paper and dairy wastes when used as "seed". It is very difficult to explain this small change.

Table 3.6. Value of Rate constant, (k) and Ultimate BOD (L) using different type of seed in presence of 0.2mM of Ni (II) ions

S. No.	Type of seed used	Rate constant, k	Ultimate BOD, L (mg/L)
1.	**Sewage**		
	Without metal ions	0.46	479.30
	With metal ions	0.23	564.4
2.	**Distillery**		
	Without metal ions	0.44	657.7
	With metal ions	0.25	400.0
3.	**Pulp & paper**		
	Without metal ions	0.37	467.3
	With metal ions	0.32	385.0
4.	**Dairy**		
	Without metal ions	0.18	474.0
	With metal ions	0.09	383.3

Rate constant values as shown above are valid only for linear part of the growth curve, which is the initial portion of the curve also.

* * *

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Chapter – 4

BOD EXERTION IN PRESENCE OF HEAVY METAL IONS USING MICROBES FROM DAIRY WASTEWATER AND DISTILLERY WASTEWATER AS SEED

In the normal BOD measurement process, microbial matter is taken from some source which is known to be rich in bacteria and capable of undergoing BOD exertion process. It was thought that the quality of seed is not very important as regards the magnitude of BOD. Recently, it has been established that the type of inoculum becomes more important (Pulgarin et al, 2003; Shrivastava et al, 2000) for a study like this where BOD exertion is measured at different concentrations of the heavy metal ions.

Ignorance of the effect of heavy metal ions could result in significant error in BOD measurement (Karube et al. 1977; Reidal and Lange, 1990; Li and chu, 1991; Li and Tan, 1994a,b). BOD biosensors generally use living microorganisms as the biosensing component and are therefore sensitive to the presence of heavy metal ions. Many environmental factors influence or repress the operation of BOD. The important factors are temperature (Seth, 1964), pH (Pilegaurd, 1979), Chloride (Baity, 1938), nitrates (Fieldman, 1956), Pharmaceutical wastes (Ajmal et al, 1980) and organic pollutants in the biological systems.

Heavy metals are transported across the cell membrane of the microbe and tend to impair the biochemical process responsible for the exertion of BOD. These metals are generally transition elements and have unfilled d-orbitals which help the metal ion to complex with chelating molecules like enzymes proteins and amino acids. These complexes are not redox active; hence, heavy metal ions whenever present in biochemical system play an important role (Nies, 1999). At higher concentration, these metal ions become toxic

towards physiological functions. Transition metals like copper and zinc which are essential for the growth at trace concentration become toxic at higher concentration till the living cell develops heavy metal resistance. Bacteria have evolved several types of mechanisms like efflux of metal ions, be, accumulation outside the cell.

In the dairy industry, large amounts of lactic acid bacteria are involved in the daily manufacturing of fermented milk products such as cheese, butter, etc. Strains belonging to the species *Lactococcus lactis* are the most important organisms in the manufacture of these products at moderate temperatures (Nicolette et al, 1995). Large-scale industrial processes rely on the use of starter cultures that have been selected for their performance during milk fermentation and product formation (Marshall, 1991). As a result, the variability among strains used in industrial dairy fermentations is low (Salama et al, 1993). Common problem-causing bacteria in the dairy industry are: *Streptococcus agalacite* and other *streptococci, coliformic* bacteria, *Pseudomonas spp*. and *corynebacterium pyogenes*. *Staphylococcus aures*, *Bacillus cerus*, *Listeria monocytogenes*, etc. are all bacterial pathogens of concern in raw milk and other dairy products and are likely to be present in the effluent generating from the industry. These microorganisms understandably receive much attention from scientific community (Bore and Langsrud, 2004).

Five heavy metals were found in trace amounts (1.0–40 μg/ml) in distillery waste. These are lead, copper, zinc, iron and manganese. Metal ions like lead, copper and zinc are toxic to the methanogenic as well as acetogenic bacteria, commonly found in distillery waste and reduce the production of methane and acetic acid, respectively. Lead, copper, and zinc in decreasing order were found to be toxic to biomethanogenesis. Lead at the concentration of 10 μg/ml completely stopped methane production. Iron did not produce any notable change in the process while manganese stimulated the rate of methane production (Ratna et al, 1990). The sugarcane molasses-based anaerobically digested distillery effluent is dark brown due to high concentration of melanoidin (amino carbonyl polymer), phenolics, heavy metals, and sulfate which do not alter even after long extended aeration (Sonal et al, 2006). Brewing, wine making and production of other

alcohlic, beverages constitute some of the oldest and largest microbiological industries. Industrial microbiological processes have been developed using specific strains, fungi (yeasts and molds), bacteria, protozoa and viruses microbial species which have potential for industrial application are continually being sought. Iindustrial application might involve the use of a microorganism in a process such as cleaning up oil spills, the microorganism degrades the oil to non-objectionable compounds. Species of *Micrococcus*, *Arthrobactor* and *Brevibacterium* are used for its industrial production. Some autotrophic, aerobic bacteria like *Thiobacillus thiooxidans*, *Thiobacillus ferroxidans* etc, when grow in the presence of copper ores produce acid and effect oxidation of the ore with subsequent precipitation of the metal (Pelczar).

MATERIALS AND METHODS

Methodology and Test Set-up

At 27°, BOD is found out after 3 days and is called BOD_3 and after 5 days at 20° it is called BOD_5. This gives the amount of oxygen used up for biochemical degradation of organic material (Carbonaceous demand) when the stoppered sample is allowed to stay in the dark at room temperature for 3/5 days. Light must be kept out of the bottle to keep algae from adding oxygen by photosynthesis and the stopper is used to keep air from replenishing DO that has been removed by biodegradation and the oxygen used to oxidize inorganic matter such as sulphide and ferrous ions. Only 3-day BOD (BOD_3) is used for carrying out the studies.

Seed: Mixed flora from dairy and distillery industries were derived and incubated at 37° separately for 24 hours and developed in the Luriya medium and were used as “seed”. Therefore, experiments were performed using dairy wastewater and distillery wastewater. During digestion process, more and more sample water was added to the concentrated waste being digested so as to grow the bacteria which could become acclimatize thoroughly in the wastewater environment. The microbiology of the digested sample was

done to locate different colonies of bacteria present in the sample. A sample is taken and grown separately in Luriya medium and then used as a seed.

Culture medium: Luriya broth was used for the growth of dairy and distillery organisms. Microbial growth was done using shaker set at 37°, 120 rpm. Spectrophotometer (U2800, Hitachi) was used for the measurement of optical density of microbial matter after 24 hours at 600nm.

Sampling and Storage: Synthetic samples were prepared by using glucose and glutamic acid mixture of fixed concentration ratio (1: 1) in distilled water and were used as a substrate. Samples from waste streams were not used as the study is of relative nature. Samples were stored at 4° and were equilibrated to 27° before use.

Apparatus

Incubation Bottles: 300 mL capacity amber glass bottles were cleaned with chromic acid, washed thoroughly with tap water followed by rinsing with distilled water and dried before use. As a precaution against drawing air from the atmosphere in the dilution bottle during incubation, a water seal was used. A foil cap was placed over the flared mouth of bottle to reduce evaporation of water seal during incubation.

BOD Incubation: BOD incubator (Indian Equipment Co., Mumbai) thermostatically controlled at 27±0.3° was used for the tests.

Reagents: Solutions were prepared according to the procedure of standard methods (APHA, 2000). Chemicals of AR grade were used.

Procedure

Preparation of Dilution Water: To a desired volume of seeded dilution water, added 1 mL/L each of phosphate buffer, $MgSO_4$, $CaCl_2$ and $FeCl_3$ solutions. Dilution water was

brought to 27° before use, saturated it with dissolved oxygen (DO) by aerators for 4-5 hours.

Substrate: Glucose-Glutamic acid (GGA), a mixture of 300mg/L glucose and 300mg/L glutamic acid, was used as a substrate. Glucose has an exceptionally high and variable oxidation rate but when it is used with glutamic acid, the oxidation rate is stabilized. Concentration of commercial mixtures was adjusted to 5mL/L in each GGA test bottle. The standard BOD value for a GGA system is 396 ± 30 mg/L (APHA, 2000).

Seed Control: BOD of seeding material was measured and dilution was made so that its DO uptake comes between 0.6 to 1.25 mgL^{-1}.

Preparation of Metal Ion Solutions: Metals selected for the study were cobalt, nickel, copper, zinc, silver and cadmium. Appropriate amounts of nitrate salts of these metals were dissolved in distilled water and diluted to 100mL to prepare 2M stock solution of each metal ion.

Estimation of BOD: A known quantity of stock solution of metal ion was added in each set of bottles so as to get a concentration range from 0.2mM to 14.0 mM and separate tests were performed to find BOD.

5 mL of synthetic wastewater sample and a known quantity of metal ion solution were added in distilled water and diluted to one litre. Since, a BOD bottle has a capacity of 300mL and three bottles are arranged in each set, all bottles are filled carefully with seeded dilution water to the top of brim, allowing overflowing for the removal of air bubble. Initial DO of first bottle of each set was taken and remaining two were capped, water sealed and incubated for three days at 27° in a BOD incubator. After three days of incubation, final DO of the samples were determined.

Estimation of Dissolved Oxygen & Calculations: Initial and final DO were determined by using Dissolved Oxygen Meter (ORION, Model 805 with DO Probe 080510). BOD_3

values were calculated using the following equation and results are used to find change in BOD (increase/ inhibition) given in Table 4.3.

$$BOD_3(mgL^{-1}) = \left[(D_1 - D_2) - (B_1 - B_2)\left(1 - \frac{1}{D_f}\right) \right] D_f$$

Where,
D_1 = DO of diluted sample immediately after preparation, mgL^{-1}
D_2 = DO of diluted sample after 3 day incubation at 27°C, mgL^{-1}
B_1 = DO of seed control before incubation, mgL^{-1}
B_2 = DO of seed control after incubation, mgL^{-1}.

Here, D_f=100, (1% of substrate).

Seed corrections were applied to all the observations.

Estimation of Optical density (OD) measurements: The microbes from different wastewater i.e., dairy and distillery were grown in luriya broth in the absence/ presence of different concentrations of each one of the cobalt, nickel, copper, zinc, silver and cadmium metal ions. The aqueous medium was maintained at 37° on shaker incubator set at 120 rpm. OD of the developed microbial mass was measured with the help of spectrophotometer at 600nm after 24 hours.

RESULTS AND DISCUSSION (Dairy wastewater as a 'seed')

Optical Density Measurements

Experiments were conducted to measure optical density of microbial medium containing different concentrations of each metal ion. Microbes show greater optical density in the absence of metal ions while in presence of the metal ions, there is a fall in the absorbance value (Table 4.1(a)). The extent of fall depends on the concentration of the metal ions added. Results of optical density measurement for each metal ion are shown also in the Figures 4.1(a) to 4.6(a) by taking the extreme right axis as origin of the plots.

Table 4.1(a) Optical density measurements for the growth of *dairy microbes from dairy wastewater* in presence / absence of different metal ions at different concentrations

Metal ion concentration (mML^{-1})	ABSORBANCE (OD)					
	Cobalt	Nickel	Copper	Zinc	Silver	Cadmium
0.0	0.7	0.9	2.0	2.3	1.7	2.1
0.2	0.7	0.9	2.0	2.3	0.0	1.1
0.4	0.7	0.9	2.0	2.3	0.0	0.9
0.6	0.7	0.8	1.8	2.1	0.0	0.6
0.8	0.6	0.9	1.4	2.1	0.0	0.2
1.0	0.4	0.8	1.4	2.2	0.0	0.2
1.2	0.2	0.8	2.3	2.2	0.0	0.2
1.4	0.1	0.8	1.5	2.1	0.0	0.2
2.0	0.0	0.8	1.9	2.0	0.0	0.2
2.5	0.1	0.8	1.9	1.7	0.0	0.2
3.0	0.0	0.7	2.0	1.5	0.0	0.2
3.5	0.1	0.7	2.0	1.4	0.0	0.2
4.0	0.1	0.7	1.2	1.1	0.0	0.2
4.5	0.1	0.6	0.1	0.2	0.0	0.2
5.0	0.1	0.5	0.1	0.2	0.0	0.1
5.5	0.1	0.3	0.1	0.2	0.0	0.2
6.0	0.1	0.1	0.1	0.2	0.0	0.2
6.5	0.1	0.0	0.1	0.2	0.0	0.2
7.0	-	0.0	-	0.2	0.0	-
7.5	-	0.0	-	0.2	0.0	-

In most of the figures, absorbance value corresponds to the relative change in BOD. This indicates that the inhibition in BOD is due to the decay of microbial matter. This hypothesis works very well for almost all metal environments. It clearly rules out the possibility of any other factor like metal oxidation, etc. playing any role in BOD suppression process.

Hence, the extent of BOD suppression/ increase is directly related to the decay/growth of the microbial matter. In case of copper, the correspondence of BOD suppression to increase in optical density value is erratic which probably is due to the reason that pH changes go uncontrolled with change in metal ion concentration for this metal ion. Buffers were not used to control the pH as they would have influenced the BOD exertion in their own way. For all other metal ions, there is no appreciable pH change of the resulting solution, because copper salts are known to be strongly acidic.

Table 4.2(a) Minimal Inhibitory concentration (MIC) of some heavy metal ion for *Dairy microbes from dairy wastewater*

Metal ions	MIC, mML^{-1}	Metal ions	MIC, mML^{-1}
Cobalt	1.4	Zinc	4.5
Nickel	6.0	Silver	0.2
Copper	4.5	Cadmium	0.8

The relatively poor toxic behavior of zinc among all other transition metal ions (Table 4.1(a)) is due to the reason that it does not undergo redox changes under biological conditions because of its completely filled 3d-subshell. Rather, it acts as a Lewis base in complexation with polypepetide chain (Coleman, 1998). Cadmium is more toxic than zinc (Rogan and Mast, 1990) because the solubility product of CdS is 1.4×10^{-29} while that of ZnS is 2.91×10^{-25}. Mechanisms of cadmium toxicity in microorganisms are still not well defined. It is reported in literature that microorganisms commonly found in dairy waste do not survive after certain concentration. The levels of concentration for different metal ions are different and are known as 'Minimal Inhibitory Concentration' (MIC). The respective values of MIC for the selected metal ions are shown in Table 4.2(a). From the table showing OD measurement for different metal ions at different concentration ranging from 0.2 to $7.5 mML^{-1}$, it is shown that absorbance values decrease sharply in the initial concentration range upto a value identified here as the MIC value (Table 4.2(a)). After further increase in metal ion concentration, the OD decreases very sharply to almost zero value. This is true for cobalt, silver and cadmium. The very low MIC values (cobalt 1.4mM, silver 0.2mM, cadmium 0.8mM) for these metal ions are observed. For other

metal ions i.e. nickel, copper and zinc, the sharp fall in absorbance occurs at metal ion concentrations: 6.0mM, 4.5mM and 4.5mM, respectively. On the molecular level, the cadmium uptake is hardly understood. In general, cadmium enters the cell only by some indirect means of transportation. (manganese uptake system; Burke and Pfister, 1988; Laddaga et al, 1985; Tynecka and Malm, 1995; and calcium uptake system; Clemens et al, 1998). There are reports where resistance to cadmium in bacteria is noticed (Olafson et al, 1979). In our observation also, some degree of resistance to cadmium toxicity is noticed. Plots of absorbance values were drawn as a function of concentration of metal ions and are shown in Figures 4.1(a) to 4.6(a). The optical density variation exactly correspond the change in BOD values measured independently in separate set of experiments. These BOD values have been plotted in the same figures for the purpose of easy comparison with OD measurement results.

BOD Measurements

Experiments were conducted to determine BOD in presence of some identified heavy metal ions i.e., cobalt, nickel, copper, zinc, silver and cadmium. Metal ions were taken in a wide concentration range from 0.2mM to 10.0mM to study their effect at different levels of concentrations. Mixed flora from dairy industry derived and incubated at 37° for 24 hours was used as 'seed'. A standard mixture of Glucose-glutamic acid was taken as food. Industrial wastewater was not used as source of food simply to avoid any inconsistency in the composition of the sample water.

BOD values were determined in replicates of three bottles for each metal ion concentration and by measuring DO levels using membrane based DO meter. Only average values were taken for further calculations. The observed BOD values were compared with the blank sample (containing no metal ion) and the difference is calculated and reported as percentage change in BOD corresponding to each metal ion in Table 4.3. It can be seen that a decrease in BOD is noticed in presence of each of the metal ion. For most of the metal ions, the concentration level upto 1.2mM is good enough to inhibit 100% BOD. However, the presence of zinc ions at these levels results in a fall in BOD to the extent 5 to 6 % only. Even at a large concentration (10.0mM) of zinc ions,

the fall in BOD is only upto 40%. For cobalt, nickel and cadmium, trends in fall in BOD are similar, i.e., a sharp fall even in the presence of small amount of the metal ion upto 1.0mM. In presence of copper ions, the inhibition effect is irregular with increasing concentration of the metal ion, but becomes constant after 1.2mM concentration.

Table 4.3(a) Percentage change in BOD_3 (27°) in the presence of heavy metal ions at different concentrations using *dairy microbes from dairy wastewater* **as a seed**

Concentration (mML^{-1})	Change in BOD (%)					
	Cobalt	Nickel	Copper	Zinc	Silver	Cadmium
0.2	(-) 15.2	(-) 14.7	(-) 25.0	(-) 2.50	(-) 108.0	(-) 39.4
0.4	(-) 48.5	(-) 44.1	(-) 75.0	(-) 3.10	(-) 108.0	(-) 53.9
0.6	(-) 75.8	(-) 73.5	(-) 60.0	(-) 2.50	(-) 108.0	(-) 55.2
0.8	(-) 87.9	(-) 85.3	(-) 50.0	(-) 2.50	(-) 108.0	(-) 72.1
1.0	(-) 97.0	(-) 94.1	(-) 55.0	(-) 1.90	(-) 108.0	(-) 84.2
1.2	(-) 100.0	(-) 100.0	(-) 100.0	(-) 5.70	(-) 108.0	(-) 95.6
1.4	(-) 103.0	(-) 108.0	(-) 100.0	(-) 5.00	(-) 108.0	(-) 98.9
2.0	(-) 103.0	(-) 108.0	(-) 100.0	(-) 4.40	(-) 108.0	(-) 102.4
3.0	(-) 103.0	(-) 108.3	(-) 100.0	(-) 5.50	(-) 108.0	(-) 97.6
4.0	(-) 103.0	(-) 108.3	(-) 100.0	(-) 41.4	(-) 108.0	(-) 105.7
5.0	(-) 103.0	(-) 108.3	(-) 100.0	(-) 43.2	(-) 108.0	(-) 111.0
6.0	(-) 103.0	(-) 108.3	(-) 100.0	(-) 47.8	(-) 108.0	(-) 112.5
7.0	(-) 103.0	(-) 108.3	(-) 100.0	(-) 37.8	(-) 108.0	(-) 110.1
8.0	(-) 103.0	(-) 108.3	(-) 100.0	(-) 33.2	(-) 108.0	(-) 110.1
9.0	(-) 103.0	(-) 108.3	(-) 100.0	(-) 40.5	(-) 108.0	(-) 110.1
10.0	(-) 103.0	(-) 108.3	(-) 100.0	(-) 38.6	(-) 108.0	(-) 106.7

Percentage change in BOD is plotted as a function of concentration of metal ion. The shapes of the curves are shown in Figures 4.1(a) to 4.6(a). The presence of silver ions results in a sharp fall (100 percent) in BOD even in the presence of the smallest amount of the metal ion i.e., 0.2mM. The extraordinary toxicity with silver, as observed in Figure 4.5(a) is due to the following reason: when in contact with any heavy metal like Ag^+,

Hg^{2+} and Cd^{2+}, the microbial cell allows transportation of the heavy metal ions across the cell wall into the cytoplasm. Once inside the cell, these ions tend to bind to -SH groups, resulting in inhibiting the activity of the sensitive enzyme. Since Ag^{+} forms the most stable precipitate of Ag_2S (K_{sp} = Ag_2S is $6.62x10^{-50}$) as compared to that CuS (K_{sp} = $1.28x10^{-36}$) etc. Silver is isoelectronic to copper. The standard electrode potential of Cu^{2+}/Cu^{+} pair is –0.26V while that of Ag^{2+}/Ag^{+} is 1.56V at pH 7. Thus main ionic form of these elements is Cu^{2+} and Ag^{+}. The monovalent silver cation forms a more stable complex with sulphur of microbial cell walls, which makes silver highly toxic. (Nies, 1999).

Transition metal ions, in general, are toxic to the extent that they lead to immobilization of the microbes through complex formation with the enzymes and aminoacids found in the microbial cells. Alternately, the microbes may also be not able to survive in presence of metal ions all together due to impaired metabolic activities. In both of these cases, their growth is hampered and BOD exertion is halted, thereby leading to inhibition in BOD. These observations are supported by the optical density measurements of the incubation of the medium and the results have already been discussed in previous paragraphs.

It can be seen clearly from Figures 4.1(a) to 4.6(a) that the change in BOD (i.e., the fall in presence of increasing concentrations of metal ions) just corresponds to the optical density measurements which shows decrease in OD due to decrease in growth of microbes for increasing concentration of the metal ions, resulting in net fall in BOD exertion.

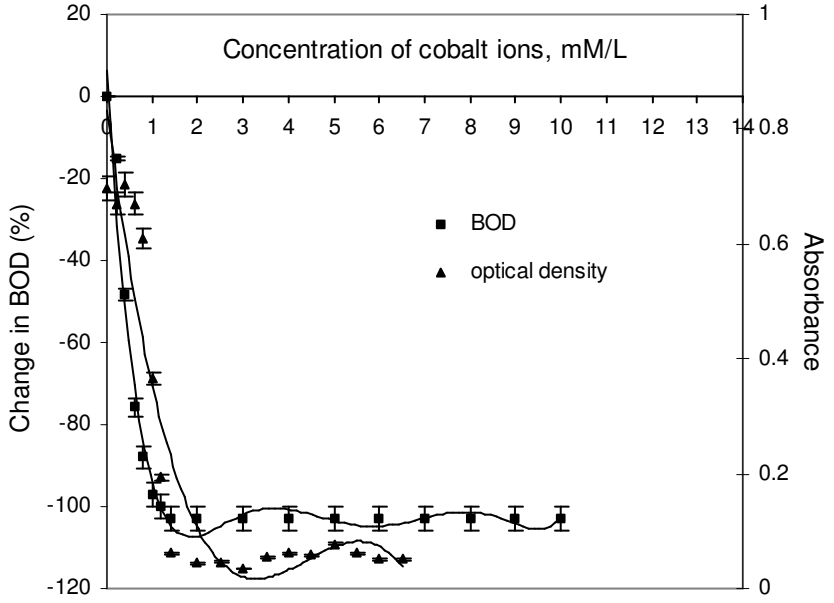

Figure 4.1(a) Plots of change in BOD_3 (%) and absorbance (OD) as a function of concentration of Co (II) ions using *dairy microbes* as a seed

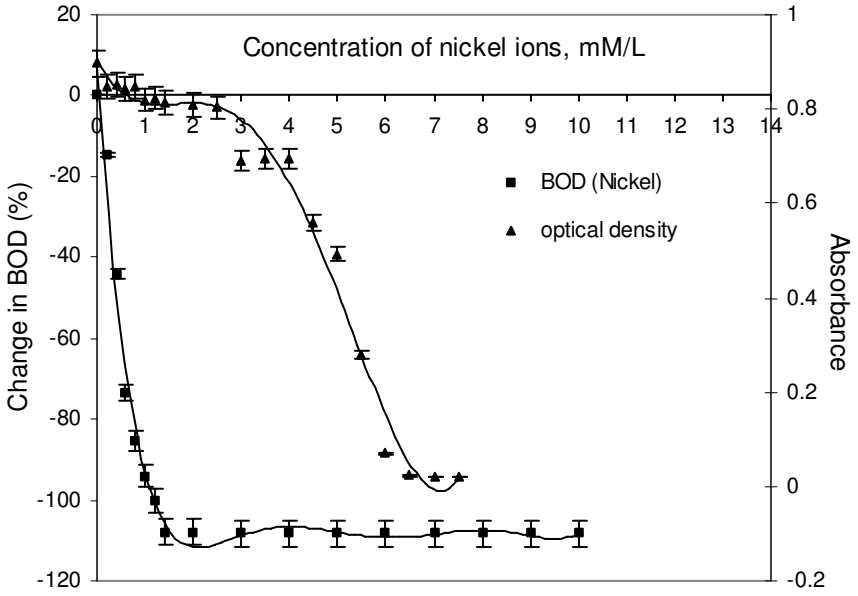

Figure 4.2(a) Plots of change in BOD_3 (%) and absorbance (OD) as a function of concentration of Ni(II) ions using *dairy microbes* as a seed

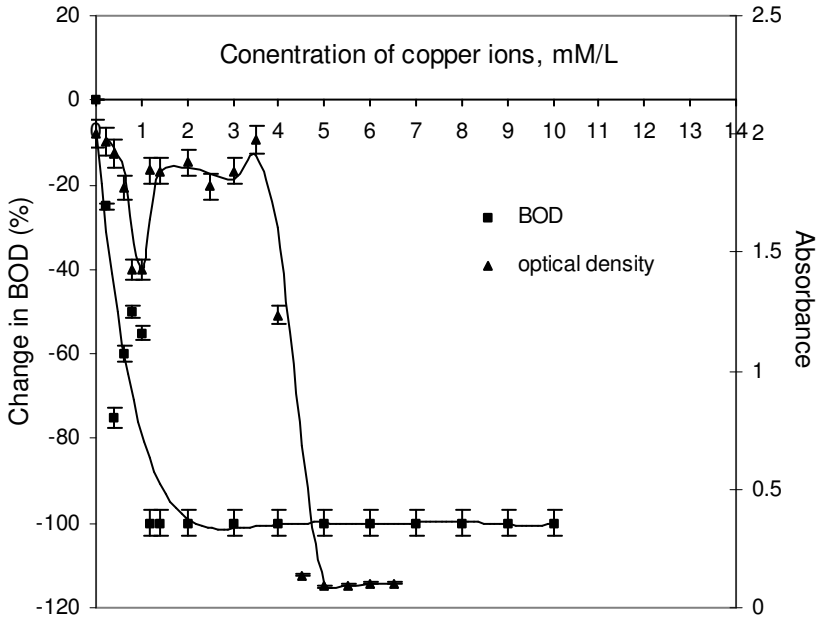

Figure 4.3(a) Plots of change in BOD_3 (%) and absorbance (OD) as a function of concentration of Cu(II) ions using *dairy microbes* as a seed

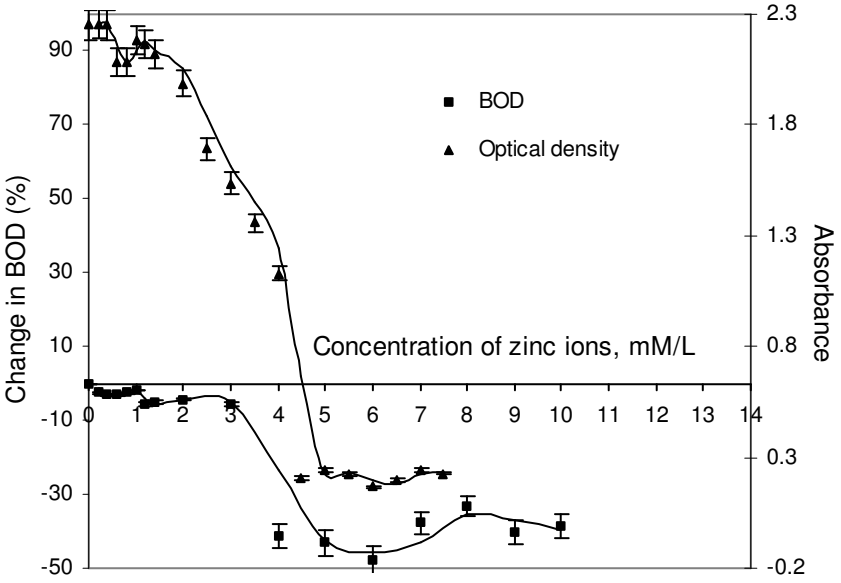

Figure 4.4(a) Plots of change in BOD_3 (%) and absorbance (OD) as a function of concentration of Zn(II) ions using *dairy microbes* as a seed

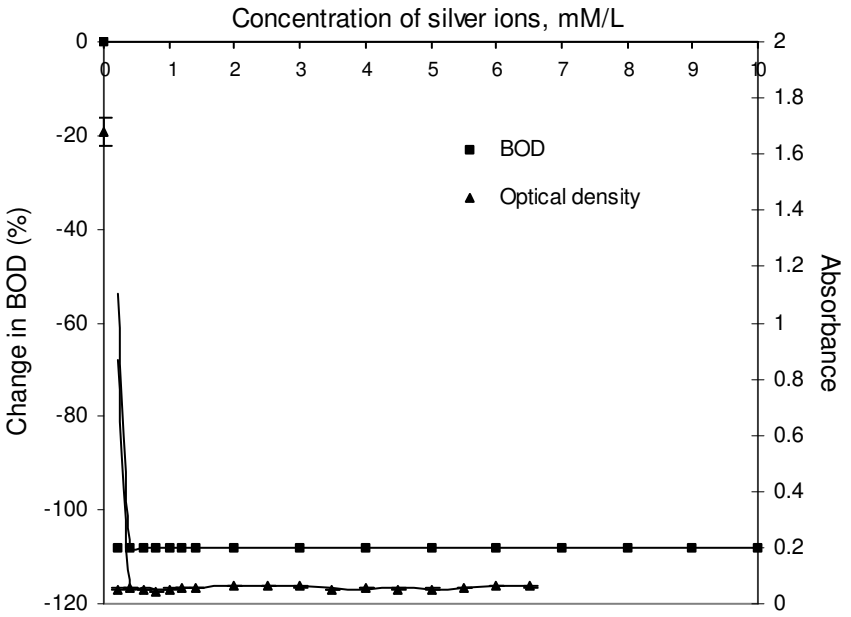

Figure 4.5(a) Plots of change in BOD_3 (%) and absorbance (OD) as a function of concentration of Ag (I) ions using *dairy microbes* as a seed

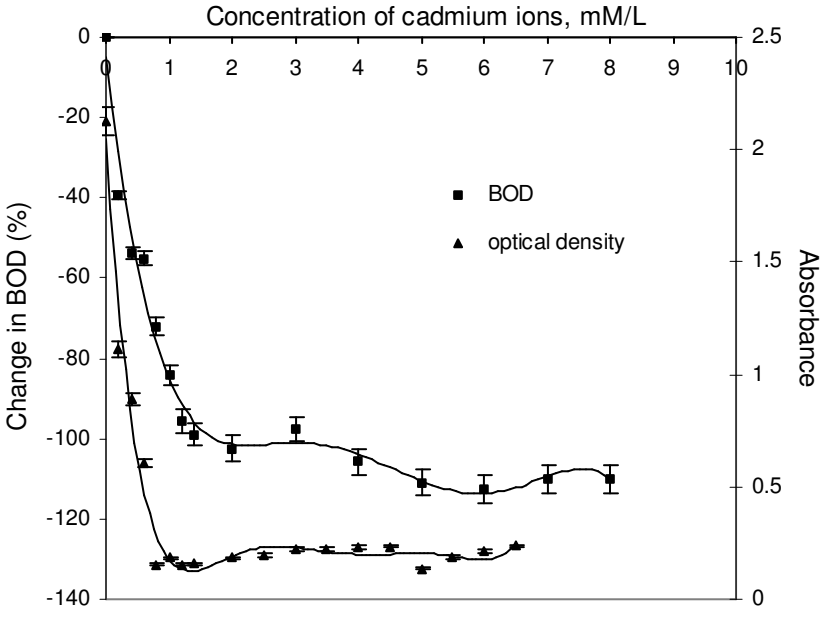

Figure 4.6(a) Plots of change in BOD_3 (%) and absorbance (OD) as a function of concentration of Cd(II) ions using *dairy microbes* as a seed

pH Effect

pH is one of the most important parameters that can influence the growth of microbes and hence the BOD. Metal ions are available as different species when observed at different pH values. For example, when pH of the metal salt solution is raised to the basic medium the metal ions precipitate as their hydroxides and hence behave differently towards the microbes. Experiments for the measurement of BOD were carried out by changing pH of the system with the help of nitric acid (1N) and sodium hydroxide (1N) as per requirement. Maximum BOD is observed for almost all metal ions between pH 5 and 6 (Table 4.4(a)). Suppression/ increase in BOD at a given pH is reported for the GGA system, containing a fixed concentration (2.0mM) of each metal ion, as the reference. The percentage fall/ increase is reported in Table 4.5(a) Figure 4.7(a) to Figure 4.8(a) represent bar charts for decrease/ increase in BOD at different pH values for each metal ion.

In the acidic range, suppression is maximum at pH 3.0 for all metal ions except copper. BOD is suppressed to the extent nearly 100% at pH 6.0 except for nickel and zinc. The behaviour of zinc ions has always been different from that of other transition elements. Here, the presence of zinc ions result in relatively less degree of inhibition (except at pH 3) in comparison to all other metal ions. Studies were extended to see the effect on BOD suppression in the basic medium. At pH 8, all transition elements do form metal hydroxides which will render the metal ions less toxic. But the theory of adsorption as reported by Nelson et al (1981) very well explains the experimental results. This mechanism suggests the metal hydroxide could provide suitable surface for the physical adsorption of microbes over the precipitates. Another reason for the fall in BOD at pH 8 may be that the microbes of dairy effluent origin do not survive at this pH, being rich in *Lactococcus genus* (Nicolette et al, 1995) these are stable only in the acidic medium.

Table 4.4(a) BOD_3 (27°) in presence/ absence of metal ions ($0.8mML^{-1}$) at different pH values using *dairy microbes* as a seed

PH	BOD_3 (mg/L)						
	GGA (without metal ion)	**Cobalt**	**Nickel**	**Copper**	**Zinc**	**Silver**	**Cadmium**
3.0	17	-1	0	13	-3	-2	-1
4.0	290	257	77	230	313	-50	27
5.0	383	260	50	33	372	-40	-20
6.0	520	63	243	-40	327	-57	-3
7.0	347	-7	-20	3	120	-17	-16
8.0	30	0	3	43	33	-2	7

Table 4.5(a) Percentage change in BOD_3 (27°) in presence of metal ions ($0.8mML^{-1}$) at different pH values using *dairy microbes* as a seed

PH	Change in BOD_3 (%)					
	Cobalt	**Nickel**	**Copper**	**Zinc**	**Silver**	**Cadmium**
3.0	(-) 106.6	(-) 102.1	(-) 25.2	(-) 104.6	(-) 108.8	(-) 106.1
4.0	(-) 11.4	(-) 73.4	(-) 20.7	(+) 8.0	(-) 117.2	(-) 90.7
5.0	(-) 32.1	(-) 86.9	(-) 91.3	(-) 2.8	(-) 110.4	(-) 105.2
6.0	(-) 87.9	(-) 53.3	(-) 107.7	(-) 37.1	(-) 110.9	(-) 100.6
7.0	(-) 102.0	(-) 105.8	(-) 99.0	(-) 65.4	(-) 104.9	(-) 105.8
8.0	(-) 100.0	(-) 90.0	(+) 44.0	(+) 11.1	(-) 105.0	(-) 77.7

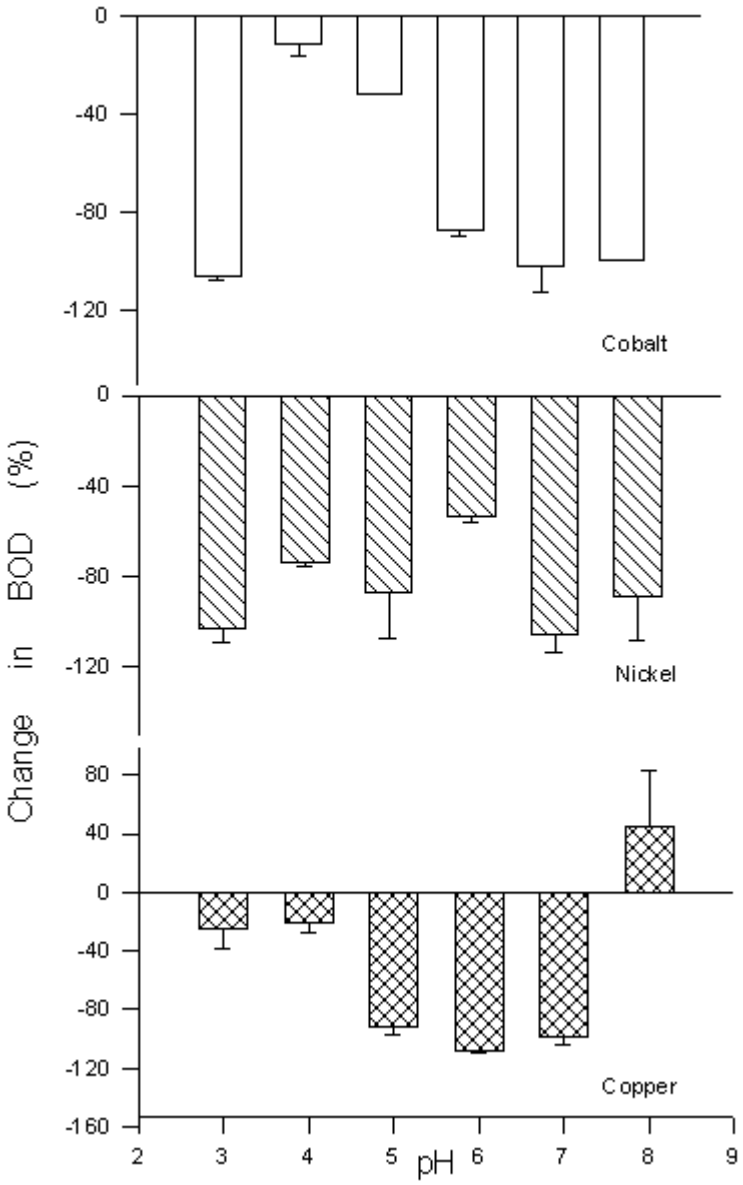

Figure 4.7(a) Bar charts for change in BOD (%) in presence of cobalt, nickel and copper ions at different pH values using *dairy microbes* **as a seed**

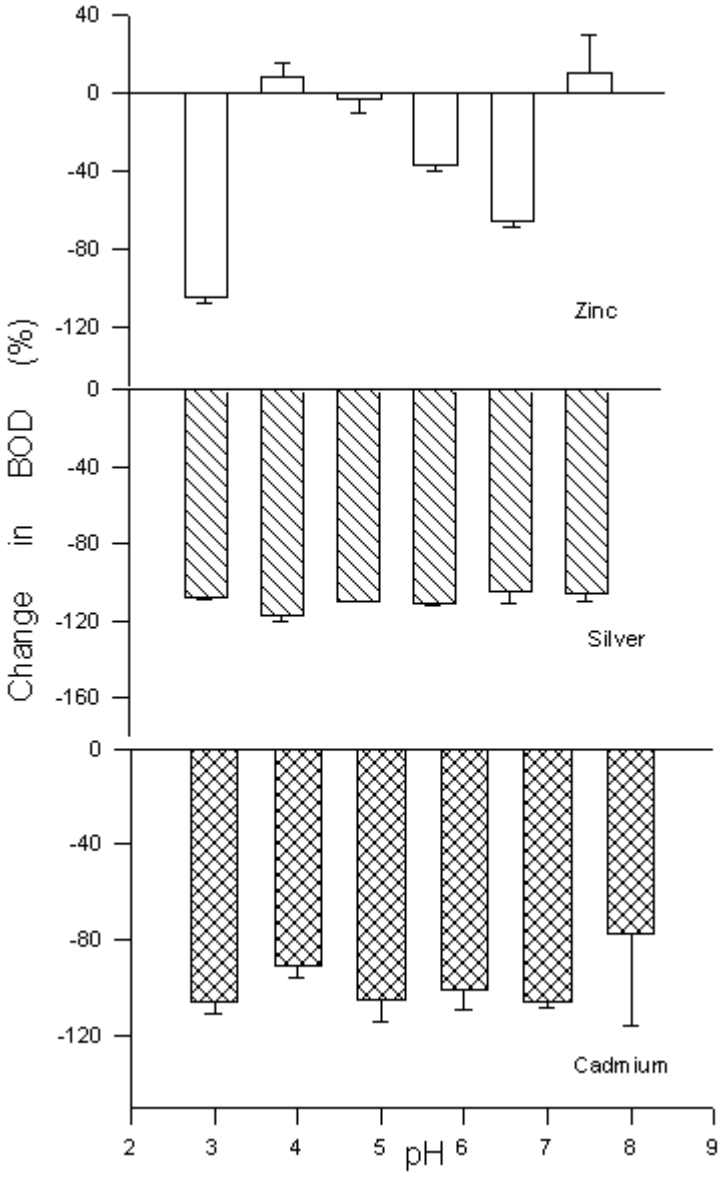

Figure 4.8(a) Bar charts for change in BOD (%) in presence of zinc, silver and cadmium ions at different pH values using *dairy microbes* **as a seed**

Temperature Effect

BOD_3 measurements were done at different temperature i.e., 15°, 20°, 25°, 30° and 35° taking GGA system as the reference. Studies were carried out in presence of constant concentration ($0.8mML^{-1}$) of cobalt, nickel, copper, zinc, silver, and cadmium. The results are shown in Table 4.6(a). BOD values of the GGA system without any metal ion also shows an appreciable increase with increase in temperature. This is certainly due to the reason that with increase in temperature solubility of oxygen in aqueous medium decreases, resulting in a corresponding increase in BOD. It is seen that the presence of metal ions do effect the BOD and result in total inhibition and even the inhibition goes beyond 100% in all of the cases under study (Table 4.7(a)). The effect of all the metal ions is almost the same at a given temperature. So, the metal ions do inhibit BOD exertion but without any preference probably because of the reason that the concentration selected for the study was very high and at this concentration most of the BOD is already inhibited. It will be worthwhile to study the effect of temperature for lower range of metal ion concentration.

An increase in BOD for the blank system may be due to greater activity of microbes with increase in temperature. No BOD exertion was noticed for GGA system as well as that for the system that contains metal ions at 15°. It can be due to reason that microbes are inactive and do not undergo biochemical processes. It is also seen that in the standard BOD curve, substantial depletion of DO takes place only after 5^{th} day of incubation (20°). BOD exertion process is likely to go still slower at 15° and BOD exertion is not expected on day-3.

Table 4.6(a) BOD_3 (27°) in presence/ absence of metal ions ($0.8mML^{-1}$) at different temperatures using *dairy microbes* as a seed

Temperature (°)	BOD (mg/L)						
	GGA (without metal ion)	**Cobalt**	**Nickel**	**Copper**	**Zinc**	**Silver**	**Cadmium**
15	0	0	0	0	0	0	0
20	160	-23	-23	0	3	-30	-20
25	307	-40	-40	-27	-20	-50	-37
30	340	-3	0	-3	-3	-3	0
35	367	-33	-20	-30	-17	-40	-20

Table 4.7(a) Percentage change in BOD_3 (27°) in presence of metal ions ($0.8mML^{-1}$) at different temperatures using *dairy microbes* as a seed

Temperature (°)	Change in BOD (%)					
	Cobalt	**Nickel**	**Copper**	**Zinc**	**Silver**	**Cadmium**
15	-	-	-	-	-	-
20	(-) 115.2	(-) 115.0	(-) 100.1	(-) 98.0	(-) 119.5	(-) 112.5
25	(-) 113.0	(-) 113.1	(-) 109.0	(-) 106.0	(-) 116.2	(-) 112.5
30	(-) 101.1	(-) 100.0	(-) 101.0	(-) 101.1	(-) 101.1	(-) 100.0
35	(-) 109.4	(-) 105.3	(-) 108.5	(-) 104.1	(-) 111.4	(-) 105.4

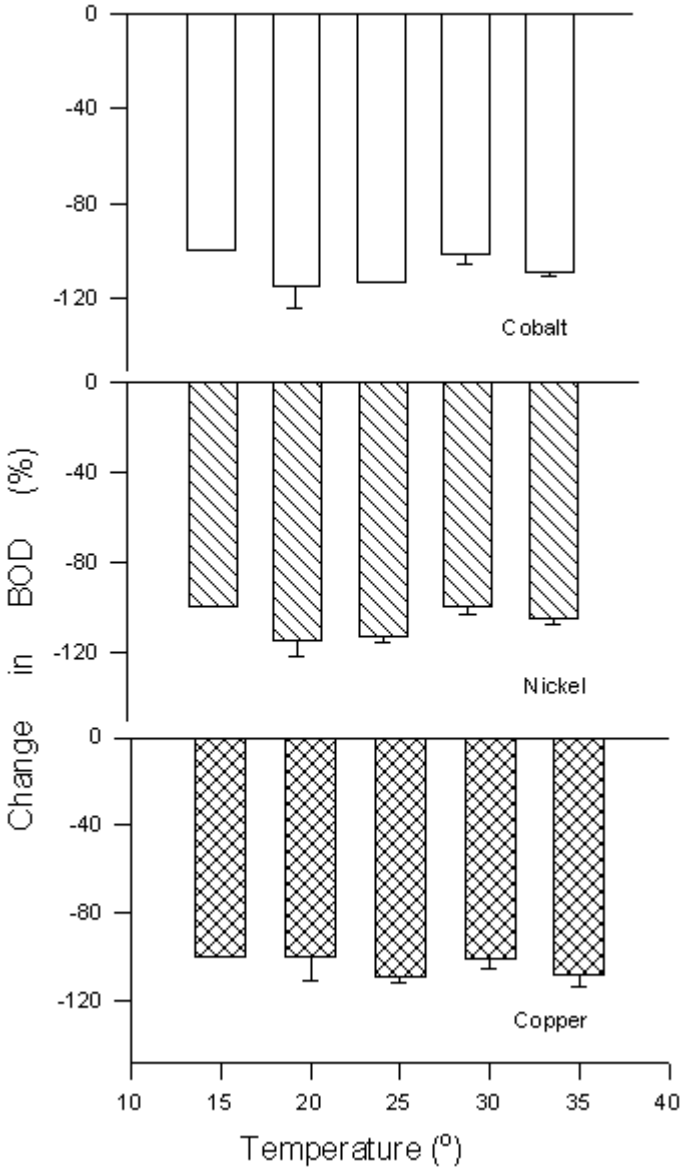

Figure 4.9(a) Bar charts for change in BOD in presence of cobalt, nickel and copper ions at different temperatures using *dairy organisms* as a seed

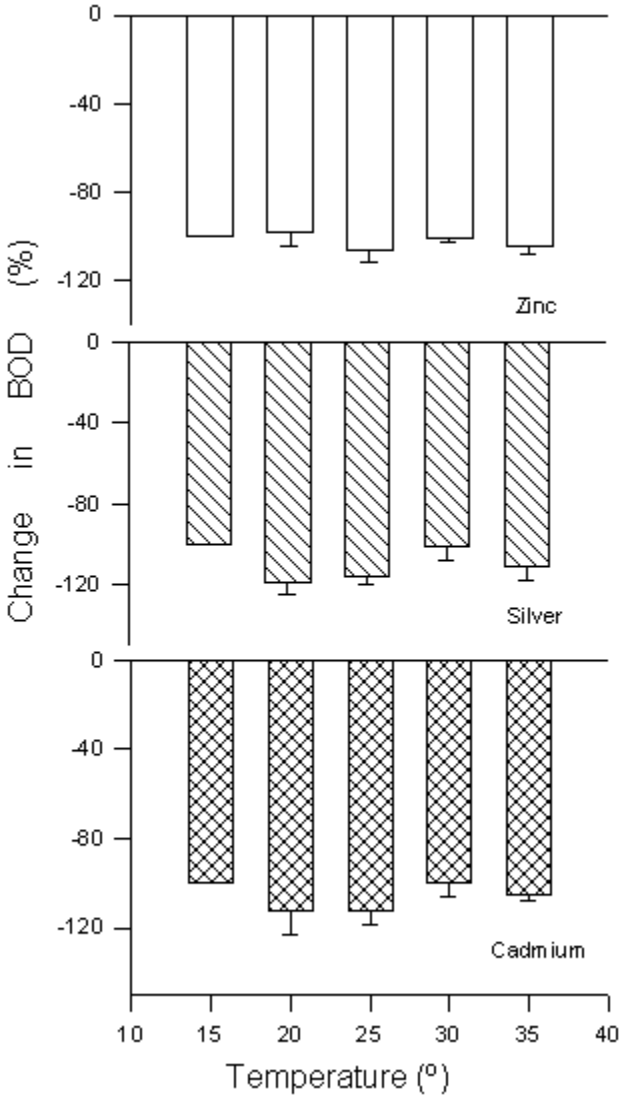

Figure 4.10(a) Bar charts for change in BOD (%) in presence of zinc, silver and cadmium ions at different temperatures using *dairy microbes* as a seed

RESULTS AND DISCUSSION (Distillery wastewater as a 'seed')

BOD Measurements and comparison with Optical Density Measurements

BOD exertion process was studied in presence of some selected heavy metal ions at different concentrations using microbes from distillery wastewater. The reason for choosing this wastewater was to study the metal ion effect in presence of a totally different class of microbes. Results are presented in terms of percentage change in BOD for different concentrations of each metal ion (Table 4.1(b)).

Experiments were also conducted to study the growth of microbes in presence of metal ions by measuring optical density (OD) of the medium. The results of OD measurement (Table 4.1(b)) and percentage change in BOD are plotted simultaneously on the same graph to co-relate change in BOD with that of microbial growth.

Minimum inhibitory concentrations (MIC) have been identified as the values of concentration at which a metal ion starts total inhibition of microbial growth. These are identified from the experimental observation of OD measurements and listed in Table 4.3(b) for each metal ion. From Figures 4.1(b) to 4.6 (b), it can be easily established that there is a good degree of correspondence between the change in BOD and optical density measurements. In general, a decrease in BOD change is accompanied by an increase in absorbance value. Any increase in the absorbance value indicates growth of microbial population and must indicate a fall in percentage inhibition of BOD.

Out of all metal ions studied, the presence of silver ions is found to be the most toxic as is also observed for system using dairy waste as a seed. It is well-established that silver ions are highly toxic to almost all types of microbes. (Nies, 1999). In general, there has been a reasonably good (with experimental error) co-relation between the absorbance values and corresponding %age changes in BOD. However, some humps are seen in the BOD curves at higher concentration range for almost all the metal ions except for silver.

These are indicative of the tendency to develop resistance towards the presence of metal ions. It is really very difficult to quantify the resistances and corelation to the

concentration levels of metal ions. At the most, it can be linked to the nature of the metal ion.

Table 4.1(b) Percentage change in BOD_3 (27°) in the presence of metal ions at different concentrations using *distillery microbes from Distillery wastewater* **as a seed**

Concentration (mML^{-1})	Change in BOD (%)					
	Cobalt	**Nickel**	**Copper**	**Zinc**	**Silver**	**Cadmium**
0.2	(-) 12.3	(-) 15.2	(-) 10.0	(+) 9.7	(-) 106.6	(-) 2.2
0.4	(-) 21.7	(-) 44.2	(-) 17.4	(+) 8.6	(-) 110.3	(-) 8.1
0.6	(-) 23.1	(-) 98.9	(-) 19.6	(+) 7.5	(-) 110.3	(-) 15.3
0.8	(+) 9.4	(-) 103.2	(-) 8.5	(+) 8.6	(-) 110.3	(-) 19.7
1.0	(+) 10.8	(-) 103.2	(-) 8.5	(+) 11.2	(-) 110.9	(-) 23.4
1.2	(+) 13.0	(-) 108.6	(-) 7.4	(+) 13.5	(-) 110.9	(-) 29.9
1.4	(+) 18.1	(-) 107.5	(-) 7.4	(+) 10.5	(-) 111.6	(-) 40.9
2.0	(+) 19.1	(-) 116.1	(+) 1.1	(+) 3.7	(-) 111.6	(-) 44.6
3.0	(+) 36.1	(-) 104.6	(-) 10.0	(-) 5.2	(-) 112.5	(-) 46.0
4.0	(+) 21.7	(-) 105.1	(-) 15.9	(-) 13.9	(-) 112.5	(-) 25.7
5.0	(+) 6.5	(-) 116.1	(-) 25.9	(-) 17.6	(-) 112.5	(-) 26.4
6.0	(-) 14.4	(-) 116.1	(-) 58.1	(-) 5.2	(-) 112.5	(-) 27.5
7.0	(-) 23.1	(-) 116.1	(-) 44.0	(-) 5.2	(-) 113.1	(-) 30.3
8.0	(-) 41.2	(-) 116.1	(-) 107.6	(+) 16.7	(-) 113.1	(-) 33.1
9.0	(-) 55.6	(-) 116.1	(-) 113.6	(+) 17.9	(-) 113.1	(-) 33.9
10.0	(-) 98.9	(-) 116.1	(-) 111.4	(+) 10.3	(-) 113.1	(-) 34.7

Table 4.2(b) Optical density measurements for the growth of *distillery microbes from distillery wastewater* **in presence of different metal ions at different concentrations**

Concentration (mML^{-1})	ABSORBANCE (OD)					
	Cobalt	Nickel	Copper	Zinc	Silver	Cadmium
0.0	2.1	2.1	2.0	2.0	2.0	2.1
0.2	2.0	2.2	1.6	2.0	0.1	1.4
0.4	1.2	2.3	1.5	1.9	0.1	0.8
0.6	1.1	1.9	1.5	2.2	0.1	0.3
0.8	0.9	2.2	1.5	1.0	0.1	0.1
1.0	-	2.2	1.3	1.0	0.1	0.1
1.2	0.8	1.7	1.3	0.9	0.1	0.1
1.4	0.7	1.9	1.2	0.8	0.1	0.1
2.0	0.5	1.5	1.2	0.8	0.1	0.1
2.5	0.2	1.0	0.8	0.8	0.1	0.1
3.0	0.1	0.9	0.3	0.7	0.1	0.1
3.5	0.1	0.5	0.1	0.7	0.1	0.1
4.0	0.1	0.2	0.1	0.6	0.1	0.1
4.5	0.1	0.1	0.1	0.1	0.1	0.1
5.0	0.1	0.1	0.1	0.1	0.1	0.1
5.5	0.1	0.1	0.1	0.1	0.1	0.1
6.0	0.1	0.1	0.1	0.1	0.1	0.1
6.5	0.1	0.1	-	0.1	0.1	0.1

Table 4.3(b) Minimal Inhibitory concentration (MIC) of some heavy metal ion in *distillery microbes from distillery wastewater*

Metal ions	MIC, mML^{-1}	Metal ions	MIC, mML^{-1}
Cobalt	2.5	Zinc	4.5
Nickel	4.5	Silver	0.2
Copper	3.5	Cadmium	0.8

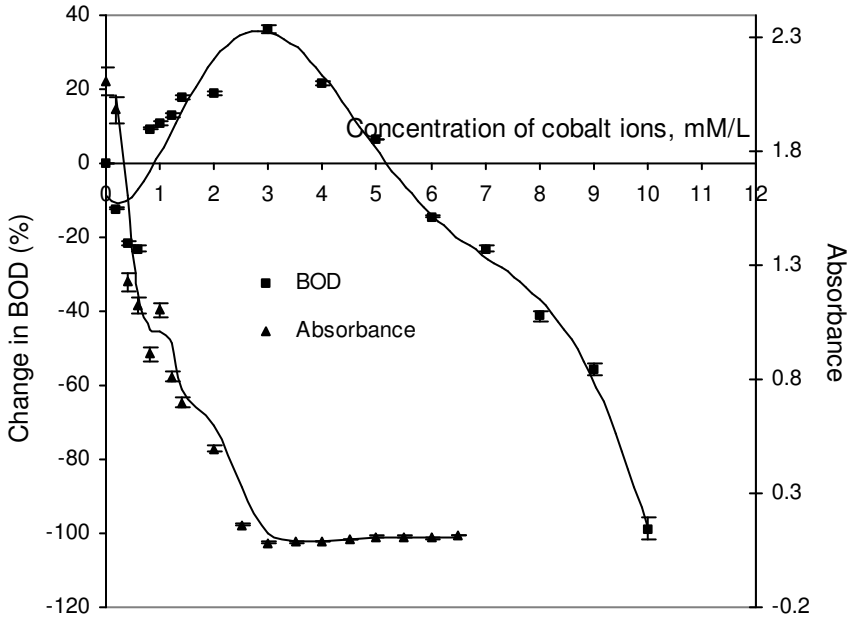

Figure 4.1(b) Plots of change in BOD (%) and absorbance (OD) as a function of concentration of Co (II) ions using *distillery microbes* as a seed

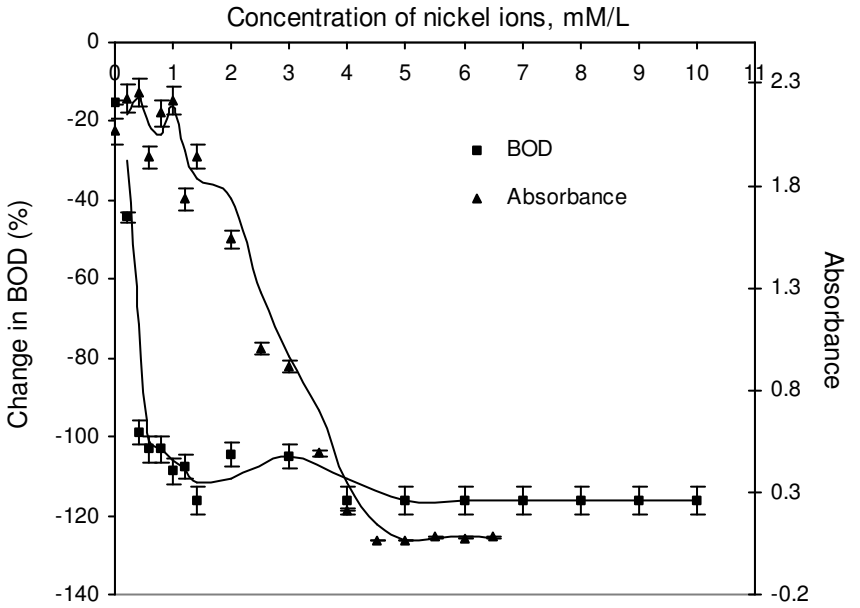

Figure 4.2(b) Plots of change in BOD (%) and absorbance (OD) as a function of concentration of Ni(II) ions using *distillery microbes* as a seed

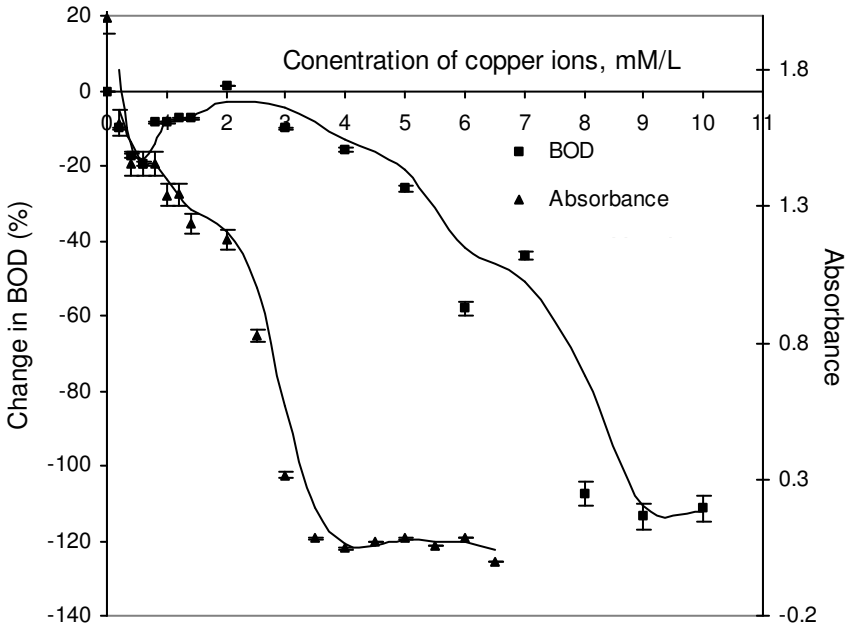

Figure 4.3(b) Plots of change in BOD (%) and absorbance (OD) as a function of concentration of Cu(II) ions using *distillery microbes* as a seed

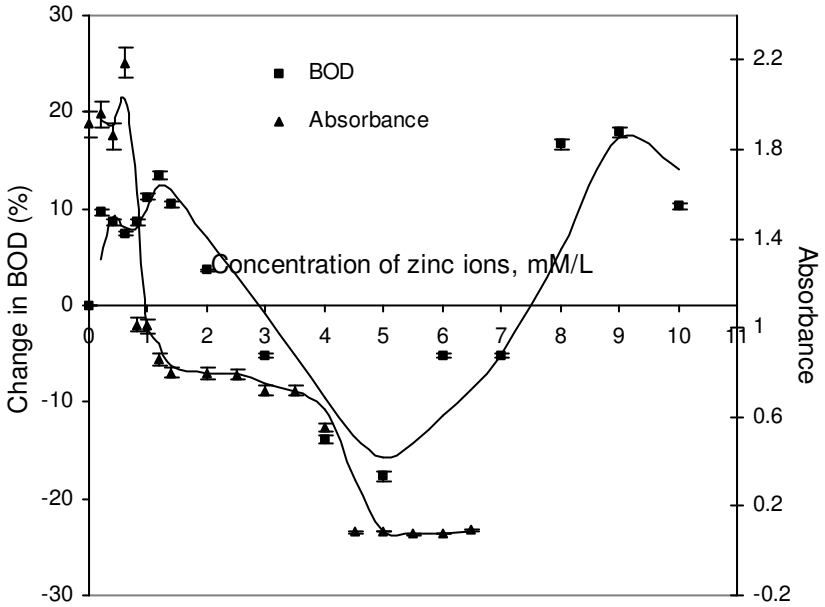

Figure 4.4(b) Plots of change in BOD (%) and absorbance (OD) as a function of concentration of Zn(II) ions using *distillery microbes* as a seed

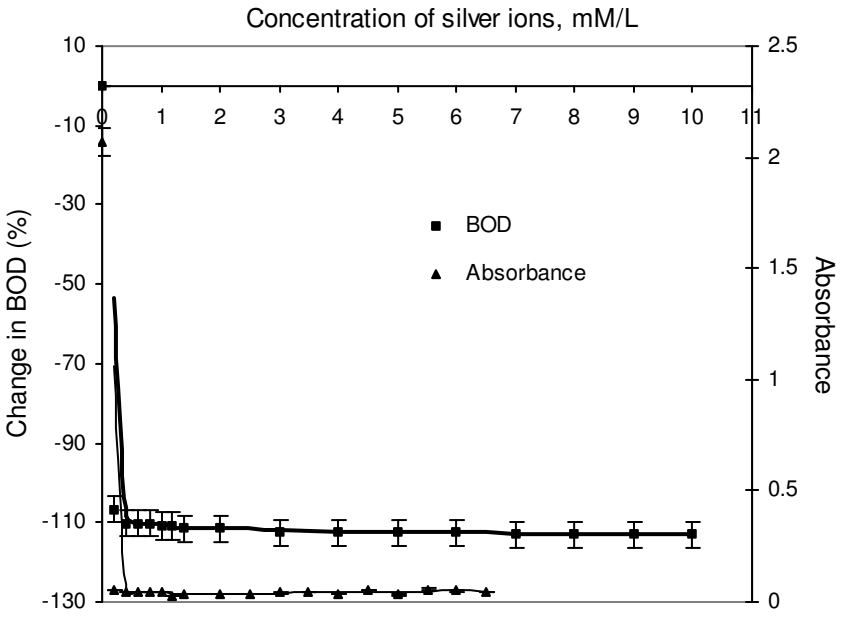

Figure 4.5(b) Plots of change in BOD (%) and absorbance (OD) as a function of concentration of Ag (I) ions using *distillery microbes* as a seed

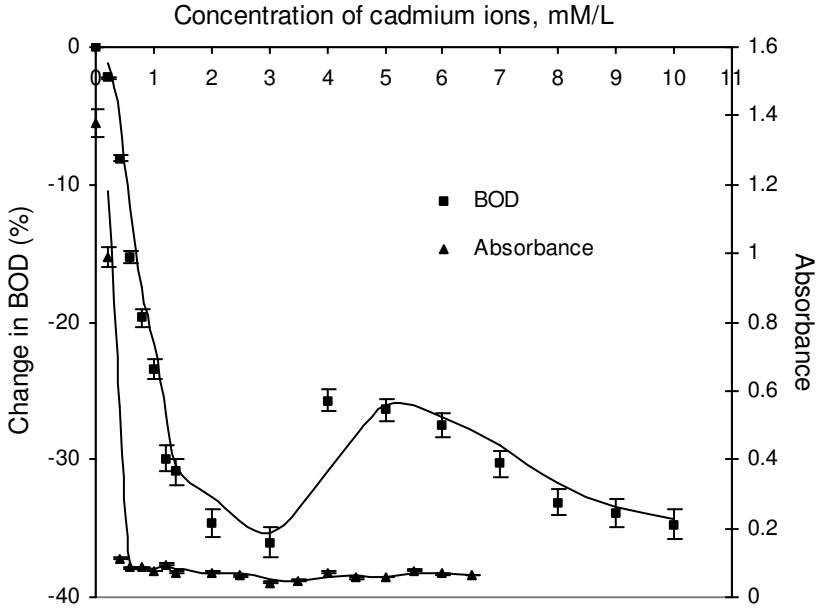

Figure 4.6(b) Plots of change in BOD (%) and absorbance (OD) as a function of concentration of Cd(II) ions using *distillery microbes* as a seed

pH Effect

pH and temperature are among the most important environmental parameters governing the activities and growth rates of microbes in biochemical processes. Toxicity of a pollutant under a given circumstance is a function of chemical structure that in turn is greatly influenced by the physicochemical state in which metal ions are present. In the aqueous environment, extreme pH values are generally not encountered except for industrial processes like electroplating, tanning, chemical processes, etc.

BOD measurement has little relevance towards the quality of wastewater of such industries. pH range 6 – 9 is generally considered acceptable to most acquatic organism.

BOD of synthetic samples maintained at different pH values were measured in the absence and presence of each metal ion, separately. Experiments were conducted by maintaining a pH in the range 3.0 to 8.0 because microbes like *E.coli, Bacillus subtilis* etc. are stable in this pH range. 2.0mML-1 of the metal ion was maintained of metal ion in each sample bottle. At this selected concentration, the metal ions exert toxicity to a reasonable extent, as observed from Table 4.4(b).

Results of change in BOD with addition of metal ions at different pH values are shown in Table 4.5(b) and Figures 4.7(b) & 4.8(b).

BOD is found to undergo a substantial decrease to the extent greater than or equal to 100% in the presence of almost all metal ions Table 4.5(b) with an exception in the presence of zinc ions (Figure 4.7(b) & 4.8(b)) where different behaviour is shown by zinc. The reasons are the same as discussed in the previous chapter.

This process of BOD exertion in presence of heavy metal ions involves the penetration of the metal ion through the cell membranes, thus inhibiting and simulating the catalytic function of the enzymes. (Stotzky, 1977).

These metal microbe complexes are sensitive to change in pH of the medium. Hydrogen ion concentration or pH is probably the single most important factor influencing the metal ion adsorption on both organic and inorganic surfaces. Metal speciation is significantly affected both from complexation and change in protonation level of the other soluble cell enzyme.

Table 4.4(b) BOD_3 (27°) in presence/ absence of metal ions (2.0mML^{-1}) at different pH values using *distillery microbes* as a seed

pH	BOD (mg/L)						
	GGA (without metal ion)	**Cobalt**	**Nickel**	**Copper**	**Zinc**	**Silver**	**Cadmium**
3.0	27	6	6	9	1	-2	-1
4.0	263	-33	-73	-43	217	-93	-63
5.0	287	-17	-23	-30	353	-27	-13
6.0	243	-7	-23	-10	113	-23	-13
7.0	290	7	0	3	47	-13	-7
8.0	133	-33	-3	-3	23	-20	-3

Table 4.5(b) Percentage change in BOD_3 (27°) in presence of metal ions (2.0 mML^{-1}) at different pH values using *distillery microbes* as a seed

PH	Change in BOD (mg/L)					
	Cobalt	**Nickel**	**Copper**	**Zinc**	**Silver**	**Cadmium**
3.0	(-) 77.6	(-) 76.6	(-) 65.0	(-) 96.8	(-) 106.0	(-) 102.0
4.0	(-) 112.5	(-) 128.0	(-) 116.3	(-) 17.5	(-) 135.4	(-) 124.0
5.0	(-) 106.0	(-) 108.0	(-) 110.0	(+) 23.0	(-) 109.0	(-) 105.0
6.0	(-) 103.0	(-) 109.0	(-) 104.0	(-) 54.0	(-) 109.5	(-) 105.0
7.0	(-) 98.0	(-) 100.0	(-) 99.0	(-) 84.0	(-) 105.0	(-) 102.0
8.0	(-) 125.0	(-) 102.0	(-) 102.0	(-) 83.0	(-) 115.0	(-) 102.0

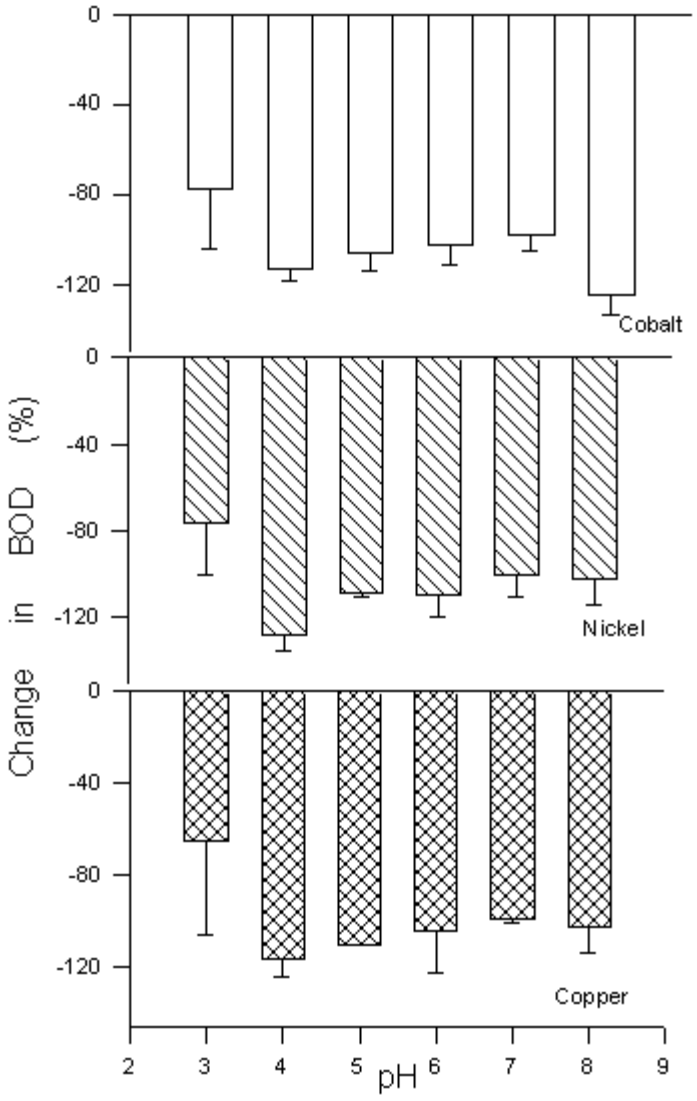

Figure 4.7(b) Bar charts for change in BOD (%) in presence of cobalt, nickel and copper ions at different pH values using *distillery microbes* **as a seed**

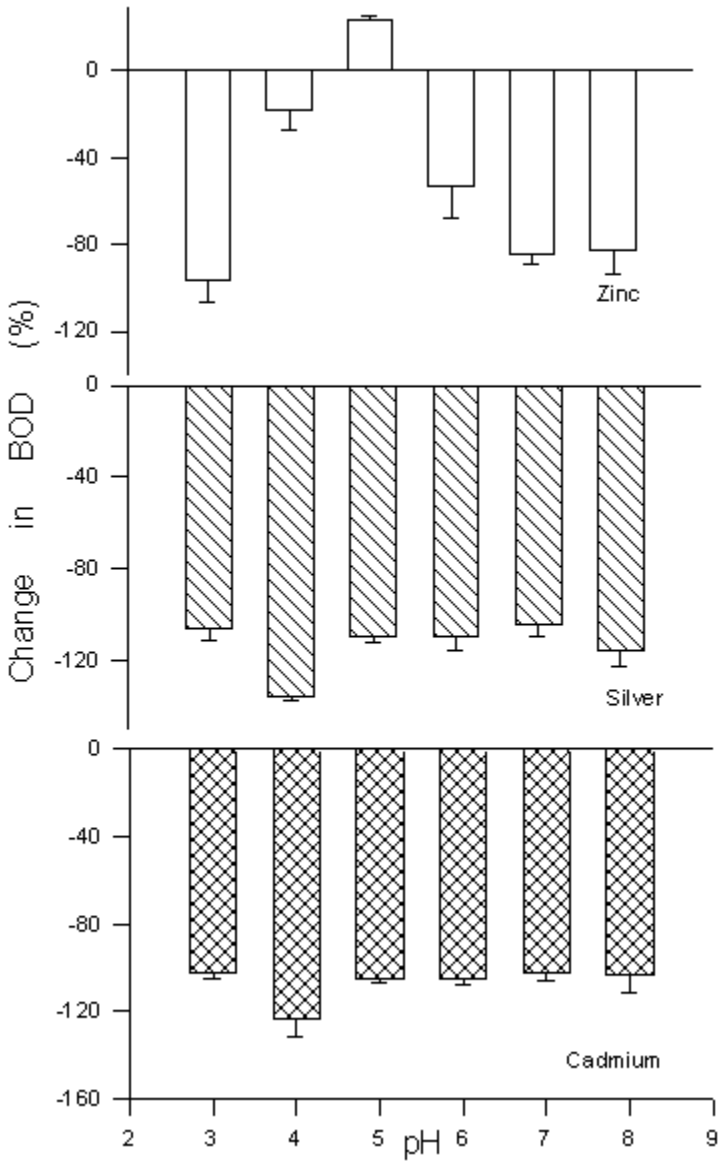

Figure 4.8(b) Bar charts of change in BOD (%) in presence of zinc, silver and cadmium ions at different pH values using *distillery microbes* **as a seed**

Temperature Effect

Studies were conducted for BOD measurement in presence of all the metal ions at different incubation temperatures (15°, 20°, 25°, 30° and 35°). As discussed in the previous section 4.3.4(a) there are primarily two sources of observed change in BOD.

The first factor is due to the variable dissolved content in the aqueous system at different temperatures and the second one is due to microbial population surviving in presence of a fixed concentration $2.0mML^{-1}$ of each heavy metal ion solution. The first reason is common to all the system under study, where as the effect of second factor is almost similar to the system using seed from dairy wastewater. The microbial composition of these two wastewaters is not much different. The BOD is inhibited to the extent 100 to 120%, there is little variation in BOD inhibition with change of the metal ion. The observation at 15° show that BOD exertion does not take place at all, which may be due the reason that 15° the microbial matter is dormant and does not undergo any biochemical process.

Table 4.6(b) BOD_3 (27°) in presence/ absence of metal ions ($2.0mML^{-1}$) at different temperatures using *distillery microbes* as a seed

Temperature (°)	BOD (mg/L)						
	GGA (without metal ions)	**Cobalt**	**Nickel**	**Copper**	**Zinc**	**Silver**	**Cadmium**
15	0	0	0	0	0	0	0
20	147	-23	-23	-23	-10	-10	-20
25	275	-40	-33	-30	-40	-27	-27
30	330	-57	-30	-33	-30	-40	-17
35	450	-23	-37	-33	-37	-37	-30

Table 4.7(b) Percentage change in BOD_3 (27°) in presence of metal ions ($2.0mML^{-1}$) at different temperatures using *distillery microbes* as a seed.

Temperature (°)	Change in BOD (%)					
	Cobalt	**Nickel**	**Copper**	**Zinc**	**Silver**	**Cadmium**
15	0	0	0	0	0	0
20	(-) 115.6	(-) 115.6	(-) 115.6	(-) 106.8	(-) 106.8	(-) 113.6
25	(-) 114.5	(-) 112.0	(-) 110.9	(-) 114.5	(-) 109.8	(-) 109.8
30	(-) 117.3	(-) 109.1	(-) 110.0	(-) 109.1	(-) 112.1	(-) 105.2
35	(-) 105.1	(-) 108.2	(-) 107.3	(-) 108.2	(-) 108.2	(-) 106.7

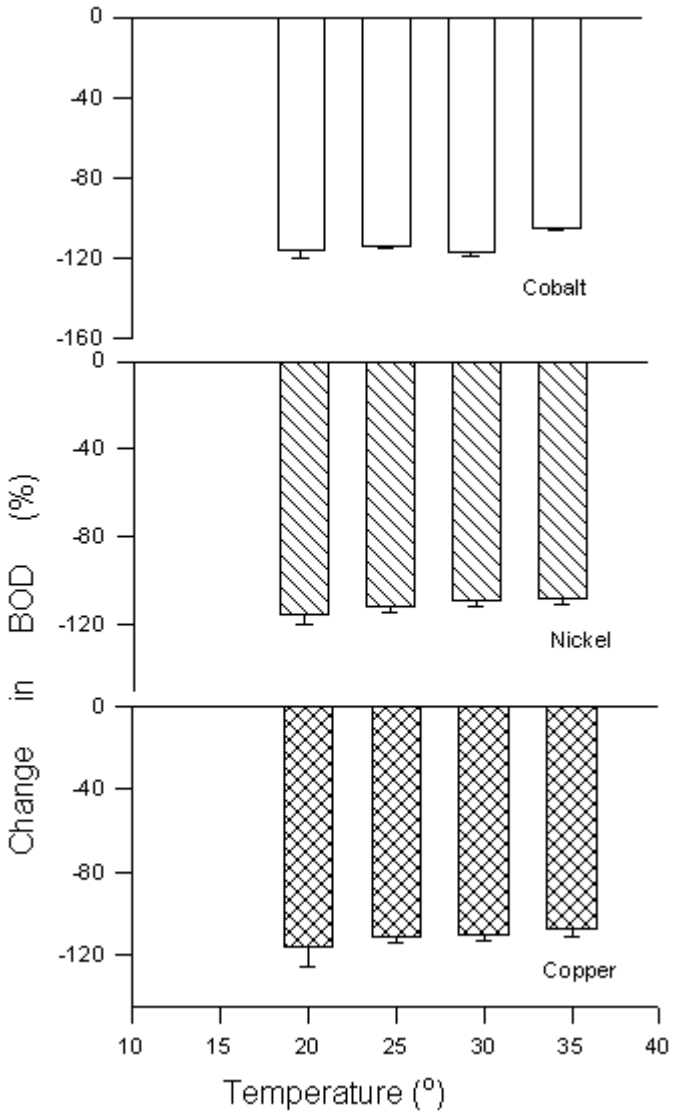

Figure 4.9(b) Bar charts for change in BOD (%) in presence of cobalt, nickel and copper ions a different temperatures using *distillery microbes* **as a seed**

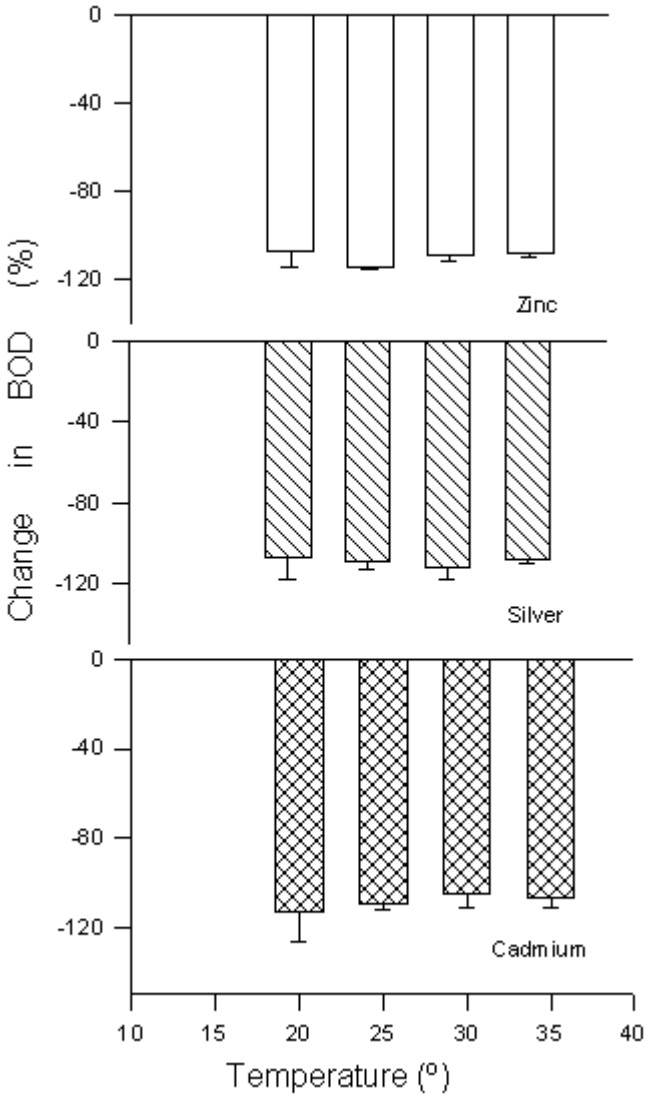

Figure 4.10(b) Bar charts for change in BOD (%) in presence of zinc, silver and cadmium ions at different temperatures using *Distillery microbes* **as a seed**

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Chapter – 5

BOD EXERTION IN PRESENCE OF HEAVY METAL IONS USING *Bacillus brevis* AND *Alcaligenes odorans* AS SEED

BOD exertion is sometimes not affected by the presence of certain levels of metal ions i.e., the threshold of tolerance of microbes towards heavy metals is different and depends on the nature of the metal ion. Stones (1981) measured the percentage suppression caused by 1 mgL^{-1} of selected heavy metals dosed in the form of inorganic salts, on the BOD of domestic sewage. Kansal and Mehrotra (1982) studied the effect of different concentrations of cadmium ions on two wastes of different nature. Schubert (1980) showed that even for easily biodegradable material sub-toxic concentrations of toxic material can inhibit biodegradation. Copper inhibition of treatment process is found to show synergetic effect (Admeroti, 1988) with nickel and zinc. Since on the addition of certain heavy metals, BOD of certain industrial waste water and domestic waste water may be suppressed, hence a systematic study on the effect of heavy metal ions on BOD of waste water is needed to understand the mechanism involved in the process of BOD.

In an industrial effluent, the microflora present is of mixed type. Different microbes have different levels of affinities for metal ions (Dean and Lynne, 1982) and it is desirable to first isolate and identify each one of all the important colonies that might be present in the effluent. The purified degrading enzymes, Nitrilase, Azoreductases and Oragnophosphate hydrolases could be effectively used in industry for the treatment of effluents (Lalithakumari, 2007).

Microbes are used to degrade the organic matter by the utilization of oxygen. The sources of heavy metals in municipal wastewaters include industrial discharges, water

distribution system corrosion and urban storm water runoff (Klein et al, 1974). As a result, municipal (and industrial) wastewater treatment plants are a logical focal point for the control of heavy metals discharged to the aquatic environment.

One of the important colonies from a domestic wastewater was isolated and identified as *Bacillus brevis*. It was grown in a suitable minimal medium. *Bacillus brevis* is found in soil, air dust, milk and cheese. It is a soil living and commonly used in bioremediation, typically endospore, naturally competent, gram positive, aerobic or facultative aerobic bacillus. *Bacillus brevis* is a gram positive, rod shaped and stable in the temperature range 25° to 55° and pH range 6.8 to 11.0. *Bacillus brevis* found in olive oil wastewater (Tenreiro et al*.,* 2006).

A bacterial strain, *Alcaligenes odorans*, has been isolated, by enrichment from soil, using lantadene A, the pentacyclic triterpenoid from lanatana plant, as sole carbon source (Sharma et al., 2000). This strain is gram negative, motile, catalase positive and is capable of utilizing LA.

MATERIAL AND METHODS

Methodology and Test Set-Up (Same as discussed in previous chapter 4)

Seed

Two wastewater samples were used as seed for BOD measurements. One was collected from local sewage treatment plant, Rajpura and another was collected from aeration tank of effluent of treatment plant of pulp & paper industry. Then they were incubated at 37° for 6 hours for acclimation. More and more sample water was added to the concentrated water being digested so as to grow the bacteria which could acclimatize thoroughly in the wastewater environment. The microbiology of the digested sample was done to locate different colonies of bacteria present in the sample. Plate count test was performed for

each sample. A number of colonies were observed on the plate. A prominent colony is picked up from each sample and slants were prepared. Slants of the specimen were sent to Institute of Microbiology Technology (IMTECH), Chandigarh for its identification. Specimens were identified as *Bacillus brevis* and *Alcaligenes odorans.* Each colony was cultured in a rich medium and grown at 37° in an incubator. *Bacillus brevis* and *Alcaligenes odorans* were used as seed for the BOD measurements and their growth were noticed through absorbance (OD) measurements in the absence / presence of identified metal ions.

RESULTS AND DISCUSSION (*Bacillus brevis* used as a seed)

Optical Density Measurements

The bacteria were grown in presence of different concentrations of each one of the cobalt, nickel, copper, zinc, silver and cadmium ions. Bacterial growth was monitored by measuring optical density at 600nm in presence of metal ions at different concentrations and results are shown in Table 5.1(a) and Figures 5.1(a)-5.6(a). In presence of larger concentration of a metal ion the optical density is decreased. This is true for all the metal ions and indicates that with increase in concentration of the metal ion larger number of microbes are complexed or are mutated and do not contribute towards the optical density values *(optical density in the absence of metal ion is the maximum).* These results establish the fact that the presence of metal ions does affect the microbial concentration in the medium. The minimum inhibitory concentration (MIC) of metal ions tolerated by *Bacillus brevis* are shown in Table 5.2(a). Similar metal – microbe complexation processes are taking place in BOD bottles.

Table 5.1(a) Optical density measurements for the growth of *Bacillus brevis* in presence/ absence of different metal ions at different concentrations

Concentration (mML^{-1})	ABSORBANCE (OD)					
	Cobalt	Nickel	Copper	Zinc	Silver	Cadmium
0.0	1.4	0.9	1.5	1.7	1.3	1.8
0.2	1.4	0.8	1.3	1.7	0.1	1.3
0.4	0.9	0.8	1.1	1.6	0.1	0.2
0.6	0.9	0.7	1.0	1.6	0.1	0.1
0.8	0.7	0.5	1.0	1.6	0.1	0.1
1.0	0.6	0.3	1.0	1.5	0.1	0.1
1.2	0.4	0.3	1.0	1.4	0.1	0.1
1.4	0.0	0.2	1.1	1.4	0.1	0.1
2.0	0.0	0.0	0.4	1.2	0.1	0.1
2.5	0.0	0.0	0.2	1.1	0.1	0.1
3.0	0.0	0.0	0.1	0.4	0.1	0.1
3.5	0.0	0.0	0.0	0.3	0.1	0.1
4.0	0.0	0.0	0.0	0.3	0.1	0.1
4.5	0.0	0.0	0.0	0.1	0.1	0.1
5.0	0.0	0.0	0.0	0.1	0.1	0.1

Table 5.2(a) Minimal Inhibitory concentration (MIC) of some heavy metal ion for *Bacillus brevis*

Metal ions	MIC, mML^{-1}	Metal ions	MIC, mML^{-1}
Cobalt	3.0	Zinc	1.0
Nickel	4.5	Silver	0.2
Copper	3.0	Cadmium	0.2

BOD Measurements

BOD (300mL) was measured by using a fixed amount of *Bacillus brevis* added to each bottle containing different concentrations of each metal ion. Results of BOD exertion (percent change in BOD) in presence of metal ions i.e., suppression/ increase is shown in Table 5.3(a). BOD suppression/ increase are plotted as a function of metal ion concentration and results are shown in Figures 5.1(a)-5.6(a). BOD inhibition as observed from the figures is supported by the optical density measurements, i.e., metal-microbe interactions taking outside the bottle. As a result of metal-microbe complexation, the available microbial matter in the BOD bottle is reduced. Lesser the microbial matter, lesser will be the exertion of BOD. Hence, suppression in BOD is observed. Besides other factors like pH and temperature, the magnitude of suppression would also depend on the extent of metal-microbe interactions.

Optical density measurements can be an indicator of the metal – microbe interactions taking place in a BOD bottle but does not give a complete picture of it, as in BOD exertion process, a sufficient amount of dissolved oxygen is ensured while in optical density measurement, there are no such arrangements. For a given metal ion, the microbial population decreases very fast as observed from optical density values upto a concentration range of 1mM - 3mM (except for silver and cadmium for which the microbial concentration falls at 0.04 mM and 0.5 mM, respectively), depending upon the metal ion.

Figures 5.1(a)-5.6(a) show plots of percentage inhibition (primary axis) and optical density (secondary axis) taken on y-axis drawn against the metal ion concentration. Each of these figures shows a trend in optical density values as well as percentage inhibition in BOD for different metal ion concentrations. It is difficult to find any relation between fall in BOD and the nature of metal ion vis a vis its position in the periodic table. However, it can be easily inferred that silver shows exceptionally high toxicity towards the microbes.

Table 5.3(a) Percentage change in BOD_3 (27°) in the presence of heavy metal ions at different concentrations using *Bacillus brevis* as a seed

Concentration (mML^{-1})	Change in BOD (%)					
	Cobalt	**Nickel**	**Copper**	**Zinc**	**Silver**	**Cadmium**
0.2	(-) 13.0	(-) 31.0	(-) 3.0	(+) 31.2	(-) 100.0	(-) 31.0
0.4	(-) 17.4	(-) 40.4	(-) 8.0	(+) 25.6	(-) 100.0	(-) 39.0
0.6	(-) 22.0	(-) 45.0	(-) 3.0	(+) 18.3	(-) 100.0	(-) 42.0
0.8	(-) 28.6	(-) 51.5	(-) 13.5	(+) 9.4	(-) 100.0	(-) 43.0
1.0	(-) 14.3	(-) 69.7	(-) 3.0	(+) 9.4	(-) 100.0	(-) 45.0
1.2	(-) 5.7	(-) 72.0	(-) 3.0	(+) 9.4	(-) 100.0	(-) 49.0
1.4	(-) 8.6	(-) 89.0	(-) 0.0	(+) 3.0	(-) 100.0	(-) 57.0
2.0	(-) 23.0	(-) 84.8	(-) 22.6	0.0	(-) 100.0	(-) 64.0
4.0	(-) 36.0	(-) 97.2	(-) 50.0	(-) 28.1	(-) 100.0	(-) 64.0
6.0	(-) 36.0	(-) 111.0*	(-) 61.9	(-) 90.6	(-) 100.0	(-) 78.0
8.0	(-) 45.5	-	(-) 76.2	(-) 115.6*	(-) 100.0	(-) 78.0
10.0	(-) 50.0	-	(-) 108.3	(-) 121.9*	(-) 100.0	(-) 80.0
12.0	(-) 59.0	-	(-) 122.6	(-) 121.9*	(-) 100.0	(-) 86.0
14.0	(-) 82.0	-	(-) 125.0	(-) 118.7*	(-) 100.0	(-) 96.0

* Percentage change in BOD more than 100% indicates that seed was immobilized or destroyed by the presence of metal ions even to the extent that 'Blank BOD' is not exerted.

In general, a fall in optical density corresponds to an increase in percentage of inhibition in BOD. These observations lead to the conclusion that *Bacillus brevis* does form complex with each of the transition metal ion and hence only an apparent BOD is observed. Metal-microbe interactions in a BOD bottle result to a reduced magnitude in comparison to the direct interaction because of the different environments for the microbes. The extent to which the presence of metal ion affects the BOD exertion process depends upon the concentration and nature of the metal ion (Mittal and Ratra, 2000).

Co(II) – *Bacillus brevis* **Interaction**

In presence of cobalt ions, optical density of *Bacillus brevis* culture shows a sharp fall upto 1.5mM and remains stable thereafter. There is no corresponding sharp decrease in BOD. But an inhibition of only 20% is observed at 2mM concentration of Co(II) ions. Percentage inhibition in BOD increases with increase in metal ion concentration upto 14mM (Figure 5.1(a)). This may be probably due to the reason that the metal forms its oxide than combining with the microbe and affects the BOD exertion process to a small extent. Hence, a strong suppression in BOD is not observed.

Ni(II) – *Bacillus brevis* **Interaction**

When Ni(II) ions are present in the BOD bottle they form complex with *Bacillus brevis* and hence reduce the biochemical activity of the microbes resulting in a corresponding decrease in the dissolved oxygen demand. Figure 5.2(a) shows an excellent correlation with the decrease in optical density with the increase in the inhibition as the metal ion concentration increases. This behaviour is prominent in the concentration range from 0.2 to 2.0mM of Ni(II) ions. The optical activity falls upto 90% of its initial value at 2mM. Correspondingly, there is an inhibition in BOD exertion upto 90% at this concentration.

Cu(II) – *Bacillus brevis* **Interaction**

In case of Cu (II) there is a sharp decline (Figure 5.3(a)) in growth of the microbes with increase in concentration (upto 0.4mM) of metal ion as observed for OD values, indicating an extensive metal-microbe interaction. But, the same trend is not observed during BOD exertion as there is a very small or almost negligible inhibition in BOD. Why such a behavior? It is probably due to an additional demand of oxygen by the uncomplexed Cu(II) ions leading to metal oxide formation. So, the fall in oxygen requirement by the microbes bound by Cu(II) ions is compensated by the oxygen demand for the metal oxide formation. Such an observation has already been reported by the

authors (Mittal and Ratra, 2000). This is due to relatively large value of oxidation potential of Cu(II) ions (i.e., its tendency to get oxidized).

Zn(II)– *Bacillus brevis* **Interaction**

In case of zinc ions, the optical density measurements indicate formation of the metal-microbe complex. But the fall in percentage inhibition decreases as the metal ion is added (Figure 5.4(a)). This trend is in opposition to the expected increase in inhibition with concentration of the metal ion. This behaviour can be related to the nature of the metal ion which is known to posses poor complexing properties (d^{10} system with no vacant d orbital to accommodate electrons from the amino acids of the cell membrane) as compared to other members of the transition metal ion series. Hence, BOD exertion is not affected by the presence of Zn(II) ions and an increase in BOD is noticed.

Ag(I) – *Bacillus brevis* **Interaction**

Results of BOD inhibition in presence of Ag(I) ions show exceptional toxicity (Figure 5.5(a)) for the *Bacillus brevis*. For a very dilute concentration of the metal ion (<0.2mM) there is about 100% inhibition of BOD. The optical density results also firmly support this trend as the optical density values fall sharply from 1.222 at 0.02 mM to 0.055 at 0.04mM. The optical density measurements show high degree of toxicity for *Bacillus brevis*.

Cd(II) – *Bacillus brevis* **Interaction**

Cd(II) forms stable complex with amino acids of microbial cell just like any other transition metal as shown in optical density measurements (Figure 5.6(a)). This fact is also supported by the fall in BOD exertion in presence of the metal ions, although the concentration levels of these two studies do not correspond exactly as the inhibition in BOD exertion cannot be quantitatively linked to optical density measurements.

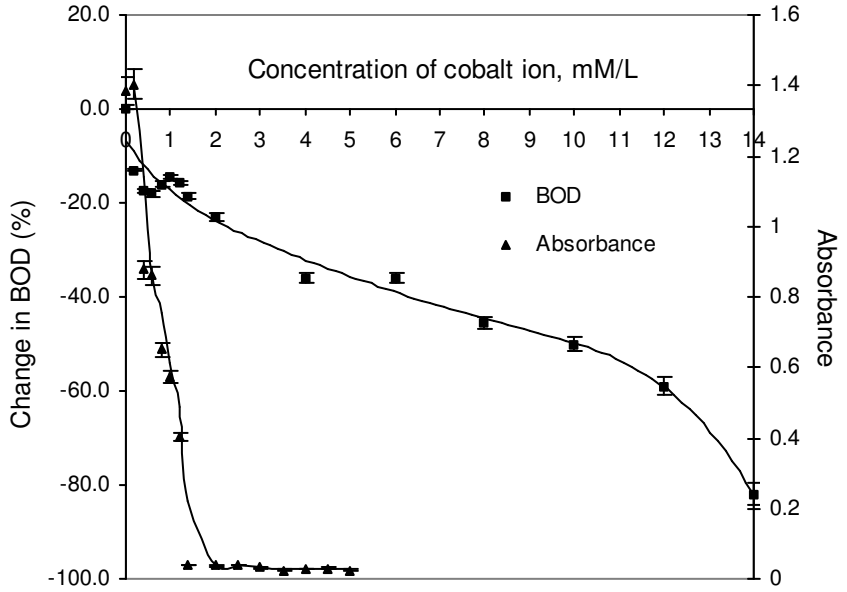

Figure 5.1(a) Plots of Change in BOD (%) and absorbance (OD) as a function of concentration of Co (II) ions using *Bacillus brevis* as a seed

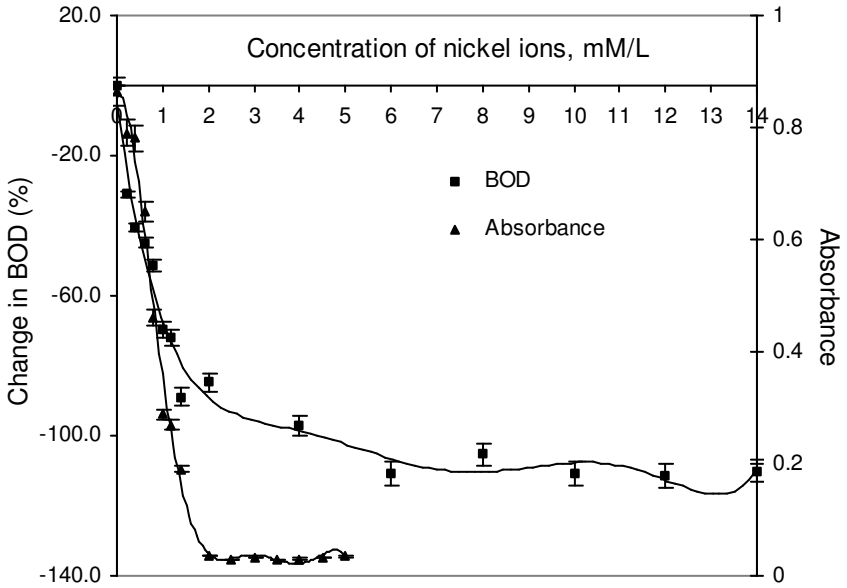

Figure 5.2(a) Plots of Change in BOD (%) and absorbance (OD) as a function of concentration of Ni(II) ions using *Bacillus brevis* as a seed

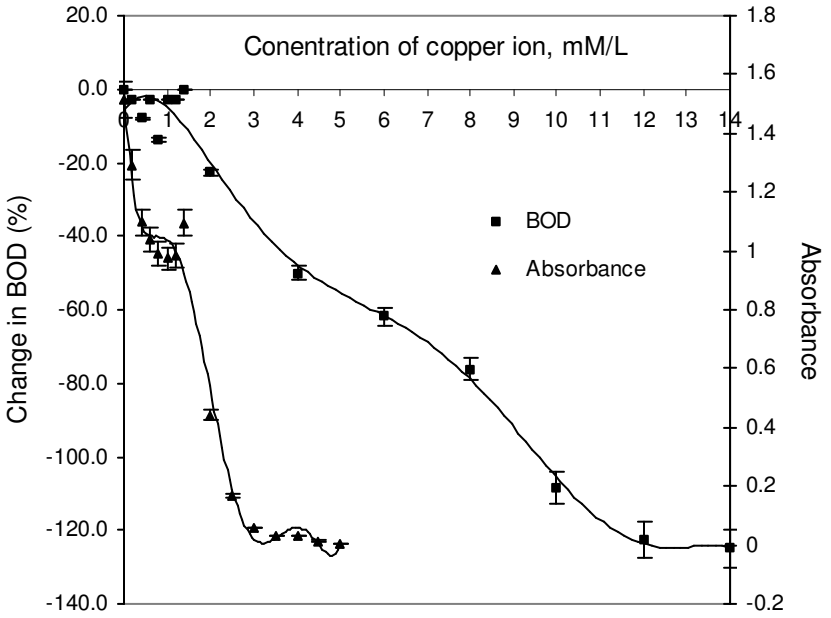

Figure 5.3(a) Plots of Change in BOD (%) and absorbance (OD) as a function of concentration of Cu (II) ions using *Bacillus brevis* as a seed

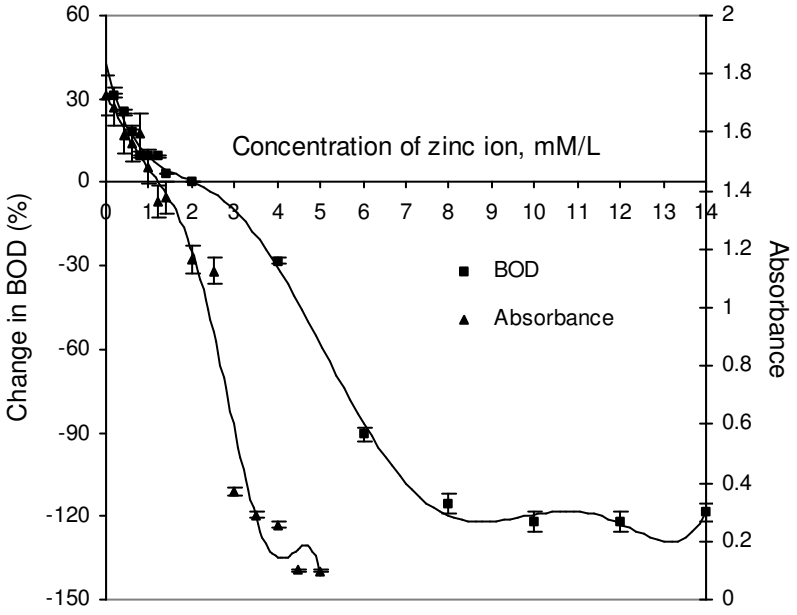

Figure 5.4(a) Plots of Change in BOD (%) and absorbance (OD) as a function of concentration of Zn(II) ions using *Bacillus brevis* as a seed

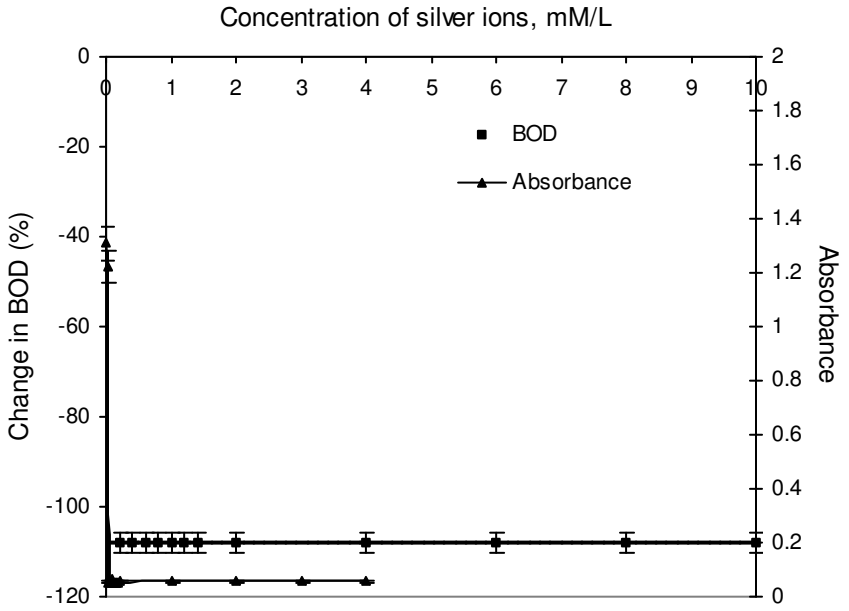

Figure 5.5(a) Plots of Change in BOD (%) and Absorbance (OD) as a function of concentration of Ag (I) ions using *Bacillus brevis* **as a seed**

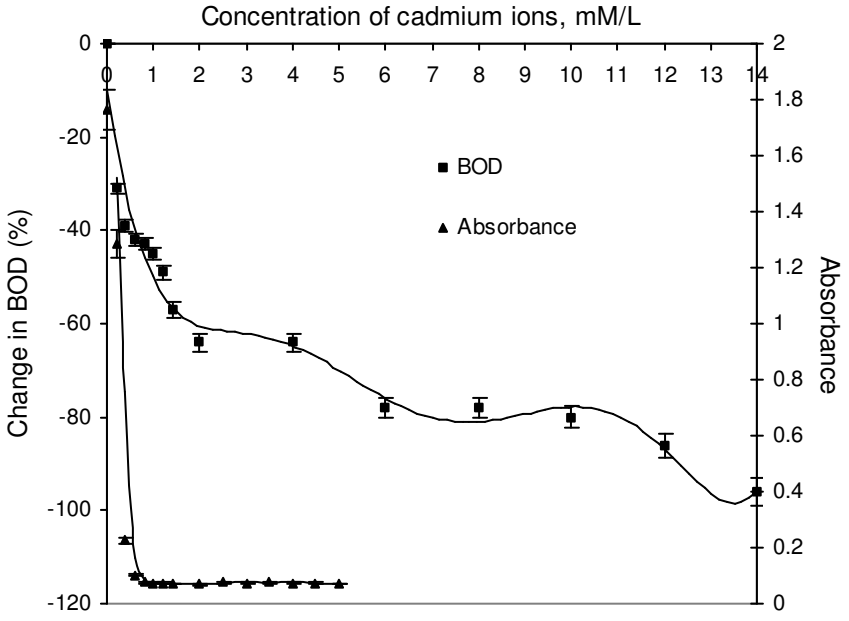

Figure 5.6(a) Plots of Change in BOD (%) and Absorbance(OD) as a function of concentration of Cd(II) ions using *Bacillus brevis* **as a seed**

pH Effect

BOD studies were carried out for *Bacillus brevis* as a seed at different pH 3.0 to 8.0 of the medium. pH of the aqueous medium was maintained by using appropriate amounts of nitric acid or sodium hydroxide. Metal salt buffers were deliberately not used to avoid the introduction of undesirable metal ions in the medium. BOD studies were conducted in presence of 2.0 mML^{-1} of each metal ion. Results are presented in Tables 5.4(a) and 5.5(a) showing the observed absolute values of BOD and the change in the BOD, respectively. Results are also plotted in terms of percentage change in BOD as a function of pH of the medium, separately for each metal ion (Figures 5.7(a) to 5.8(a)). At pH 7, almost all metal ions inhibit BOD to the maximum. Although no regular trend in BOD inhibition is noticed with change in pH but in the basic range a larger inhibition in BOD is observed in comparison to the acidic medium. In the acidic range, minimum inhibition is observed at pH 5.0. Except for zinc, there is a large decrease in BOD when the pH of the medium is 4.0.

It is rather interesting to observe a greater fall in BOD when pH of the medium is maintained in the range >6.0 because metal ions will not be available for complexation with microbes as they are precipitated as metal hydroxides.

It can be explained by proposing a mechanism of adsorption of the microbes on to the metal hydroxides (Jianmin et al 1999). This physical process inhibits the bacteria from its microbial action, hence resulting in less BOD exertion. At a given pH in the acidic range, different extent of inhibition by different metal ion species is due to their different chemical nature.

Table 5.4(a) BOD_3 (27°) in presence of metal ions ($2.0mL^{-1}$) at different pH values using *Bacillus brevis* as a seed

pH	BOD (mg/L)						
	GGA (without metal ion)	**Cobalt**	**Nickel**	**Copper**	**Zinc**	**Silver**	***Cadmium***
3.0	127	73	-10	80	57	0	0
4.0	240	-20	-50	-63	270	-67	-43
5.0	247	70	37	210	259	-47	290
6.0	280	-56	-77	-33	201	-73	-37
7.0	277	-107	-117	-107	-57	-57	-93
8.0	237	-20	-17	30	20	-30	-10

Table 5.5(a) Percentage change in BOD (27°) in presence of metal ions ($2.0mL^{-1}$) at different pH using *Bacillus brevis* as a seed

pH	Change in BOD (%)					
	Cobalt	**Nickel**	**Copper**	**Zinc**	**Silver**	**Cadmium**
3.0	(-) 42.5	(-) 107.9	(-) 37.0	(-) 55.1	(-) 100.0	(-) 100.0
4.0	(-) 108.3	(-) 120.8	(-) 126.3	(+) 12.5	(-) 128.0	(-) 118.0
5.0	(-) 74.1	(-) 86.3	(-) 14.8	(+) 4.8	(-) 119.0	(+) 17.4
6.0	(-) 120.6	(-) 125.1	(-) 110.7	(-) 28.3	(-) 126.1	(-) 112.0
7.0	(-) 139.0	(-) 142.0	(-) 139.0	(-) 120.5	(-) 120.6	(-) 133.6
8.0	(-) 108.4	(-) 107.6	(-) 87.3	(-) 91.6	(-) 112.7	(-) 104.2

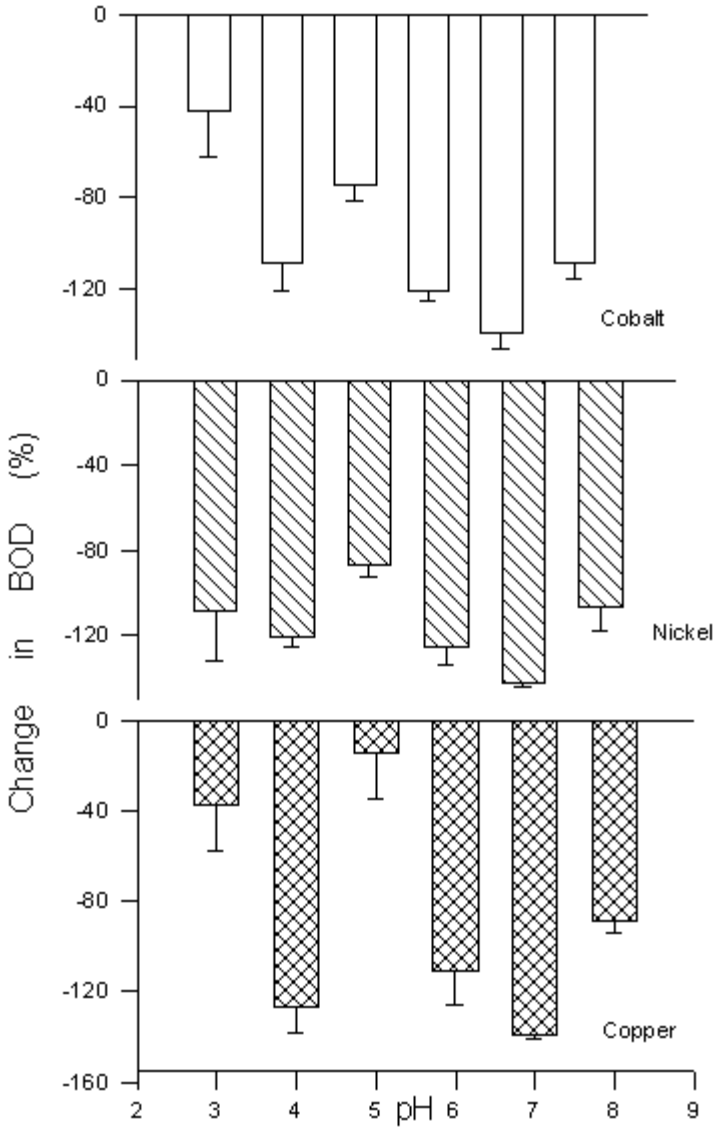

Figure 5.7(a) Bar charts for change in BOD (%) in presence of cobalt, nickel and copper ions at different pH values using *Bacillus brevis* as a seed

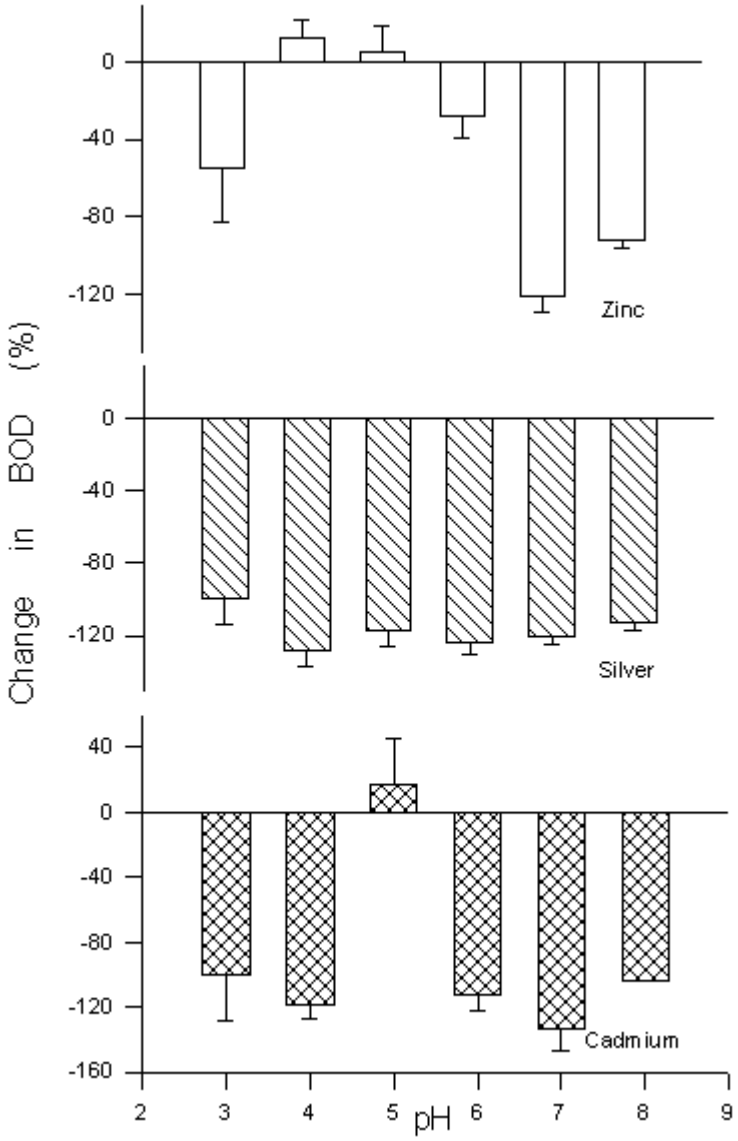

Figure 5.8(a) Bar charts for change in BOD (%) in presence of zinc, silver and cadmium ions at different pH values using *Bacillus brevis* **as a seed**

Temperature Effect

Temperature is one of the important environmental factor known to influence the operation of BOD process (Seth, 1964). The temperature lower than room temperature is expected to slow down the biochemical process, hence a fall in BOD exertion. Studies on BOD measurement were carried out at different temperatures and the effect of each metal ion was observed. Although BOD exertion is likely to be affected due to changed levels of dissolved oxygen at different temperatures (10.07mg/Lat 15°, 9.08mg/L at 20°, 8.27mg/L at 25°, 7.59mg/L at 30°, 7.05mg/L at 35°), the presence of metal ions do have further influence on the BOD exertion. Results of BOD for the system not containing any metal ion are compared at different temperatures in Table 5.6(a). BOD inhibition due to the presence of the metal ions (2.0 mML^{-1}) of each is also reported in Table 5.7(a). Studies were carried out with *Bacillus brevis* as a seed and GGA as the nutrient medium.

BOD exertion increases from 120mg/L at 20° to 540mg/L at 35°. With these values as references, the presence of all metal ions result in a fall in BOD exertion to the extent greater than 100%. There is not much difference in the level of BOD inhibition for different metal ions, however, maximum inhibition is observed at 25° for all the metal ions (Table 5.6(a)).

For all the metal ions, an increase in BOD inhibition is observed when the temperature raised from 20° to 25°. A fall in inhibition observed from 25° to 30° and 35° should be termed as “apparent fall”, as this inhibition is observed besides the effect of nitrifying bacteria which are known to be most active in the temperature range 30° to 40°. In the absence of any such effect the inhibition would have been much stronger than observed. Although no additional nutrients of nitrogen base were added, the bacteria must have used nitrogen of glutamic acid to exert additional BOD. Bacillus brevis bacteria are known to behave as nitrifying microbe as well. (Kenneth Todar). In the primary process, the bacteria exert BOD by stabilizing the glucose as a food. In addition, it fixes nitrogen of glutamic acid which is an amino acid and hence increases oxygen requirement, thereby, resulting in an increase in BOD.

The extent of suppression at different temperature does not change with the nature of the metal ions. Almost similar behaviour is observed for all the metal ions (Figure 5.9(a) to 5.10(a)) It was interesting to note the results of experiments for BOD exertion at 15°. There was practically no change in DO levels at this temperature either in the presence or in the absence of metal ions. It may be due to the reason that microbes are quite dormant below 20°.

Table 5.6(a) BOD_3 (27°) in presence/ absence of metal ions (2.0mML^{-1}) at different temperatures using *Bacillus brevis* as a seed

Temperature (°)	BOD (mg/L)						
	GGA (without metal ions)	Cobalt	Nickel	Copper	Zinc	Silver	Cadmium
15	0	0	0	0	0	0	0
20	120	-16	-30	-27	-27	-23	-27
25	290	-93	-107	-100	-110	-107	-100
30	400	-47	-60	-60	-63	-57	-60
35	540	-67	-77	-73	-63	-83	-67

Table 5.7(a) Percentage change in BOD_3 (27°) at different temperatures for different metal ions (2.0mML^{-1}) using *Bacillus brevis* as a seed

Temperature (°)	Change in BOD (%)					
	Cobalt	Nickel	Copper	Zinc	Silver	Cadmium
15	0	0	0	0	0	0
20	(-) 113.2	(-) 125.0	(-) 122.5	(-) 122.5	(-) 119.2	(-) 122.5
25	(-) 132.1	(-) 136.9	(-) 134.5	(-) 137.9	(-) 136.9	(-) 134.5
30	(-) 111.8	(-) 115.0	(-) 115.0	(-) 115.8	(-) 114.2	(-) 115.0
35	(-) 112.4	(-) 114.3	(-) 113.5	(-) 111.7	(-) 115.4	(-) 112.4

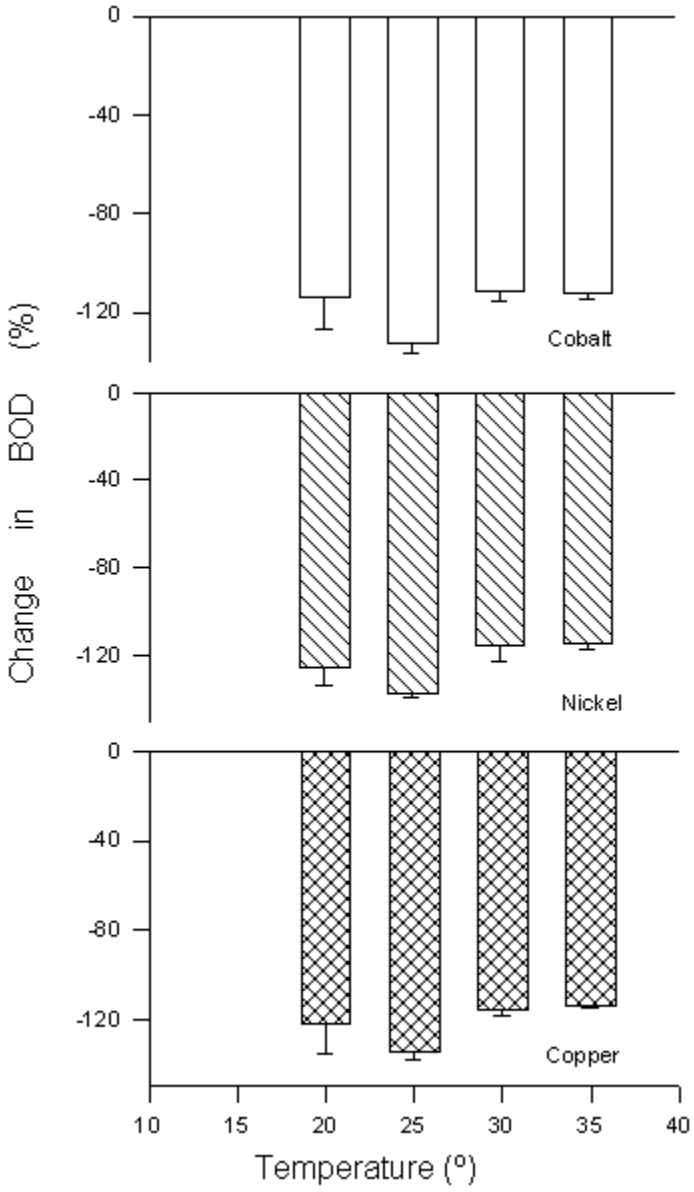

Figure 5.9(a) Bar charts for change in BOD (%) in presence of cobalt, nickel and copper ions at different temperatures using *Bacillus brevis* as a seed

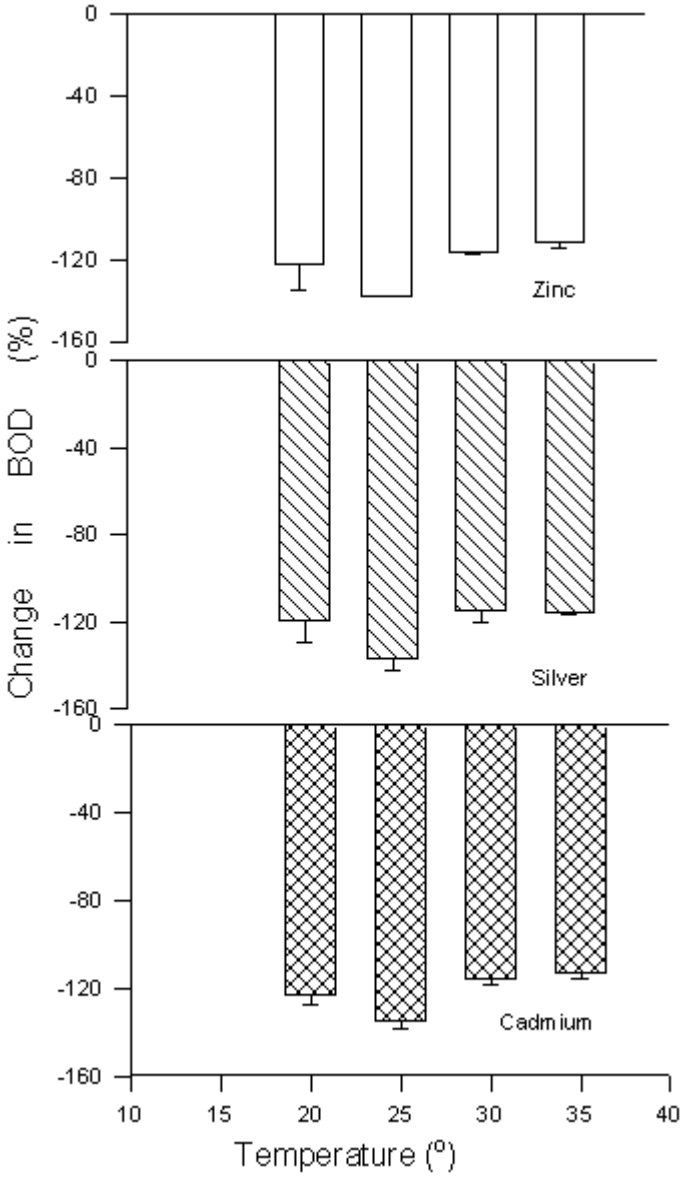

Figure 5.10(a) Bar charts for change in BOD (%) in presence of zinc, silver and cadmium ions at different temperatures using *Bacillus brevis* **as a seed**

RESULTS AND DISCUSSIONS (*Alcaligenes odorans* used as a seed)

Optical Density Measurement

In this study, a representative bacteria from the wastewater of pulp & paper industry was used as a seed for the determination of BOD in the presence of heavy metal ions.

The MIC of certain metal ions was determined using *A odorans* as the microbe. (Table 5.2(a)). Studies were carried out using Luriya medium. Table showing these values indicate that different metal ions behave toxic at different concentration levels. MIC referred to the smallest concentration necessary to inhibit the growth. Thus, lower MIC values indicate more toxicity of metals and higher MIC values represent less toxicity. It is very difficult to explain the order in which the metals exert toxic behavior. It can be due to the reason that the microbes might have developed resistances to different degrees towards such heavy metal ions as showing less toxicity.

Table 5.1(b) Optical density measurements for the growth of *Alcaligens odorans* in presence of different metal ions at different concentrations

Concentration (mML^{-1})	ABSORBANCE (OD)					
	Cobalt	**Nickel**	**Copper**	**Zinc**	**Silver**	**Cadmium**
0.0	1.7	0.8	0.8	2.0	1.4	1.4
0.2	2.2	0.9	0.8	1.8	0.1	1.0
0.4	2.2	0.8	0.8	1.9	0.1	0.1
0.6	1.9	0.8	0.8	1.2	0.1	0.1
0.8	1.8	0.8	0.8	0.2	0.1	0.1
1.0	2.0	0.8	0.8	0.1	0.0	0.1
1.2	1.9	0.7	0.7	0.1	0.0	0.1
1.4	1.8	0.7	0.7	0.1	0.0	0.1
2.0	1.4	0.3	0.8	0.1	0.0	0.1
2.5	0.7	0.3	0.7	0.1	0.0	0.1
3.0	0.1	0.1	0.1	0.1	0.0	0.0
3.5	0.1	0.2	0.1	0.1	0.0	0.0
4.0	0.1	0.1	0.1	0.1	0.0	0.1
4.5	0.1	0.0	0.1	0.1	0.1	0.1
5.0	0.2	0.1	0.1	0.1	0.0	0.1
5.5	0.1	0.1	0.1	0.1	0.1	0.1
6.0	0.1	0.1	0.1	0.1	0.1	0.1
6.5	0.2	0.0	0.1	-	0.0	0.1

Table 5.2(b) Minimal Inhibitory concentration (MIC) of some heavy metal ion for *Alcaligenes odorans*

Metal ions	MIC (mML^{-1})	Metal ions	MIC (mML^{-1})
Cobalt	3.0	Zinc	1.0
Nickel	4.5	Silver	0.2
Copper	3.0	Cadmium	0.2

BOD Measurement

Experiments were conducted to measure inhibition in BOD on the addition of different concentration levels of the heavy metal ions. *Alcaligenes odorans* is a rod like structure and is known to grow in simple nitrogenous environment and is stable upto 42°. It is an aerobic microbe. Our studies reveal that the presence of heavy metal ions like cobalt, nickel, copper, zinc, silver and cadmium show behaviour much different when *A. odorans* is used as a seed in comparison to the wastewater from dairy or distillery. The levels of inhibition in BOD are much lower in presence of copper, zinc and cadmium. The bacteria seem to tolerate difficult concentration levels of metal ions as shown in Table 5.2(b). Cobalt and nickel are more toxic, as about 100% decrease in BOD is noticed in presence of 2.0mM and 1.4mM of these metal ions, respectively. Silver is known to be highly inhibitory in its action even at the smallest concentration of it.

Different levels of tolerance of metal ions may be due to the different complexing nature and their different biochemical preferences for the respective metal ions (Nies, 1999). It can also be due to the different electrochemical nature of metal ions. The metal ions with high reduction potential shall preferably get reduced and will be less toxic for the microbe.

Reduction potential of a metal ion is its tendency to get reduced. It has widely been used as a quantitative parameter of the oxidizing nature of the metal ion. Greater the value of the reduction potential, greater is its oxidizing power, or it can be interpreted in terms of its toxicity power for a given microbial matter. Results as shown in Table 5.3(b) indicate that different concentrations of the metal ions are required to inhibit the BOD exertion. Here, tolerance is compared when BOD is suppressed to the extent of about 100%.

Metal ions can be arranged in the following order of toxicity when BOD inhibition to the extent 100% is taken as a reference for determining the toxicity.

$$Ag^{+}\ (0.2) > Ni^{2+}\ (1.4) > Co^{2+}\ (2.0) > Zn^{2+}(7.0) > Cu^{2+}\ (8.0) > Cd^{2+}\ (10.0)$$

Silver is known to posses extraordinary toxicity towards microbes and reasons for this behavior are much beyond their reduction potential values. Copper due to its variable valency shows inconsistent toxicity towards the microbes. Hence, its toxicity behavior and relation to its reduction potential is not easy to predict. Reduction potential values of nickel, cobalt and cadmium very well explain their toxicity towards the microbes as observed from the levels of inhibition.

Metal ions can be arranged in the following increasing order of their reduction potentials (Skoog and Leavy) i.e., the decreasing order of their toxicity:

Zn^{2+} (-0.763V) >Cd^{2+} (-0.403V) Co^{2+} (-0.277V)>Ni^{2+} (-0.25V) > Cu^{2+} (0.337V) > Ag^{+} (0.799V)

The unexpected low tolerance level for zinc can be correlated to its poor complex forming ability to metal ions like cadmium and mercury.

Table 5.3(b) Percentage change in BOD_3 (27°) in presence of metal ions at different concentrations using *Alcaligenes odorans* as a seed

Concentration (mML^{-1})	Change in BOD (%)					
	Cobalt	**Nickel**	**Copper**	**Zinc**	**Silver**	**Cadmium**
0.2	(+) 17.1	(+) 2.9	(+) 5.7	(+) 6.4	(-) 109.0	(+) 4.7
0.4	(+) 14.3	(-) 11.4	0.0	(+) 2.4	(-) 109.0	(+) 4.7
0.6	(+) 8.6	(-) 45.7	(+) 5.7	(+) 3.3	(-) 109.0	(+) 2.3
0.8	(-) 5.7	(-) 57.1	(-) 2.9	(+) 0.9	(-) 109.0	(+) 4.7
1.0	(-) 17.1	(-) 65.7	(+) 5.7	(-) 0.7	(-) 109.0	(+) 2.3
1.2	(-) 34.4	(-) 88.6	(+) 5.7	(-) 2.4	(-) 109.0	0.0
1.4	(-) 68.6	(-) 97.1	(+) 5.7	(+) 1.6	(-) 109.0	(-) 11.6
2.0	(-) 97.1	(-) 114.3	(+) 5.7	(+) 2.4	(-) 109.0	(-) 18.6
3.0	(-) 108.6	(-) 117.1	(+) 6.1	(-) 28.4	(-) 109.0	(-) 23.3
4.0	(-) 108.6	(-) 108.6	0.0	(-) 69.3	(-) 109.0	(-) 30.2
5.0	(-) 108.6	(-) 117.1	(-) 64.0	(-) 91.3	(-) 109.0	(-) 32.6
6.0	(-) 108.6	(-) 117.1	(-) 91.4	(-) 92.9	(-) 109.0	(-) 32.6
7.0	(-) 108.6	(-) 117.1	(-) 94.0	(-) 108.3	(-) 109.0	(-) 58.1
8.0	(-) 108.6	(-) 117.1	(-) 97.6	(-) 115.8	(-) 109.0	(-) 83.7
9.0	(-) 108.6	(-) 117.1	(-) 108.6	(-) 114.9	(-) 109.0	(-) 89.0
10.0	(-) 108.6	(-) 117.1	(-) 108.6	(-) 115.8	(-) 109.0	(-) 95.6

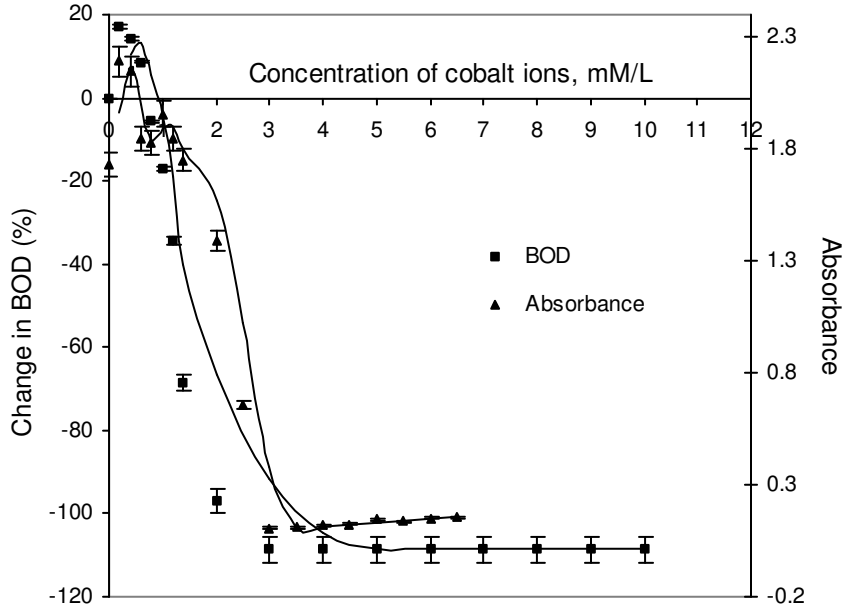

Figure 5.1(b) Plots of Change in BOD (%) and Absorbance (OD) as a function of concentration of Co (II) ions using *Alcaligenes odorans* as a seed

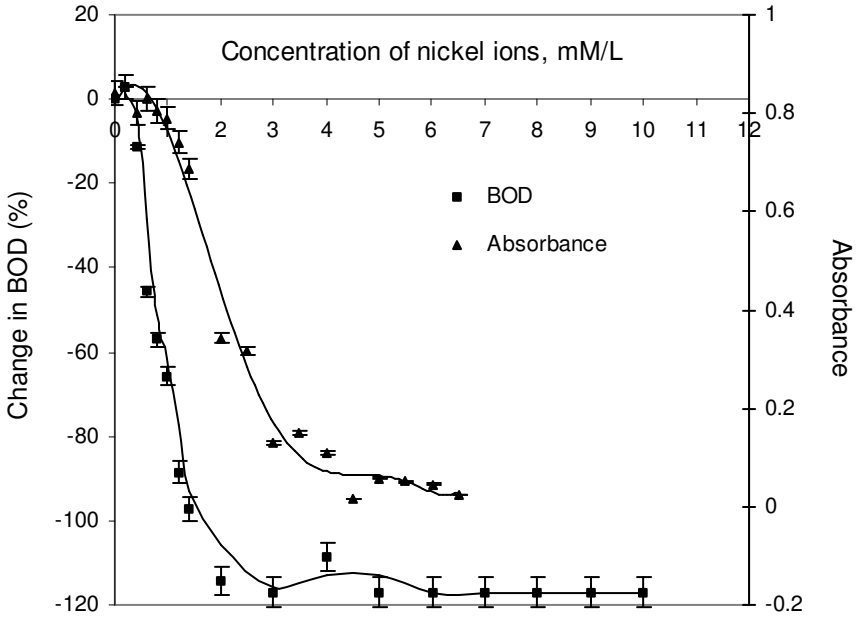

Figure 5.2(b) Plots of Change in BOD (%) and Absorbance (OD) as a function of concentration of Ni(II) ions using *Alcaligenes odorans* as a seed

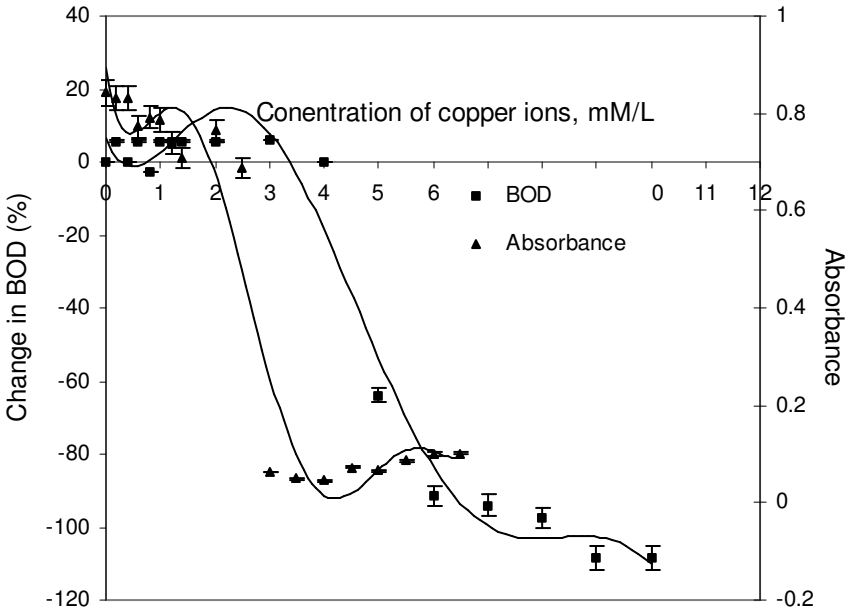

Figure 5.3(b) Plots of Change in BOD (%) and Absorbance(OD) as a function of concentration of Cu(II) ions using *Alcaligenes odorans* **as a seed**

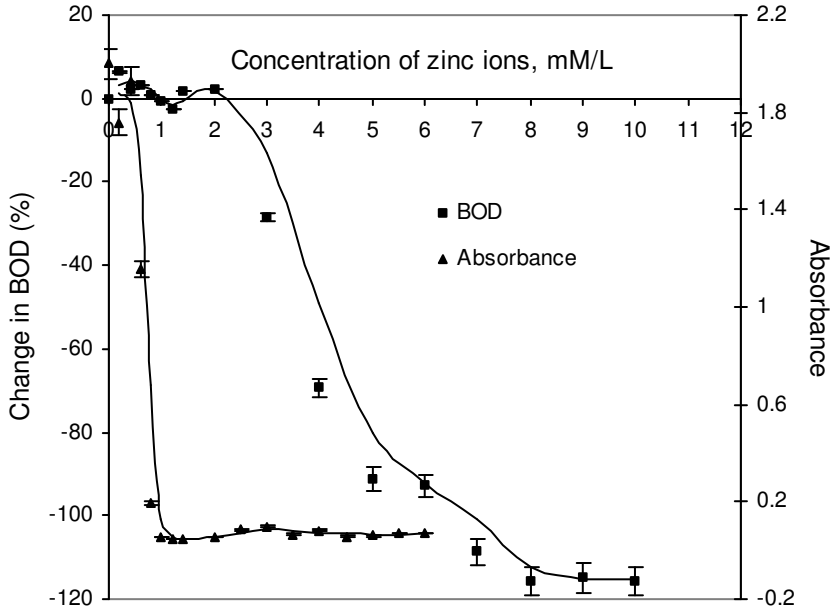

Figure 5.4(b) Plots of Change in BOD (%) and Absorbance(OD) as a function of concentration of Zn(II) ions using *Alcaligenes odorans* **as a seed**

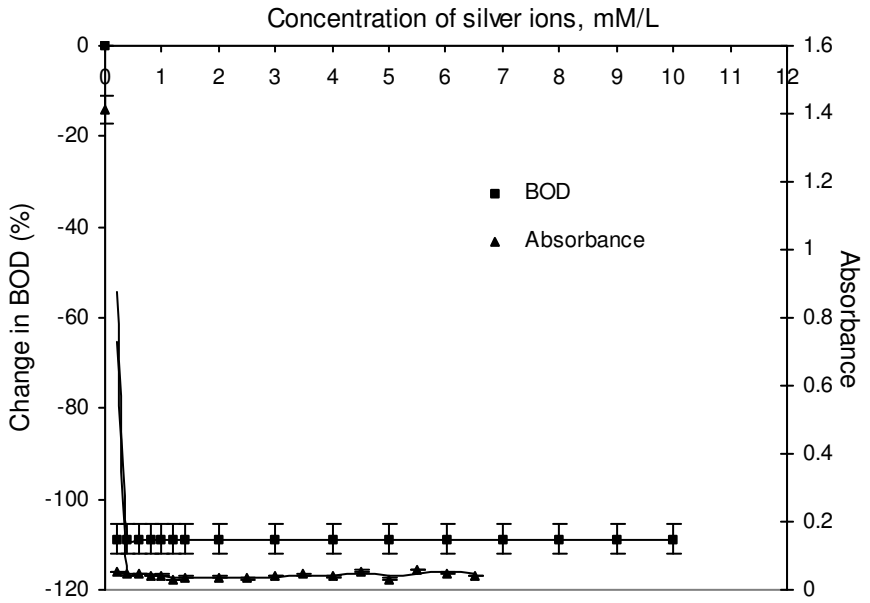

Figure 5.5(b) Plots of Change in BOD (%) and Absorbance (OD) as a function of concentration of Ag (I) ions using *Alcaligenes odorans* **as a seed**

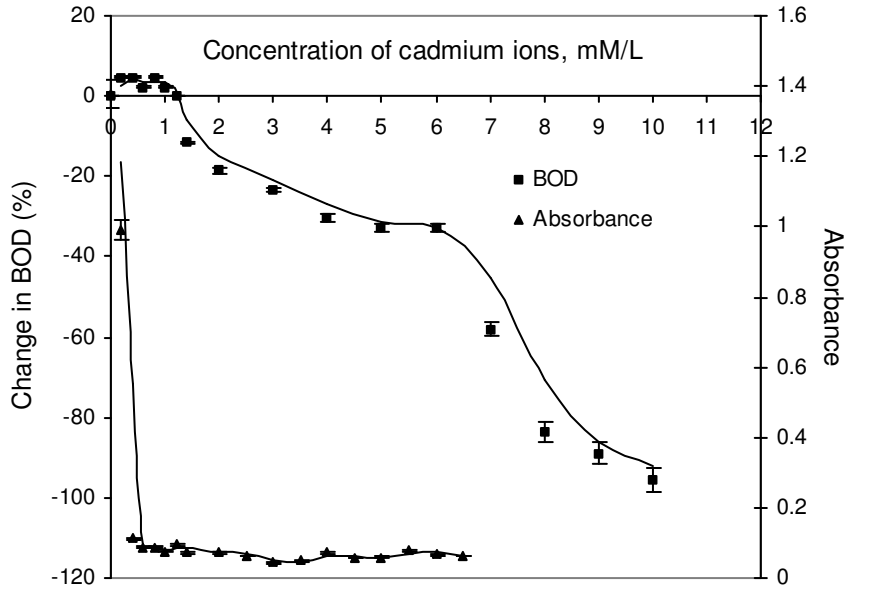

Figure 5.6(b) Plots of Change in BOD (%) and Absorbance (OD)as a function of concentration of Cd(II) ions using *Alcaligenes odorans* **as a seed**

pH Effect

Effect of metal ions on BOD exertion was carried out at different pH values of the medium. pH of the dilution water was controlled by adding requisite amount of HNO_3 or NaOH. BOD in the presence of metal ion was determined by controlling the pH in the range 3.0-8.0. Buffer solutions were not used to maintain the required pH so as to avoid the addition of unnecessary chemicals like nutrients, alkali and alkaline earth metal salts. Results indicate that the presence of metal ions significantly decrease the BOD. Each metal ion behave in a different way because of its different chemical stability at different pH values. From Table 5.5(b), it can be seen very easily that at a given pH, the degree of inhibition vary in very close range except for Zinc and Copper. These two metals have always shown inhibitory effects which are quite different from the rest of the elements.

Bar charts (Figures 5.7(b) and 5.8(b)) indicate that inhibition in BOD is not affected by change in pH in the range 6.0-8.0. Probably, because of similar chemistry of heavy metal ions in this pH range. One observation in acidic medium is that all metal ion species behave in a similar way at pH 3. The results for zinc are anomalous and are difficult to explain.

Table 5.4(b) BOD_3 (27°) in presence/ absence of metal ions ($1.2mML^{-1}$) at different pH values using *Alcaligenes odorans* as a seed

PH	BOD (mg/L)						
	GGA (without metal ion)	**Cobalt**	**Nickel**	**Copper**	**Zinc**	**Silver**	**Cadmium**
3.0	223	-57	-57	-77	183	-67	-77
4.0	440	407	397	165	407	-50	-60
5.0	402	362	55	240	382	-52	145
6.0	480	45	-67	-73	380	-77	-80
7.0	453	-40	-43	-50	263	-37	-43
8.0	430	-57	-57	-27	-13	-33	-53

Table 5.5(b) Percentage change in BOD (27°) in presence of metal ions ($1.2mML^{-1}$) at different pH values using *Alcaligenes odorans* as a seed

PH	Change in BOD (%)					
	Cobalt	**Nickel**	**Copper**	**Zinc**	**Silver**	**Cadmium**
3.0	(-) 125.6	(-) 125.6	(-) 134.5	(-) 17.9	(-) 130.0	(-) 134.5
4.0	(-) 7.5	(-) 9.8	(-) 62.5	(-) 7.5	(-) 111.4	(-) 113.6
5.0	(-) 9.9	(-) 86.3	(-) 40.1	(-) 5.0	(-) 112.9	(-) 63.9
6.0	(-) 90.6	(-) 114.0	(-) 115.2	(-) 20.83	(-) 116.0	(-) 117.0
7.0	(-) 109.0	(-) 109.5	(-) 111.0	(-) 42.0	(-) 108.2	(-) 109.5
8.0	(-) 113.3	(-) 113.3	(-) 106.3	(-) 103.0	(-) 107.7	(-) 112.3

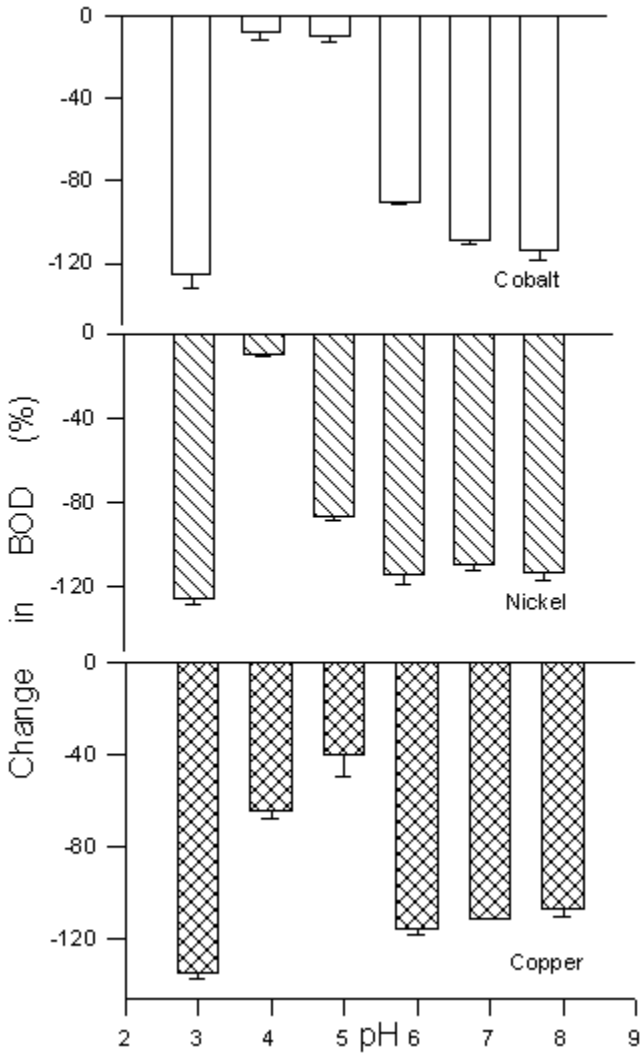

Figure 5.7(b) Bar charts for change in BOD (%) in presence of cobalt, nickel and copper ions at different pH values using *Alcaligenes odorans* **as a seed**

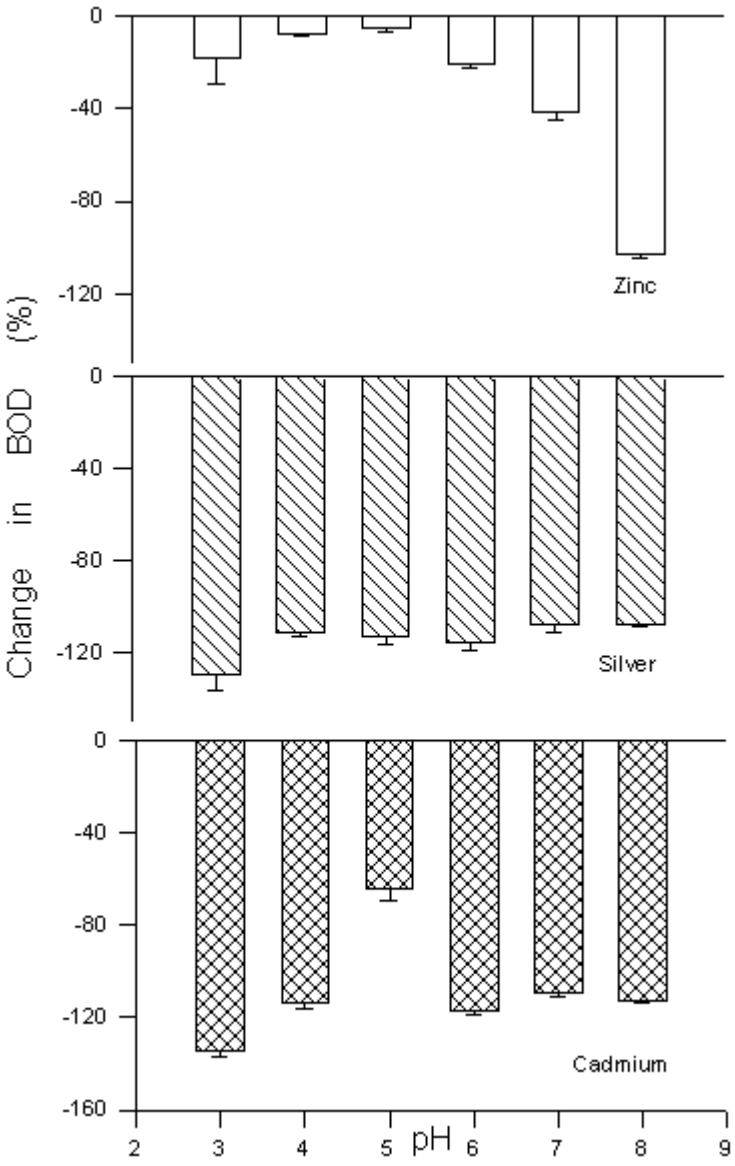

Figure 5.8(b) Bar charts for change in BOD (%) in presence of zinc, silver and cadmium ions at different pH values using *Alcaligenes Odorans* **as a seed**

Temperature Effect

As discussed in previous section, the study of temperature effect on extent of inhibition/increase in BOD is very complicated. This involves in a change in BOD due to the presence of heavy metal ions at different temperatures as well. This change has to be viewed in the background of different DO levels at different temperatures. In the present studies this was observed that BOD exertion does not take place at all at temperatures 15° & 20°. It can be due to the reason that the microbial matter used as a seed in these experiments i.e., A odorans do not survive at these temperatures. From the studies at temperatures 25°, 30° and 35°, BOD inhibition takes place almost to the same extent for all the heavy metal ions.

Bar charts (Figures 5.9(b) and 5.10(b)) clearly show that the presence of all the metal ions lead to an inhibition of about 120% at 25°, 30° and 35°. It can be due to the similar chemistry of the metal ions that does not change with temperature in this range.

Table 5.6(b) BOD_3 (27°) in presence/ absence of metal ions ($1.2mML^{-1}$) at different temperatures using *Alcaligenes odorans* as a seed

Temperature (°)	BOD (mg/L)						
	GGA (without metal ion)	**Cobalt**	**Nickel**	**Copper**	**Zinc**	**Silver**	**Cadmium**
15	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0
25	257	-83	-80	-83	-80	-73	-73
30	285	-47	-53	-53	-47	-57	-57
35	373	-70	-87	-50	-50	-70	-70

Table 5.7(b) Percentage change in BOD (27°) in presence of metal ions ($1.2mML^{-1}$) at different temperatures using *Alcaligenes odorans* as a seed

Temperature (°)	Change in BOD (%)					
	Cobalt	**Nickel**	**Copper**	**Zinc**	**Silver**	**Cadmium**
15	0	0	0	0	0	0
20	0	0	0	0	0	0
25	(-) 132.3	(-) 131.1	(-) 132.3	(-) 131.1	(-) 128.4	(-) 128.4
30	(-) 116.5	(-) 118.6	(-) 118.6	(-) 116.5	(-) 120.0	(-) 120.0
35	(-) 118.8	(-) 123.3	(-) 113.4	(-) 113.4	(-) 118.8	(-) 118.8

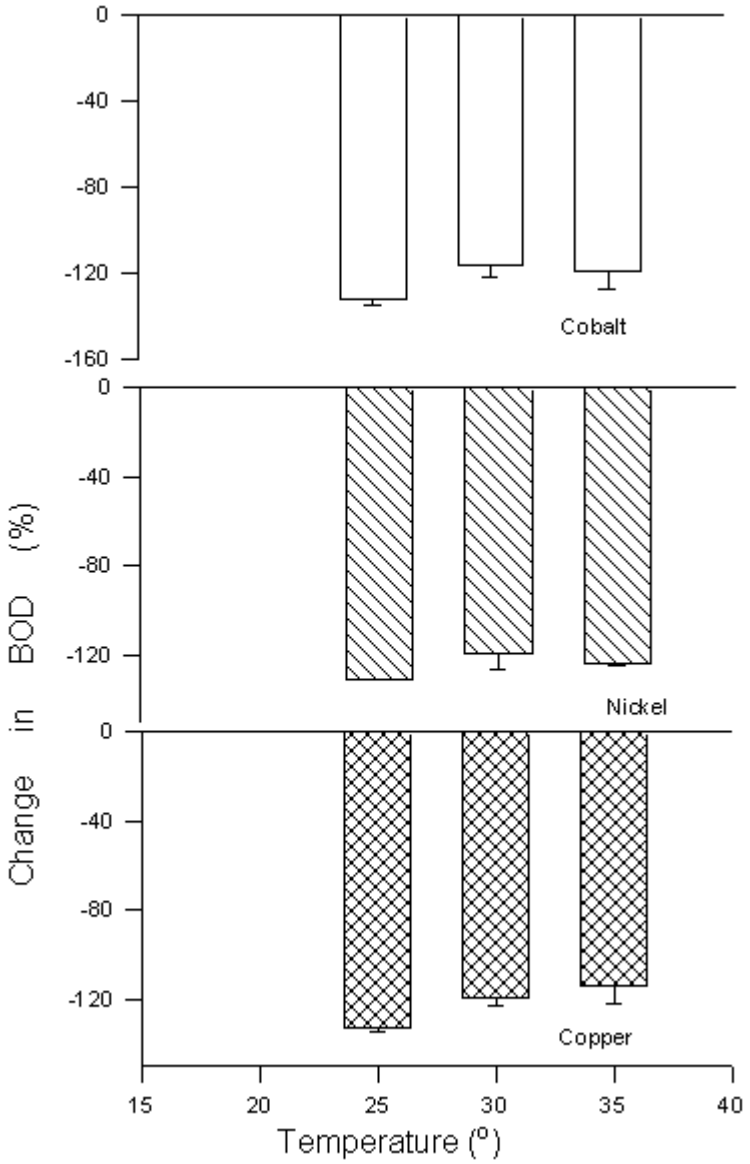

Figure 5.9(b) Bar charts for change in BOD (%) in presence of cobalt, nickel and copper ions at different temperatures using *Alcaligenes odorans* **as a seed**

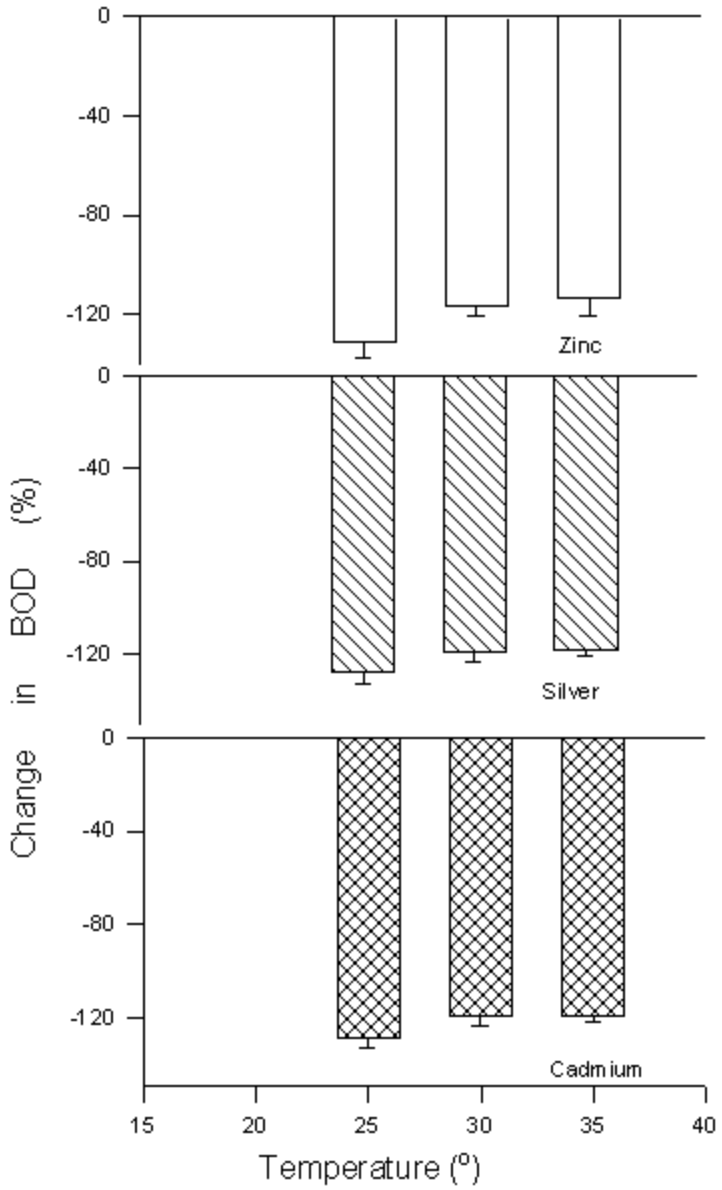

Figure 5.10(b) Bar chart for change in BOD (%) in presence of zinc, silver and cadmium ions at different temperatures using *Alcaligenes odorans* **as a seed**

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Summary

BOD Exertion in Presence of Metal Ions Using Microbes from Dairy and Distillery Wastewater as Seed

Dairy Wastewater

Metal Ion Effect

In the normal BOD measurement process, microbial matter is taken from some source which is known to be rich in bacteria and capable of undergoing BOD exertion process. It has been established that the type of inoculum becomes important for a study where BOD exertion is measured at different concentrations of heavy metal ions. Wastewaters were digested to grow the bacteria, separately, in Luria broth (LB) medium and then used as seed.

Experiments were conducted to determine BOD in presence of some identified heavy metal ions i.e. cobalt, nickel, copper, zinc, silver and cadmium using mixed flora as seed collected from dairy wastewater and distillery wastewater developed in the LB medium.

The metal ions were taken in a wide concentration range from 0.2 mM to 10.0 mM to study their effect at different levels of concentrations. A standard mixture of Glucose-glutamic acid (1:1) was taken as food. Industrial wastewater was not used as source of food simply to avoid any inconsistency in the composition of the sample water.

Experiments were also conducted to measure optical density at 600nm of microbial medium containing different concentrations of the metal ion. Microbes are expected to show greater optical density in the absence of metal ions while in presence of the metal ions, there is a fall in the absorbance values. The extent of fall depends on the concentration of the metal ions added. In most of the cases, changes in absorbance values correspond to the corresponding changes in BOD. This indicates that the inhibition in BOD is due to the decay of microbial matter. This hypothesis works very well for almost all metal ion environments. Hence, the extent of BOD suppression/ increase is directly related to the decay/ growth of the microbial matter.

BOD values were determined in replicates of three bottles for each set of combination of metal ion concentration and by measuring DO levels using membrane based DO meter. Only average values were taken for further calculations. A decrease in BOD is noticed in presence of all the metal ions. For most of the metal ions concentration level upto 1.2mM is good enough to inhibit 100% BOD. However, a presence of zinc ions at these levels results in very less fall in BOD to the extent 5 to 6 % only. Even the larger concentration (10.0 mM) results a decrease in BOD only to the 50%.

Percentage change in BOD is plotted as a function of concentration of the metal ion. The presence of silver ions results in a sharp fall in BOD as more than 100 percent inhibition is observed even in the presence of the smallest amount of the metal ion. An extraordinary toxicity with silver is observed in comparison to other transition metal ions like Co^{2+}, Ni^{2+} Cu^{2+}, Zn^{2+}, Cd^{2+}. The monovalent silver cation forms a more stable complex with sulphur of microbial cell walls and solubility product of Ag_2S is $6.62x10^{-50}$ but for CuS, it is $1.28x10^{-36}$, which makes silver highly toxic. The relatively poor toxic behavior of zinc may be due to its chemical nature. Metal ions generally complex through -SH functional groups of enzymes present in the cell wall. Cadmium is more toxic than zinc because the solubility product of CdS is 1.4×10^{-29} while that of ZnS is 2.91×10^{-25}. Mechanisms of cadmium toxicity in microorganisms are still not well defined. In general, cadmium enters the cell only by some indirect means of transportation like manganese uptake system and calcium uptake system. There are reports where resistance to cadmium in bacteria is noticed. In our observation also, some degree of resistance to cadmium toxicity is noticed. After showing an increase in inhibition upto 3mM of cadmium concentration, a decrease in inhibition from 35% to 25% is observed.

pH Effect

pH is one of the most important parameters that can influence the growth of microbes and hence the BOD. Metal ions are available as different species when observed at different pH values. For example, when pH of the metal salt solution is raised to the basic medium the metal ions precipitate as hydroxides and behave differently towards the microbes. Experiments were carried out by changing pH of the system in the range 3 – 8. Maximum BOD is observed for almost all metal ions between pH 5 and 6. Suppression in BOD at a

given pH is reported for each metal ion (0.8 mML^{-1}) taking BOD for GGA system as the reference.

In the acidic range, suppression is maximum at pH 3.0 for all metal ions except copper. BOD is suppressed to the extent nearly 100% at pH 6.0 except for nickel and zinc. The behaviour of zinc ions has always been different from that of other transition elements. Studies were extended to see the effect of medium on the BOD suppression in the basic medium.

Temperature Effect

Effect of temperature was studied by carrying out incubation at different temperatures i.e., 15°C, 20°C, 25°C, 30°C and 35°C. Because of the variable solubility of oxygen in water, different values of BOD were observed for the GGA system. Different metal ions influence BOD to different extent at a given temperature. Although all metal ions inhibit BOD exertion at all temperatures except 15°C to the extent of 100%, the inhibition is maximum at 25° C.

The increase in BOD with the increase in temperature for the blank system may be due to greater activity of microbes at higher temperatures. There seems to be significant effect on BOD inhibition due to the presence of metal ion. The little variation in BOD exertion from 15° C to 35° C can be associated to the microbial population, which is dependent on change in temperature at a given temperature.

At 15°C, no BOD exertion was noticed for the blank system as well as the system that containing metal ions. It is also seen that in BOD curve, substantial depletion of DO takes place only after 5^{th} day of incubation (20°C).

Distillery Wastewater

Metal Ion Effect

Results of optical density (OD) measurement and percentage change in BOD are presented simultaneously to corelate change in BOD with that of microbial growth.

Out of all metal ion studied, the presence of silver ions is found to be most toxic as also observed for system using distillery waste as a seed. In general, there has been a reasonably good co-relation between the OD value and corresponding %age change in BOD. However, there are indications of resistance being developed towards the presence of metal ions. It is really very difficult to quantify the resistances and co-relation to the concentration levels of metal ions. At the most, it can be linked to the nature of the metal ion. The minimum inhibitory concentration (MIC) of metal ions tolerated by mixed flora from dairy and distillery wastewater through OD and BOD measurements.

pH Effect

BOD of synthetic samples maintained at different pH values were measured in the absence and presence of each metal ion separately. Experiments were conducted by maintaining a pH in the range 3.0 to 8.0. 2.0mML^{-1} of the metal ion was maintained in each sample bottle. At this selected concentration, the metal ions exert toxicity to a reasonable extent, as observed. BOD undergoes a substantial decrease to the extent greater than or equal to 100% in the presence of the almost all metal ions. The exception in the observation is in the presence of zinc ions which has already been discussed previously.

The metal - microbe complexes are sensitive to change in pH of the medium. Hydrogen ion concentration or pH is probably the single most important factor influencing the metal ion adsorption on both organic and inorganic surfaces. Metal speciation is significantly affected both from hydroxyl complexation and change in protonation level of the complex.

Temperature Effect

Results of temperature effect on BOD for both the seeds are similar. BOD is inhibited to the extent 100 to 120 % and there is a little variation with change of the metal ion. At 15°C, BOD exertion does not take place at all which may be due to the reason that microbes are not active at this temperature.

BOD Exertion in Presence of Metal Ions using *Bacillus brevis* and *Alcaligenes odorans* as Seed

Bacillus brevis

Metal Ion Effect

In an industrial effluent, the microflora present is of mixed type. Different microbes have different levels of affinities for metal ions and therefore, it is desirable to first isolate and identify each one of all the important colonies that might be present in the effluent. Samples were collected from local sewage treatment plant, Rajpura and pulp & paper industry. Then it was incubated at 37°C for 6 hours for acclimation. 1 mL of each sample is taken for plate count test. A number of colonies are observed on the plates. Important and prominent colonies from both types of wastewaters were isolated and got identified as *Bacillus brevis* from sewage treatment plant and *Alcaligenes odorans* from pulp & paper industry. The microbes were cultured in a rich medium and grown at 37°C in incubator. *Bacillus brevis* and *Alcaligenes odorans* were used as seed for the BOD measurements. Separate experiments were conducted to monitor the growth of microbes in the presence of different concentration of metal ions added to LB medium by measuring optical density of each system. Measurements were also made in the absence of metal ions.

In presence of large concentration of a metal ion, the optical density is decreased. This is true for all the metal ions and indicates that with increase in concentration of the metal ion larger number of microbes are complexed or are mutated and do not contribute towards the optical density values. The minimum inhibitory concentration (MIC) of metal ions tolerated by *Bacillus brevis* and *Alcaligenes odorans* are shown in Table 1. Similar metal – microbe complexation processes are taking place in BOD bottles.

Results of BOD inhibition are supported by the optical density measurements, i.e., metal-microbe interactions taking outside the bottle. As a result of metal-microbe complexation, the available microbial matter in the BOD bottle is reduced. Lesser the microbial matter, lesser will be the exertion of BOD. Hence, suppression in BOD is observed. Besides

other factors like pH, temperature, etc., the magnitude of suppression would also depend on the extent of metal-microbe interactions.

Optical density measurements may not give a complete picture of metal – microbe interactions, as in BOD exertion process a sufficient amount of dissolved oxygen is ensured while in optical density measurement, there are no such arrangements. For a given metal ion, the microbial population decreases very fast as observed from optical density values upto a concentration range of 1mM - 3mM (except for silver and cadmium for which the microbial concentration falls at 0.04 mM and 0.5 mM, respectively), depending upon the metal ion. It can be easily inferred that silver shows exceptionally high toxicity towards the microbes.

In general, a fall in optical density corresponds to an increase in percentage of inhibition in BOD. These observations lead to the conclusion that *Bacillus brevis* does form complex with each of the transition metal ion and hence only an apparent BOD is observed. When Ni(II) ions are present in the BOD bottle they form complex with *Bacillus brevis* and an excellent correlation is seen with the decrease in optical density with the increase in the inhibition as the metal ion concentration increases. In case of Cu (II), there is a sharp decline in growth of the microbes with increase in concentration of metal ion, indicating an extensive metal-microbe interaction. But, the same trend is not observed during BOD exertion as there is a very small or almost negligible inhibition in BOD. In case of zinc ions, the optical density measurements indicate formation of the metal-microbe complex. But the fall in percentage inhibition decreases as the metal ion is added. This trend is in opposition to the expected increase in inhibition with concentration of the metal ion.

Results of BOD inhibition in presence of Ag(I) ions show exceptional toxicity for the *Bacillus brevis*. The optical density measurements also show high degree of toxicity of Ag(I) for *Bacillus brevis*.

Cd(II) forms stable complex with amino acids of microbial cell just like any other transition metal as shown in optical density measurements. This fact is also supported by the fall in BOD exertion in presence of the metal ions, although the concentration levels

of these two studies do not correspond as the inhibition in BOD exertion cannot be quantitatively linked to optical density measurements.

pH Effect

BOD studies were carried out for *Bacillus brevis* as a seed at different pH 3.0 to 8.0 of the medium. pH of the aqueous medium was maintained by using appropriate amounts of nitric acid or sodium hydroxide. Metal salt buffers were deliberately not used to avoid the introduction of undesirable metal ions in the medium. BOD studies were conducted in presence of 2.0 mML^{-1} of each metal ion. At pH 7, almost all metal ions inhibit BOD to the maximum. Although no regular trend in BOD inhibition is noticed with change in pH but in the basic range a larger inhibition in BOD is observed in comparison to the acidic medium. In the acidic range, minimum inhibition is observed at pH 5.0. Except for zinc, there is a large decrease in BOD when the pH of the medium is 4.0. It is rather unexpected to observe a greater fall in BOD when pH of the medium is maintained in the basic range (i.e., pH 7 & 8) because metal ions will not be available for complexation with microbes as they are precipitated as metal hydroxides.

Temperature Effect

The lower temperature is expected to slow down the biochemical process and hence a fall in BOD exertion. Studies on BOD measurement were carried out at different temperatures and the effect of each metal ion was observed. Although BOD exertion is likely to be affected due to changed levels of dissolved oxygen at different temperature, the presence of metal ions do have further influence on the BOD exertion. Results of BOD for the system not containing any metal ion are compared at different temperatures. BOD inhibition due to the presence of the metal ions (2.0 mML^{-1}) of each is also reported. Studies were carried out *Bacillus brevis* as a seed and GGA as the nutrient medium.

For all the metal ions, an increase in BOD exertion when the temperature raised from 20°C to 25°C. A fall in inhibition from 25°C to 30°C and 35°C should be termed as

"apparent fall" as this inhibition exists besides the effect of nitrifying bacteria which are known to be most active in the temperature range 30°C to 40°C.

The extent of suppression at different temperature does not change with the nature of the metal ions. Almost similar behaviour is observed fall all the metal ions. It was interesting to note the results of experiments for BOD exertion at 15°C. There was practically no change in DO levels at this temperature either in the presence or in the absence of metal ions. It may be change due to the reason that *Bacillus brevis* are quite dormant below 20°C. In reports, microbial growth is active in the temperature range 25°C to 55°C *Bacillus brevis* (IMTECH).

Alcaligenes odorans

Metal Ion Effect

Experiments were conducted to measure inhibition in BOD on the addition of different concentration levels of the heavy metal ions. The MIC of metal ions was determined using *A. odorans* as the microbe (Table 1). Studies were carried out using LB medium in *A. odorans*. MIC refers to the smallest concentration necessary to inhibit the growth. Thus, lower MIC values indicate more toxic metals and higher MIC values less toxicity.

Alcaligenes odorans is a rod like structure and is known to grow in simple nitrogenous environment and is stable upto 42°C. It is an aerobic microbe. Our studies reveal that the presence of heavy metal ions like cobalt, nickel, copper, zinc, silver and cadmium show behaviour much different when *A. odorans* is used as a seed in comparison to the wastewater from dairy or distillery. The levels of inhibition in BOD are much lower in presence of copper, zinc and cadmium. The bacteria seem to tolerate different concentration levels of metal ions as shown in Table 1. Cobalt and nickel are more toxic, as about 100% decrease in BOD is noticed in presence of 2.0mM and 1.4mM of these metal ions, respectively. Silver is known to be highly inhibitory in its action even at the smallest concentration of it.

Different levels of tolerance of metal ions may be due to the different complexing nature and their different biochemical preferences for the respective metal ions. It can also be

due to the different electrochemical nature of metal ions. The metal ions with high reduction potential shall preferably get reduced and will be less toxic for the microbe.

pH Effect

Effect of metal ions on BOD exertion was carried out at different pH values of the medium in the range 3-8. The results indicate that the presence of metal ions significantly decrease the BOD. Each metal ion behaves in a different way because of its different chemical stability at different pH values in the range 3 – 6. Results indicate that inhibition in BOD is not affected by change in pH in the range 6 – 8, probably, because of similar chemistry of heavy metal ions in this pH range. In acidic medium, all the metal ion species behave in a similar nature at pH = 3.

Temperature Effect

As discussed in previous chapters, the effect of temperature on the extent of inhibition/ increase in BOD is very complicated. This involves a change in BOD due to the presence of heavy metal ions at different temperatures as well as different concentrations. This change has to be viewed in the background of different DO levels at different temperatures. In the present studies it was observed that BOD exertion does not take place at all at 15°C to 20°C. From the studies at temperatures 25°C, 30°C, 35°C, BOD inhibition takes place almost to the same extent for all the heavy metal ions used.

The presence of the metal ions leads to an inhibition of 120% at 25°C, 30°C, 35°C. It can be due to the similar chemical ions which do not change with temperature in this range. In reports, microbial growth is active in the temperature range 25°C to 45°C for *Alcaligenes odorans*.

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List of Publications

1. S K Mittal and Siloni Goel, 2005 "BOD exertion in presence of some heavy Metal Ions: The pH effect", Pollution Research 24(2): 1-5.

2. S K Mittal, Ajay Sharma and Siloni Goel, 2004 "Effect of temperature on BOD exertion in presence of some heavy metal ions" Int. J. of Environmental Research and Public Health 1(2) 132-137.

3. S K Mittal and Siloni Goel, 2003 "Role of living organisms in BOD exertion in presence of heavy metal ions", Indian Journal of Environment Protection 23(7): 782-784.

4. S K Mittal and Siloni Goel, "Developments In BOD Measurement Techniques", (Submitted).

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Conclusions

Metal Ion Effect

In the normal BOD measurement process, microbial matter is taken from some source which is known to be rich in bacteria and capable of undergoing BOD exertion process. It has been established that the type of inoculum becomes important for a study where BOD exertion is measured at different concentrations of heavy metal ions. Wastewaters were digested to grow the bacteria, separately, in Luria broth (LB) medium and then used as seed.

Experiments were conducted to determine BOD in presence of some identified heavy metal ions i.e. cobalt, nickel, copper, zinc, silver and cadmium using mixed flora as seed collected from dairy wastewater and distillery wastewater developed in the LB medium.

The metal ions were taken in a wide concentration range from 0.2 mM to 10.0 mM to study their effect at different levels of concentrations. A standard mixture of Glucose-glutamic acid (1:1) was taken as food. Industrial wastewater was not used as source of food simply to avoid any inconsistency in the composition of the sample water.

Experiments were also conducted to measure optical density at 600nm of microbial medium containing different concentrations of the metal ion. Microbes are expected to show greater optical density in the absence of metal ions while in presence of the metal ions, there is a fall in the absorbance values. The extent of fall depends on the concentration of the metal ions added. In most of the cases, changes in absorbance values correspond to the corresponding changes in BOD. This indicates that the inhibition in BOD is due to the decay of microbial matter. This hypothesis works very well for almost all metal ion environments. Hence, the extent of BOD suppression/ increase is directly related to the decay/ growth of the microbial matter.

BOD values were determined in replicas of three bottles for each set of combination of metal ion concentration and by measuring DO levels using membrane based DO meter. Only average values were taken for further calculations. A decrease in BOD is noticed in presence of all the metal ions. For most of the metal ions concentration level upto 1.2mM

is good enough to inhibit 100% BOD. However, a presence of zinc ions at these levels results in very less fall in BOD to the extent 5 to 6 % only. Even the larger concentration (10.0 mM) results a decrease in BOD only to the 50%.

Percentage change in BOD is plotted as a function of concentration of the metal ion. The presence of silver ions results in a sharp fall in BOD as more than 100 percent inhibition is observed even in the presence of the smallest amount of the metal ion.

- An extraordinary toxicity with silver is observed as silver cation forms a more stable complex with sulphur of microbial cell walls.

- The solubility product of Ag_2S is 6.62×10^{-50} as compared to for CuS, is 1.28×10^{-36}, which makes silver highly toxic.

- The relatively poor toxic behavior of zinc may be due to its chemical nature. Metal ions generally complex through -SH functional groups of enzymes present in the cell wall. Cadmium is more toxic than zinc because the solubility product of CdS is 1.4×10^{-29} while that of ZnS is 2.91×10^{-25}.

- Mechanisms of cadmium toxicity in microorganisms (not well defined) it enters the cell only by some indirect means There are reports where resistance to cadmium in bacteria is noticed. After showing an increase in inhibition upto 3mM of cadmium concentration, a decrease in inhibition from 35% to 25% is observed.

- In the acidic range, suppression is maximum at **pH** 3.0 for all metal ions except copper. BOD is suppressed to the extent nearly 100% at pH 6.0 except for nickel and zinc.

- The increase in BOD with the increase in temperature may be due to greater activity of microbes at higher temperatures. There seems to be significant effect on BOD inhibition due to the presence of metal ion.

•The presence of heavy metal ions like cobalt, nickel, copper, zinc, silver and cadmium show behaviour much different when *A. odorans* is used as a seed in comparison to the wastewater from dairy or distillery.

Distillery Wastewater

Metal Ion Effect

Results of optical density (OD) measurement and percentage change in BOD are presented simultaneously to co-relate change in BOD with that of microbial growth.

Out of all metal ion studied, the presence of silver ions is found to be most toxic as also observed for system using distillery waste as a seed. In general, there has been a reasonably good co-relation between the OD value and corresponding %age change in BOD. However, there are indications of resistance being developed towards the presence of metal ions. It is really very difficult to quantify the resistances and co-relation to the concentration levels of metal ions. At the most, it can be linked to the nature of the metal ion. The minimum inhibitory concentration (MIC) of metal ions tolerated by mixed flora from dairy and distillery wastewater through OD and BOD measurements.

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BOD of synthetic samples maintained at different pH values were measured in the absence and presence of each metal ion separately. Experiments were conducted by maintaining a pH in the range 3.0 to 8.0. 2.0mML^{-1} of the metal ion was maintained in each sample bottle. At this selected concentration, the metal ions exert toxicity to a reasonable extent, as observed. BOD undergoes a substantial decrease to the extent greater than or equal to 100% in the presence of the almost all metal ions. The exception in the observation is in the presence of zinc ions which has already been discussed previously.

The metal - microbe complexes are sensitive to change in pH of the medium. Hydrogen ion concentration or pH is probably the single most important factor influencing the metal ion adsorption on both organic and inorganic surfaces. Metal speciation is significantly affected both from hydrooxyl complexation and change in protonation level of the complex.

Temperature Effect

Results of temperature effect on BOD for both the seeds are similar. BOD is inhibited to the extent 100 to 120 % and there is a little variation with change of the metal ion. At 15°C, BOD exertion does not take place at all which may be due to the reason that microbes are not active at this temperature.

BOD Exertion in Presence of Metal Ions using *Bacillus brevis* and *Alcaligenes odorans* as Seed

Bacillus brevis

Metal Ion Effect

In an industrial effluent, the microflora present is of mixed type. Different microbes have different levels of affinities for metal ions and therefore, it is desirable to first isolate and identify each one of all the important colonies that might be present in the effluent. Samples were collected from local sewage treatment plant, Rajpura and pulp & paper industry. Then it was incubated at 37°C for 6 hours for acclimation. 1 mL of each sample is taken for plate count test. A number of colonies are observed on the plates. Important and prominent colonies from both types of wastewaters were isolated and got identified as *Bacillus brevis* from sewage treatment plant and *Alcaligenes odorans* from pulp & paper industry. The microbes were cultured in a rich medium and grown at 37°C in incubator. *Bacillus brevis* and *Alcaligenes odorans* were used as seed for the BOD measurements. Separate experiments were conducted to monitor the growth of microbes in the presence of different concentration of metal ions added to LB medium by measuring optical density of each system. Measurements were also made in the absence of metal ions.

In presence of large concentration of a metal ion, the optical density is decreased. This is true for all the metal ions and indicates that with increase in concentration of the metal ion larger number of microbes are complexed or are mutated and do not contribute towards the optical density values. The minimum inhibitory concentration (MIC) of metal ions tolerated by *Bacillus brevis* and *Alcaligenes odorans* are shown in Table 1. Similar metal – microbe complexation processes are taking place in BOD bottles.

Results of BOD inhibition are supported by the optical density measurements, i.e., metal-microbe interactions taking outside the bottle. As a result of metal-microbe complexation, the available microbial matter in the BOD bottle is reduced. Lesser the microbial matter, lesser will be the exertion of BOD. Hence, suppression in BOD is observed. Besides other factors like pH, temperature, etc., the magnitude of suppression would also depend on the extent of metal-microbe interactions.

Optical density measurements may not give a complete picture of metal – microbe interactions, as in BOD exertion process a sufficient amount of dissolved oxygen is ensured while in optical density measurement, there are no such arrangements. For a given metal ion, the microbial population decreases very fast as observed from optical density values upto a concentration range of 1mM - 3mM (except for silver and cadmium for which the microbial concentration falls at 0.04 mM and 0.5 mM, respectively), depending upon the metal ion. It can be easily inferred that silver shows exceptionally high toxicity towards the microbes.

In general, a fall in optical density corresponds to an increase in percentage of inhibition in BOD. These observations lead to the conclusion that *Bacillus brevis* does form complex with each of the transition metal ion and hence only an apparent BOD is observed. When Ni(II) ions are present in the BOD bottle they form complex with *Bacillus brevis* and an excellent correlation is seen with the decrease in optical density with the increase in the inhibition as the metal ion concentration increases. In case of Cu (II), there is a sharp decline in growth of the microbes with increase in concentration of metal ion, indicating an extensive metal-microbe interaction. But, the same trend is not observed during BOD exertion as there is a very small or almost negligible inhibition in BOD. In case of zinc ions, the optical density measurements indicate formation of the metal-microbe complex. But the fall in percentage inhibition decreases as the metal ion is added. This trend is in opposition to the expected increase in inhibition with concentration of the metal ion.

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reported. Studies were carried out *Bacillus brevis* as a seed and GGA as the nutrient medium.

For all the metal ions, an increase in BOD exertion when the temperature raised from 20°C to 25°C. A fall in inhibition from 25°C to 30°C and 35°C should be termed as "apparent fall" as this inhibition exists besides the effect of nitrifying bacteria which are known to be most active in the temperature range 30°C to 40°C.

The extent of suppression at different temperature does not change with the nature of the metal ions. Almost similar behaviour is observed fall all the metal ions. It was interesting to note the results of experiments for BOD exertion at 15°C. There was practically no change in DO levels at this temperature either in the presence or in the absence of metal ions. It may be change due to the reason that *Bacillus brevis* are quite dormant below 20°C. In reports, microbial growth is active in the temperature range 25°C to 55°C *Bacillus brevis* (IMTECH).

Alcaligenes odorans

Metal Ion Effect

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metal ions, respectively. Silver is known to be highly inhibitory in its action even at the smallest concentration of it.

Different levels of tolerance of metal ions may be due to the different complexing nature and their different biochemical preferences for the respective metal ions. It can also be due to the different electrochemical nature of metal ions. The metal ions with high reduction potential shall preferably get reduced and will be less toxic for the microbe.

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Effect of metal ions on BOD exertion was carried out at different pH values of the medium in the range 3-8. The results indicate that the presence of metal ions significantly decrease the BOD. Each metal ion behaves in a different way because of its different chemical stability at different pH values in the range 3 – 6. Results indicate that inhibition in BOD is not affected by change in pH in the range 6 – 8, probably, because of similar chemistry of heavy metal ions in this pH range. In acidic medium, all the metal ion species behave in a similar nature at pH = 3.

Temperature Effect

As discussed in previous chapters, the effect of temperature on the extent of inhibition/ increase in BOD is very complicated. This involves a change in BOD due to the presence of heavy metal ions at different temperatures as well as different concentrations. This change has to be viewed in the background of different DO levels at different temperatures. In the present studies it was observed that BOD exertion does not take place at all at 15°C to 20°C. From the studies at temperatures 25°C, 30°C, 35°C, BOD inhibition takes place almost to the same extent for all the heavy metal ions used.

The presence of the metal ions leads to an inhibition of 120% at 25°C, 30°C, 35°C. It can be due to the similar chemical ions which do not change with temperature in this range. In reports, microbial growth is active in the temperature range 25°C to 45°C for *Alcaligenes odorans*.

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SEED	Metal ion concentration (mM)					
	Co^{2+}	Ni^{2+}	Cu^{2+}	Zn^{2+}	Ag^{+}	Cd^{+}
Dairy	1.2	1.2	1.2	>10.0	0.2	4.0
Distillery	10.0	0.8	8.0	-	0.2	-
B. brevis	>14.0	6.0	10.0	8.0	-	0.2
A. odorans	3.0	2.0	9.0	7.0	0.2	10.0