ISTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

EFFECTS OF HEAVY METAL INHIBITION ON THE METABOLISM OF VARIOUS ACTIVATED SLUDGE BACTERIA

M. Sc. Thesis by

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ÖNSÖZ

Yüksek lisansım boyunca yapılan titiz çalışmalar sonucu ortaya çıkan bu tezde başta danışmanlarım Doç. Dr. Candan Tamerler ve Prof. Dr. Derin Orhon olmak üzere tüm çalışma arkadaşlarım ve hocalarıma teşekkürü bir borç bilirim.

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ABBREVIATIONS

CAC **:** COD Adsorption Capacity
HRT **:** Heterotrophic Retention Time
SBR **:** Sequencing Batch Reactor
COD **:** Chemical Oxygen Demand
BOD **:** Biological Oxygen Demand
MLSS **:** Mixed Liqeur Suspended Solid
ATP **:** Adenozine Triphosphate
MIC **:** Minimum Inhibition Concentration (mM)
OTR **:** Oxygen Transfer Rate (mg/cm^2sec)
F/M **:** Food per microorgansim ratio (gr COD/gr SS)
VSS **:** Voltile Suspended Solid (gr/L)
SS **:** Suspended Solid (gr/L)
CDW **:** Cell Dry Weight (gr/L)
So/Xo **:** Substrate per microorganism ratio (grCOD/grSS)
Rpm : Rotates per minute
HPLC : High Pressure Liquid Chromatography

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μ_{max}	**:** Maximum growth rate (d^{-1})
X_h	**:** Heterotrophic biomass (g/L)
t	**:** Time
Y_{xs}	**:** Biomass yield accourding to the substrate consumed (g/g)
Y_h	**:** Heterotrophic yield
Y_{XO}	**:** Biomass yield accourding to the oxygen consumed (g/g)
K_S	**:** Half velocity constant
S	**:** Substrate concentration (gr/L)
K_I	**:** Inhibition constant
k'_d	: Death coefficient
OD_{600}	: Optical Density at 600 nm of wavelength
b_h	**:** Death coefficient (d^{-1})
X_{max}	**:** Maximum biomass concentration of the culture during the experiment
F/M	**:** Ratio of gr food used for gr microorganism (g/g)

AĞIR METALLERİN YARATMIŞ OLDUĞU STRES KOŞULLARININ ÇEŞİTLİ AKTİF ÇAMUR BAKTERİLERİNİN METABOLİZMALARINA OLAN ETKİLERİ

ÖZET

Ağır metallerin, endüstriyel yollarla atılmaları sonucu biyolojik arıtma sistemleri üzerinde yarattıkları stres koşullarının genel etkileri sıkça incelenmiştir. Ancak bu stres koşullarının yaratmış olduğu metabolik değişiklikler henüz anlaşılamamıştır. Toksik seviyelerin üzerindeki ağır metal konsantrasyonlarının, bakteriyel metabolizma üzerinde inhibisyon etkisi vardır. Özellikle evsel atık sulardaki yüksek metal konsantrasyonları biyolojik arıtımın etkinliğini düşürmektedir. Bu durum, sadece ağır metallerin denizlere ve göllere karışmasiyla sonuçlanmaz, aynı zamanda biyolojik arıtmanin verimini de düşürür. Buna karşın, bazı metallerin de eser mikarlarda kullanıldığında, mikroorganizmalarda optimum metabolik aktivitenin gerçeklesmesini sağladığı bilinmektedir. Atik sular icin biyolojik arıtma sistemleri gelistirmeden önce, toksisite ve inhibisyonun, mikroorganizmalarin limit değerleri ve mikrobiyal metabolizma toleransları açısından incelenmeleri gerekir.

Bu çalışmada, bakır, kobalt ve nikel metallerinin aktif çamur sistemlerinde sıkça rastlanan *Paracoccus pantotrophus, Microlunatus phosphovorus,* ve *Escherichia coli* bakteri suşları üzerindeki etkileri incelenmistir. Büyüme profilleri boyunca her metalin, her suş uzerindeki minimum inhibisyon konsantrasyonları bulunmuştur. Bu konsantrasyonlar, hem katı, hem de sıvı ortamda kimyasal olarak tanımlı bir besiyeri olan M9'da belirlenmiştir. Katı ortamdaki deneyler Petri kutularında, sıvı ortamlardaki deneyler de erlen, biyoreaktör ve respirometrede gerçekleşmiştir. Yapılan deneylerde üç metalin de bakterilerin büyüme verimleri, maksimum büyüme hızları ve substrat tüketim seviyeleri üzerindeki etkileri belirlenmiş ve karsilastirilmiştir. Bunun için spektrofotometrik yöntemlerin yanında artık substratın belirlenmesi için yüksek basınçlı likit kromatografisi de kullanılmıştır. Respirometrik deneyler, Escherichia coli kültürü üzerinde yapılmıştır. Bu deneylerin sonucunda, biyoreaktör deneylerinde elde edilen sonuçların yanında OUR profilleri de elde edilmiştir. Respirometre denyleri sonucunda K_S, μ_{max}, ve Y_h gibi parametreler de elde edilmiştir. Elde edilen büyüme verimi, maksimum büyüme hızı gibi sonuçlar biyoreaktör deney sonuçları ile karşılaştırılmıştır.

Tüm bu sonuclar, ağır metal kosullarinda, secilen bakterilerin metabolik faaliyetlerinde meydana gelebilecek degisiklikler hakkinda görüş açısı kazandırmaktadır. Bu sayede ağır metal konsantrasyonu bilinen atık suların arıtılması için gereken biyolojik arıtma sistemlerinin dizaynı ve modellenmesi mümkün olacaktır.

EFFECTS OF HEAVY METAL INHIBITION ON THE METABOLISM OF VARIOUS ACTIVATED SLUDGE BACTERIA

SUMMARY

The general effects of the stress caused by industrial discharge of heavy metals on the biological treatment processes have been well studied. However, the biological mechanisms of these stress conditions are still not well understood. Heavy metal concentrations above toxic levels have inhibition effects on bacterial metabolism. Especially high metal concentrations in municipal wastewater reduce the efficiency of sewage treatment operations. This does not only result in disposal of heavy metals into the seas and lakes, but also in inefficient biological treatment. In the contrary, some of these metals when supplied in trace amounts are essential for the optimum metabolic activities of microorganisms. Before improving the biodegradation processes for wastewater treatment systems, toxicity and inhibitory effects need to be well investigated in terms of the limits and the mechanisms of microbial tolerance.

In this study, three of the commonly found in activated sludge bacteria, *Paracoccus pantotrophus*, *Microlunatus phosphovorus*, and *Escherichia coli*, and the inhibitory effects of three different heavy metals, copper, cobalt and nickel were investigated in detail. The minimum inhibition concentrations of each metal on each strain were determined through growth profiles obtained. These inhibition levels were tested on both the liquid and solid medium conditions using a chemically defined minimal medium, M9. Solid medium experiments were carried out in Petri plates, while the liquid medium experiments were carried out in shake flasks, bioreactor and respirometer. The inhibition effects of these three metals on the growth yields, maximum growth rates and substrate uptake levels were determined and compared. High pressure liquid chromatographic analysis were carried out for residual substrate measurements while spectrophotometric analysis were made for the calculation of the growth yields and the maximum growth rates in the batch bioreactor experiments. Respirometric analysis were carried out for the *Escherichia coli* culture. Kinetic parameters like K_S, μ_{max}, and Y_h were calculated by use of the oxygen uptake rate data. Maximum growth rate and growth yield results were found compatible with the results of the bioreactor experiments.

These results provide some insight into metabolic activities of the chosen activated sludge model organisms under heavy metal stress conditions. By the help of these analysis, it will be possible to model the biological treatment plants with the known metal tolerances.

1. INTRODUCTION

Environmental biotechnology can be considered as the discipline, which applies biological methods to clean up environmental pollutants. Use of biological systems and processes is advantageous over physical and chemical processes, since such processes have lower costs and have fewer disturbances to the environment. Also biological treatment systems can degrade the organic pollutants down to CO_2 and H_2O, while chemical and physical systems (e.g. vaporization, adsorption and extraction) can only transfer the pollutants to different locations. Biological treatment of domestic and industrial waste water is the largest biotechnological industry in volumetric terms. The activated sludge wastewater treatment systems have become a common practice for the last 80 years (Eckenfolder, 1995). On the other hand, bioremediation of polluted soil, groundwater and marine environments has only been recently worked on (Head, 1998).

Environmental biotechnology relies on the capacity of various microorganisms, of which bacteria plays the major role, to degrade the pollutants. This is the reason why researches are focused on pollutant degrading bacteria, which inhabit polluted environments. These studies include the isolation of the bacteria from the environment, their classification, and physiological characterization, molecular analyses of their degradative enzymes and sometimes the construction of 'super bugs'. By the help of these researches, extensive knowledge of bacterial physiology and the molecular features of degradative enzymes can be obtained. (Chadhry, 1991). However, discrepancies between the physiology of isolated pollutant-degrading bacteria and the nature of *in situ* pollutant biodegradation have been reported (Watanabe, 1997).

Laboratory science focuses on isolated bacteria, which is then studied as pure cultures and the mixture of the bacteria. Only the fusion of these two can fulfill certain unknowns which are necessary to improve efficiency of biological systems used in environmental biotechnology.

There are some challenges against achieving this aim. Insufficient knowledge obtained from laboratory studies to interpret what is happening in the real environment. The broad range of microbial variety is another challenge that makes it difficult to analyze the structures of relevant microbial consortia. The bacteria, which can be used for, activated sludge and soil consortia are generally very complex which enables them to act on different kinds of pollutants.

In order to solve this problem several studies have been made since the early 1990s, when good molecular biological techniques were developed to study microbial ecology (called molecular microbial ecology). With this new discipline, we can understand natural microbial consortia in a more realistic manner.

2. LITERATURE REVIEW

2.1. Heavy Metals Effect on Environment

2.1.1. Chemistry of Heavy Metals

The toxicity of the heavy metals depends upon the chemical form in which they are present (Rochow, 1964). Organometallic complexes may well show the phenomenon of bioconcentration due to the greater solubility, and hence preferential uptake, of the substance in lipid rather than water.(Mara, 2003)

2.1.2. Effects of Heavy Metals on the Environment

The world population increases evey year, which brings an increase in the total natural resource requirements. Industrial activities pollute these resources and reduce the future capacity. The effects of heavy metals on the environment and public health have been of concern for nearly a century. Microorganisms, aquatic flora and fauna are the habitats where heavy metal residues may accumulate in.

Some of the heavy metals are micronutrients and are required in trace amounts by living organisms for their normal metabolic function. At elevated concentrations in the environment they exhibit toxic effects. Elements such as cadmium and mercury have no known metabolic function and are considered to exhibit some toxicity at all concentrations. The inability of most living organisms to cope with the toxic effects of heavy metals is due to lack of evolutionary selection pressures to develop these mechanisms.(Mara, 2003)

Besides directly causing non-infectious diseases in man, heavy metals may have a significant impact when released into the environment. They are a serious and persistent pollutant of terrestrial and aquatic ecosystems (Bourg, 1995). These heavy metals may also enter into the human food chain and result in health problems. For instance, chromium poisoning causes skin disorders and liver damages (Chua, 1998).

Many organic compounds may be degraded, while there is no possibility of destruction of these elements in the biosphere. Contamination of soil by heavy metals

is a long-term problem. Estimation of the half-lives of some of the elements in soil range from 15 to 1100 years for cadmium, 310 to 1500 years for copper and 740 to 5900 years for lead, the wide range of values being due to differing soil conditions (Alloway, 1995).

2.1.3. Sources of Heavy Metals in Wastewater

Heavy metals are present as trace contaminants in most materials; more concentrated sources of these elements are found in fossil fuels and mineral ores. Anthropogenic* sources have greatly accelerated biogeochemial cycles of heavy metals (Lombi, Wenzel, Adriano, 1998).

Atmospheric release may generally disperse contaminants, such as lead from the, now banned, use of tatraethyl lead as an "anti-knocking" agent in petrol of pollutants may be more concentrated on a local level, e.g. cadmium from preindustrial mining and smelting operations (Mara and Horan, 2003).

Heavy metals, which include copper, zinc, nickel, cobalt, silver, cadmium and chromium, can also be spread out during electroplating, metal processing industries, mining operations; from metal-work and metal finishing industrial effluents and corrosion of galvanised pipings. These heavy metals and others, such as barium, lead, iron and mercury, are also used in the manufacture of printed circuit boards, paints, plastics, batteries, alloys, refractors, scientific instruments and paper (Chua, 1998).

Undesired amount of nickel is spread out especially from mining and metallurgy of nickel, stainless steel, nickel electroplating, battery and accumulator manufacturing, pigments and ceramic industries wastewaters contain undesired amount of nickel (II) ions.

All of these industrial processes are reliant on the use of heavy metals as process reagents, or they are present as contaminants in their raw materials. Elimination of compounds from many sources of waste is often either impossible or needlessly expensive. But, fortunately, pollution from heavy metals can generally be traced back to a point source.

2.1.4. Legislation

Concern over the impact of the activities of man on the natural environment has led international agreements limiting resource usage and environmental pollution. With these concerns in mine legislation is now being implemented which not only requires an installation to meet specific discharge criteria, but also to consider to Best Environmental Practice, (BEP), Best Available Technology (BAT), and Integrated Pollution Prevention and Control (IPPC). In order to enforce these regulations enforcing bodies require some form of criteria and indicators to measure environmenatl damage, and to conform to such regulations, the operators of polluting or potentially polluting installations will most probably have to implement some form of environmental management programme. The heavy metals of concern, as have been identified in European Union and US legislation, are those which are present in sufficient quantity in the earth's crust and are of sufficient solubility in water to be determined an environmental hazard. European Union Directives concerning these dangerous substances list the pollutants as being of concern in the aquatic environment (Mara and Horan, 2003).

The use of heavy metals in industry is governed by health and safety, transportation and storage legislation. These will also cover operators of wastewater treatment facilities. (Mara and Horan, 1998)

The Directive 2000/60/EC will integrate existing water quality legislation (EU, 2000a). An obligaiton is placed on member states to ensure that surface and ground waters reach a good condition by 2010. Polluters will be required to pay the full cost of the damage they cause, rather than leave it to society to bear the cost (Mara and Horan, 1998).

2.2. Activated Sludge Systems

Sewage treatment relies to a large extent on settling and screening to remove solids; this physical separation step is called primary treatment. Most municipalities also carry out secondary treatment, which harnesses bacteria to metabolize organic compounds, converting them to CO_2. In this way, the BOD is substantially decreased (Figure 2.1). If the sewage is not metabolized in this way, then the wastewater BOD can overwhelm the oxidizing capacity of receiving waters, leading to anoxic

conditions. In secondary treatment, the wastewater is sprayed over a bed of sand or gravel that is covered by aerobic microorganisms, or else agitated with the microbes, which is called activated sludge, in a reactor.

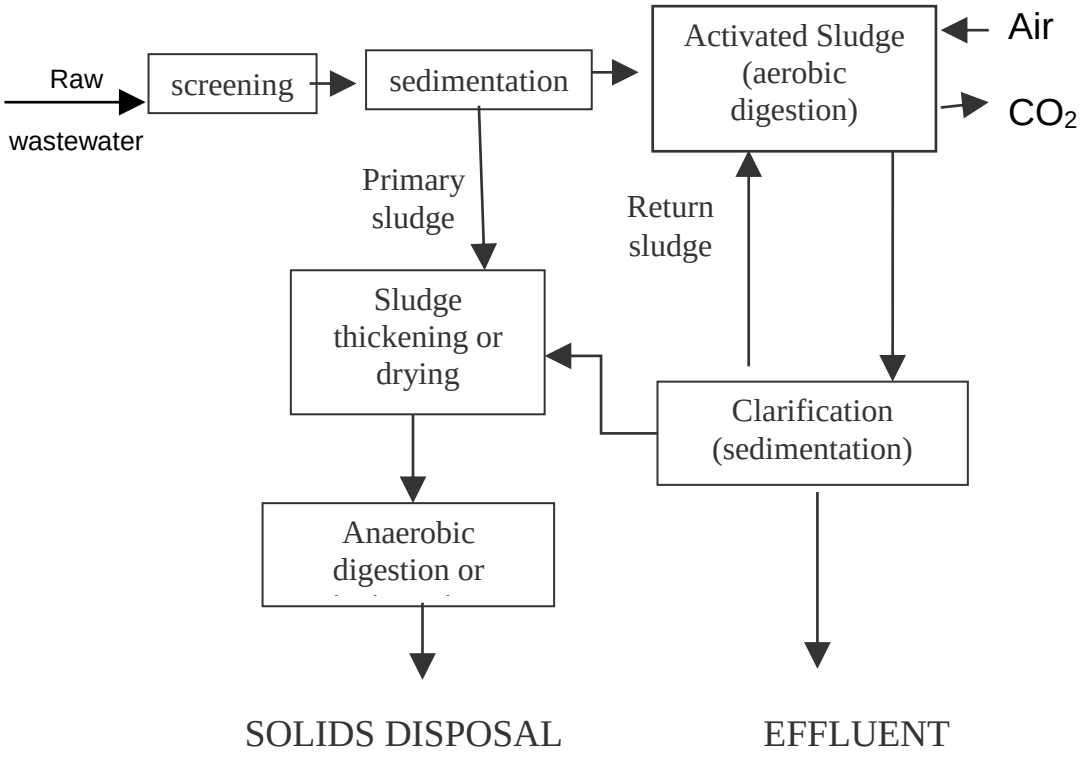

Figure 2.1. An example of primary and secondary treatment of municial wastewater

The activated sludge process is currently the most popular biological system for the treatment of domestic and industrial wastewater. Important features of this process are: (i) heterotrophic bacteria oxidatively digest organic pollutants in an aeration tank, and (ii) microorganisms form flocs, so that most of the microorganisms are separated from the treated water in a settling tank. At the end of this process, the BOD is lowered by as much as 90 percent (Spiro and Stigliani, 2003). Although microbes convert most of the organic matter to CO_2, they also incorporate some of it into new cells as the culture grows. These cells must be harvested from time to time, and are added to the sludge from the primary settling tank.

Biological treatment is a big issue in waste management. Activated sludge is an attractive biosorbent for heavy metals, dyes and organic compounds such as phenol because of its low cost and availability. Biosorption is potentially an attractive technology for treatment of municipal or industrial wastewaters or the separation and

recovery of heavy metal ions. On the other hand, since it is composed mainly of organic matter, sludge is an excellent fertilizer in principle. Unfortunately, in practice, its application to cropland is restricted by the presence of toxic metals that are flushed into wastewater from domestic and industrial sources or from urban runoff. Alternatively, the sludge can be incinerated and provide energy for heating or electricity production. Another alternative is to convert the sludge to methane by digesting it with anaerobic bacteria. However, poor economics and local opposition often militates against these options. Consequently the sludge ends up in landfills. But as landfills become full, pressure for cropland application increases, so the issue of metal hazards and metals removal is again examined more closely. If the metal accumulation problem can be solved, sludge can become a valuable resource as fertilizer, in stead of an environmental disposal problem (Spiro and Stigliani, 2003).

2.2.1. Microbial Composition of Activated Sludge

Most bacteria may be placed into one of three groups based on their response to gaseous oxygen. **Aerobic** bacteria thrive in the presence of oxygen and require it for their continued growth and existence. Other bacteria are **anaerobic**, and can not tolerate gaseous oxygen, such as those bacteria which live in deep underwater sediments, or those which cause bacterial food poisoning. The third group are the **facultative anaerobes**, which prefer growing in the presence of oxygen, but can continue to grow without it.

Bacteria may also be classified according to the mode by which they obtain their energy. Classified by the source of their energy, bacteria fall into two categories: heterotrophs and autotrophs. **Heterotrophs** derive energy from breaking down complex organic compounds that they must take in from the environment, which includes saprobic bacteria found in decaying material, as well as those that rely on **fermentation** or **respiration**.

The other group, the **autotrophs**, fix carbon dioxide to make their own food source; this may be fueled by light energy (**photoautotrophic**), or by oxidation of nitrogen, sulfur, or other elements (**chemoautotrophic**). While chemoautotrophs are uncommon, photoautotrophs are common and quite diverse. They include the cyanobacteria, green sulfur bacteria, purple sulfur bacteria, and purple nonsulfur

bacteria. The sulfur bacteria are particularly interesting, since they use hydrogen sulfide as hydrogen donor, instead of water like most other photosynthetic organisms, including cyanobacteria (UCMP).

Many different species have been reported to be present in activated sludge. At least for a while, it was thought that *Zooglea ramigera* was the only heterotrophic organism of any consequence in activated sludge, since this bacterium produces a copious extracellular slime matrix and in pure culture grows in aggregates of cells embedded in the matrix forming a structure which looks like an activated sludge floc. It is now generally accepted that there may be several of different species present in an activated sludge community (Lester and Birkett, 1999). Some biological treatment processes contain multiple classes of aerobic organisms. Such is the case with nitrifying activated sludge, in which heterotrophic and nitrifying microorganisms co-exist.

Table 2.1 shows some members of the genera commonly found in activated sludge systems. It shows the heterotrophic members of the sludge community, but it should be kept in mind that autotrophs, particularly the nitrifying organisms, will frequently be present, although their isolation is rarely reported (21 Lester and Birkett, 1999).

Table 2.1 Microorganisms commonly found in the activated sludge systems.(Lester and Birkett, 1999)

Major Genera	Minor Genera
Zooglea	Aeromonas
Pseudomonas	Aerobacter
Comomonas	Micrococcus
Flavobacterium	Spirillum
Alcaligenes	Acinetobacter
Brevibacterium	Gluconobacter
Bacillus	Cytophaga
Achromobacter	Hyphomicrobium
Corynebacterium	
Sphaerotilus	

It can be argued that certain certain species of microorganisms are now proven to be associated with certain functions of the activated sludge systems. For example, a close relationship between *Acetinoabacter sp.* and biological phosphorus removal is now well documented. (Wntzel *et al* 1992). Eikelboom, et al., 1975 concluded that several groups are responsible for flocs formed and several others for the filaments. A good blend of the two is absolutely necessary for the good settling and non-bulking activated sludge (Sezgin and Jenkins 1978,).

Aerobic heterotrophs represent the carbonaceous BOD removing microorganisms. Nitrifiers (Nitrosomonas and Nitrobacter) represent the microorganisms responsible for the oxidation of ammonia nitrogen to nitrite and nitrate to nitrogen (1-5). Juretschko *et al.* have suggested Nitrosococcus and Nitrospiro-like populations to be the dominant ammonia and nitrite-oxidizers, respectively (22), although Nitrosomonas and Nitrobacter are the most frequently isolated counterparts. Nitrifiers result in being more inhibited than the aerobic heterotrophs to a large variety of toxicants, so that in this case failure of the nitrifying process occurs before failure of the carbonaceous BOD removal process (Blum and Speece, 1992).

Bond and coworkers have found that populations affiliated with the Rhodocyclus group in the beta subclass of Proteobacteria were abundantly detected only in the efficient sludge, suggesting that these populations are involved in phosphate removal (Watanabe and Parker, 2000).

Yeasts and algae are also rarely reported and these appear to have only a minor role, if any, in the process. Fungi belong to the common population of activated sludge but their role has not been studied in detail except their possible significance in bulking. (Lester and Birkett, 1999).

In this study, three representative microorganisms belong to activated sludge cultures were selected to study in detail. These are *Escherichia coli, Paracoccus pantotrophus* and *Microlunatus phosphovorus*.

Escherichia coli is a rod-shaped (Figure 2.2) Gram (-) strain, which has the risk group 1. Its optimum growth temperature is 37^{o}C. It belongs to the family *Enterobacteriaceae* named *Escherichia* (Genus) *coli* (Species). *Escherichia coli* K-12 strain MG1655, which has served for decades as a model organism for basic

studies of biochemistry, physiology, genetics and biotechnology and it serves as a model for comparative studies (Brown, J.).

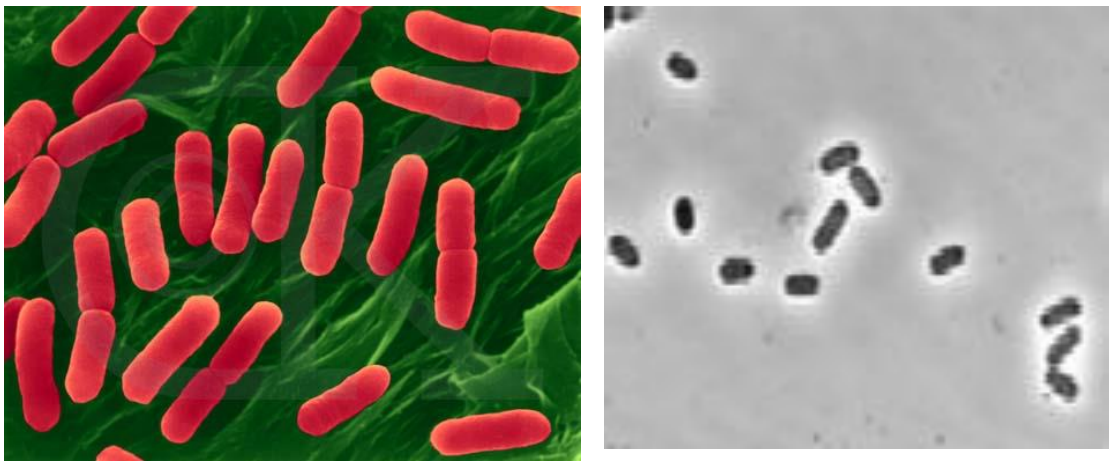

Figure 2.2 Pictures of two different kinds of *Escherichia coli* cultures taken by microscope. (Maricopa University)

Paracoccus pantotrophus is a Gram (-) strain which is found in the activated sludge systems. Its optimum growth temperature is 37 ^{o}C. *P. pantotrophus is known to synthesize* nitrous oxide reductase which reduces nitrate to nitrogen gas. (36) Strains of the genus *Paracoccus* are facultatively lithoautotrophic, neutrophilic bacteria able to grow with various organic compounds and inorganic electron donors such as molecular hydrogen or thiosulfate for autotrophic carbon dioxide fixation. *Paracoccus pantotrophus*, latterly isolated as *Thiosphaera pantotropha*, is accessible to gene transfer via conjugation. The strain was later reclassified as *Paracoccus denitrificans* (UCMP)

Microlunatus phosphovorus is a Gram (+) strain which is found in the biological phosphor removing systems. Its optimum growth temperature is 30 ^{o}C. Polyphosphate-accumulating bacteria that were previously isolated from activated sludge and exhibited high phosphate removal activity were studied taxonomically and phylogenetically. *Microlunatus phosphovorus* is a coccus-shaped, aerobic chemoorganotroph that has a strictly respiratory type of metabolism in which oxygen is a terminal electron acceptor. They accumulate large amounts of polyphosphate under aerobic conditions (NCBI).

2.2.2. Heavy Metals in Activated Sludge Systems

Heavy metals may enter the activated sludge systems, which are designed to remove soluble and colloidal organic matter, from the municipal wastewaters. Metal-laden municipal sewage above specific metal concentrations is toxic to the microorganisms in biological processes in municipal sewage treatment works and affects the organic removal efficiency (Lamb and Tollefson, 1998).

The presence of various heavy metals does not only affect the efficiency of the plant, but also the discharges containing metals may have a serious effect on the environment or man. This is reflected in the legislation concerning such emissions.

Toxicity is expressed as 'inhibition of respirotory activity of microorganisms' present in the activated sludge. The toxicity of the metal is directly related to its solubility in the presence of the sludge. If the affinity of the metal for the sludge is high, then the toxicity is reduced (Mueller and Steiner, 1992).

In recent years, with more stringent environmental control, industries are obliged to pre-treat the wastewaters to substantially reduce heavy metal concentrations before discharge. Metal toxicity in such systems may be reduced by precipitating the metal out of solution as the insoluble sulphide or hydroxide, etc (Mara and Horan, 2003). For instance, the metals in the effluent from printed circuit board manufactories and in the waste bath solution and rinse water from electroplating industries are removed either by chemical precipitation or ion exchange techniques. However, a small fraction of the metals inevitably escape these pre-treatment processes and end up in the municipal sewage works in trace concentrations (Chua, 1998).

Heavy metals, such as iron, copper and zinc, have relatively low toxicity if kept below concentrations of a few milligrams per litre, although copper at 1 mg/L has been reported to inhibit the growth of specific algal species (Sawyer and Mc- Carty, 1978). In trace concentrations, heavy metals are commonly believed to have no detrimental environmental impact and in the contrary, many of these metals are essential as micro-nutrients for microbial growth (Chua, 1998).

Metals such as Ni, Co and Mo, however, are known to promote anaerobic digestion (Shonheit et al., 1979; Whitman and Wolfre, 1980; Murray and van den Berg, 1981; Bitton, 1994) However, Tan and Chua. (1997) observed that heavy metals, including

copper and zinc, at sub-toxic concentrations {as low as 1 mg/L} also affected COD removal in activated sludge. It has been postulated that, besides the toxicity and inhibitory effects on the microorganisms, heavy metals could also physically affect activated sludge in the adsorption and uptake of organic compounds (6). A recent study have shown that, the presence of heavy metals can considerably affect the efficiency of the plant, reducing the chemical oxygen demand adsorption capacity (CAC). The presence of heavy metals may also affect the settling characteristic of sludge. This may be due to a direct effect upon the extracellular biopolymer or to the relative proportion of filamentous bacteria. (Mara and Horan, 2003)

Trace levels of Cu^{+2} and Zn^{+2} have been investigated on the performance of activated sludge. Adsorption onto batch flocs followed both Langmuir and Freundlich isoterms. The presence of these two metals affected organic matter adsorption and the CAC. The metal ions reduced CAC by competition for adsorption sites on bioflocs in a sequence batch reactor (SBR) depending on hydraulic retention time (HRT). Heavy metals were adsorbed quicker in shorter HRTs as opposed to longer. (Sin et al., 2000)

Cu^{2+} , Pb^{2+} , Cd^{2+} ,Ni^{2+} , Zn^{2+} and Cr^{2+} are known to inhibit anaerobic digestion (Lin, 1992; Mueller and Steiner, 1992). The relative degree of anaerobic inhibition in a municipal sludge has been found to be: (Mara and Horan, 2003)

$$Ni^{2+} > Cu^{2+} > Cd^{2+} > Cr^{2+} > Pb^{2+}$$

Suthirak and Sherrard (1981) reported that microorganisms in the activated sludge were inhibited by chromium and nickel at concentrations above 10 mg/l and COD removal efficiency of the process was adversely affected. A chromium concentration of 20 mg/l caused severe problems in biological processes. Heavy metal concentrations in wastewater discharged into municipial sewers that are considered unacceptable for activated sludge processes are given as an example in Table 2.2 based on Hong Kong Drainage Services Department registrations (Chua, 1998).

The removal of COD by activated sludge is proceeded by a rapid adsorption of organic matters on certain active sites on the bioflocs, followed by metabolic destruction of organic compounds. Metal ions acted as a strong competitor against organic compounds for active sites on the bioflocs, in stead of acting as a toxic microbial inhibitor.

Table 2.2 Heavy metal discharge standards (mg/L) for flow rates in m3/day (Chua, 1998).

Heavy metal	Discharge standard (mg/l) for flowrates in m^3/day				
	< 10	10–100	101–600	601–1000	1001–6000
Iron	30	25	25–15	12.5–10	7.5–1.5
Barium	8	7	6–4	3–2.4	1.6–0.4
Mercury	0.2	0.15	0.1–0.001	0.001	0.001
Cadmium	0.2	0.15	0.1–0.001	0.001	0.001
Copper	4	4	4–1.5	1.5–1	
Nickel	4	3	3–1.5	1	0.8–0.6
Chromium	2	2	2–1	0.7–0.6	0.4–0.1
Zinc	5	5	4–1.5	1.5–1	0.8–0.6
Silver	4	3	3–1.5	1.5–1	0.8–0.6
Other toxic metals	2.5	2.2	2–1	0.7–0.6	0.4–0.1

Such competitive behaviour not only hampered the organic adsorption, but also affected the COD removal (Chua, 1999).

As a general trend, the CAC of activated sludge decreased as an increase of metal ions of concentration. Figure 2.3 shows the CACs of activated sludge with and without copper at 1 mg/l and zinc at 5 mg/l. (Chua, 1999)

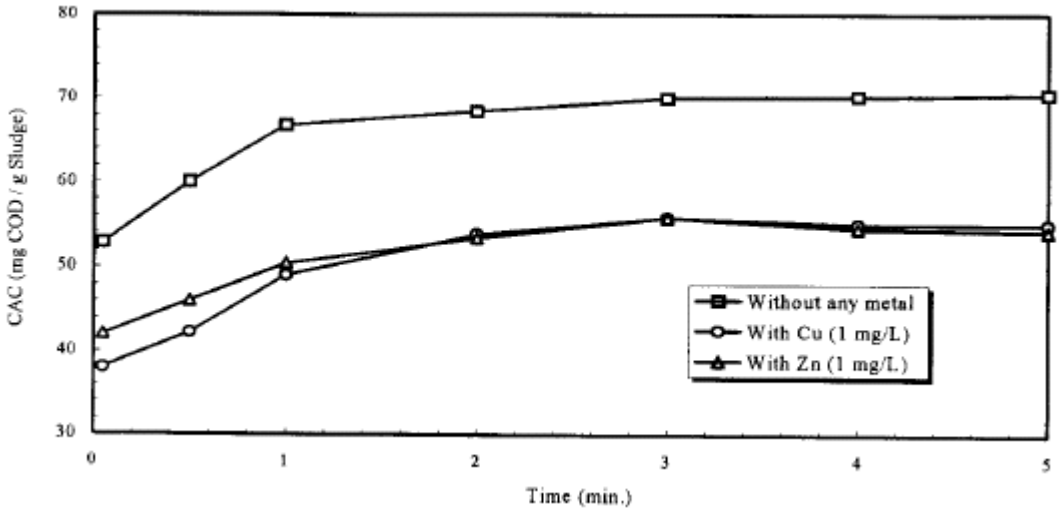

Figure 2.3. CACs of activated sludge with and without copper at 1 and 5 mg/l zinc (Chua, 1999)

The results illustrated that municipal sewage with metal ions at sub-lethal concentration affected the adsorption rate of organic matters and the CAC of activated sludge. When metals, particularly copper and zinc presented in the activated sludge process, the CAC almost 20% lower than the usual value (Chua, 1998).

Unfortunately the effect of heavy metals on the activated sludge processes are complex and exceedingly difficult to study because of the diversity of factors affecting the system as well as the varying composition of the activated sludge microbial population. Because of the heterogenous composition of the cultures the toxicity effects on individual species are not exactly known.

An activated sludge study made on a chemically defined medium with four different concentrations of Cu and Zn indicates that, there was no significant change neither the acclimation, nor in the exponential phase for all the metal concentrations studied if compared with the reference reactor (Cabrero et al., 1998)

In a work performed by Cabrera et al. (1998), on activated sludge process, comparison to the control reactor, a lower MLSS concentration was observed for all of the experiments except that carried out with 1 mg/L of zinc, where a stimulating effect was detected Figure 2.4.

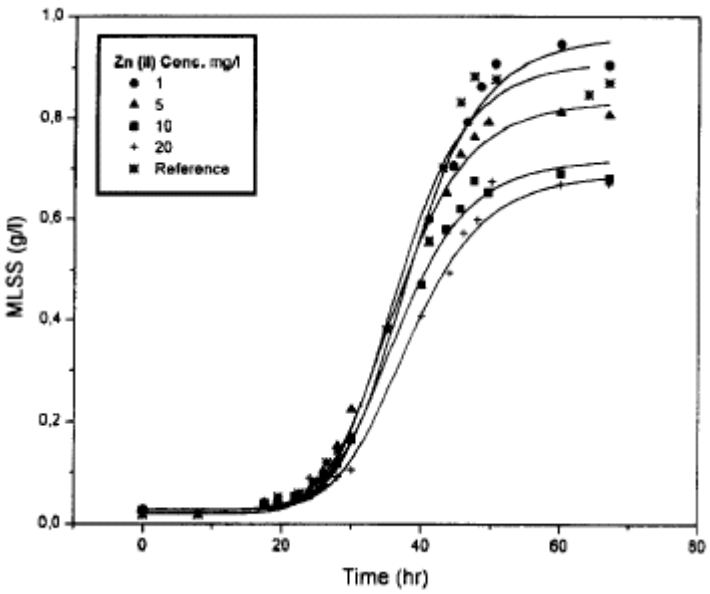

Figure 2.4. Batch growth experiments in presence of Zn(II). (Cabrero et al., 1998)

This seems to indicate that 10 mg/L of zinc are more than enough to inhibit sludge growth, however, up to this metal concentration, a precipitation and/or complexation process may occur (24).Whereas, when the effect of copper was considered in the same activated sludge study, the maximum MLSS concentration was lower than in the reference reactor. There was no copper concentration that caused any stimulating effect as happens with 1 mg/L of zinc Figure 2.5 (Cabrero et al., 1998).

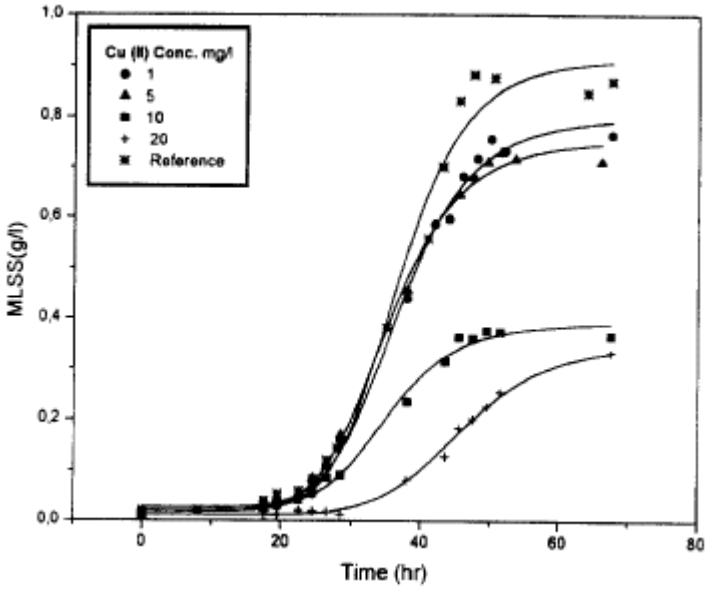

Figure 2.5. Batch growth experiments in presence of Cu(II) (Cabrero et al., 1998)

The results Cabrero et al. (1998) showed that, comparatively, the toxic inhibitory effect of copper was substantially higher than zinc. Growth yields, maximum growth rates and maximum biomass concentrations reported by the authors are summarized in Table 2.3.

Table 2.3 Mean value parameters of logistic model fit to data on the bacteria cell growth and standart deviations (Cabrero et al., 1998).

Metal concentration (mg/l)	$Y_{x/s}$ (g/g)	C_{xm} (g/l)	μ_m (h^{-1})
Reference	0.448 ± 0.024	0.916 ± 0.048	0.102 ± 0.014
Cu (II)			
1	0.378 ± 0.004	0.770 ± 0.008	0.096 ± 0.003
5	0.357 ± 0.006	0.759 ± 0.012	0.094 ± 0.005
10	0.188 ± 0.006	0.394 ± 0.012	0.091 ± 0.010
20	0.171 ± 0.005	0.353 ± 0.010	0.078 ± 0.005
Zn (II)			
1	0.469 ± 0.013	0.965 ± 0.027	0.098 ± 0.008
5	0.412 ± 0.007	0.852 ± 0.015	0.097 ± 0.006
10	0.348 ± 0.004	0.723 ± 0.009	0.099 ± 0.004
20	0.333 ± 0.008	0.693 ± 0.016	0.094 ± 0.006

2.3. Bioaccumulation Mechanisms of Trace Metals

Microorganisms exhibit great powers of trace metal accumulation and immobilization. They may be frequently encountered in extreme metal contaminated environments, both natural and man-made (Adriano, 1992). Some researches made on the the kinetics of metal uptake shows that it takes place in two stages as the physical adsorption and the chemisoprtion processes. The first stage, thought to be physical adsorption or ion exchange at the cell surface, is very rapid and occurs in a

short time (min) after the alga comes into contact with the metal (Sag and Kutsal, 2000).

This metabolically independent uptake of metals may account for the most significant proportion of total uptake. It is found that up to 90% of total Cd uptake by *Bacillus subtilis* was located in the cell wall, 3 to 4% on the cell membrane, and the remainder in the soluble fraction of the cell (Adriano, 1992).

Gram positive bacteria possess cell walls with powerful chelating properties. Initial interaction between metal ions and reactive groups is followed by inorganic deposition of additional metal. Metals can accumulate in greater than stoichiometric amounts, which cannot be solely accounted for by ion-exchange processes. Metal-loaded bacterial cells have been shown to act as nuclei for crystalline metal deposits when mixed with metalliferous sediments (Adriano, 1992).

The cellular envelopes of Gram-negative bacteria have a very different structure from the cell walls of Gram-positive organisms. Studies with purified cell envelopes of *E. coli* K-12 show most metals to be deposited at the polar head group regions of membranes or along the peptidoglycan layer. Again, a set hierarchy for metal affinity is evident.

Cellular distribution analysis showed that relatively large amounts of metal ions were bound to the cell wall, and to an intracellular insoluble fraction (Garnham *et al*., 1992) namely, Polyphosphates (Jensen *et al*., 1982), and/or phytochelation (Robinson, 1988). There are some aquatic organisms that can accumulate heavy metals into their protoplasmic structure without marked toxic effects. For example, *Euglena gracilis* could accumulate Zn^{2+} ions until 5 mg/g dry weight (Fukami, 1988). In other cases, toxic effects could inhibit the enzymatic system affecting the biochemical and physiological processes (Sag and Kutsal, 2000).

Cu^{2+} , Pb^{2+} , Zn^{2+} and Cr^{+2} have found to adsorb onto biomass much faster than organic competitors. This is most probably due to the increased mobility of the metal ion over the organic molecules due to their relative size and greater charge. The metal ions, being much more mobile, are able to seek out adsorption sites quicker than their larger organic competitors on the bioflocs (Mara and Horan, 2003).

Studies have shown that heavy metals are transported across the outer microbial cell membrane via two types of transport system:

1. Fast unspecific uptake driven by a chemostatic gradient across a cell membrane. These are constitutionally expressed mechanisms of which two general types have been recognized. Divalent metal cations enter through one type of channel that is present to allow the ingress of micronutrient metal cations, eg Zn^{+2}. Oxyanions, e.g. chromate gain entry through unspecific uptake systems allowing the passage of phosphate and sulphate ions.
2. Systems showing high substrate specificity. This may be utillized against a concentration gradient, sometimes use an ATP source as an energy source; they are inducable (Niess, 1999,).

Second stage, related to metabolic activity, is slower and called chemisorption. Metal uptake process in the second stage is slower than passive adsorption (first stage), demanding the presence of suitable energy sources and ambient conditions. The physiological state of the bacterial cells is also important, as is the nature and composition of the culture of the culture medium. Several of the elements classed as trace metals are essential to life, and cells may have more or less specific mechanisms for the uptake of elements such as Cu, Co, Fe, Ni, and Zn. Mechanisms for such metals are not known with certainity, may involve specific carrier systems associated with active ionic fluxes across the cell membranes. If the process is not saturated, there is the potential for significant metal accumulation via this route, greater than for a purely passive association. This may not be true, however, for those organisms producing copious extracellular deposits of polysaccharides with high biosorption capacities (Adriano, 1992).

Providing that the overall enthalpy of the interactions is not zero, a temperature dependence of metal ion biosorption by microorganisms will occur. Temperature changes will affect a number of factors which are important in heavy metal ion biosorption. Some of the factors include: (i) the stability of the metal ion species initially placed in solution; (ii) the stability of the microorganism–metal complex depending on the biosorption sites; (iii) the effect of temperature on the microorganism cell wall configuration; (iv) the ionization of chemical moieties on the cell wall (Sag and Kutsal, 2000).

Long-term studies on metal uptake relate to the ability of growing and multiplying cultures of bacteria to accumulate metals. This process can again be modified by a range of factors including the nature of the medium, metal-independent changes in morphology and physiology of the growing cells (2). Differences in growth phase can be important in metal uptake in batch cultured bacteria. Different metals show different growth-dependent uptake patterns. Cu uptake in *E. coli* increases at the end of exponential growth phase, reached a maximum at the end of deceleration phase, and declined in the stationary phase of colony growth (Adriano, 1992).

Studies made on activated sludge processes, using synthetic waste water (780 mg COD/L reconstituted milk) has shown that the ultimate adsorption capacities for copper, chromium, zinc and lead were 0.24, 0.18, 0.15, and 0.14 mg/g-sludge, respectively (Figure 2.6). These results illustrated that the adsorption capacity by activated sludge for these metal ions was saturated very quickly and the process was completed within 0.5 minutes (Chua, 1999).

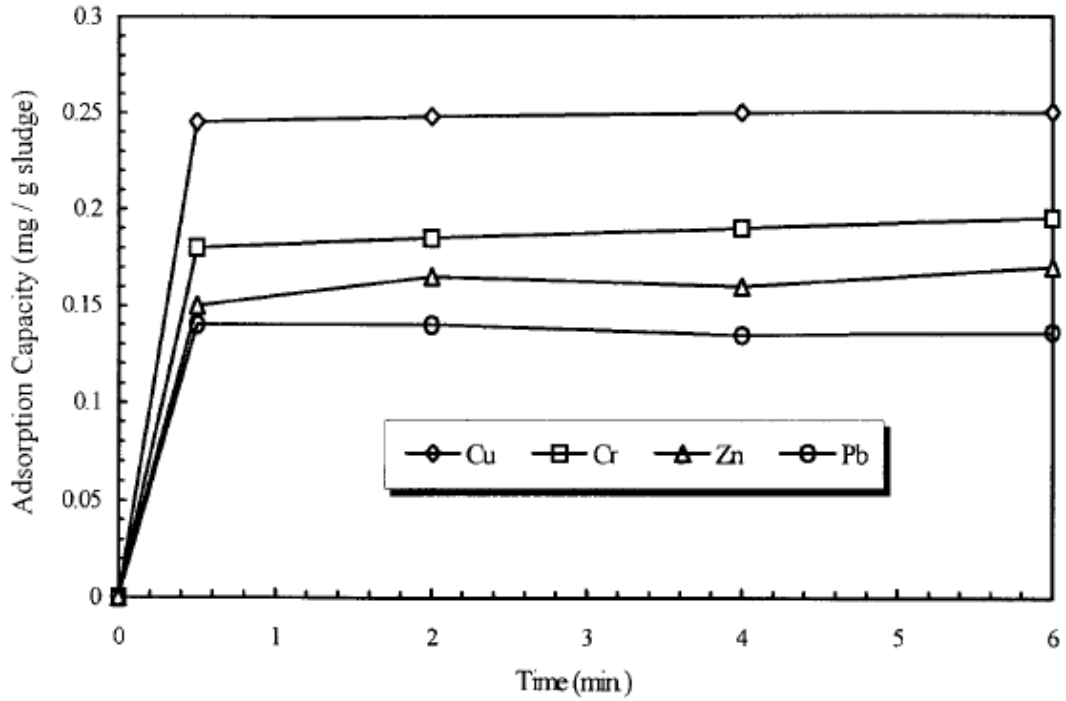

Figure 2.6 Adsorption capacities for copper, chromium, zinc and lead were 0.24, 0.18, 0.15, and 0.14 mg/g-sludge, respectively (Chua, 1999).

From the same study, adsorption isotherm of metal ions obtained from the activated sludge was given in Figure 2.7. The isotherms were derived at 25 ^{o} C with the concentrations of copper, chromium, lead at 2 mg/l and zinc at 4 mg/l. The

adsorption capacities were in the order of Cu>Cr>Zn>Pb. The results demonstrated that activated sludge adsorbed copper more readily and to the higher extend than other metal ions, and that these metal ions were adsorbed readily particularly at low concentrations (Chua, 1999).

The adsorption capacities of the given metals are almost in the same order as the adsorption capacities of various pure bacteria cultures.

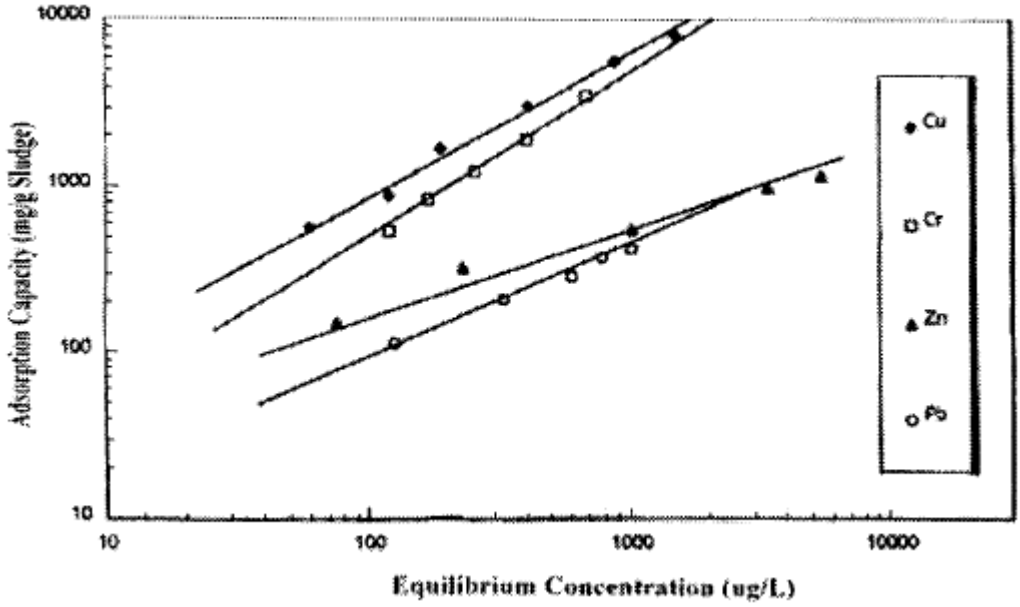

Figure 2.7. Adsorption isotherm of metal ions by activated sludge (Chua, 1999).

Also, dead cells of the activated sludge complex are found to work as an ion exchange resin composed of a network of cell membrane, cell wall, cytoplasm and nucleus. The equilibrium uptake and yields of Cu ion by the growing cells are found to be lower than those of both the resting and dead cells of activated sludge, while the equilibrium uptake capacity for Pb(II) of the resting cells of activated sludge was substantially higher than that of the dead cells. The Pb(II) biosorption yields of the resting cells decreased with increasing initial Pb(II)ion concentration (Sag et al., 2003).

Although the search for new and innovative treatment technologies has focused attention on the metal binding capacities of various microorganisms, the exact interactions between the ligands on the cell walls and the heavy metal ions, the kinetics of the metal uptake process and the description of the thermal properties of the biosorption remain essentially unknown (Sağ and Kutsal, 2000).

2.4. Pure Culture Studies in Representing Activated Sludge Processes

Microbial consortia has usually been studied on as it was a 'black box', without analyzing the constituent microbial populations. Recently, the importance of the pure culture studies has been understood as the molecular techniques developed and researches were made on the activated sludge cultures.

Bacteria in natural waters and sediments mediate the chemical transformations and biogeochemical cycling of nutrients and toxic substances, besides being important links in aquatic food chains; therefore, inhibition or stimulation of these organisms by pollutants may have far reaching consequences for aquatic ecosystems (Vernet, 1991).

The effects may vary with environmental conditions, which control the chemical speciation, bio-availability, and toxicity of pollutants as well as microbial growth and activities. The complexity of these processes is compounded if there is a mixture of pollutants, which may interact synergistically or antagonistically, or if a pollutant can act as a nutrient as well as a toxic agent, as is the case with copper and zinc (Vernet, 1991).

2.4.1. Effects of Trace Metals on Various Bacteria

Heavy metals generally exert an inhibitory action on microorganisms by blocking essential functional groups, displacing essential metal ions, or modifying the active conformations of biological molecules; however at relatively low concentrations, some heavy metals may be essential for some microorganisms (eg. Co, Cu, Zn, Ni) since they provide vital cofactors for metallo-proteins and enzymes (Hassen et al., 1997).

The toxic mode of action of heavy metals is observed generally within the cell. This may be by the substitution of the metal ion for a structural homolog micronutrient or binding to material within the cell, especially heavy metals with a high atomic mass showing a great affinity for thiol constituent groups on amino acids, such as cysteine. Both mechanisms can cause considerable oxidative stress on the cell (Mara and Horan, 2003). The effects may be deleterious, if heavy metals' concentrations are above the level which naturally occurs in the organisms' environment. In general, heavy metals tend to have a bacteriostatic effect, but increasing concentrations lead

to mortality. Some microorganisms have developed mechanisms to cope with elevated concentrations of heavy metals in their environment (Mara and Horan, 2003).

The effect of heavy metal concentration on the microorganisms makes the minimum inhibitory concentration necessary in all studies. The minimal inhibitory concentrations (MIC) is defined as the lowest concentration of metal that completely prevents bacterial growth (Hassen et al., 1997). In order to determine the minimal inhibitory concentrations (MIC) for bacterial strains found in various natural habitats such as soil, water, sediments and sewage-amended soil, generally the medium that best supports the growth of the microorganism or a group of microorganisms were chosen in the most of the studies. Frequently media have been amended with various quantities of heavy metal salts, inoculated with the appropriate microorganism, and the microbial growth measured to determine the MIC. A number of problems are associated with this approach. Metal-binding capacity of the microorganisms, chelation to various components of the media, and formation of complexes can each cause a reduction in the activities of free metals. As a consequence, MICs determined with traditional media approach can not be related to actual metal concentrations in the habitat from which bacteria were isolated. In spite of these limits, the technique of MICs remains a valid approach to evaluate the action of heavy metals on the microbial activity in polluted habitats such as agricultural soils, sludge-amended soils, marine sediments and municipal refuse (Mara and Horan, 2003).

There are some studies indicating that growth rate decreases as the metal concentration increases as in the case of Zn^{2+} concentration increase in *Euglena gracilis culture.*. Growth stimulation by low concentrations of heavy metals may be due to their involvement in the mechanisms by which metabolites are retained inside the cells or their involvement in the production of lipid (Omar 2002). In another study, Cu^{+2}, Ni^{+2}, Zn^{+2} were found to inhibit β-glucosidase enzyme acivity. Also, the pH and buffer type were found to have considerable effect on the enzyme activity. Under optimal concentrations, a metal concentration of 0,6 mM and a pH of 5, the presence of Zn^{+2} and Ni^{+2} decreased the enzyme activity by 25 to 30%, whereas Cu^{+2} reduced it by more than 90%. The citrate buffer was not inhibited at all, even at higher Cu concentrations. The inhibition by Zn^{+2} and Ni^{+2} was found to be less pH dependent-range 4 to 5,5. Chemical speciation models were used to describe

inhibition by buffer and pH for all three metals. Here it was assumed that the enzyme activity depended on protonation of the amino acid at the reactive site complexation by the heavy metal cation. (Geiger et al., 1999).

In another study, toxicity experiments made on solid media have shown that copper and mercury were found to be the most tolerated and most toxic metals, respectively, whereas chromium, zinc, cobalt and cadmium gave intermediate results. Figure 2.8 and Figure 2.9 show the effects of copper and mercury inhibition respectively on various strains (Hassen et al., 1997).

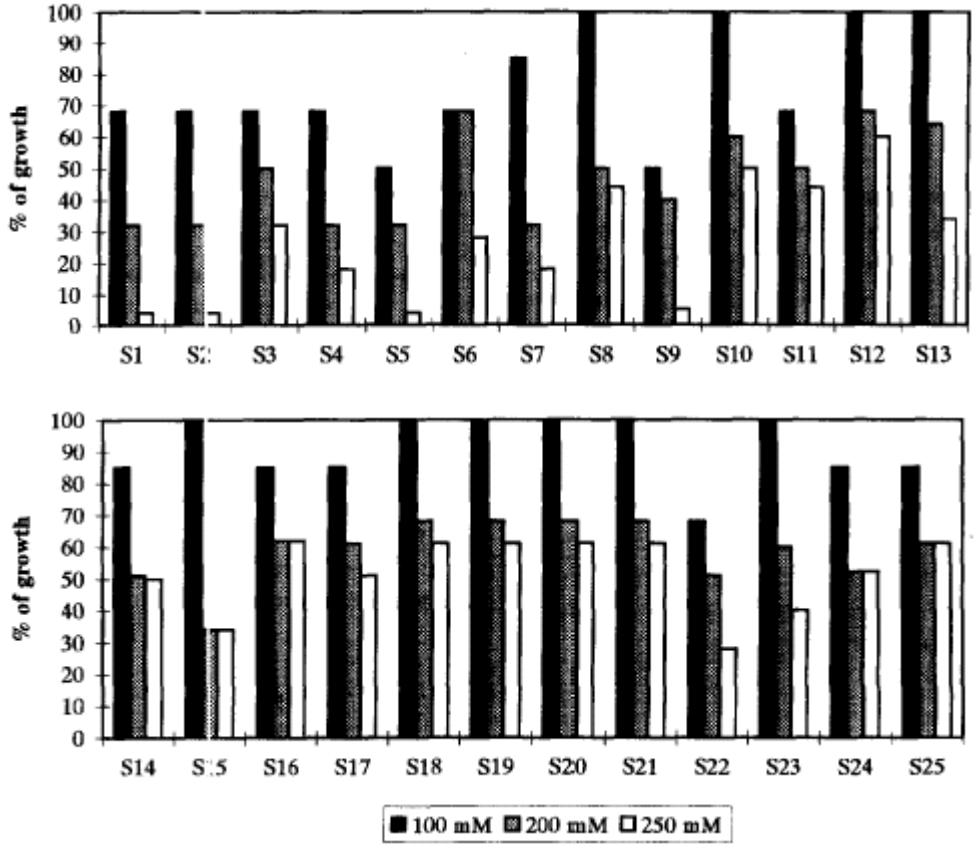

Figure 2.8.Toxicity of copper to various bacterial strains, which are shown in Table 4 (A nad B), in liquid medium cultures (Hassen et al., 1997)

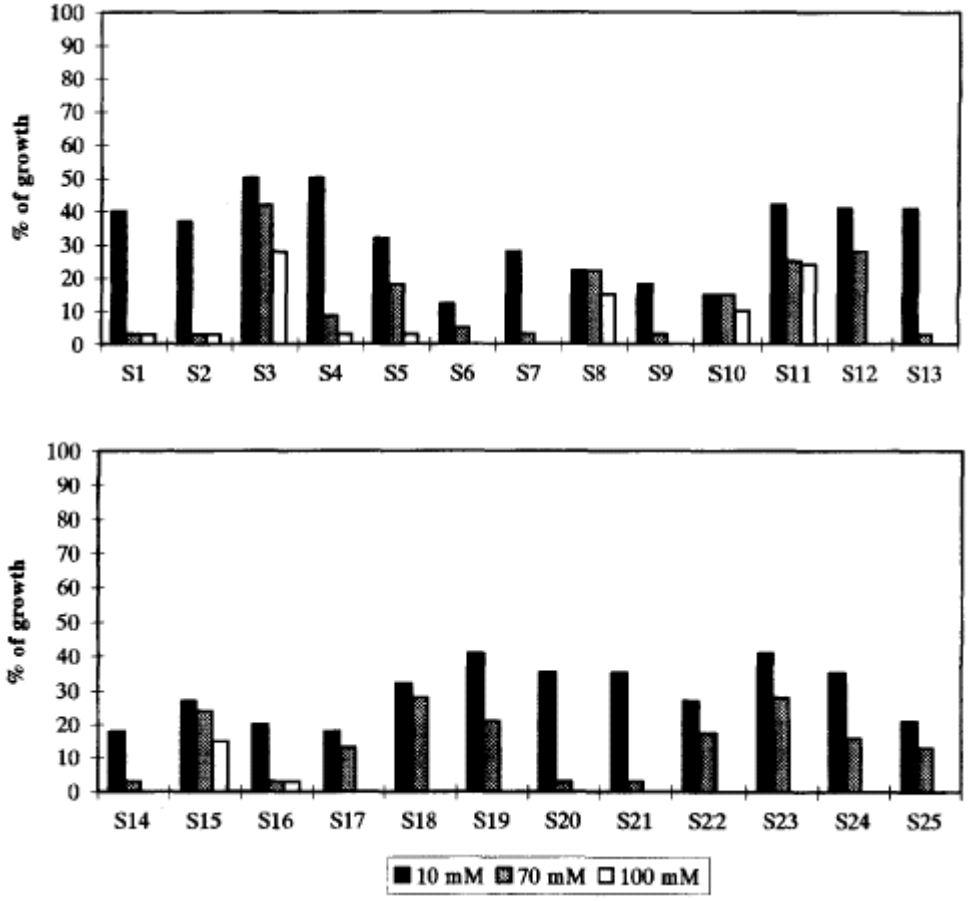

Figure 2.9. Toxicity of mercury to bacterial strains, which are shown in Table 4, tested in agar media (Hassen et al., 1997)

Comparison between the solid and liquid media experiment results have shown that bacteria tolerate higher concentrations of heavy metals in solid media than they do in liquid media. As an example, Table 2.4 shows that three different strains of *Pseudomonas aeraginosa* have very different MIC values for cobalt and mercury in the liquid media than in the solid media, shown in Figure 2.8 and Figure 2.9. In solid media, *Pseudomonas aeraginosa* can tolerate 200mM of copper and 10 mM of mercury, while in the liquid media these values are 0,6 and 0,05 mM respectively (Hassen et al., 1997).

Table 2.4 MIC values expressed in mM/L in nutrient broth (Oxoid). (ND: none detected) (Hassen et al., 1997)

Strain no.	Identification	MIC* of metal					
		Cu	Cr	Co	Cd	Zn	Hg
S1	*Escherichia coli* K12	0.5	1.5	0.2	0.2	0.2	0.05
S2	*Bacillus thuringiensis* (serotype I)	0.5	1.0	0.05	1.2	0.5	0.06
S3	*Streptococcus* sp.	0.02	0.8	0.05	0.2	0.2	0.05
S4	*Streptococcus* sp.	0.05	0.5	0.05	0.2	0.2	0.005
S5	*Staphylococcus aureus*	0.2	0.2	0.2	0.1	0.2	0.005
S6	*Pseudomonas aeruginosa*	1.2	1.2	0.4	1.5	1.5	0.08
S7	*Pseudomonas aeruginosa*	1.6	1.5	0.6	1.5	1.5	0.05
S8	*Pseudomonas aeruginosa*	0.1	3	0.8	0.6	1	0.1
S9	*Pseudomonas paucimobilis*	1.5	1.2	0.4	0.2	0.2	0.08
S10	*Pseudomonas paucimobilis*	0.2	1.8	0.4	0.2	0.2	0.05
S11	*Pseudomonas paucimobilis*	1.2	1.2	0.4	1.5	0.8	0.08
S12	*Pseudomonas paucimobilis*	1.5	1	0.2	0.2	0.5	0.05
S13	*Pseudomonas paucimobilis*	0.8	1.2	0.4	0.2	0.2	0.05
S14	*Pseudomonas cepacia*	1.2	1.2	0.2	0.2	0.2	0.04
S15	*Providencia rettgeri*	1.8	1.2	0.4	0.5	0.5	0.05
S16	*Providencia rettgeri*	1.2	1.5	0.6	0.2	0.8	0.05
S17	*Proteus mirabilis*	ND	ND	ND	ND	ND	0.1
S18	*Proteus mirabilis*	1.5	1.5	0.2	1.2	1	0.08
S19	*Proteus mirabilis*	1.2	ND	0.2	1.2	0.8	0.08
S20	*Proteus mirabilis*	1.5	1.5	0.2	1.2	1.2	0.1
S21	*Proteus mirabilis*	1.5	1.2	0.2	1.2	1	0.1
S22	*Aeromonas hydrophila*	1.2	1.5	0.6	0.2	0.2	0.05
S23	*Acinetobacter calcoaceticus*	ND	ND	ND	ND	1.2	ND
S24	*Citrobacter freundii*	0.2	0.8	0.2	0.2	1.5	0.04
S25	*Klebsiella rhinoscleromatis*	ND	1.2	ND	ND	ND	ND

Many studies have shown that the order of metal toxicity is Hg>Co>Cd>Cu-Cr>Zn. In spite of its limits, liquid media experiments allow us a good evaluation of metal toxicity in polluted environments, such as industrial effluents, incinerator residues, landfill municipial refuse and sewge sludge leachates. In spite of the toxicity order given above, the power of metal adsorption and complexation is classified as , Hg^{+2}> Cu^{2+} >Cd^{+2}-Zn^{+2} > Co^{+2} –Cr^{+2} (Hassen et al., 1997).

Studies made on the effects of heavy metals on *Pseudomonas aeruginosa* and *Bacillus thuringiensis* with nutrient broth (Oxoid) medium have shown that growth of *Pseudomonas aeruginosa* in the presence of metals have shown a lag phase much longer than in case of the absence of metals (Hassen et al. ,1998). On the other hand, there was a short lag phase for *Bacillus thuringiensis*. Cobalt and copper, at 0,2 mM inhibited the bacterial growth by approximately %30 with regard to controls. Figures 2.10 to 2.13 show that the growth of *Bacillus thuringiensis* is more sensitive to Co and Cu than *Pseudomonas aeruginosa* (Hassen et al. ,1998).

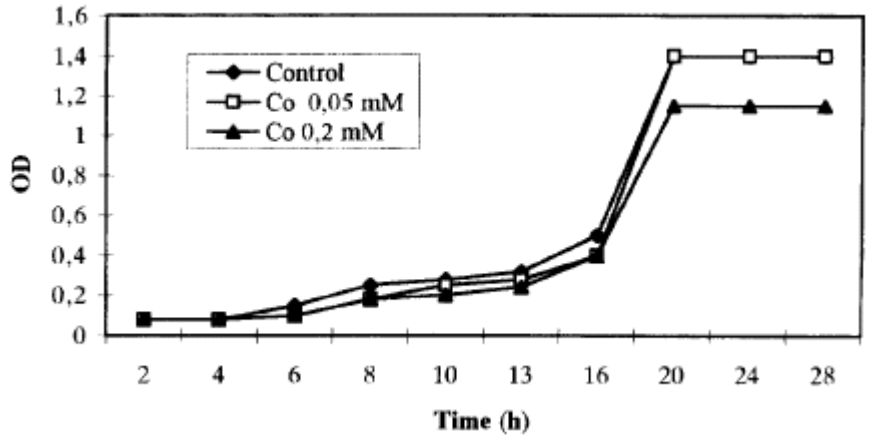

Figure 2.10. The growth curve of *Pseudomonas aeruginosa* under Co^{+2} inhibiton (Hassen et al. ,1998)

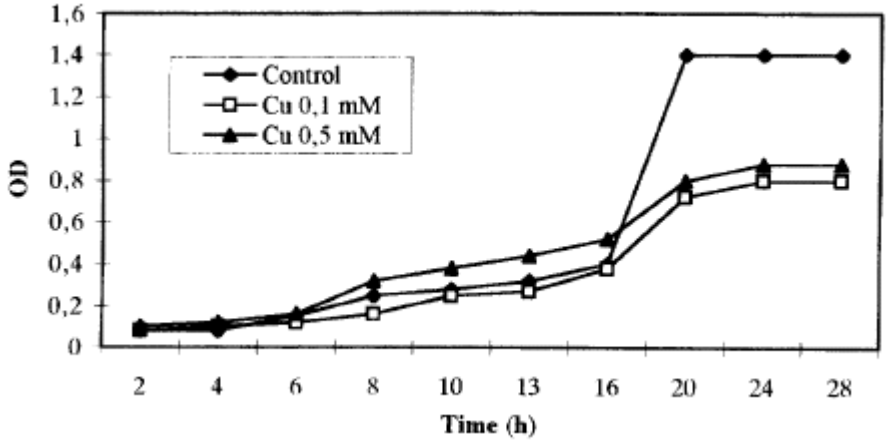

Figure 2.11 The growth curve of *Pseudomonas aeruginosa* under Cu^{+2} inhibiton (Hassen et al. ,1998)

Figure 2.10 and Figure 2.11 show that 0,2 mM of Co and 0,1 mM of Cu have viable inihibitory effects on *Pseudomonas aeruginosa.* Similarly, 0,2 mM of Co and 0,05 mM of Cu have negative effects on *Bacillus thuringiensis* (Figure 2.12 and Figure 2.13) (Hassen et al. ,1998).

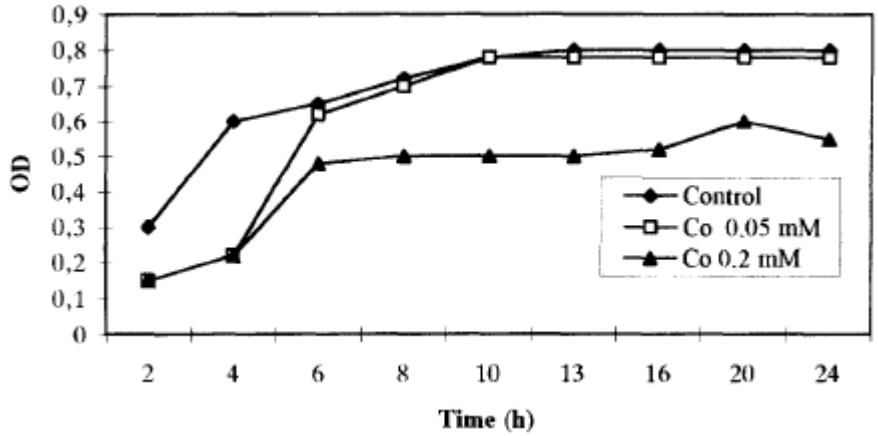

Figure 2.12. The growth curve of *Bacillus thuringiensis* under Co inhibiton (Hassen et al. ,1998)

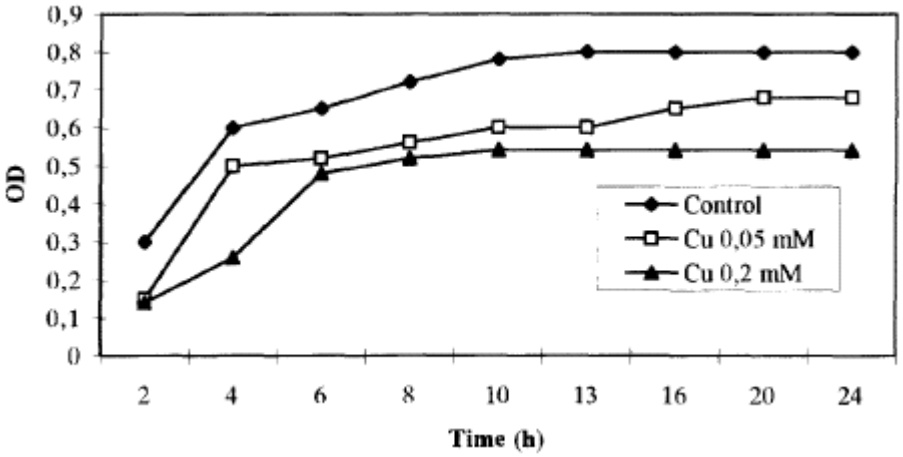

Figure 2.13. The growth curve of *Bacillus thuringiensis* under Cu inhibiton (Hassen et al.,1998)

Since the responses of the activated sludge to metals are different for various species of organisms making up the culture, suppression of certain species may lead to the overgrowth of others. It was found that tin mixed culture of four strains isolated from activated sludge, alcaligenes was the most susceptible organism and Brevi bacterium the most resistant to heavy metal inhibition. The researcher also postulated that a metal at a given concentration may be toxic to a certain species while serving as growth stimulant to others (Lester et al.1979).

There is some evidence that microbial residence to Ni may occur in environments polluted with this metal, as strains of Pseudomonas and Fluorescens isolated from metal polluted areas showed resistance to elevated concentrations of Ni. (Olson et al 1979). Similarly, an increase in the tolerance of microorganisms to heavy metals

upon exposure to sublethal doses of these metals have been demonstrated by a number of workers (Malaney et al, 1959, Ghosh and Zugger, 1979, Bonomo 1974).

2.4.2. Natural Microbial Resistance or Detoxification Mechanisms

There are three general mechanisms are available to the microbial cell for detoxification:

1. Reduction to a less toxic form
2. Effusion from the cell generally requiring a specific transport mechanism working against a concentration gradient coupled to an energy releasing reduction.
3. Complexing the heavy metal to form an inert relatively non-reactive species.

Microbial cells generally adopt two or all three of the above mechanisms. The metabolic activity of microorganisms can result in solubilization, precipitation, chelatation, biomethylation or volatilizaiton of heavy metals (Iverson and Brinckman, 1978). Microbial activity may result in the following:

Strong acid production, such as sulphiric acid by chemoautotrophic bacteria, e.g. *Thiobacillus*, which dissolves minerals.

Weak organic acid production, such as citric acid, which dissolves and chelates metals forming organometallic compounds. Metals may also be complexed by carbonyl groups found in microbial polysaccharides and other polymers. This is of importance in wastewater treatment plants, particularly those using the activated sludge process, where industrial wastes are treated ((Bitton and Freihoffer, 1978; Brown and Lester, 1979, 1982; Rudd et al., 1984; Sterrid and Lester, 1986; McLean et al., 1990) *Pseudomonas putida* has a cystein-rich protein that readily binds with Cd (Higham et al., 1984).

Ammonia production or organic bases, which precipitate heavy metals as the insoluble hydroxides. Some bacteria promote metal precipitaion producing ammonia, organic bases, or hydrogen sulphide, which precipitate metals as hydroxides or sulphides.

Hydrogen sulphide production by sulphate-reducing bacteria, which precipitate heavy metals as insoluble sulphides. Sulphate-reducing bacteria transform SO_4 to H_2S, which promotes the extracellular precipitation of metals from solution. *Klebsiella aerogenes* is able to detoxify Cd to CdS, which precipitates as electron-

dense granules at the cell surface. This process is induced by Cd (Aiking et al., 1982).

Extracellular polysaccharide production, which can chelate heavy metals and reduce toxicity (Bitton and Freihoffer, 1978). These extracellular polymers may form either capsules or loose aggregates around individual cells. In many cases, these are of a polysaccharide nature, with anionic properties, and are capable of significant metal cation binding. These polymers are strongly implicated in the removal of soluble metal ions by activated sludge biomass; the removal of extracellular polymers from cultures of *Klebsiella aerogenes* considerably reduces its ability to sorb metals. Polysaccharides produced by the common sewage treatment organisms, *Zooglea ramigera* have significant metal binding properties (Adriano, 1992).

Fixing metal ions; certain bacteria (sheated filamentous) fix Fe and Mn on their surface in the form of hydroxides or other insoluble metals salts.

Biotransformation of certain bacteria that have the ability biomethylate or volatilize heavy metals. Some metals are transformed to volatile species as a result of microbial action. Bacterially methylation converts Hg^{+2} to $(CH3)_2Hg$, a volatile compound. Some bacteria are able to detoxify Hg by transforming Hg+2 to Hg0, which again is volatile. This detoxificaiton process is plasmid-encoded and is regulated by an operon consisting pf several genes. The most important gene is the merA gene, which is responsible for the production of the mercuricreductase, the enzyme that catalyses the transformation Hg^{+2} to Hg^0 (Adriano, 1992)

Microbial crystallization of metals is a well-known phenomenon. The bacterium Gallionella can grow using energy derived from Fe^{+2}oxidation; these organisms form long twisting stalks on which iron oxides are deposited Sulfate-reducing bacteria, such as Desulphovibrio, form sulfide deposits that can immobilize large quantities of heavy metals. Some bacteria produce iron-sequestering organic molecules (siderophores) as part of their overall Fe^{+2} uptake strategy (Adriano, 1992).

2.5. Microbial Growth and Inhibition Kinetics

2.5.1 Microbial Growth Kinetics

The rate of microbial growth is characterized by the *specific growth* rate, which is defined in Equation 2.1

$$\mu = \frac{1}{x} . \frac{dX}{dt} \tag{2.1}$$

where X_h is the cell mass concentration (g/L), t is itme (h), and μ is specific growth rate (h^{-1}).

Batch growth refers to culturing cells in a vessel with an initial charge of medium that is not altered by further nutrient addition or removal. This form of cultivation is simple and widely used both in the laboratory and industry. When a liquid nutrient medium is inoculated with a seed culture, the organisms selectively take up dissolved nutrients from the medium and convert them into energy and biomass. A typical batch growth curve includes lag phase, logaritmic exponential growth phase, deceleration phase, stationary phase, and death phase. The lag phase occours immediately after inoculation and is a period of adaptation of cells to a new environment. In the exponential growth phase, the cells have adapted to their environment. The cells multiply rapidly. This is a period of balanced growth in which all components of a cell grow with the same rate.

The deceleration growth phase follows the exponential phase. The rapidly changing environment results in unbalanced growth. The stationary phase starts at the end of the deceleration phase, when the net growth rate is zero (no cell division) or when the growth rate is equal to the death rate. Even tough the net growth rate is zero during the stationary phase, cells are still metabolically active and produce secondary metabolites. During the course of stationary phase, one or more of the following phenomena may take place:

1. Total cell mass concentration may stay constant, but the number of viable cells may decrease.
2. Cell lysis may occour and vialble cell mass may drop. A second growth phase may pccour and cells may grow on lysis products of lysed cells (cryptic growth).

3. Cells may not be growing but many have active metabolism to produce secondary metabolites. Cellular regulation changes when concentrations of certain metabolites (carbon, nitrogen, phosphate) are low. Secondary metabolites are produced as a result of metabolite deregulation.

During the stationary phase, the cell catabolizes cellular reserves for new building blocks and for energy-producing monomers. This is called *endogenous metabolism.* The reason for termination of growth may be either exhaustion of an essential nutrient or accumulation of toxic products. In case of the presence of an inhibitor, the growth rate slows down or stops accourding to the inhibitor concentration.

Some stochiometrically related parameters were defined in order to describe growth kinetics. For organisms growing aerobically on glucose, Y_{xs} is typically 0,4 to 0,6 g/g for most yeast and bacteria, while Y_{XO} is 0,9 to 1,4 g/g. (Schuler and Kargı, 1992)

Dissolved oxygen (DO) is also an important substrate in aerobic fermentations and may be a limiting substrate, since oxygen is a sparingly soluble gas in water. At high cell concentrations, the rate of oxygen consumption may exceed the rate of oxygen supply, leading to oxygen limitations. Oxygen is usually introduced to fermentation broth by sparging air through the broth. Oxygen transfer from gas bubbles to cells is usually limited by oxygen transfer through the liquid film surrounding the gas bubbles. The rate of oxygen transfer from the gas to liquid phase is given by:

$$N_{O_2} = k_L.a.(C^* - C_L) = OTR \qquad (2.2.)$$

where k_L is the oxygen transfer coefficient (cm/h), a is the gas-liquid interfacial area (cm^2/cm^3), k_La is the volumetric oxygen transfer coefficient (h^{-1}), C* is saturated DO concentration (mg/L), C_L is the actual DO concentration in the broth (mg/L), and N_O, is the rate of oxygen transfer (mg O_2/L.h) (Schuler and Kargı, 1992).

The quantity of oxygen consumed per unit of time and per unit of volume, called respiration rate (mg/l.h) is one of the most important parameters for the biological activity of activated sludge. Consequently, the determination of the respiration rate is of great importance in gaining insight in activated sludge plants. For the on-line determination of the respiration rate of activated sludge, or any other oxygen

consuming liquid the measured values must be available with short intervals, and must be reproducible and of high accuracy (Applitek).

The quality and the kind of kinetic information contained in these batch respirometric tests, are governed by the ratio of the initial substrate concentration to the initial biomass concentration F/M (Chudoba et al., 1992; Ekama et al., 1986; Kappeler and Gujer, 1992; Spanjers and Vanrolleghem, 1995). The use of a low So/Xo ratio (<0.2 gCOD/gVSS) leads to short-term experiments, as the pollutants are rapidly assimilated and no significant biomass growth occurs. During these measurements, even the optimized respirometric systems do not provide sufficiently high measuring frequency for correct observation of the rapid oxydation of readily biodegradable components (Ekama et al., 1986; Spanjers and Vanrolleghem, 1995). On the other hand, at high So/Xo (>1 gCOD/gVSS), the degradation of these components is easily observable, but growth of micro-organisms occurs, making the interpretation of the multicomponent kinetics complex (Chudoba et al., 1992; Wentzel et al., 1995).

Therefore, in most of the procedures proposed, a simple method for distinguishing between the oxygen consumption associated with the use of rapidly or slowly biodegradable substrate requires an optimal So/Xo ratio, which needs to be determined by trial and error (Ekama et al., 1986). This optimal value appears to be greatly dependent on origin and characteristics of the wastewater and biomass (Dold

and Marais, 1986b; Ekama et al., 1986; Kappeler and Gujer, 1992; Sollfrank and Gujer, 1991; Xu et al., 1996).

It can be noted that the heterotrophic yield is a constituent of most parameter combinations. If the heterotrophic yield is known, all the kinetic parameters and initial concentrations are identifiable in growth conditions. therefore, even if the endogenous activity varies, model identification should theoretically permit an estimate of the oxygen consumption associated with degradation of rapidly biodegradable and hydrolysable substrate ($(1-Y_H)S_{So}$ and $(1-Y_H)X_{So}$). In addition, in growth conditions, maximum specific growth rate and initial biomass concentration can be identified separately if Y_H is known (Sperandio and Paul, 1999).

The mathematical models developed recently to simulate these processes (Gujer et al., 1995; Henze et al., 1987) are based on the division of the wastewater into various

fractions: readily biodegradable, slowly biodegradable or hydrolysable material, soluble and particulate inert fractions, and heterotrophic biomass fraction (Sperandio and Paul, 1999).

In this study, it is supposed that part of the hydrolysable components were not adsorbed in the condition of excess substrate, as the maximum adsorption capacity of biomass is completed (fixed by f_{ma} in the model). When the substrate is partially degraded and totally adsorbed, maximum activity is reached. Hence, modelling results are greatly improved by taking into account this adsorption mechanism. On the contrary, when activated sludge are in excess (low So/Xo), all the hydrolysable components were adsorbed and therefore making an adsorption model is not necessary (Sperandio and Paul, 1999).

The kinetic parameters in activated sludge systems are calculated by using software programs which utilize the data obtained from respirometric analysis. Aquasim is a software program, which was designed for the identification and simulation of aquatic systems in the laboratory, in technical plants and in nature. It performs the four tasks of simulation, identifiability, parameter estimation and uncertainity analysis.

By comparing calculated results with measured data, such simulations reveal, whether certain model assumptions are compatible with measured data.Identifiability analysis allows the user to calculate linear sensitivity functions of arbitrary variables with respect to each of the parameters included in the analysis. Parameter estimations are important for obtaining neutral estimates of parametes besides a main prerequisite for efficiently comparing different models (EAWAG, 1998)

2.5.2 Inhibition Kinetics and Kinetic Constants

At high concentrations of substrate or product and in the presence of inhibitory substances in the medium, growth becomes inhibited, and the growth rate depends on the inhibitor concentration. The inhibiton pattern of microbial growth is analogous to enzyme inhibiton. If a single-substrate enzyme-catalyzed reaction is the rate-limiting step in microbial growth, then the kinetic constants in the rate expression are biologically meaningful. Often, the underlying mechanism is complicated, and kinetic constants do not have biological meanings and are obtained from experimental data by curve fitting (Schuler and Kargı, 1992).

When inhibition is caused by toxic compounds, there can be three inhibition anology for the microorganisms' growth kinetics:

(i) competitive inhibiton, where

$$\mu = \frac{\mu_m S}{K_s.(1+\frac{I}{K_I})+S} \tag{2.3.}$$

(İİ) noncompetitive inhibition where,

$$\mu = \frac{\mu_m}{(1+\frac{K_s}{S}).(1+\frac{I}{K_I})} \tag{2.4}$$

(iii) uncompetitive inhibiton where,

$$\mu = \frac{\mu_m S}{\left(\frac{K_s}{(1+\frac{I}{K_I})}+S\right).\left(1+\frac{I}{K_I}\right)} \tag{2.5}$$

In some cases, the presence of toxic compounds in the medium results in the inactivation of cells or death. The net specific rate expression in the presence of death has the following form:

$$\mu = \frac{\mu_m S}{K_s+S} - k'_d \tag{2.6.}$$

where k'_d is the death coefficient.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Strains

E. coli K12 (DSM 498), *Microlunatus phosphovorus* (DSM 10555) and *Paracoccus pantotrophus* (DSM 2944) were selected to investigate the effects of heavy metals on bacterial mechanism. Phosphate removing bacteria, *Microlunatus phosphovorus* was selected since the heavy metal effects on this microorganism was not investigated in the literature. *Paracoccus pantotrophus* was choosen which is known as an activated sludge bacterium species which reduces nitrate to nitrogen. All of the strains were purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). The experiments were carried out in the optimum living temperatures of the strains which were: 37°C for *Eschericia coli*, 30°C for *Microlunatus phosphovorus*, and 37°C for *Paracoccus pantotrophus*,.

3.1.2. Medium

A chemically defined medium, M9 was used as the growth medium. Ingredients of solid and liquid M9 medium contain the ingredients given in Table 3.1 per one liter of medium. Glucose was used as the only carbon source. The ingredients were mixed, completed to one liter of distilled water and then autoclaved for 15 min. at 121°C. Also, 0.1 M $CaCl_2$ and 1 M $MgSO_4$ stock solutions were autoclaved separately. Stock glucose solution was also separately autoclaved for 10 min. at 121°C.

Table 3.1. Ingredients of solid and liquid standart M9 medium.

Ingredients	Quantity (gr/L)
$Na_2HPO_4.2H_2O$	8.421
KH_2PO_4	3
NaCl	0.5
NH_4Cl	1
D-Glucose	4
Agar	15

3.1.3. Heavy Metals

Sulphate salts of copper, nickel and cobalt (Merck Co.) were selected as heavy metal sources. These metals were selected because of their common existance in both municipial and industrial wastewaters. Heavy metal solutions were filter sterilized using 0.20 μm (Schleicher & Schuell, Germany) filters. Each metal was added to the culture medium before the inoculations.

3.1.4. Reactor

A reactor with 1,5 L capacity is used as the bioreactor (Biostat B, Braun Biotech International) equipped with a control unit with integrated digital measurement. Dissolved oxygen, temperature and pH were controlled in-line throughout the experiment. The jacketed glass vessel provided the constant growth temperatures. Internal concave bottom section provided optimum mixing. Off-gas was also filtered through a 20 μm filter.

3.1.5. Respirometer

A diagram of the respirometer is presented in Figure 3.1. The experiments were performed with a cylindrical aerated reactor working volume of 4L. It was equipped with a peristaltic pump and a santrifuge type stirring pump. A water jacket was used to keep the temperature of the liquid constant at the microorganisms' optimum temperatures. The water in the jacket was provided by a water circualtor (Heto). Also, the pH was measured continously by a pH meter (WTW inolab).

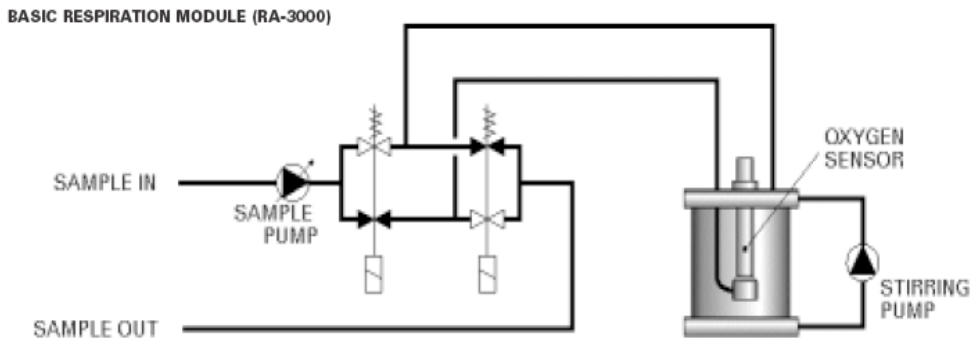

Figure 3.1. A basic respiration module (38)

Temperature and dissolved oxygen were measured in the respirometric chamber by an oxygen meter (WTW inolab). A peristaltic pump was used to keep the inlet air constant. Air was blown into the reactor through a stone sparger. The oxygen uptake rate, (OUR) was determined by measuring of the dissolved oxygen concentration in the air-tight vessel.

3.1.6. High Pressure Liquid Chromatography, (HPLC)

High Pressure Liquid Chromatography system is composed of mobile phase reservour, Shimadzu SCL-10A; system controller, LC-10A; pump, DGU-14A; degasser, SPD-10A; refractive index detector, RID-10A; autoinjector, SIL-10AD; oven CTO-10AC; and the data software, Class-VP.

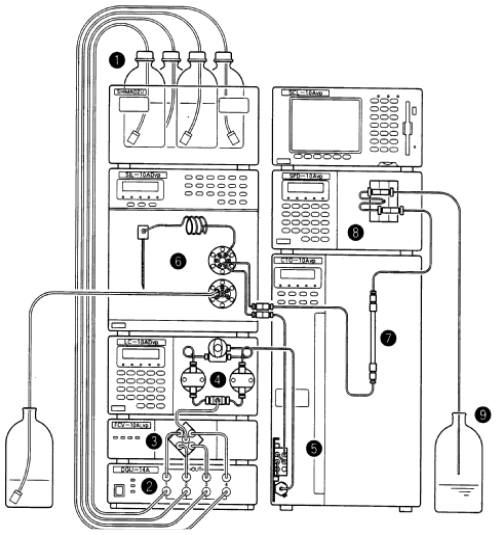

Figure 3.2. A simple diagram of High Pressure Liquid Chromatography system

3.1.7. Tools for Analysis

OD_{600} values were measured at 600 nm absorbance by a spectrophotometer, Shimadzu (UV 1601). pH of the media were controlled using a pH meter, WTW inolab. Shake flask experiments which were carried out in order to determine the

MIC values were made in baffled shake flasks, Nalgene Baffled Fasks. For all of the weight measures Precisa 125 A SCS microbalance were used.

3.2. Methods

3.2.1. Culture inoculation

The liophilized pure cultures were inoculated in 15 ml of thier own growth media, suggested by DSMZ. After the growth of the cultures, stock cultures were prepared in eppendorf tubes by adding 0,5 ml of glycerol to each 0,5 ml of culture. Some of the stocks were kept in -80°C, while some others were kept in -20°C. Also from the grown culture, petri plate stocks were prepared using LB agar media. LB agar medium was prepared in petri plates, where the cultures were inoculated and incubated overnight at their optimum growth temperatures and then kept in the refrigirator. The solid culture stocks were renewed once every 15 days.

Solid M9 media prepared in petri plates were inoculated from preprepared liquid cultures grown in M9 medium, using a loop. Precultures were prepared by inoculating 10 ml of liquid M9 medium with 1 ml of stock culture. During the shake flask experiments each flask was inoculated with 1 ml of the preculture prepared in M9 medium. Bioreactor and respirometer experiments required more biomass for inoculation. In order to maintain a certain F/M ratio precultures were prepared in shake flasks, then the required volume was calculated using the OD_{600}-CDW corelations. Required volume of preculture was then centrifuged and the pellet was washed with M9 medium without glucose, then vortexed and inoculated to the medium. In respirometer experiments, the substrate was added after the sludge reached the b_h level.

3.2.2. Solid medium experiments

Solid medium experiments were carried out in order to determine the MIC values in solid medium. Experiments were performed in petri plates. Freshly autoclaved M9 agar medium was poured to the glass petri plates containig various heavy metal concentrations. When the agar became solid, the bacteria were incubated with a loop. Wide range of heavy metal concentrations were studied until the MIC values were obtained.

3.2.3. Liquid Medium Experiments

One liter baffled flasks with a working volume of 250 ml were used in order to determine the MIC values in the liquid medium. 1 ml of glyserol stock culture which was taken from -20°C were used to inoculte the solid medium. Different concentations of heavy metal were added from the stock solutions. Then the flasks were incubated in orbital shakers at the microorganisms' optimum living tempratures with 250 rpm. Both in solid and liquid medium experiments, different concentrations of various heavy metals were investigated until the MIC value were reached.

3.2.4. Reactor Experiments

During the fermentation the pressure was kept at 1 atm and the stirrer speed at 250 rpm. Filter sterilized heavy metal solutions were added to the medium before the inoculations of the cultures. OD_{600} of the fully grown preculture was taken for calculating the total biomass in order to keep the F/M ratio constant. The preculture was was centrifuged and washed in order to wash away the residual glucose left from the preculture. After the preculture was inoculated, samples were taken once every hour for residual glucose, OD_{600} and cell dry weight analysis.

3.2.5. Respirometer experiments

Respirometric experiments were carried out with only *E. coli* K12 culture. Dynamic oxygen respiration rates of the activated sludge system were modeled using the estimates obtained with batch respirometric tests (39). A working sludge volume of 3 L was put into the jacketed vessel. The sludge included the liquid M9 medium and the washed biomass. A volume of sample is pumped through the measuring vessel with the peristaltic sample pump. The speed of the pump was adjusted to 3 which corresponds to 37,2 L/h. The closed measuring vessel was completely mixed by the stirring pump. 750 ml of sample was pumped through the measuring vessel with the sample pump, so the level of the sludge decreases in the vessel. Prior to the entering of the sample to the measuring vessel, the oxygen concentration was measured with a single oxygen sensor. After 30 seconds, the flow through the measuring vessel was reversed by switching the pinch valves. A difference in oxygen concentration was measured and leads to the respiration rate in mg O_2/l.h. 38 Air was provided by an air compressor. Air pressure was kept constant at 2 bars.

After the microorganism reaches the b_h level, where the oxygen uptake rate of the culture becomes constant, the substrate is added to the vessel, and samples are taken once every hour from the vessel. Also pH changes were determined by using a separate pH meter (WTW E90). The temperature was maintained at the microorganisms' optimum living conditions.

3.2.5.1 Modelling Respirometric Data

A simple Petersen model matrix for growth of *E.coli* was used in order to obtain the kinetic parameters by using the data obtained from the respirometric analysis. Table 3.2 shows the Petersen model matrix for growth of *E.coli.*

Table 3.2 A simple Petersen model matrix for growth of *E.coli*

Process	S_{O2}	S_S	S_A	X_{STO}	X_H	**Process rate**
Growth on S_S	$-\frac{1-Y_{H1}-Y_{Hac}}{Y_{H1}}$	$-\frac{1}{Y_{H1}}$	Y_{Hac}		1	$\mu_H \frac{S_S}{K_S+S_S} X_H$
Growth on X_{STO}	$-\frac{1-Y_{H2}}{Y_{H2}}$			$-\frac{1}{Y_{H2}}$	1	$\mu_H \frac{X_{STO}/X_H}{K_{SX}+X_{STO}/X_H} X_H$
Storage of S_A	$1-Y_{STO}$		-1	Y_{STO}		$q_{PHA} \frac{S_A}{K_{STO}+S_A} \frac{K_I}{K_I+S_S} X_H$
Decay of X_{STO}	-1			-1		$b_{STO}X_{STO}$
Decay of X_H	$1-f_p$				-1	$b_H X_H$
Aeration	1					$K_La\ (S_{O2}^*-S_{O2})$

The OUR data obtained from the respirometer is applied on the software program, AQUASIM. Then both the kinetic parameters in the Petersen model and the model simulation of the growth and substrate uptake were obtained. During the modelling construction, the following assumptions were made:

A1: During glucose degradation a part of glucose (S_S) is transformed into acetate S_A (aerobic fermentation)

A2: During the first log-growth phase, growth only takes place upon S_S

A3: Acetate uptake starts when S_S is completely degraded (ref: K_I).

A4: The growth rates on X_{STO} and S_S are kept as the same

A5: Storage and growth model was adopted from ASM3

A6: No glycogen is stored during S_S degradation (experimental evidence!)

3.2.6. Determining the OD_{600}-CDW Corelat ions

10 ml of culture samples from the reactor was taken once every hour. 0,20 μm pore size filters were dried in 105^oC oven for an hour, cooled in the dessicator and weighed. Following the filtration of the samples filters were dried again for an hour at 105^oC, cooled in the desicator and weighted. The weight difference of the filter and the sample resulted in the cell dry weight values. These values are corelated with the corresponding OD_{600} values. The line in the OD_{600}-CDW graph gives the OD_{600}-CDW corelation. Thus, at any time for a given OD_{600} value, it's possible to reach the CDW values.

3.2.7. Residual Substrate Analysis

In order to analyse the residual substrate concentrations in the growth medium, HPLC analysis were made. The column temperature of the HPLC was kept at 60^oC, and the Injection volume was chosen as 20 μl. Deionaized water used as the mobile phase in stead of 5 M of H_2SO_4, since the latter interferes with the glucose peak. Flow rate of the mobile phase was chosed as 0,6 ml/min.

3.3. Calibration of the Instruments Used For The Analysis

3.3.1. Calibration of HPLC

In order to determine the glucose concentration quantitatively, the HPLC was calibrated. Standards at various concentrations were prepared to make the calibration. The peak areas of these standards were measured and were plotted against their corresponding concentrations. The linear function of the trendline gives the concentrations of any peak measured. Figure 3.3 shows 1000 mg/L glucose calibration chromatogram

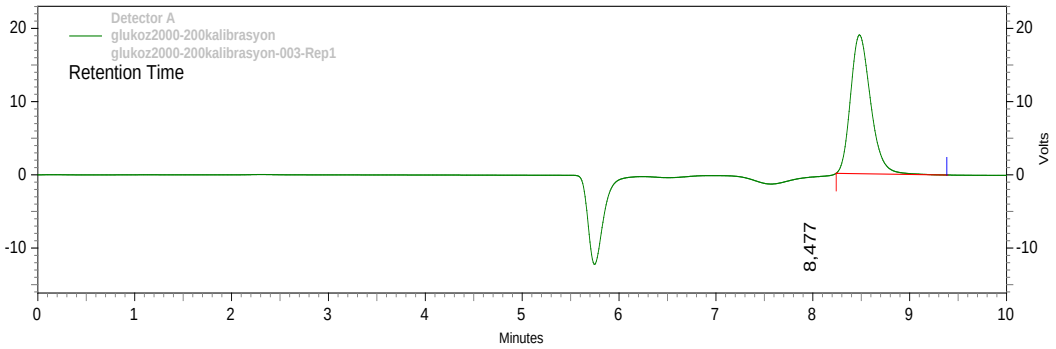

Figure 3.3. 1000 mg/L glucose calibration chromatogram

The peak at the 5,75 minutes of retention time and 8,477 minutes of retention time belonged to water and glucose respectively.

Glukoz (Detector A)
Average RF: 0,00369536 RF StDev: 0.00010162 RF %RSD: 2,74995
Scaling: None LSQ Weighting: None Force Through Zero: Off
Replicate Mode: Replace
Fit Type: Linear
y = 0,00364278x + 4,92133
Goodness of fit (r^2): 0,999949

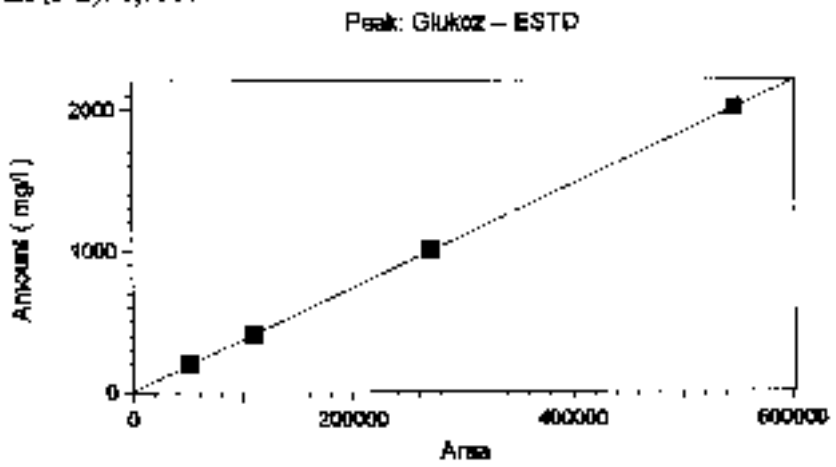

Figure 3.4. 200-2000 mg/L calibration line for glucose in HPLC

Figure 3.4. demonstrates the calibration line of glucose standards at various concentrations (200-2000 mg/L)

3.3.2. Calculation of the Kinetic Parameters

Kinetic parameters, like heterotrophic growth yields, maximum growth rate, K_S can be calculated by the help of the OUR profiles and biomass profiles.

3.3.2.1. Oxygen Uptake Rate (OUR)

The rate of oxygen uptake is denoted as OUR (oxygen uptake rate) in mg/Lh and simply formulated as,

$$OUR = q_{O_2} X_H = \frac{\mu X_H}{Y_{XO_2}} \tag{3.1.}$$

It can directly be measured with respirometer. In this study, since glucose is the only substrate, hydrolization is not considered among the processes. A simple model matrix for heterotrophic growth is given in Table 3.3. b_h and f_i values were assumed to be 0,2 and 0 respectively.

Table 3.3. Simple model matrix for heterotrophic growth

Process	S_s	Y_h	S_{O_2}	Process rate
1. growth	$-\frac{1}{Y_h}$	1	$-\frac{1-Y_h}{Y_h}$	$\mu_{max} \frac{S_S}{K_S + S_S} X_h$
2. decay		-1	$-(1-f_i)$	$b_h X_h$

Thus the effect of the decay on the yield is very low. By the help of the data obtained from the respirometer, Y_h, μ_{max}, Ks can be obtained using the software program.

3.3.2.2. Calculation of the Growth Yields (Y_h)

The substrate consumed by the microorganisms can be utilizied for different metabolic activities (equation 3.2).

$$\Delta S = \Delta S_{biomass} + \Delta S_{product} + \Delta S_{growthenegy} + \Delta S_{maintenanceenegy} \tag{3.2}$$

Biomass yields, $Y_{XS,}$ can be calculated by the growth yields of the cultures accourding to the substrate consumptions, ΔS. This calculation was made by making the assumption that the $\Delta S_{product}$ and $\Delta S_{maintenanceenegy}$ were neglected. $Y_{XS,}$ was calculated with Equation 3.3.

$$Y_{xs} = -\frac{\Delta X}{\Delta S} \tag{3.3}$$

where Y_{XS} is in g biomass/g substrate.

Heterotrophic yield, Y_h, can be calculated by using the OUR profiles provided by the respirometer accourding to equation (3.4)

$$OUR = \frac{1 - Y_H}{Y_H} \mu_\mu \frac{S}{S + K_S} X_H + b_h (1 - f_i) X_H \qquad (3.4)$$

because of the assumptions made above, Y_{XS} may not be equal to Y_h. During the bioreactor experiments, only Y_{XS} could be calculated, since OUR profiles can not be obtained.

3.3.2.3. Calculation of the Maximum Growth Rate (μ_{max})

With a simplistic approach, unstructured models defining the macroscopic environment can be described by monod kinetics. (Equation 3.5):

$$\mu = \frac{\mu_m S}{K_s + S} \qquad (3.5)$$

where μ_m is the maxiumum growth rate when $S >> K_s$. Maximum growth rate can be calculated by both the biomass concentrations and OUR profiles. Considering only the exponential growth, b_h can be neglected and equation 3,6 is obtained

$$\frac{dX_H}{dt} = \mu X_H \qquad (3.6)$$

and integrating equation 3.6,

$$X_H = X_{H_0} e^{\mu t} \qquad (3.7)$$

where X_H and X_{H0} are cell concentrations (mg/L) at time t and t_0. These equations help us obtain the maximum growth rates when OUR profile is not available. Equation 3.7 is applied for the exponential phase of the growth and the maxiumum growth rate is found plotting $\ln(X/X_0)$ values against time. The maxiumum growth rate is calculated from the slope of the OUR profile.

3.3.2.4. Half Velocity Constant (Ks)

The constant K_s is known as the saturation constant or half-velocity constant and is equal to the concentration of the rate-limiting substrate when the specific rate of growth is equal to one-half of the maximum. That is, $K_s = S$ when $\mu = 1/2\mu_{max}$. In general, $\mu = \mu_{max}$ for $S >> K_s$ and; $\mu = \mu_{max}/K_s$ for $K_s >> S$. K_S can be calcualted through the OUR experiments.

4. EXPERIMENTAL RESULTS

Heavy metal effects on pure cultures of activated sludge model organisms were studied under the chemically defined media conditions. *Escherichia coli, Microlunatus phosphovorus* and *Paracoccus pantotrophus* were chosen as the activated sludge model organisms during the studies. Cobalt, nickel and copper inhibition on the selected microorganisms were examined. The inhibition experiments were carried out both on solid and liquid media environment to observe if any tolerance difference existed between the different cultivation conditions. In liquid media environment, first shake flask experiments were performed to find out MIC values of each microorganism, and then the cultivations were repeated under bioreactor environment for detailed analysis. Finally, respirometric analyses were carried out for the microorganisms.

4.1. Determination of MIC Values of model organisms in Solid Medium

4.1.1. Determination of MIC Values in *Eschericia coli* cultures

Copper inhibition on *E. coli* cultures was studied over a range of concentrations varying between 0,01 and 0,1 mM of copper. In Figure 4.1, cultures grown on petri dishes were shown for four different $CuSO_4$ concentrations between 0.04-0.09 M.

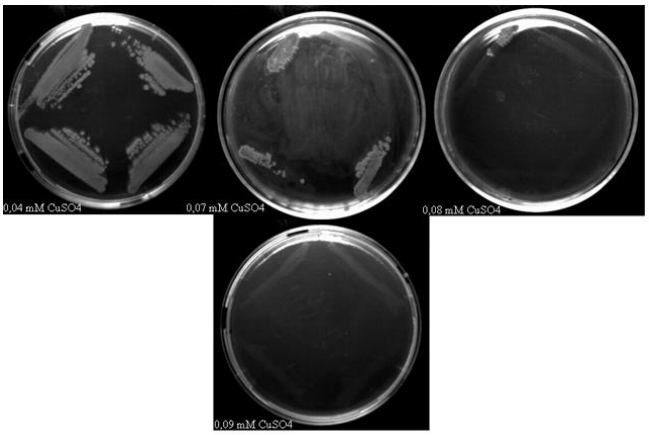

Figure 4.1: Growth inhibition of *E. coli* by various concentrations of Copper (0,04 mM, 0,07 mM, 0,08 mM, 0,09 mM of Cu^{+2})

No growth was observed above the 0.08M concentrations, therefore the MIC value of Cu^{+2} on *E. coli* culture on solid M9 medium is found to be 0,08 mM. Similarly, cobalt inhibition on *E. coli* cultures was studied over a range of concentrations varying between 0,01 and 0.3. In Figure 4.2, cultures grown on Petri dishes were shown for five different $CoCl_2$ concentrations between 0.02-0.2 M. No growth was observed above 0.15 M concentrations, therefore the MIC value of Co^{+2} on *E. coli* culture on solid M9 medium is found to be 0,15 mM

Figure 4.2: Growth inhibition of *E. coli* by various concentrations of cobalt. (0,02 mM, 0,04 mM, 0,1 mM, 0,15 mM, 0,2 mM of Co^{+2})

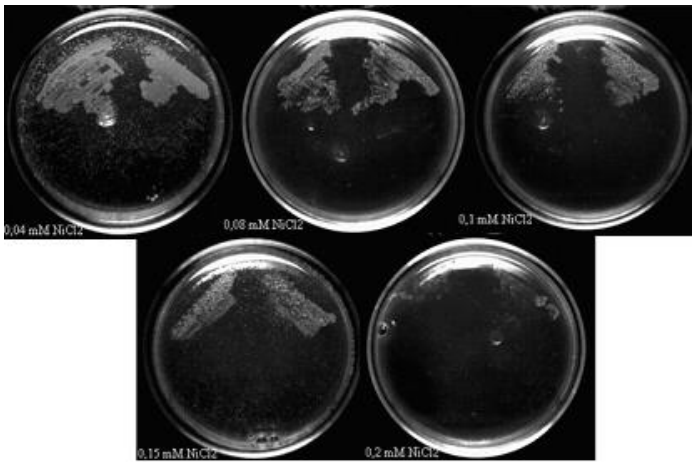

Figure 4.3: Growth inhibition of *E. coli* by various concentrations of Nickel (0,04 mM, 0,08 mM, 0,10 mM, 0,15 mM, 0,20 mM)

Nickel inhibition on *E. coli* cultures was studied over a range of concentrations varying between 0.04 and 0.2. In Figure 4.3, cultures grown on Petri dishes were shown for five different $NiCl_2$ concentrations between 0.04-0.2 mM. No growth was observed above 0.2mM concentrations, therefore the MIC value of Ni^{+2} on *E. coli* culture on solid M9 medium is found to be 0,2 mM

4.1.2. Determination of MIC Values in *Microlunatus phosphovorus* cultures

Copper inhibition on *M. phosphovorus* cultures was studied over a range of concentrations varying between 0.01 and 0.1 mM. In Figure 4.4, cultures grown on Petri dishes were shown for four different $CuSO_4$ concentrations between 0.02-0.06 mM. No growth was observed above 0.06 mM concentrations, therefore the MIC value of Cu^{+2} on *M. phospohvorus* culture on solid M9 medium is found to be 0,06 mM

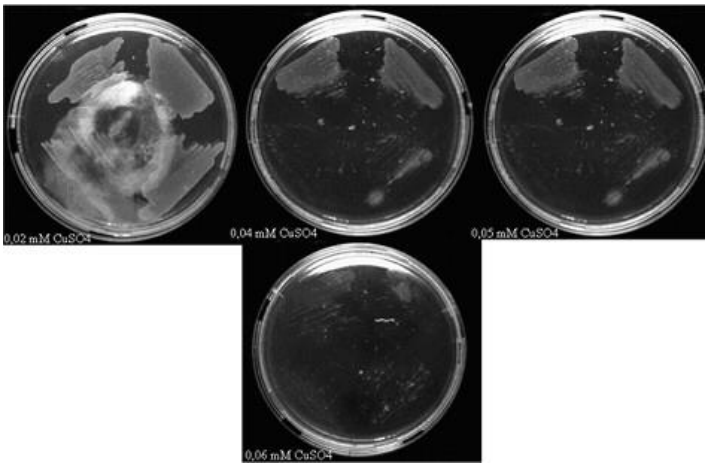

Figure 4.4: Growth inhibition of *M. phospohvorus* by various concentrations of Copper (0,02 mM, 0,04 mM, 0,05 mM, 0,06 mM of Cu^{+2})

Similarly, cobalt inhibition on *M. phospohvorus* cultures was studied over a range of concentrations varying between 0,02 and 0,2 mM. In Figure 4.5, cultures grown on Petri dishes were shown for three different CoCl2 concentrations between 0.08-0.15 M. No growth was observed above 0.15M concentrations, therefore the MIC value of Co^{+2} on *M. phospohvorus* culture on solid M9 medium is found to be 0,15 mM

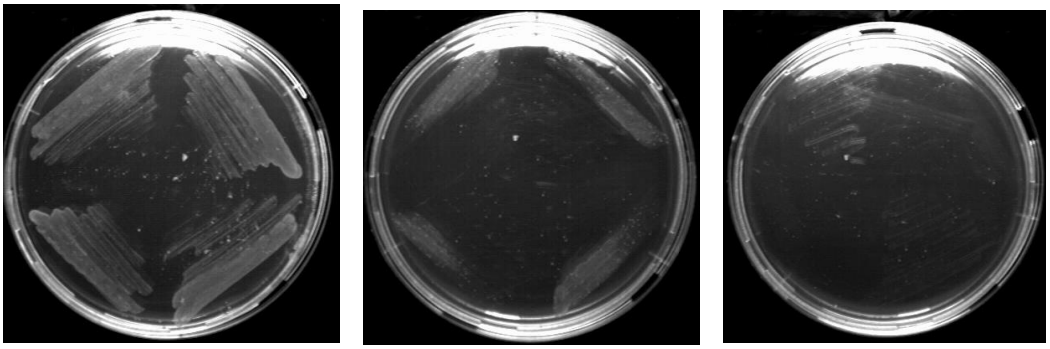

Figure 4.5: Growth inhibition of *M. phospohvorus* by various concentrations of Cobalt (0,08 mM, 0,10 mM, 0,15 mM of Co^{+2})

Nickel inhibition on *M. phospohvorus* cultures was studied over a range of concentrations varying between 0,02 and 0,2 mM. In Figure 4.6, cultures grown on Petri dishes were shown for two different NiCl2 concentrations between 0.15-0.2 mM. No growth was observed above 0.2mM concentrations, therefore the MIC value of Ni^{+2} on *M. phospohvorus* culture on solid M9 medium is found to be 0,2 mM

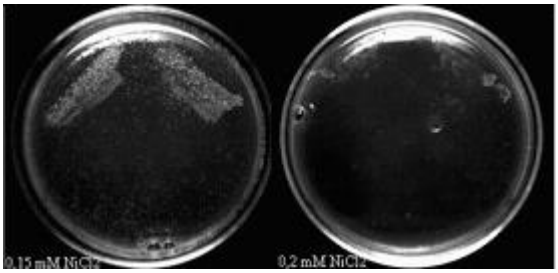

Figure 4.6: Growth inhibition of *M. phospohvorus* by various concentrations of Nickel (0,15 mM, 0,2 mM of Ni^{+2})

4.1.3. Determination of MIC Values in *Paracoccus pantotrophus* cultures

Copper inhibition on *P. pantotrophus* cultures was studied over a range of concentrations varying between 0,01 and 0,1 mM. In Figure 4.7, cultures grown on Petri dishes were shown for two different $CuSO_4$ concentrations between 0.02-0.025 mM. No growth was observed above 0.025 mM concentrations, therefore the MIC value of Cu^{+2} on *P. pantotrophus* culture on solid M9 medium is found to be 0,025 mM

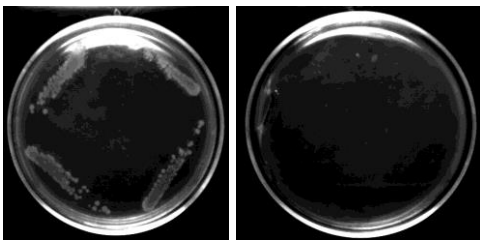

Figure 4.7: Growth inhibition of *P. pantotrophus* by various concentrations of Copper (0,02 mM, 0,025 mM of Cu^{+2})

Nickel inhibition on *P. pantotrophus* cultures was studied over a range of concentrations varying between 0,01 and 0,1 mM. In Figure 4.8, cultures grown on Petri dishes were shown for four different NiCl2 concentrations between 0.01-0.04 mM. No growth was observed above 0.04mM concentrations, therefore the MIC value of Ni^{+2} on *P. pantotrophus* culture on solid M9 medium is found to be 0,04 mM.

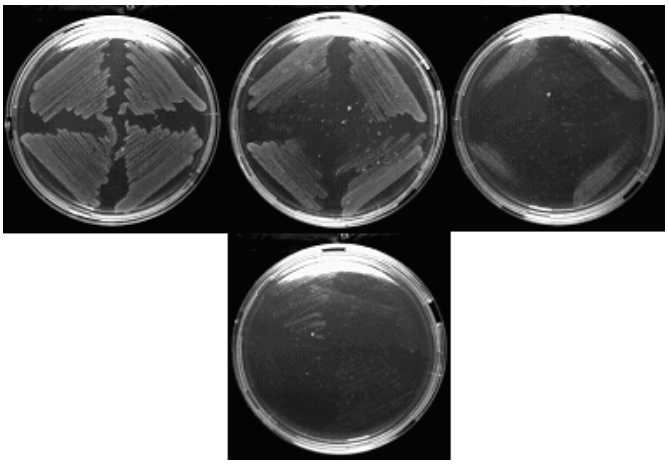

Figure 4.8: Growth inhibition of *P. pantotrophus* by various concentrations of Nickel (0,01 mM, 0,02 mM, 0,03 mM, 0,04 mM of Ni^{+2})

Similarly, cobalt inhibition on *P. pantotrophus* cultures was studied over a range of concentrations varying between 0,01 and 0,2 mM. In Figure 4.9, cultures grown on Petri dishes were shown for three different CoCl2 concentrations between 0.01-0.02 M. No growth was observed above 0.02M concentrations, therefore the MIC value of Co^{+2} on *P. pantotrophus* culture on solid M9 medium is found to be 0,02 mM.

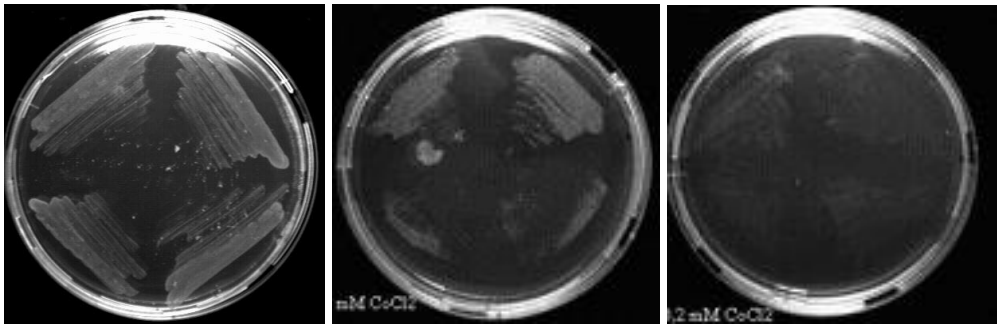

Figure 4.9: Growth inhibition of *P. pantotrophus* by various concentrations of Cobalt (0,01 mM, 0,015 mM, 0,02 mM of Co^{+2})

The MIC values determined throughout the solid media experiments are shown in Table 4.1 As seen in Table 4.1; the toxic inhibitory effect of copper was relatively higher than cobalt and nickel in solid M9 medium. As the overall heavy metal tolerance of the cultures, *P. pantotrophus* showed the least resistance. Although *E. coli* is a gram negative and *M.* p*hosphovorus* is a gram-positive bacterium, they showed nearly the same levels of tolerance to the studied heavy metal concentrations.

Table 4.1: The MIC values of Cu^{+2}, Co^{+2}, and Ni^{+2} for *E. coli, M. phosphovorus, and P. pantotrophus* in solid M9 media.

	Cu^{+2} (mM)	Co^{+2} (mM)	Ni^{+2} (mM)
E. coli	0,08	0,15	0,2
M. phosphovorus	0,06	0,15	0,2
P. pantotrophus	0,025	0,02	0,04

4.2. Determination of MIC Values in Liquid Cultures

The growth profiles of *E. coli*, *M.* p*hosphovorus* and *P. pantotrophus* were obtained in the presence and absence of heavy metals in the shake flask cultures. Baffled shake flasks were used during the experiments to minimize the oxygen limitation. The MIC values determined in the solid medium were taken as the starting concentrations in shake flask cultures. Microorganisms were found to be less tolerant to metal inhibition in liquid culture; lower metal concentrations were applied to liquid media.

4.2.1. Determination of MIC Values in *Eschericia coli* cultures

Nickel inhibition were tested over a wide range of metal concentrations but even at concentration of 0,005 mM of Ni^{+2}, the growth of *E. coli* is totally inhibited. Only the growth of control culture can be seen in Figure 4.10

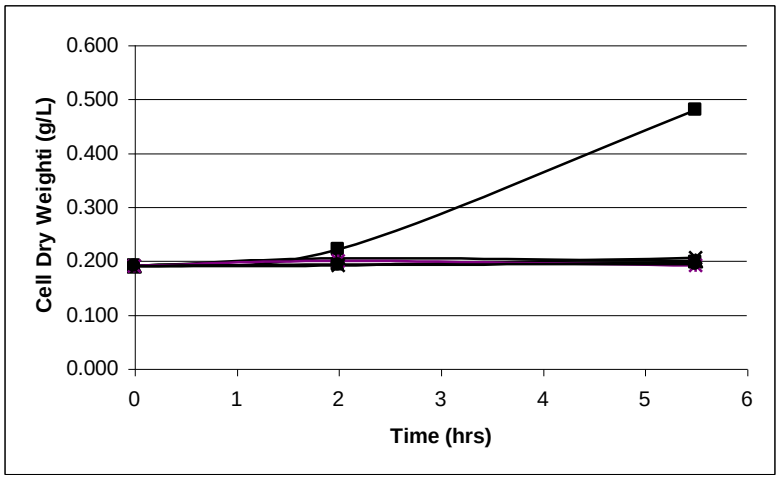

Figure 4.10: Growth inhibition of *E. coli* by various concentrations of Ni^{+2} (■control, ● 0,05, ♦0, 01, ▲0,02, *0,03, **x** 0,04, *0,05)

Below 0.02 mM Cobalt concentration, growth of *E.coli* was completely inhibited. The MIC value of Co^{+2} for *E. coli* in liquid M9 medium is found to be 0,02 mM.

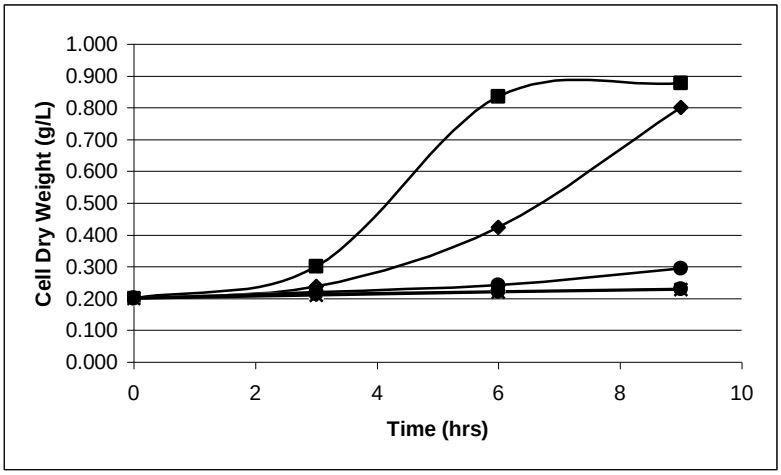

Figure 4.11: Growth inhibition of *E. coli* by various concentrations of Co^{+2} (■control, ♦0,01, ●0,02, ▲0,03, *0,04)

Below 0.04 mM of copper concentration, growth of *E.coli* was completely inhibited. The MIC value of Cu^{+2} for *E. coli* in liquid M9 medium is found to be 0,04 mM.

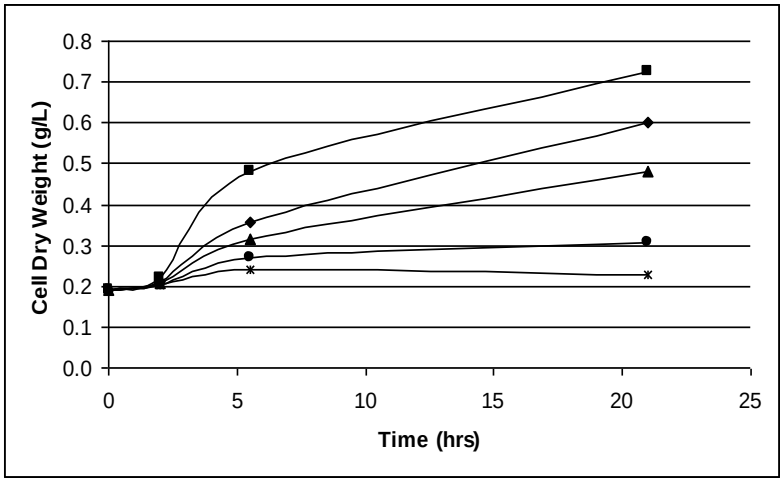

Figure 4.12: Growth inhibition of *E. coli* by various concentrations of Cu^{+2} (control, ♦ 0,01, ▲ 0,02, ● 0,03, x 0,04

4.2.2. Determination of MIC Values in *Microlunatus phosphovorus* cultures

Below 0.02 mM Cobalt concentration, growth of *M. phosphovorus* was completely inhibited. The MIC value of Co^{+2} for *M. phosphovorus* in liquid M9 medium is found to be 0,01 mM.

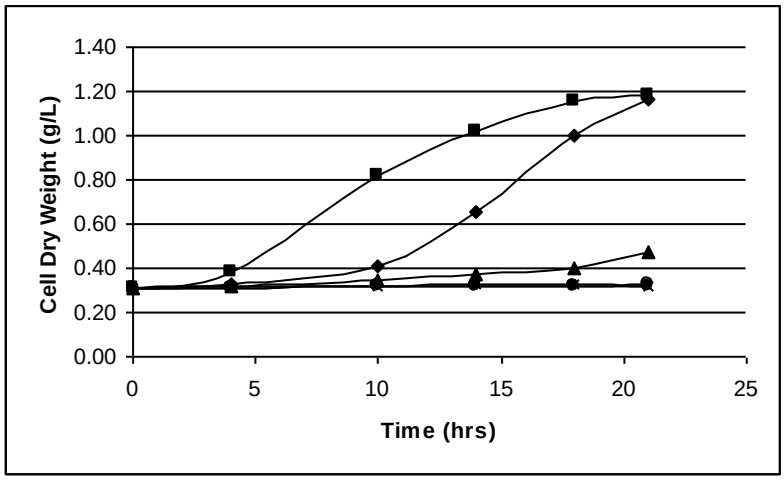

Figure 4.13: Growth inhibition of *M. phosphovorus* by various concentrations of Co^{+2} (■ control, ♦ 0,005 mM ▲0,01, ● 0,02, o 0,03, * 0,04)

Below 0.02 mM Nickel concentration, growth of *M. phosphovorus* was completely inhibited. The MIC value of Ni^{+2} for *M. phosphovorus* in liquid M9 medium is found to be 0,02 mM.

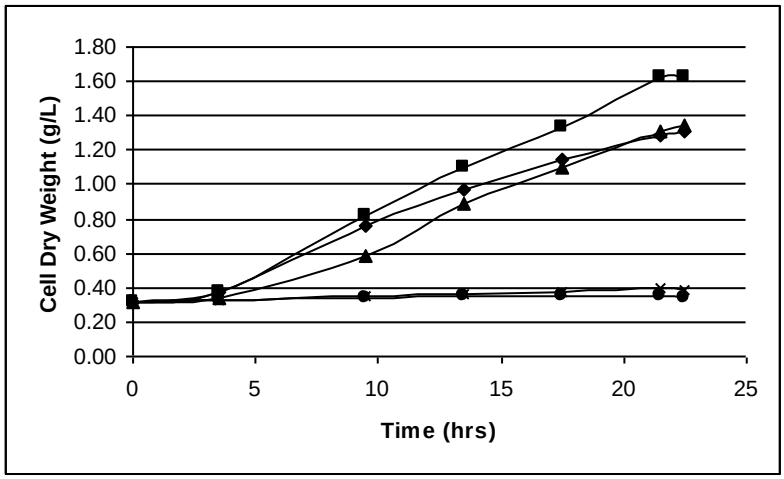

Figure 4.14: Growth inhibition of *Microlunatus phosphovorus* by various concentrations of Ni^{+2} (■ control, ♦ 0,005 mM ▲0,01, *0,02, ●0,03)

Below 0.02 mM Cupper concentration, growth of *M. phosphovorus* was completely inhibited. Even at concentration of 0,005 mM of Cu^{+2}, the growth of *Microlunatus phosphovorus* is totally inhibited.

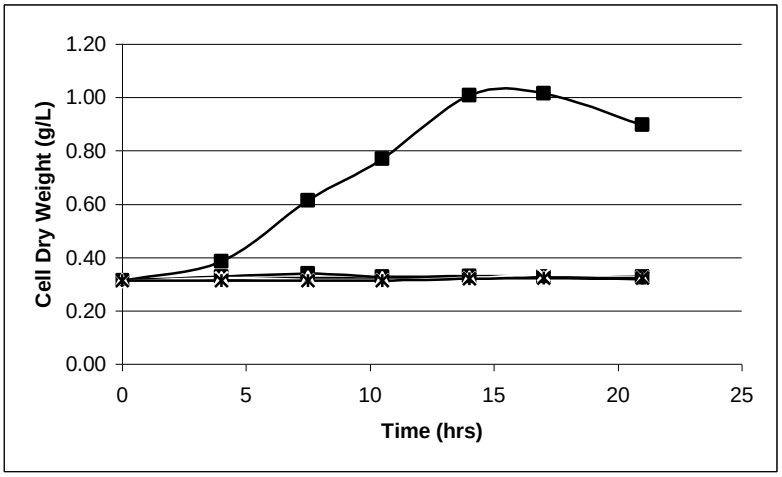

Figure 4.15: Growth inhibition of *Microlunatus phosphovorus* by various concentrations of Cu^{+2} (■ control, ♦ 0,005 mM ▲0,01, *0,02, ●0,03)

4.2.3. Determination of MIC Values in *Paracoccus pantotrophus* cultures

Below 0.02 mM of copper, the growth of *P. pantotrophus* was completely inhibited. The MIC value of Cu^{+2} for *P. pantotrophus* in liquid M9 medium is found to be 0,02 mM.

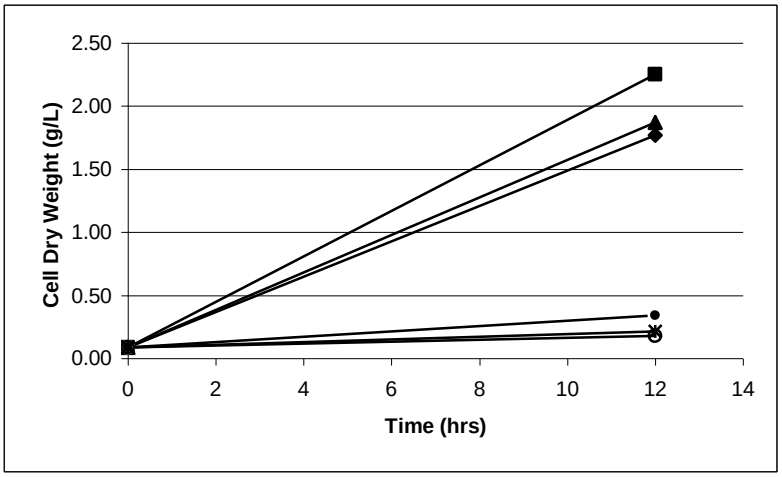

Figure 4.16: Growth inhibition of *Paracoccus pantotrophus* by various concentrations of Cu^{+2} (■ control, ▲ 0,005 mM, ♦ 0,01 mM, ● 0,02 mM, *0,03 mM, o 0,04mM)

Below 0.03 mM Nickel concentration, growth of *P. pantotrophus* was completely inhibited. The MIC value of Ni^{+2} for *P. pantotrophus* in liquid M9 medium is found to be 0,03 mM.

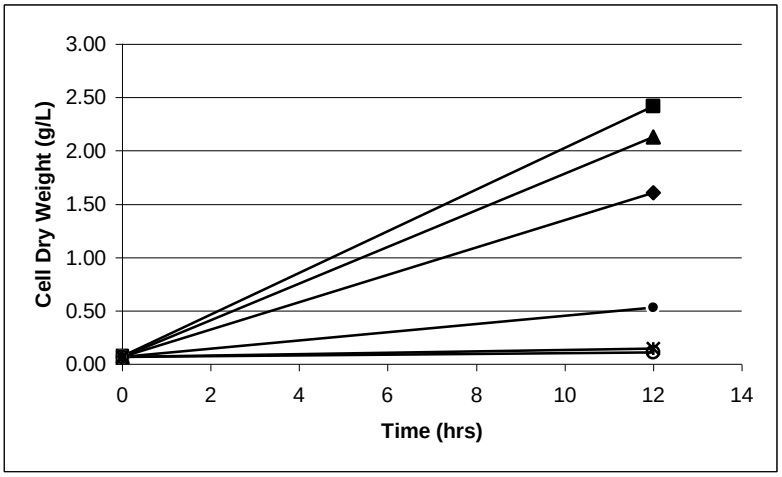

Figure 4.17: Growth inhibition of *Paracoccus pantotrophus* by various concentrations of Ni^{+2} (■ control, ▲ 0,005 mM, ♦ 0,01 mM, ● 0,02 mM, *0,03 mM, o 0,04mM)

Below 0.02 mM of cobalt, growth of *P. pantotrophus* was completely inhibited. The MIC value of Co^{+2} for *P. pantotrophus* in liquid M9 medium is found to be 0,02 mM.

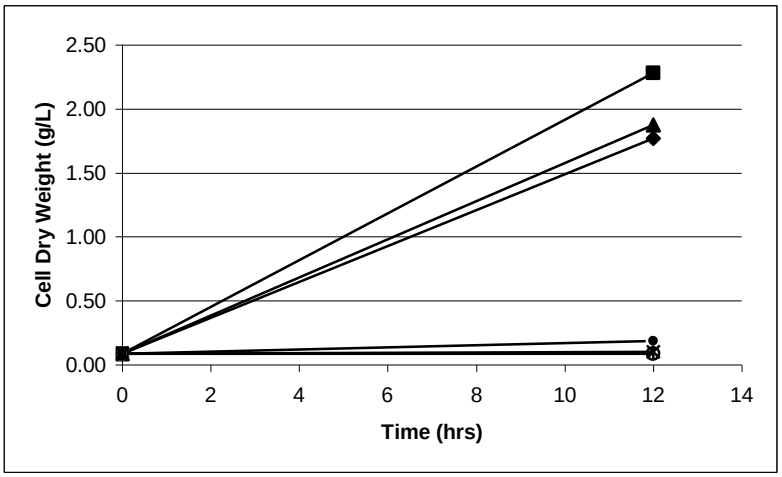

Figure 4.18: Growth inhibition of *Paracoccus pantotrophus* by various concentrations of Co^{+2} (■ control, ▲ 0,005 mM, ♦ 0,01 mM, ● 0,02 mM, *0,03 mM, o 0,04mM)

The MIC values of Cu^{+2}, Co^{+2}, Ni^{+2} for *E. coli, , M. phosphovorus and P. pantotrophus* grown in the liquid M9 medium are summarized in table 4.2.

Table 4.2: MIC values of Cu^{+2}, Co^{+2}, Ni^{+2} for *E. coli, , M. phosphovorus and P. pantotrophus* in liquid M9 medium.

	Cu^{+2} (mM)	Co^{+2} (mM)	Ni^{+2} (mM)
E. coli	0,04	0,02	-
M. phosphovorus	-	0,01	0,02
P. pantotrophus	0,02	0,02	0,03

4.3. Batch Reactor Experiments

Microorganisms were grown in bioreactor environment for detailed analysis of their metabolic tolerance to different metal concentrations. Bioreactor experiments were carried out considering the MIC values determined in the shake flasks. Bioreactor conditions were provided controlled environment compared to the shake flasks cultures, therefore the growths of microorganisms were studied under non-oxygen limiting conditions and the kinetic parameters of the system were obtained. Similar to the shake flask experiments, the growth profiles of *E.coli, M.phosphovorus,* and *P.pantotorpus* under metal inhibition were found to be different from the control cultures' growth profiles. The lowest possible heavy metal concentrations, which can inhibit the growth of the cultures, were used in the batch experiments.

Heavy metal inhibition did not only affect the bacterial growth, but also the substrate uptake as expected. Although the control and the inhibited cultures had the same feed/microorganism ratio and lag phase time, the final CDW values were different for *E.coli* K12, *M.phosphovorus* and *P.Pantotropus*.

During the control experiments, which carried out under uninhibited conditions, OD_{600}-CDW correlations were also made along with the determination of the metabolic values.

In order to see the metabolic shifts on the cultures of *E. coli, M. phosphovorus and P. pantotrophus* cultures caused by Cu^{+2}, Co^{+2}, Ni^{+2} metals, batch reactor experiments were carried out in heavy metal concentrations lower than the minimum inhibition concentrations. Thus the following heavy metal concentrations were studied during the batch reactor experiments.

Table 4.3. Heavy metal concentrations studied for each microorganism in bioreactor experiments

	Cu^{+2} (mM)	Co^{+2} (mM)	Ni^{+2} (mM)
E. coli	0,01	0,01	-
M. phosphovorus	-	0,005	0,02
P. pantotrophus	0,01	0,01	0,01

The baffled shake flasks cannot provide as much oxygen transfer to the culture medium as a well-aerated fermentor. Thus the microorganisms cultivated in a shake flask may respond in different ways to the metal inhibitions when incubated in a well-aerated fermentor.

4.3.1. Batch Experiments in *Eschericia coli* cultures

In experiments carried out on *E. coli* cultures, the metal inhibited cultures consumed the substrate by different levels. All the inhibited and non-inhibited cultures reached the stationary phase at 7^{th} hour. It should be noted that the same cobalt and copper concentrations caused different levels of inhibitory effects on *E. coli* cultures. This can be seen both in the growth yields, the residual substrate levels and the maximum growth rates. As a consequence the cultures ended up with different biomass concentrations.

4.3.1.1. Control Experiments in *Eschericia coli* cultures

First control experiments were carried out for *E.coli* cultures in M9 liquid media. OD versus cell dry weight calibration was obtained (Figure 4.19). The OD_{600}-CDW correlation of *E. coli* culture is found to be:

$$CDW\ (g/L) = 0{,}460.(\ OD_{600}) \qquad (4.1)$$

Using Equation 3.7, the maximum growth rate, μ_{max} was found to be 0,6802 h^{-1}, which equals to 16,3 d^{-1} for *E. coli* culture under uninhibited conditions (Figure 4.20).

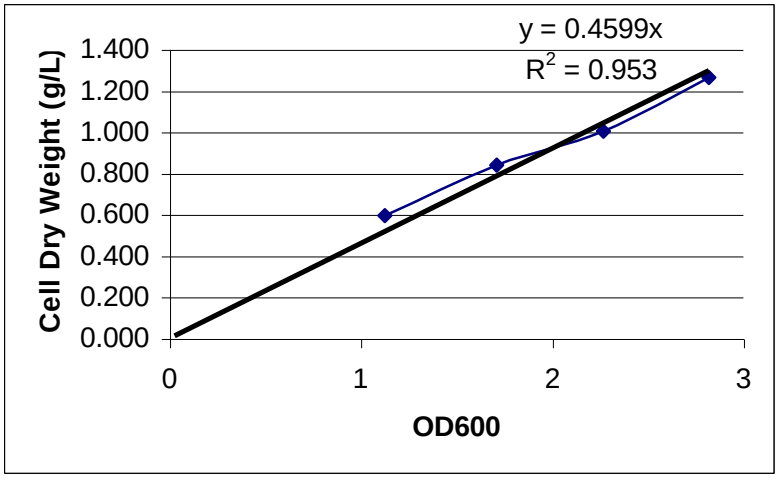

Figure 4.19: OD_{600}-CDW correlation of *E. coli* culture.

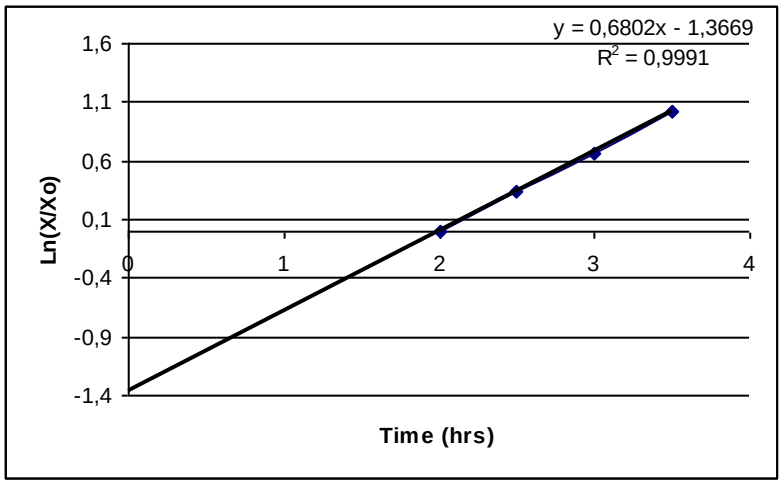

Figure 4.20: Maximum growth rate of *E. coli* culture under uninhibited conditions.

4.3.1.2 Copper inhibition in *Eschericia coli* cultures

The lowest possible heavy metal concentration, which can inhibit the growth of Eschericia coli, was used in the batch experiments. 0,01 mM of Cu^{+2} is a concentration, which is high enough to inhibit the growth of *E. coli* and low enough to observe the metabolic shifts

Figure 4.21 shows the effect of the 0,01 mM of Cu^{+2} on the maximum growth rate, μ_{max} of *E. coli* culture. Using equation 3.7, the maximum growth rate, μ_{max} was found to be 0,331 h^{-1}, which equals to 7,95 d^{-1}.

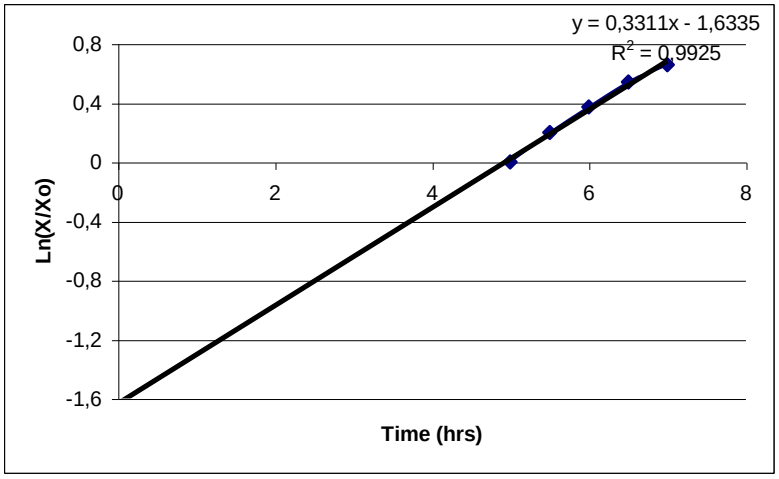

Figure4.21: Maximum growth rate of *E. coli* culture inhibited with 0,01 mM of Cu^{+2}

Figure 4.22 shows the effect of the 0,01 mM of Cu^{+2} on the Cell dry weight level of *E. coli* culture during the experiment. The log phase delayed for 3 hours under Co^{+2} inhibition. Copper caused the final Cell dry weight concentration to decrease (Figure 4.22)

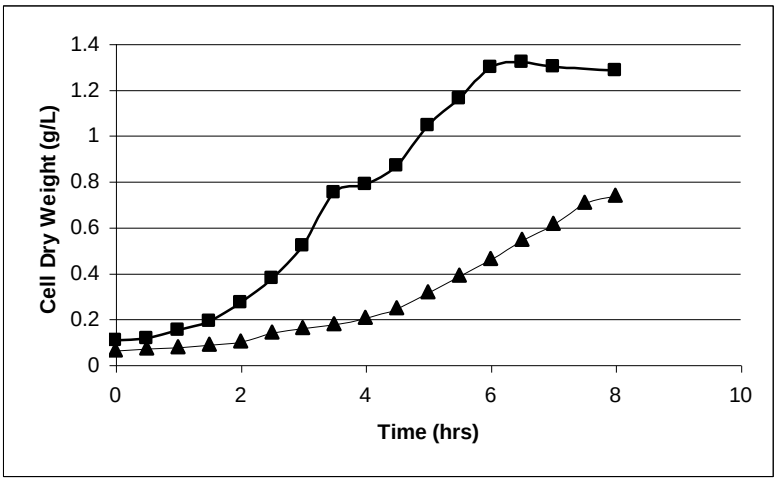

Figure 4.22: Cell dry weight in the *E. coli* culture medium under inhibited and non-inhibited conditions. (■ control, ♦ 0,01 mM Cu)

Figure 4.23 shows the effect of the 0,01 mM of Cu^{+2} on the residual substrate level of *E. coli* culture during the experiment. The inhibited culture consumes the substrate slower than the uninhibited culture and reaches the stationary phase before it consumes all of the substrate.

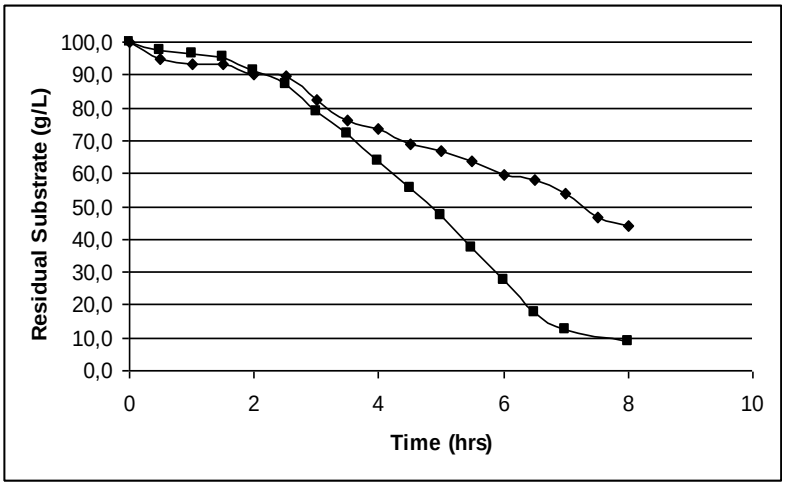

Figure 4.23: Residual substrate in *E. coli* culture medium under inhibited and non-inhibited conditions. (■ control, ♦ 0,01 mM Cu)

4.3.1.3. Cobalt inhibition in *Eschericia coli* cultures

The lowest possible heavy metal concentration, which can inhibit the Eschericia coli growth, was used in the batch experiments. 0,01 mM of Co^{+2} is a concentration, which is high enough to inhibit the growth of *E. coli* and low enough to observe the metabolic shifts.

Figure 4.24. shows the effect of the 0,01 mM of Co^{+2} on the maximum growth rate, μ_{max} of *E. coli* culture. Using Equation 3.7, the maximum growth rate, μ_{max} was found to be 0,329 h^{-1}, which equals to 7,9 d^{-1}.

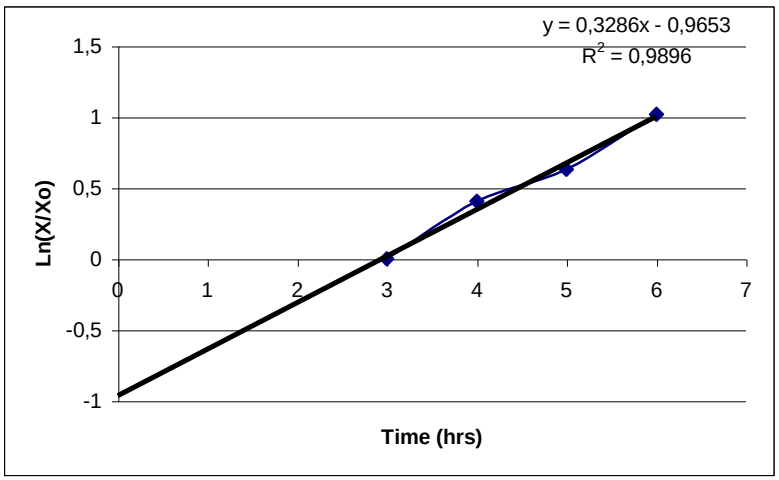

Figure 4.24: Maximum growth rate of *E. coli* culture inhibited with 0,01 mM of Co^{+2}

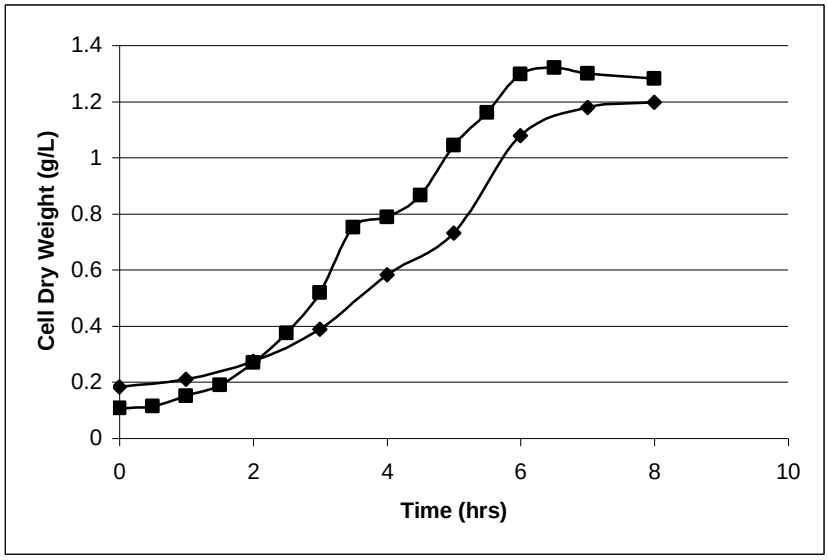

Figure 4.25: Cell dry weight in the *E. coli* culture medium under inhibited and non-inhibited conditions (■ control, ● 0,01 mM Co)

Figure 4.25 shows the effect of the 0,01 mM of Co^{+2} on the cell dry weight level of *E. coli* culture during the experiment. The log phase delayed for 1 hour under Co^{+2} inhibition. Figure 4.26 shows the effect of the 0,01 mM of Co^{+2} on the residual substrate level of *E. coli* culture during the experiment. The inhibited culture consumes the substrate slightly faster than the uninhibited culture and reaches the stationary phase as it consumes all of the substrate.

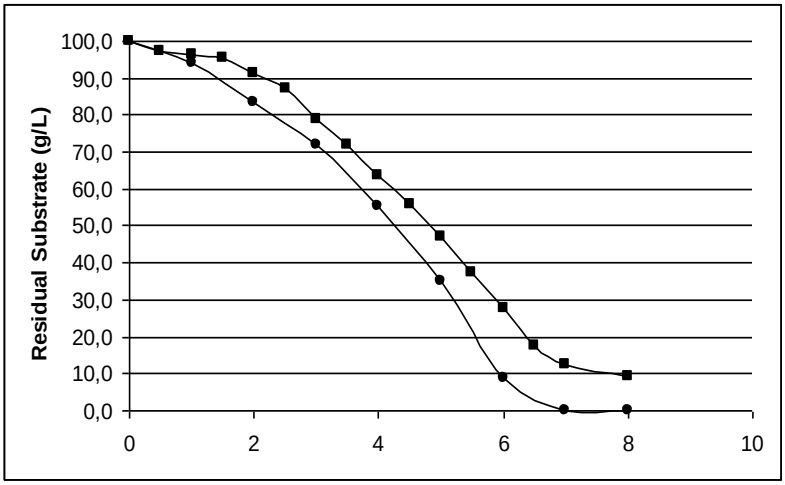

Figure 4.26. Residual glucose in *E. coli* culture medium under inhibited and uninhibited conditions. (■ control, ● 0,01 mM Co)

The overall results of inhibited and uninhibited *E. coli* cultures are shown in Table 4.4. copper and cobalt caused a shift by %20 and %30 in the biomass yields, respectively while the maximum growth rates decreased by a 50%.

Table 4.4: Kinetic parameters calculated for E.coli culture for metal inhibited and uninhibited conditions

	Y_{xs} (g/g)	μ_{max} (d^{-1})	X_m (g/L)	S_c (mg/L)
Control	0,37	16,32	1,32	3640
Cu^{+2}	0,30	7,94	0,78	2128
Co^{+2}	0,25	7,89	1.196	4000

4.3.2. Batch Experiments in *Microlunatus phosphovorus* cultures

Experiments carried out on *Microlunatus phosphovorus* cultures showed that, the inhibited cultures consumed the substrate by different levels. All the inhibited and non-inhibited cultures reached the stationary phase at 7^{th} hour. It should be noted that the same cobalt and copper concentrations caused different levels of inhibitory effects on *Microlunatus phosphovorus* cultures. This can be seen both in the growth yields, the residual substrate levels and the maximum growth rates. As a consequence the cultures ended up with different biomass concentrations.

4.3.2.1. Control Experiments in *Microlunatus phosphovorus* Cultures

First control experiments were carried out for *Microlunatus phosphovorus* cultures in M9 liquid media. OD versus cell dry weight calibration was obtained (Figure4.27) The OD_{600}-CDW correlation of *M. phosphovorus* culture is found to be:

$$CDW\ (g/L) = 0{,}469.(\ OD_{600}) \qquad (4.2.)$$

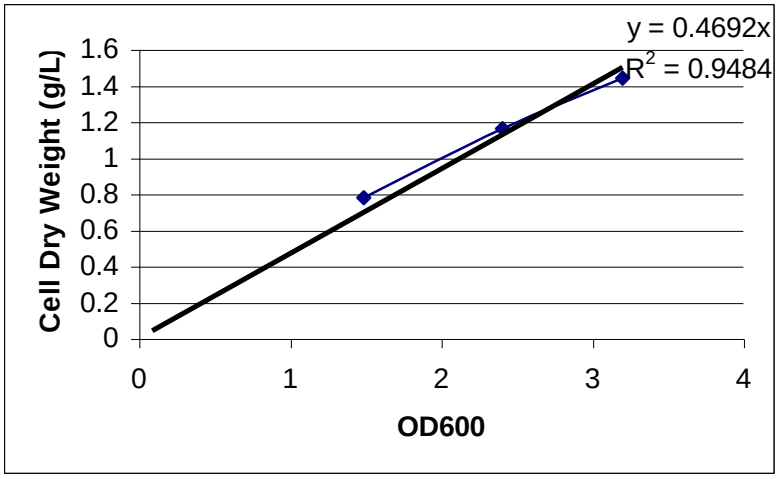

Figure 4.27: OD_{600}-CDW correlation of *M. phosphovorus* culture.

Using equation 3.7, the maximum growth rate, μ_{max} was found to be 0,2405 h^{-1}, which equals to 5,78 d^{-1} for *M. phosphovorus* culture under uninhibited conditions (Figure 4.28).

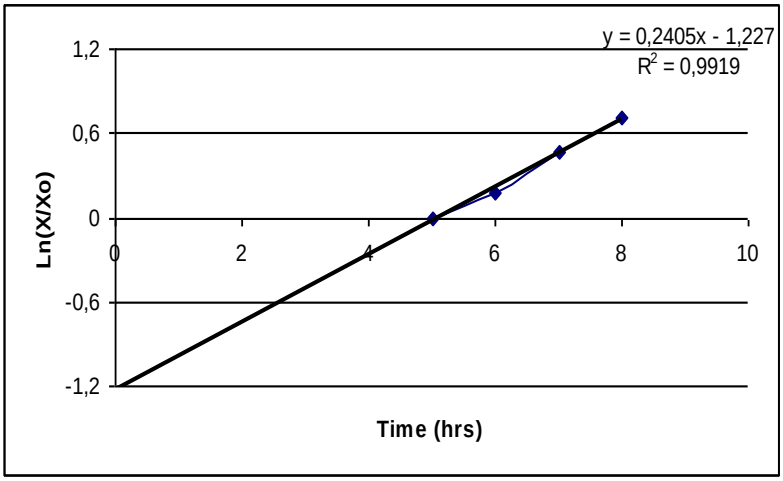

Figure 4.28. Maximum growth rate of *Microlunatus phosphovorus* culture under uninhibited conditions.

4.3.2.2 Cobalt inhibition in *Microlunatus phosphovorus* cultures

Figure 4.29 shows the effect of the 0,005 mM of Co^{+2} on the maximum growth rate, μ_{max} of *M. phosphovorus* culture. Using equation 3.7 the maximum growth rate, μ_{max} was found to be 0,229 h^{-1}, which equals to 5,5d^{-1}.

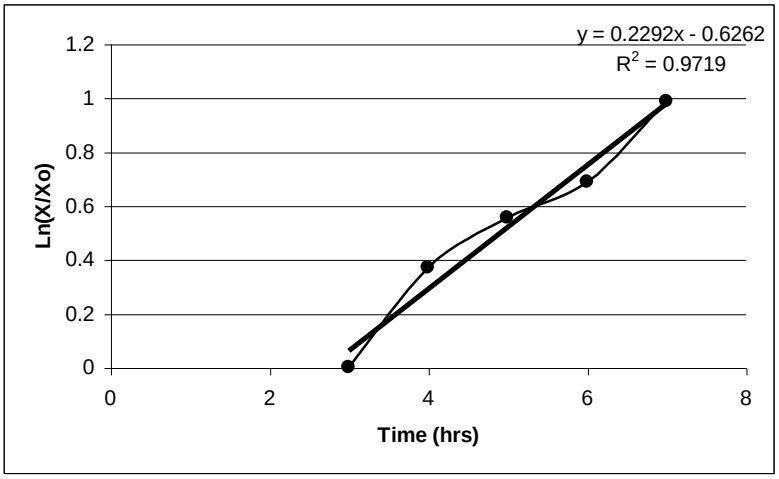

Figure 4.29: Maximum growth rate of *M. phosphovorus* culture inhibited with 0,005 mM of Co^{+2}

Figure 4.30 shows the effect of the 0,005 mM of Co^{+2} on the cell dry weight level of *M. phosphovorus* culture during the experiment. Although the growth rate of Co^{+2} inhibited *M. phosphovorus* culture is almost the same as the uninhibited culture, the inhibition effect is seen on the final biomass concentration

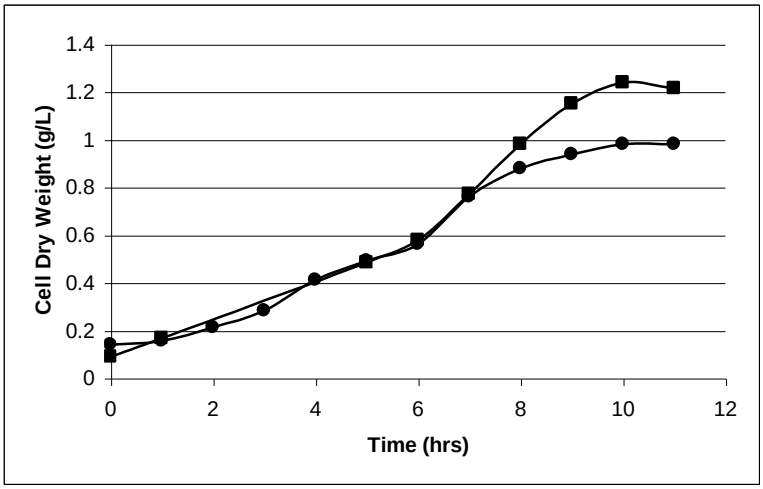

Figure 4.30: Cell dry weight in the *M. phosphovorus* culture medium under inhibited and non-inhibited conditions. (■ control, ● 0,005 mM Co)

.Figure 4.31 shows the effect of the 0,005 mM of Co^{+2} on the residual substrate level of *M. phosphovorus* culture during the experiment. The inhibited culture consumes the substrate as faster as the uninhibited culture and reaches the stationary phase at the same level of substrate uptake.

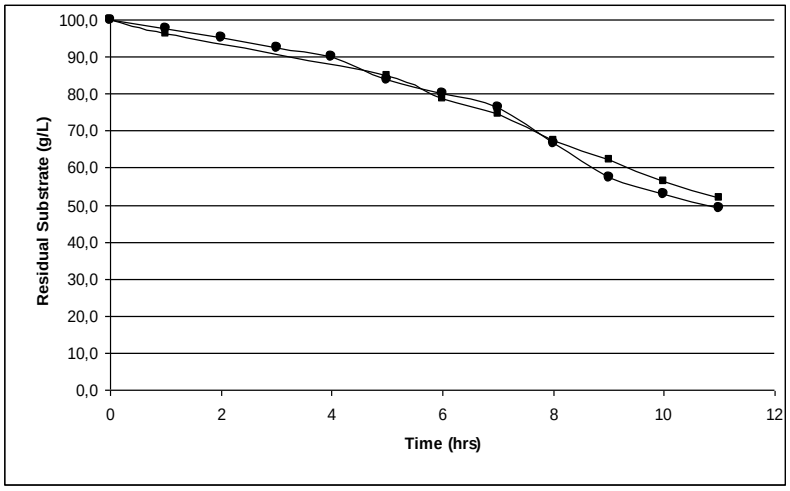

Figure 4.31: Residual glucose in *M. phosphovorus* culture medium under inhibited and non-inhibited conditions. (■ control, ● 0,005 mM Co)

4.3.2.3. Nickel inhibition in *Microlunatus phosphovorus* cultures

The response of *Microlunatus phosphovorus* to the Ni^{+2} inhibition in the well-aerated conditions of the bioreactor was different from the shake flask experiments. Although, 0,02 mM of Ni^{+2} was found to be enough to inhibit the growth of the culture completely in the shake flask experiments, *M. phosphovorus* could grow in the bioreactor experiments under this concentration of nickel. The log phases delayed by 2 hours under Ni^{+2}inhibited conditions.

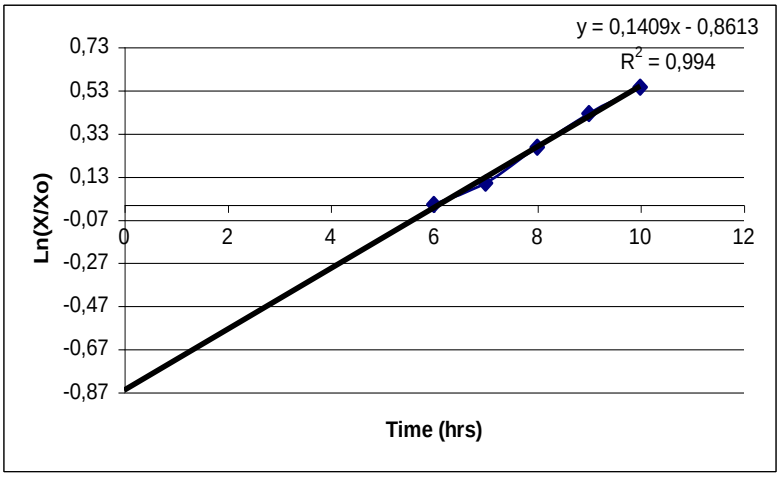

Figure 4.32: Maximum growth rate of *M. phosphovorus* culture inhibited with 0,02 mM of Ni^{+2}

Figure 4.32 shows the effect of 0,02 mM of Ni^{+2} on the maximum growth rate, μ_{max} of *M. phosphovorus* culture. Using equation 3.7, the maximum growth rate, μ_{max} was found to be 0,141 h^{-1}, which equals to 3,38 d^{-1}. Figure 4.33 shows the effect of the

0,02 mM of Ni^{+2} on the cell dry weight level of *M. phosphovorus* culture during the experiment. Both the general growth rate and the final biomass concentration of the inhibited culture are lower than the control values.

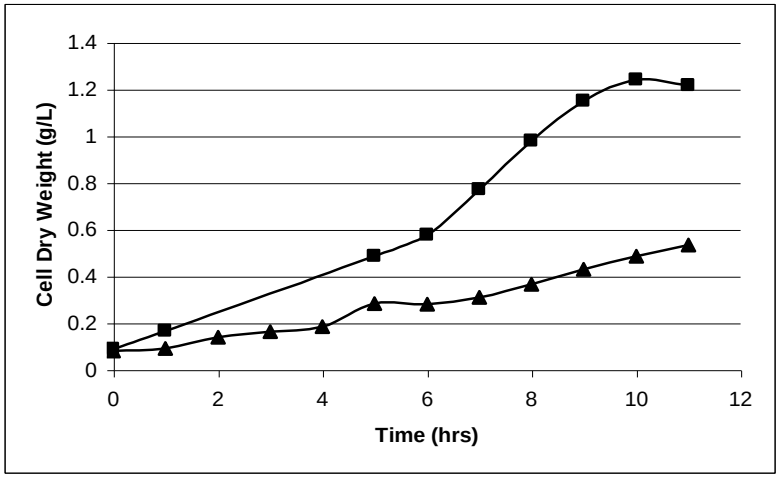

Figure 4.33: Cell dry weight in the *M. phosphovorus* culture medium under inhibited and non-inhibited conditions. (■ control, ▲ 0,02 mM Ni^{+2})

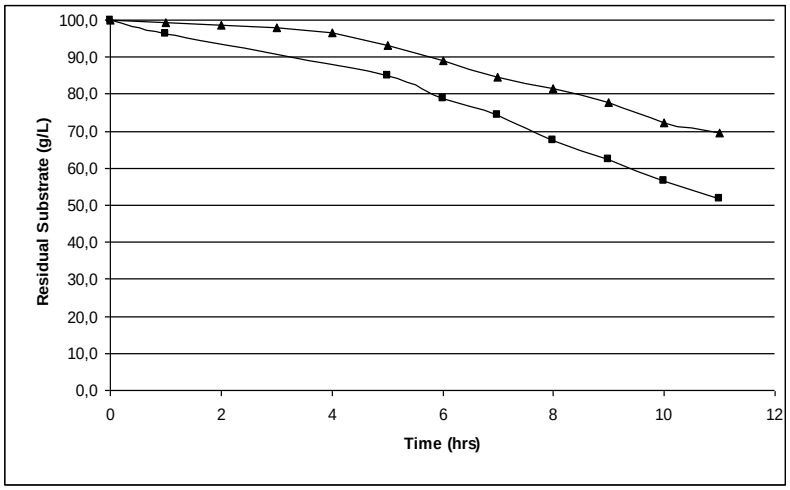

Figure 4.34: Residual glucose in *M. phosphovorus* culture medium under inhibited and non-inhibited conditions. (■ control, ▲ 0,02 mM Ni^{+2})

Figure 4.34 shows the effect of the 0,02 mM of Ni^{+2} on the residual substrate level of *M. phosphovorus* culture during the experiment. The inhibited culture consumes the substrate slower than the uninhibited culture the overall results of inhibited and uninhibited *M. phosphovorus* cultures are shown in Table 4.5

Table 4.5: Kinetic parameters obtained for *M. phosphovorus* cultures under metal inhibited and uninhibited conditions

	Y_{xs} (g/g)	μ_{max} (d^{-1})	X_m (g/L)	S_c (mg/L)
Control	0,64	5,78	1,24	1792
Co^{+2}	0,45	5,49	0,98	1880
Ni^{+2}	0,36	3,38	0,53	1172

According to the data obtained from *M.phosphovorus* bioreactor experiments; the microorganisms showed more tolerance to 0,005 mM Cobalt than to 0,02 mM of Nickel.

As can be seen on Table 4.5, both the maximum growth rate and the final biomass concentrations of the nickel-inhibited culture are lower than the control values by %42 and by %58 respectively. The growth yields of *M. phosphovorus* were inhibited by 30% under cobalt inhibition and by 44% under nickel inhibition with respect to the control set.

4.3.3. Batch Experiments in *Paracoccus pantotrophus* cultures

The inhibitory effects of 0,01 mM nickel, 0,01mM cobalt and 0,01 mM copper on *P.pantotrophus* were almost the same with each other, considering the growth and substrate uptake profiles. Y_{xs}, μ_{max}, and X_m values of the inhibited *P.pantotrophus* cultures are close to each other.

Nickel, cobalt and copper had the same level of inhibitory effects on *P.pantotrophus.* Neither *M. phosphovorus* nor *P.pantotrophus* control sets could consume the substrate completely till the end of the experiment.

4.3.3.1 Control Experiments in *Paracoccus pantotrophus* cultures

First control experiments were carried out for ***Paracoccus pantotrophus*** cultures in M9 liquid media The OD_{600}-CDW correlation of *P. pantotrophus* culture is found to be:

$$CDW\ (g/L) = 0,686.(\ OD_{600}) \qquad (4.3)$$

Using equation 3.7, the maximum growth rate, μ_{max} was found to be 0,462 h^{-1}, which equals to 11,1 d^{-1} for *M. phosphovorus* culture under uninhibited conditions (Figure 4.36).

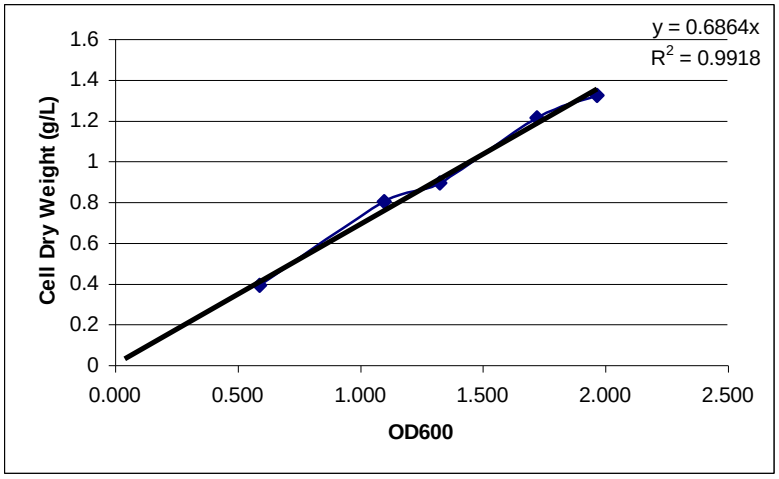

Figure 4.35: OD_{600}-CDW correlation of *P. pantotrophus* culture.

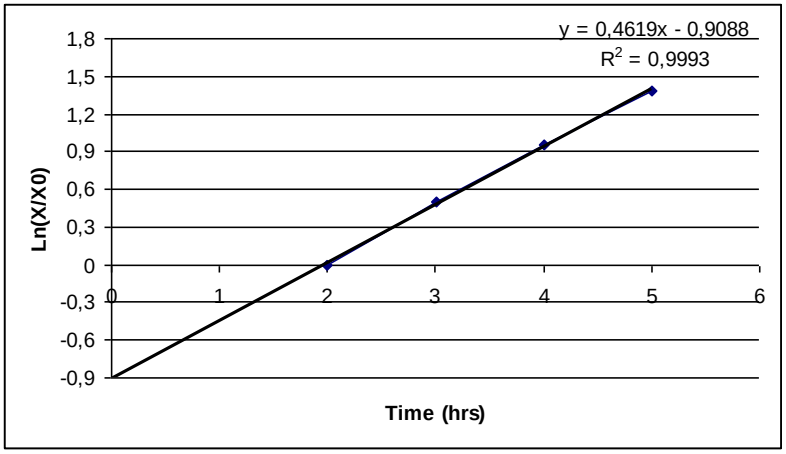

Figure 4.36: Maximum growth rate of *P. pantotrophus* culture under uninhibited conditions.

4.3.3.2 Copper inhibition in *Paracoccus pantotrophus* cultures

Figure 4.37 shows the effect of 0,01mM of Cu^{+2} on the maximum growth rate, μ_{max} of *P. pantotrophus* culture. Using equation 3.7, the maximum growth rate, μ_{max} was found to be 0,408 h^{-1}, which equals to 9,8 d^{-1}.

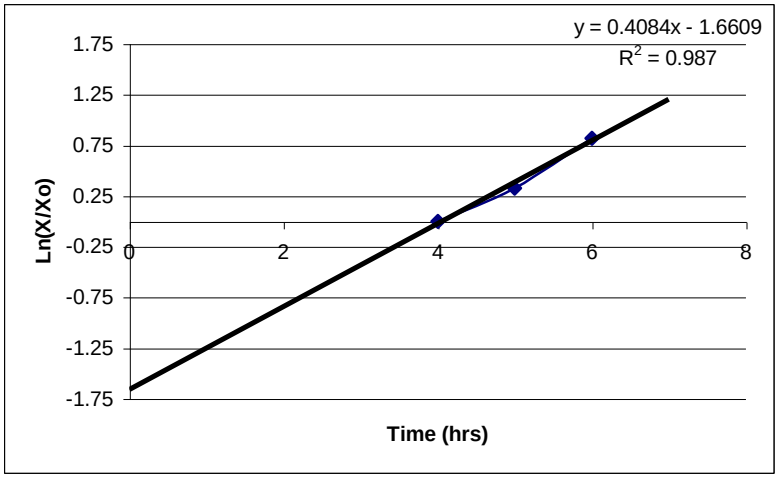

Figure 4.37: Maximum growth rate of *P. pantotrophus* culture inhibited with 0,01mM of Cu^{+2}

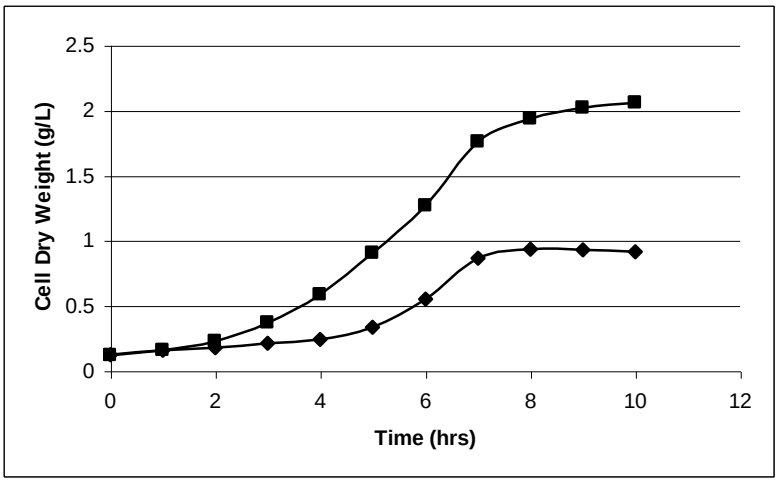

Figure 4.38: Cell dry weight in the *P. pantotrophus* culture medium under inhibited and non-inhibited conditions. (■ control, ♦ 0,01 mM Cu)

Figure 4.39 shows the effect of the 0,01mM of Cu^{+2} on the cell dry weight level of *P. pantotrophus* culture during the experiment. Both the general growth rate and the final biomass concentration of the inhibited culture are lower than the control values. Figure 4.40 shows the effect of the 0,01 mM Cu^{+2} on the residual substrate level of *M. phosphovorus* culture during the experiment. The inhibited culture consumes the substrate slower than the uninhibited culture.

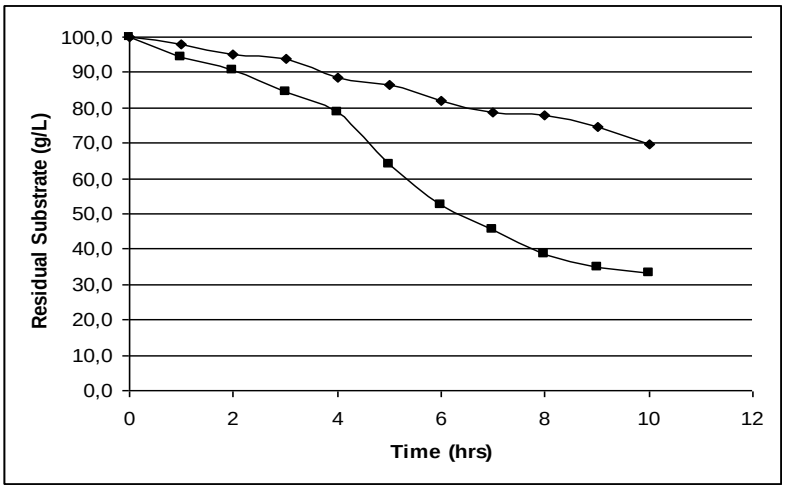

Figure 4.39: Residual glucose in *P. pantotrophus* culture medium under inhibited and non-inhibited conditions. (■ control, ♦ 0,01 mM Cu^{+2})

4.3.3.3. Cobalt inhibition in *Paracoccus pantotrophus* cultures

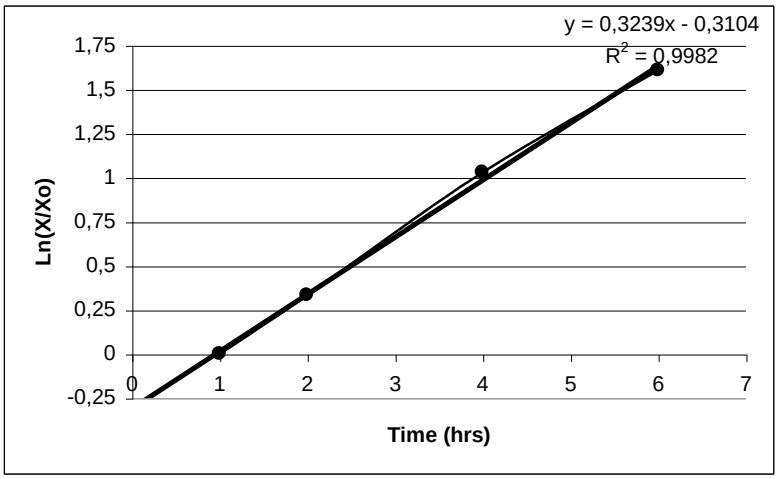

Figure 4.40: Maximum growth rate of *P. pantotrophus* culture inhibited with 0,01mM of Co^{+2}

Figure 4.41 shows the effect of 0,01mM of Co^{+2} on the maximum growth rate, μ_{max} of *P. pantotrophus* culture. Using equation 3.7 the maximum growth rate, μ_{max} was found to be 0,324 h^{-1}, which equals to 7,8 d^{-1}. Figure 4.42 shows the effect of the 0,01mM of Co^{+2} on the cell dry weight level of *P. pantotrophus* culture during the experiment. Both the general growth rate and the final biomass concentration of the inhibited culture are lower than the control values

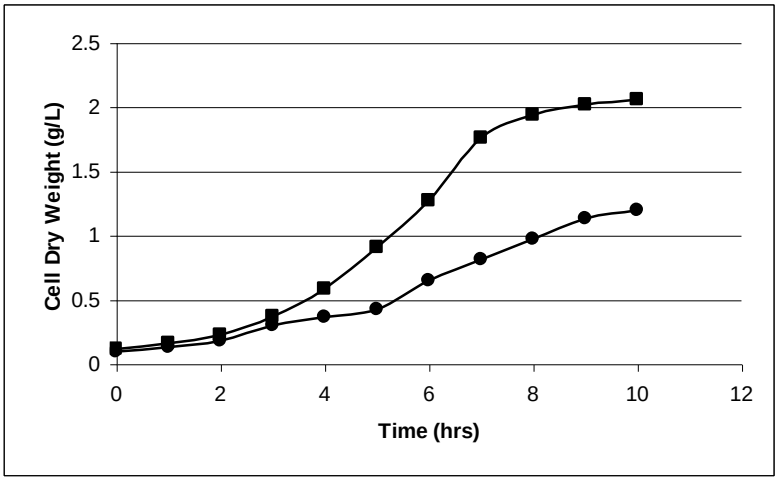

Figure 4.41: Cell dry weight in the *P. pantotrophus* culture medium under inhibited and non-inhibited conditions. (■ control, ● 0,01 mM Co)

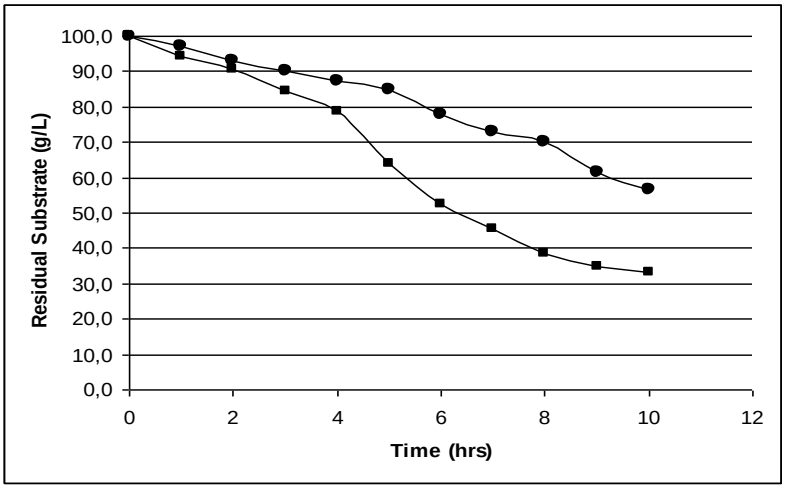

Figure 4.42: Residual glucose in *P. pantotrophus* culture medium under inhibited and non-inhibited conditions. (■ control, ● 0,01 mM Co)

Figure 4.43 shows the effect of the 0,01 mM Co^{+2} on the residual substrate level of *M. phosphovorus* culture during the experiment. The inhibited culture consumes the substrate slower than the uninhibited culture.

4.3.3.4. Nickel inhibition in *Paracoccus pantotrophus* cultures

Figure 4.44 shows the effect of 0,01mM of Ni^{+2} on the maximum growth rate, μ_{max} of *P. pantotrophus* culture. Using equation 3.7, the maximum growth rate, μ_{max} was found to be 0,439 h^{-1}, which equals to 10,54 d^{-1}.

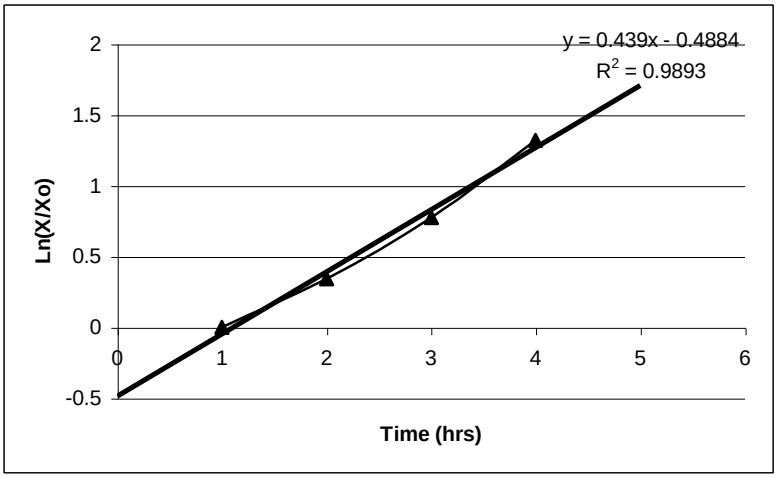

Figure 4.43: Maximum growth rate of *P. pantotrophus* culture inhibited with 0,01 mM of Ni^{+2}

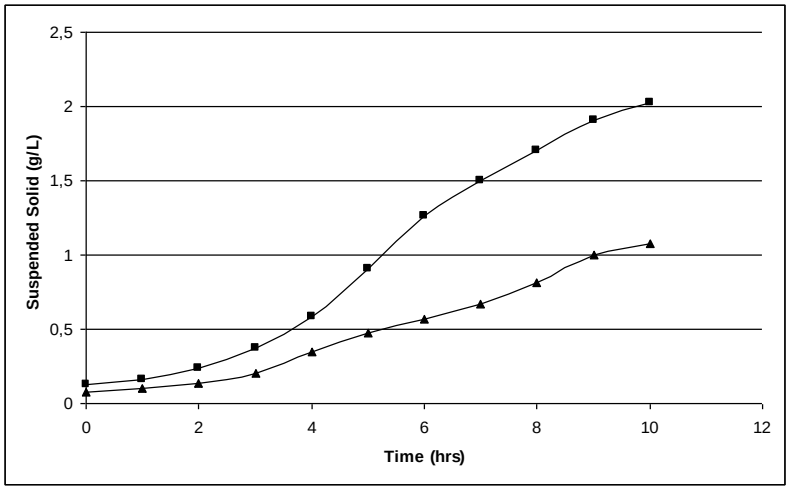

Figure 4.44: Cell dry weight in the *P. pantotrophus* culture medium under inhibited and non-inhibited conditions. (■ control, ▲ 0,01 mM Ni^{+2})

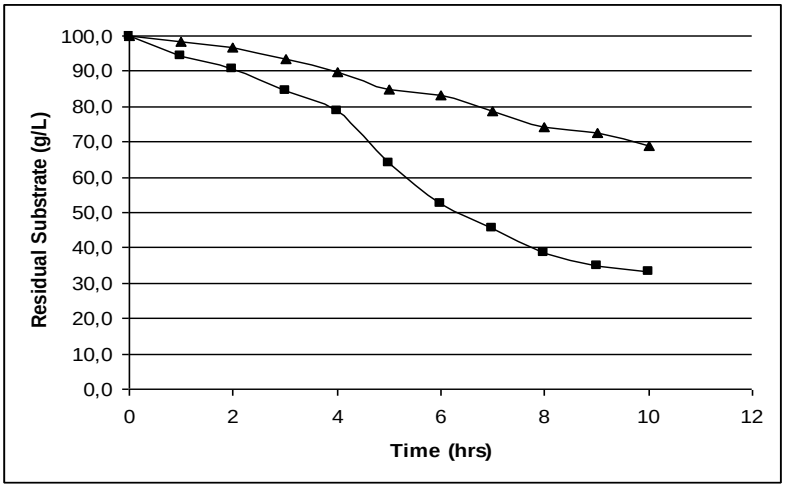

Figure 4.45: Residual glucose in *P. pantotrophus* culture medium under inhibited and uninhibited conditions. (■ control, ▲ 0,01 mM Ni^{+2})

Figure 4.45 and 4.46 shows the effect of the 0,01 mM Ni^{+2} on the growth and residual substrate level of *P. pantotrophus* culture during the experiment, respectively together with the control cultures. The inhibited culture consumes the substrate slower than the uninhibited culture. The overall results on kinetic evaluation of inhibited and uninhibited *P. pantotrophus* cultures are shown in Table 4.6

Table 4.6: Kinetic parameters obtained for *P. pantotrophus* cultures for metal inhibited and uninhibited conditions

	Y_{xs} (g/g)	μ_{max} (d^{-1})	X_m (g/L)	S_c (mg/L)
Control	0,57	11,1	2,06	3383
Cu^{+2}	0,60	9,8	0,93	1320
Co^{+2}	0,56	7,8	1,19	1980
Ni^{+2}	0,64	10,5	1,03	1509

Although X_m values of *P.pantotrophus* were decreased approximately by 50% under the inhibition of the studied heavy metals, it was observed that the growth yields of both the inhibited and the uninhibited cultures were the same. The log phases of the inhibited cultures delayed by and lower final CDW values than the control set.

4.4. Respirometric Analysis of the Model Organisms

Respirometric studies were also performed on the selected microorganisms for obtaining kinetic parameters, such as oxygen utilization uptake rate, specific growth rate, K_S, and Y_h. During the experiments, OUR increases during the first hours as readily biodegradable substrate is consumed and growth of heterotrophic biomass occurs. After this period, OUR decreases suddenly as the readily biodegradable fraction has been completely depleted. Then, OUR exhibits a slow decrease as microorganisms, which can be interpreted as a substrate-limited reaction, degrade the hydrolysable material. (39)

4.4.1. Control Experiments

4.4.2. *E.coli*

Two different control sets of experiments were made, one with F/M ratio of 30 and another with F/M ratio of 10. The growth profile and the OUR profile of *E. coli* control culture with F/M ratio 30 are given together with the substrate consumption profile in Figure 4.47 and Figure 4.48 respectively. Table 4.7 shows the kinetic parameters obtained from *E. coli* control experiments

Table 4.7. Kinetic parameters obtained from *E. coli* control experiments

Parameters	Values
Y_h	0,85
μ	$14{,}02 d^{-1}$
K_S	18,54
F/M	30 (g/g)

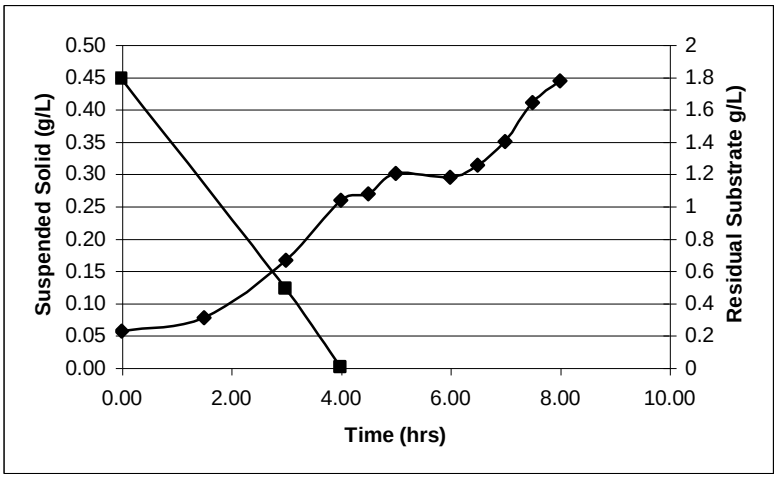

Figure 4.46: The growth profile and the residual substrate profile of *E. coli* culture. (■ residual substrate, ♦ cell dry weight)

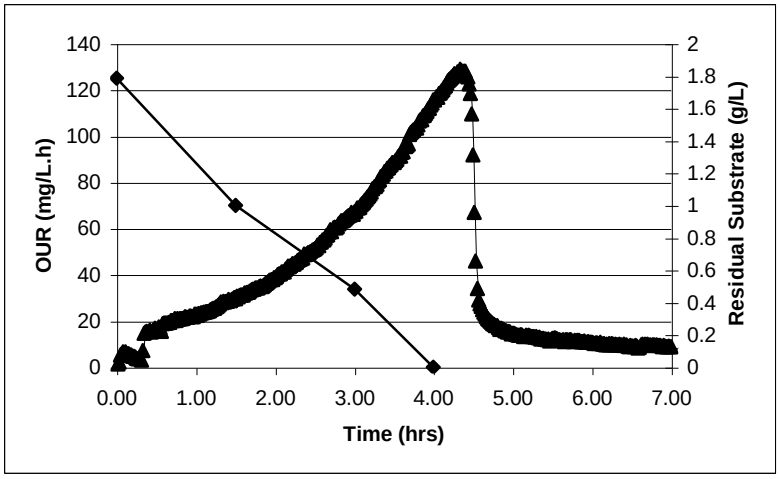

Figure 4.47: OUR profile of E. coli control experiments (♦residual substrate, ▲Oxygen uptake rate)

The substrate was used up at 4th hour of growth and the culture reaches the stationary phase at 8th hour of growth. The growth profile and the OUR profile of the *E. coli* control culture, when the F/M ratio was taken as 10, is given in Figure 4.48 and Figure 4.49, respectively.

Table 4.8. Kinetic parameters obtained from *E. coli* control experiments

Parameters	Values
Y_h	0,68
μ	21,4 d^{-1}
K_S	2,43
F/M	10

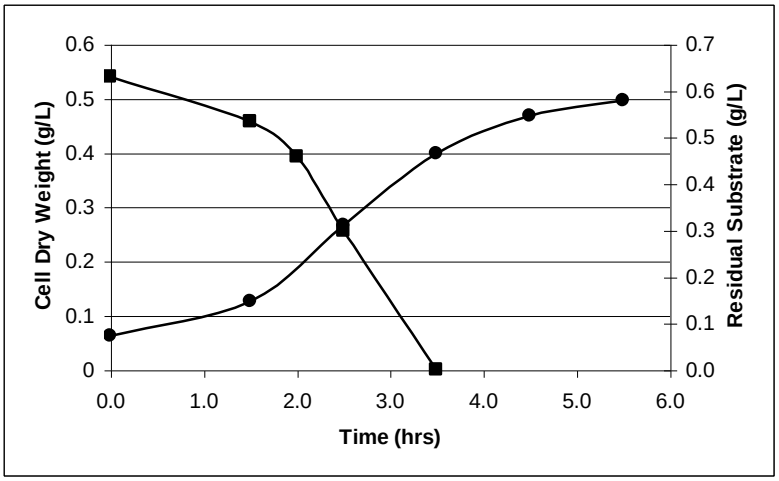

Figure 4.48. Cell dry weight and residual Substrate profiles of the second control culture. (■ residual substrate, ♦ cell dry weight)

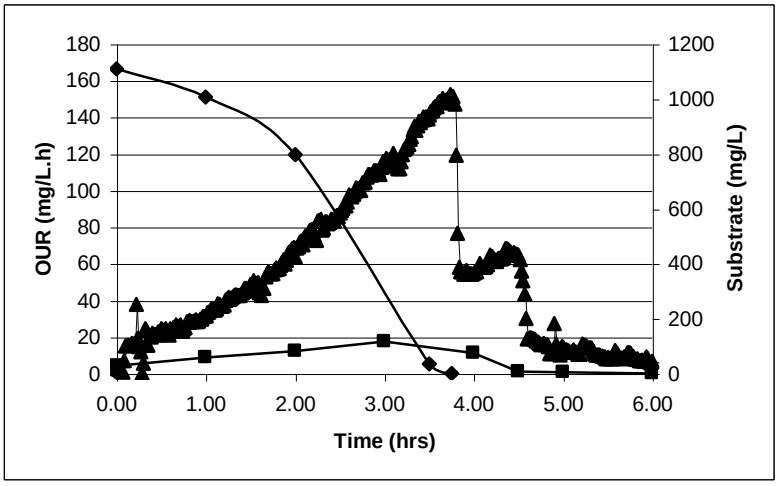

Figure 4.49. OUR profile of the *E. coli* culture under uninhibited conditions. (■ acetate concentration, ♦ residual glucose concentration, ▲OUR value)

The substrate was used up 3,5 hours and the culture reaches the stationary phase in 6 hours. It was observed that no glycogen was stored and acetate was synthesized during the growth on glucose.

When the kinetic parameters of the *E. coli* growth with two different F/M ratios were compared, it was seen that high F/M ratios cause substrate inhibition on *E.coli* culture. This effect could be followed by μ_{max}, Y_h, K_S, and X_{max} values.

4.5.2. Inhibition experiments

4.5.2.1 Cobalt Inhibited *Eschericia coli* Culture Experiments

The growth of *Eschericia coli* is inhibited also under respirometric conditions where there is no oxygen limitation. The growth profile and the OUR profile of the inhibited culture are given together with substrate and acetate uptake in Figure 4.50 and Figure 4.51 respectively. Kinetic parameters obtained from the OUR profiles of *E. coli* cultures under inhibited and uninhibited media, are presented and compared in Table 4.9. The kinetic model used during the inhibition studies is different than the one used for control experiments. The main difference between the former and the later models used comes from the fact that the second peak of the OUR profile was neglected in the first one.

Table 4.9 The kinetic parameters obtained from *E. coli* experiments under uninhibited and cobalt inhibited conditions.

Parameters	Cobalt Inhibition	Uninhibited
Y_h	0,76	0,73
μ_{max}	17,6 d^{-1}	15 d^{-1}
K_S	10,18	9
F/M	10 (g/g)	10(g/g)

The second control experiment has the same initial conditions with the cobalt-inhibited experiment. Unlike the fermentor experiments in the well-aerated respirometer, the heavy metal concentrations didn't inhibit the bacterial growth.

Although Ks value was lower than cobalt inhibited culture under inhibited conditions, maximum growth rate of inhibited culture was higher than the uninhibited one.

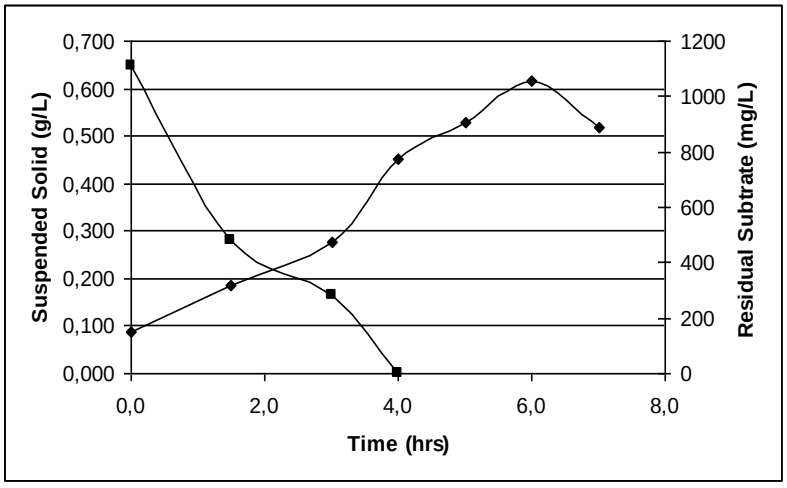

Figure 4.50 Cell dry weight and Residual Substrate profiles of *E. coli* culture under cobalt inhibited conditions. (■ residual substrate, ♦ cell dry weight)

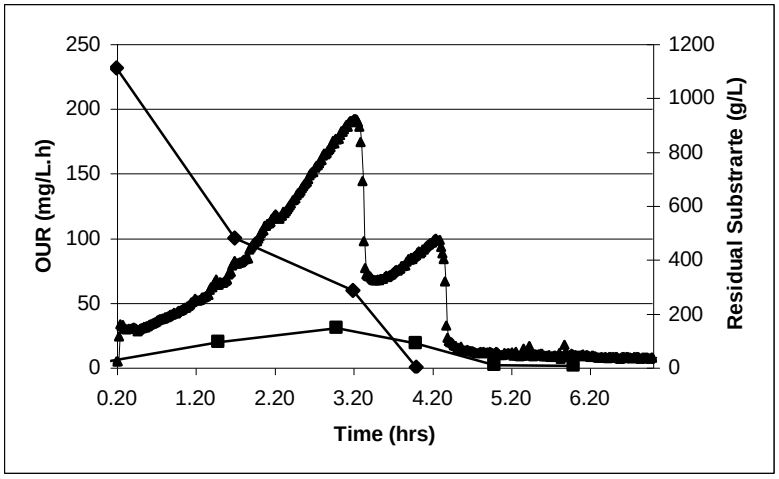

Figure 4.51. OUR profile of the *E. coli* culture under cobalt inhibited conditions. (■ acetate concentration, ♦ residual glucose concentration, ▲OUR value)

Cobalt inhibition caused an increase in Ks and a decrease in maximum growth rate values. The substrate was used up in 4 hours and the culture reaches the stationary phase in 6 hours.

4.5.2.1 Copper Inhibited *Eschericia coli* Culture Experiments

The growth of *Eschericia coli* is inhibited also under respirometric conditions where there is no oxygen limitation. The growth profile and the OUR profile of the inhibited culture together with glucose and acetate consumption are given in Figure 4.52 and Figure 4.53, respectively. Here, the growth continues on acetate following the consumption of glucose.

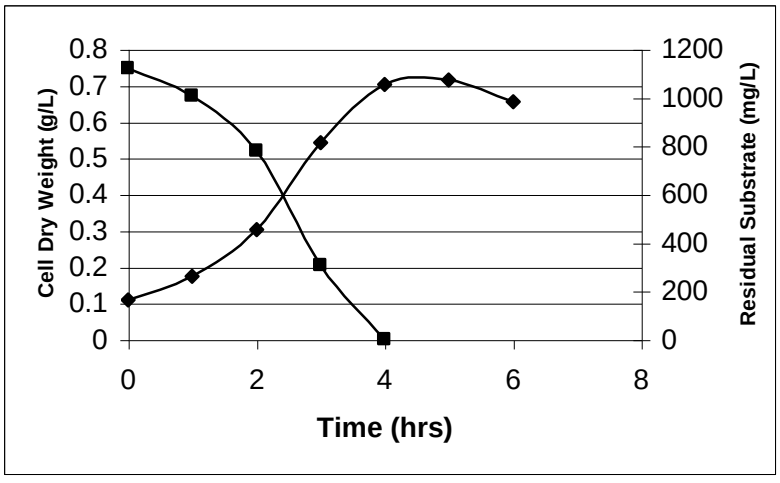

Figure 4.52. Cell dry weight and Residual Substrate profiles of *E. coli* culture under cobalt inhibited conditions. (■ residual substrate, ♦ cell dry weight)

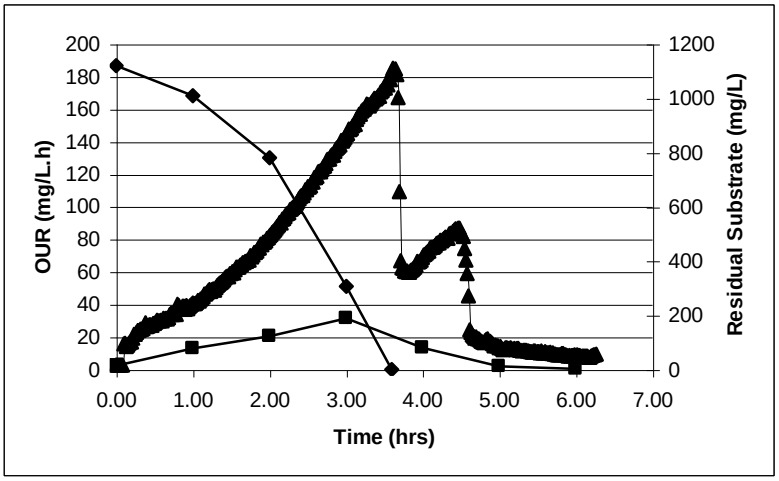

Figure 4.53. OUR profile of the *E. coli* culture under copper inhibited conditions. (■ acetate concentration, ♦ residual glucose concentration, ▲OUR value)

The substrate was used up in 3,5 hours and the culture reaches the stationary phase in 6 hours.

The total protein values were also affected by the heavy metals. Metabolic protein production was stimulated under copper inhibition, while it was inhibited under cobalt inhibition as it can be followed in Figure 4.54. In the figure, the protein production profile throughout the experiments was given.

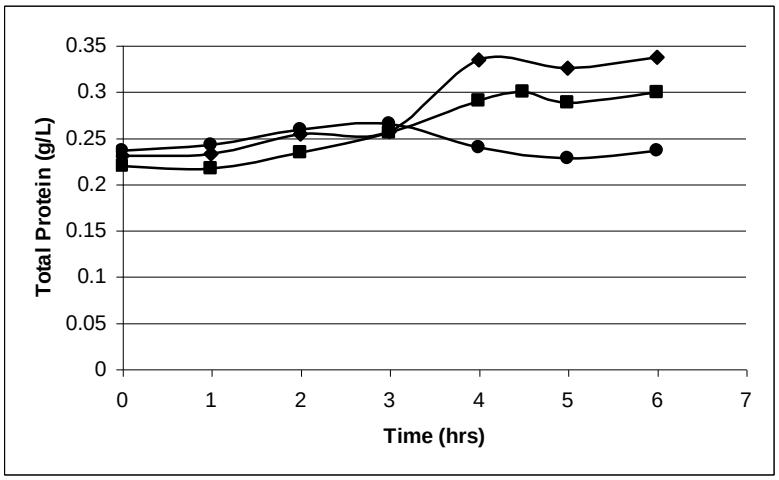

Figure 4.54 Total protein profile of *E. coli* under inhibited and uninhibited conditions. (■ control, ♦ copper inhibition, ● cobalt inhibition)

4.5.3 Modeling Studies

Three model studies were made considering the OUR profile of the *E. coli* culture. According to the first model, the second peak of the OUR profile was neglected. Thus the model simulation in Figure 4.55 was obtained.

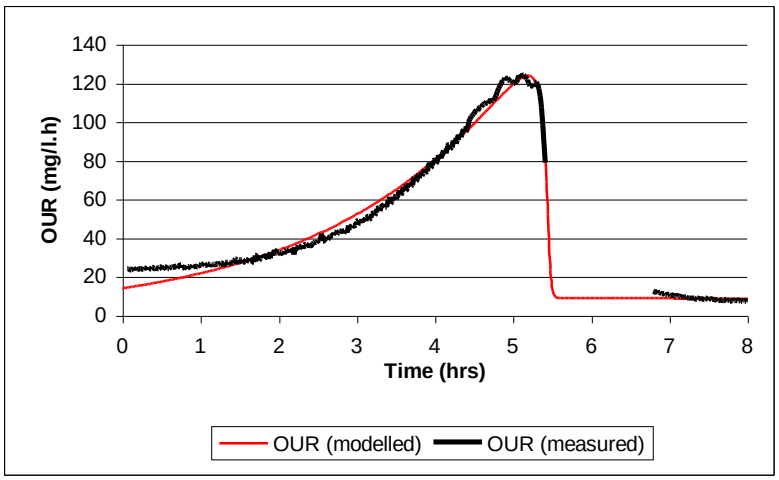

Figure 4.55 The first model made on *E. coli* OUR profile. The second peak in the profile was neglected.

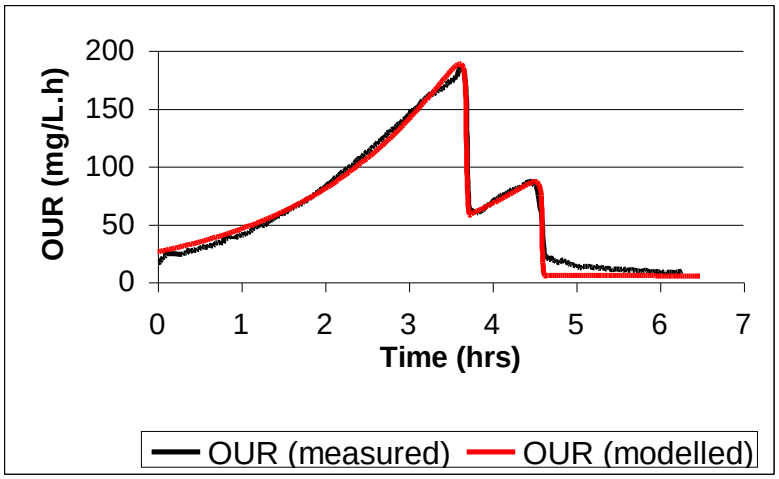

Figure 4.56 The second model made on *E. coli* OUR profile.

Whereas in the second model (Figure 4.56), the second peak appearance was taken into consideration. Experimental studies showed the second peak formation occurs because of the growth on acetate. Still, even the second model doesn't exactly fit the experimental OUR profile. The tailing part of the second peak remains unfitted. This part indicates the existence of another substrate besides acetate. The model is based on one substrate, which is glucose in our case, whereas here we have byproducts,

which can also be utilized as substrates in case of the depletion of the main one in the environment. Figure 4.57 shows the model fits of glucose and acetate uptake of *E. coli* beside the modeled biomass profile.

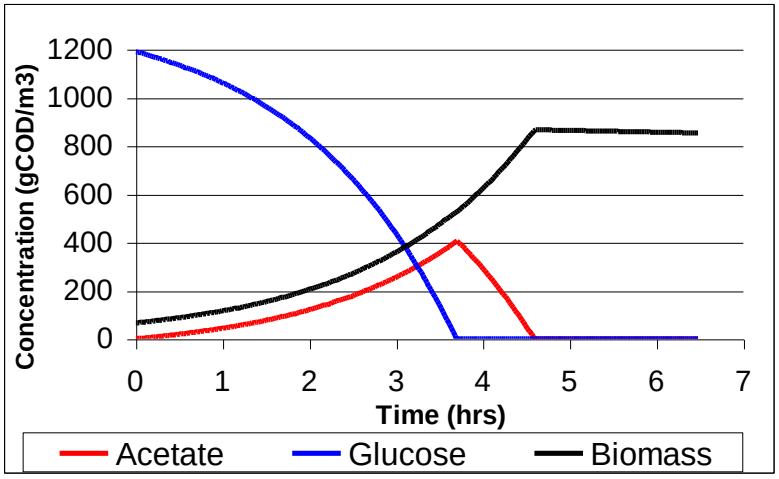

Figure 4.57 The second model of glucose and acetate uptake of *E. coli* and the modeled biomass profile.

The third model includes the first peak, which is from the glucose consumption, the second peak, which is from the acetate consumption, and the tailing, which is from another substrate. The curve fit of the third model is shown in Figure 5.56

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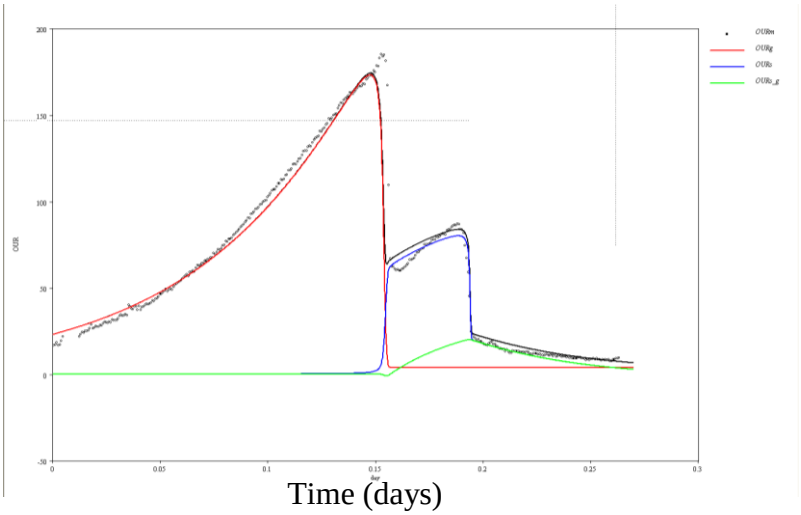

Figure 4.58 The third model of OUR profile of *E. coli*

In the third model, the tailing was also considered. Thus the modeled OUR profile and the estimated profile were completely in competence with each other. The red line indicates the growth on glucose, the blue line indicates the growth on acetate and

the green line indicates the growth on the second byproduct. The modeled residual substrate and biomass curves are presented in Figure 4.58.

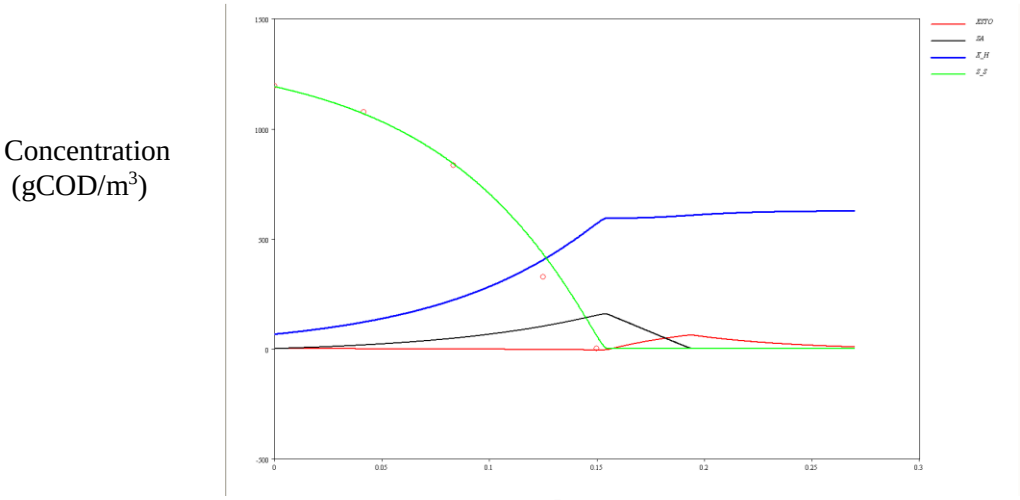

Figure 4.59 The modeled glucose and acetate uptake of *E. coli* beside the modeled biomass profile.

The third model shows less acetate than the second model, since it includes a another substrate rather than acetate. The kinetic parameters of these three models are shown in Table 4.10

Table 4.10 The kinetic parameters of Model 1, Model 2, Model 3

	μ_{max} (d^{-1})	Y_{h1}	Y_{h2}	K_S	q_{sto}
Model 1	15	0,73	-	9	-
Model 2	14	0,63	0,84	3	13
Model 3	15	0,24	0,66	5,8	13

The kinetic parameters obtained using the three different models were given in Table 4.10. Although the maximum growth rates are comparable with each other, the growth yields vary from each other reflecting the different predicting levels of the models used during the studies.

5. DISCUSSION AND CONCLUSION

Discharge of heavy metals is one of the most important problems that may significally reduce the efficiency of biological treatment processes. Heavy metals cause stress conditions which negatively affect on the metabolism of the activated sludge bacteria. Especially high metal concentrations in municipal wastewaters reduce the efficiency of sewage treatment operations. This causes the heavy metals from the industrial wastes to interfere with the seas and lakes, and reduces the efficiency of the biological treatments. In the contrary, it is known that some of these metals, when supplied in trace amounts have a stimulating effect on the metabolic activities of microorganisms. Since there is a vast number of parameters which cause heavy metals to make metabolic changes in the bacterial metabolism, toxicity and inhibitory effects of the wastewater need to be well investigated before modelling a biological treatment plant, in terms of the limits and the mechanisms of microbial tolerance.For this purpose, three model organisms of activated sludge systems, *E. coli* K12 , *Microlunatus phosphovorus* and *Paracoccus pantotrophus* were chosen and their minimum inhibitory concentrations (MIC) were determined in liquid and solid media. MIC values all of the studied strains in these two media were found to be different. Studies made by Hassen et al. (1997) show that metal binding capacity of the microorganisms, chelation to various components of the media and formation of complexes can each cause a reduction in the activities of free metals. Thus the MIC determination techniques require a standard valid approach, to evaluate the action of heavy metals in the growth medium.

In order to see the direct effects of heavy metals on the studied bacteria, the experiments were carried out in chemically defined media, M9. M9 medium has a relatively simple composition as compared with other traditional media and therefore a lower metal-binding capacity with constituents of the media.

The experiments were carried out in both solid and liquid M9 medium, since studies also show that the phase of the medium directly affects the heavy metals' effects on the bacterial metabolism. Hassen et al. (1997) reported that 0,5 mM of Cu^{+2} and 0,2 mM of Co^{+2} inhibited E. coli K 12 in nutrient broth. In our study, a simple media was used, thus the MIC values for the Hassen's study were lower (0,08 mM and 0,15 mM for Cu^{+2} and Co^{+2} respectively on the solid medium.

Studies made on many kinds of bacteria indicate that both Gram-positive and Gram-negative bacteria have tolerance to heavy metals. Duxbury et al. (1981) reported that generally, Gram-negative soil species appeared to be more tolerant than Gram-positive. However, Mahler et al. (1986) have found that all isolates tolerant for cadmium and mercury were Gram-positive bacteria. Also, in our study, the Gram-negative bacteria, *E. coli* K12 , and *Paracoccus pantotrophus* had more tendency to tolerate heavy metals than the Gram-positive bacteria, *Microlunatus phosphovorus*, in the liquid medium; while in solid medium, Gram-positive *Microlunatus phosphovorus* showed higher tolerance to cobalt and nickel than Gram-negative *Paracoccus pantotrophus.*

In mixed culture studies, Barth et. al. found that at 10 mg/L of copper caused inhibitory effects on microorganisms while Pettet found that only 1 mg/L Cu inhibits the microorganisms. Similarly, Malaney et. al. found that 16 mg/L Ni inhibited mixed culture growth. Albek et. al. showed that in the presence of 25 mg/L nickel, mixed cultures continued to grow.

All these differences in results could be explained by the conditions of each bacterial isolation and the selectivity of microbial culture techniques adopted in each study; particularly with respect to the nature and specificity of the growth media.

5.1. Determination of MIC Values of model organisms in Solid Medium

Since there are no literature studies on the inhibitory effects of heavy metals on either of the studied cultures, it is not possible to compare the results with those of other studies. In our study, the toxic inhibitory effects of copper was relatively higher than the inhibitory effects of cobalt and nickel in solid M9 medium. According to the overall heavy metal tolerance of the cultures, it was observed that *P. pantotrophus* showed the least resistance

while *E. coli* showed the highest tolerance compared with the other cultures. A generalization of MIC values in increasing order in solid medium can be made for both *E. coli* K12 and *Microlunatus phosphovorus* as:

$$Cu^{+2}<Co^{+2}<Ni^{+2}$$

For, *Paracoccus pantotrophus* as:

$$Cu^{+2},Co^{+2}<Ni^{+2}$$

It can be concluded that the general tolerance of the studied bacteria for the three heavy metals are in the same order in the solid M9 medium.

5.2. Determination of MIC Values in Liquid Cultures

The microorganisms except *P. pantotrophus* showed less tolerance in shake flasks experiments, *P.pantotrophus* showed the same metal tolerance in liquid and solid medium while it was observed that *E. coli* and *M. phosphovorus* showed less resistance to heavy metals in liquid medium. Comparatively, *E. coli* had more tolerance to Cu^{+2} than *P. pantotrophus* and more tolerance to Co^{+2} than *M. phosphovorus.* Since, there are no literature studies made on the metal inhibition of *P. pantotrophus* and *M. phosphovorus*, it was not possible to compare our results with those of the former studies.

The studies made on metal-inhibited *E.coli* cultures gave different results. Hassen et al.(1998), determined the minimum inhibition concentrations of copper and cobalt for *E.coli* as 0,5 mM and 0,2 mM respectively. These values are ten fold higher than those found in this study. This is because of the enriched medium used in that study, containing nutrient broth, yeast extract and peptone. In another study made by Nies et al., (2003) on *E. coli* K38 culture, MIC values of copper, cobalt and nickel were almost the same (about 10 mM) in Tris-buffered mineral salts medium.

Although the exact MIC values were different from those found in the literature, the ratio of the MIC values of copper and cobalt found in this study were the same for *E. coli*.

Nies et al. (1997) have found that both in Gr(-) *P. aeruginosa* and Gr(+) *B. thuringiensis*, cobalt inhibition was more effective than copper inhibition.

In liquid medium, the inihibitory effects of heavy metals could be listed as $Ni^{+2}>Co^{+2}>Cu^{+2}$ for *E.coli*; $Cu^{+2}>Co^{+2}>Ni^{+2}$ for *M. phosphovorus,* and $Cu^{+2}=Co^{+2}>Ni^{+2}$ for *P. pantotrophus*. These indicate the effects of the state of the medium on the tolerance of the bacteria to different concentrations of heavy metals.

MIC values were lower in liquid medium than in solid medium. As also supported by the literature, these results show that our chemically defined medium did not cause metal precipitation and the minimum inhibition concentrations that were obtained from the experiments indicate the true effects of the studied heavy metal concentrations.

As observed from both our experiments and literature, the order of inhibition levels in solid and liquid media are different. This is because of different chelating properties of the heavy metals. In our study, both in liquid and solid media, Gr(-) *P. pantotrophus* tolerated heavy metals more than Gr(+) *M. phosphovorus*, A study made by Duxbury et. al(1981) supports this idea, while another study, made by Mahler et. al(1986) opposes it.This result provides another evidence for the observation that gram properties of bacteria do not seem to have a direct effect on their tolerance or resistance against heavy metals.

5.3. Batch Reactor Experiments

Studies made in batch reactors under inhibited conditions are very limited. However, there are many studies made on the kinetic constants of *Escherihcia coli* culture under different media.

5.3.1. Batch Experiments with *Eschericia coli* cultures

Wirtz et al. (2002) found the maximum growth rate of *Escherichia coli* as 32,4 d^{-1} under euthrophic batch conditions with glucose as the carbon source. Studies made by Panda et al.(1999), in a medium including yeast extract indicate that the final OD_{600} value can reach up to 13, making a maximum growth rate of 17,76 d^{-1}. In our study, under batch conditions, the maximum growth rate was found to be 16, 32 d^{-1}which is similar to the results of Panda et al.

It was observed that equal concentrations of copper and cobalt caused different levels of inhibition on *E.coli* K12 culture. Although the final cell dry weight concentrations under cobalt inhibition and control values were almost the same, *E.coli* K12 culture reaches the log phase later under cobalt inhibition than uninhibited conditions. The log phase was delayed by 3 hours under Cu^{+2} inhibition and 1 hour under Co^{+2} inhibition. Copper caused the final cell dry weight concentration to decrease obviously. Copper and cobalt caused a shift by 20% and 30% in the biomass yields, respectively while the maximum growth rates were decreased by 50%.

The Cu^{+2} inhibited culture consumes the substrate slower than the uninhibited culture and reaches the stationary phase before it consumes all of the subtrate. Surprisingly, the cobalt inhibited culture consumes the substrate slightly faster than the uninhibited culture and reaches the stationary phase as it consumes all of the subtrate. Since the growth yield is lower than the control set, the maximum biomass concentration remained lower than the control.

5.3.2. Batch Experiments with *Microlunatus phosphovorus* cultures

Since there are no inhibition studies with *M.phosphovorus* in the literature, it was not possible to compare our results with those of other studies. The response of *M.phosphovorus* to the Ni^{+2} inhibition in the well-aerated conditions of the bioreactor was different from those of the shake flask experiments. Although 0,02 mM of Ni^{+2} was found to be sufficient to inhibit the growth of the culture completely in shake flask experiments, *M. phosphovorus* could grow in the bioreactor experiments at this concentration of nickel.

Altough the growth rate of the Co^{+2} inhibited *M. phosphovorus* culture is almost the same as the uninhibited culture, the inhibition effect is seen on the final biomass concentration. The log phases were delayed by 2 hours for Ni^{+2}. The maximum growth rate and the final biomass concentrations of the nickel-inhibited culture are lower than the control values by 42% and by 58% respectively. The growth yields of *M. phosphovorus* were inhibited by 30% under cobalt inhibition and by 44% under nickel inhibition with respect to the control set.

As in *E. coli* experiments, the cobalt-inhibited culture consumes the substrate slightly more than the uninhibited culture and reaches the stationary phase at the same level of substrate uptake, but the nickel-inhibited one consumes the substrate slower than the uninhibited culture.

5.3.3. Batch Experiments with *Paracoccus pantotrophus* cultures

Since there are no inhibition studies with *Paracoccus pantotrophus* cultures in the literature, it was not possible to compare our results with those of other studies. Although X_m values of *P. pantotrophus* decreased approximately by 50% under the inhibition of the studied heavy metals, it was observed that the growth yields of both the inhibited and the uninhibited cultures were the same. The log phases of the inhibited cultures were delayed by and lower final CDW values were obtained than the contol set. Both the general growth rate and the final biomass concentration of the inhibited culture are lower than the control values.

The substrate uptake of the inhibited *P. pantotrophus* cultures can be seen in Figure 6. Unlike the other two microorganisms, the amount of utilized substrate by the cobalt inhibited *P. pantotrophus* culture was nearly the same as those of the other metal inhibited *P. pantotrophus* cultures.

Because of different experimental conditions used in other studies, it is not possible to compare our results with these results. However, in many studies, it was shown that, cobalt, copper and nickel inhibit the growth of both pure cultures and mixed cultures. These findings suggested that heavy metals caused different inhibitory effects according to the microorganisms, the growth medium and the growth conditions.

5.4. Respirometric Analysis of the Model Organisms

There are no respirometric inhibition studies made on *E.coli* K12 culture under inhibited conditions in the literature, thus we cannot compare our results with other studies.

The experimental assesment of μ_{max} is based on the assumption that the experiments were performed under well aerated and perfectly stirred conditions where substrate concentration is not rate-limiting ($S_s >> K_S$)

According to our results, under cobalt inhibition, K_S value increased, while maximum growth rate decreased. The difference between the inhibited and uninhibited K_S values was much higher than the difference between the inhibited and uninhibited μ_{max} values. It can be concluded that cobalt causes a competitive inhibition under the studied conditions on *E. coli*.

Our results show stimulation of protein production under copper-inhibited conditions in *E.coli* culture and less protein production under cobalt inhibited conditions show a similarity to the studies made by Hassen et. al(1997). They found that below MIC values in Gr(-) *P. aeruginosa* the total protein (TP) production was as follows:

$$TP_{copper} > TP_{control} > TP_{cobalt}$$

Stimulation of protein production under copper inhibition in *E.coli* culture can be explained by the CopA protein which is synthesized to transport the heavy metal cation from the cytoplasm, into the periplasm (Nies, 2003). It was also found that cobalt was used as a cofactor in some proteins, as well.

A second peak could be observed in the second and third experiments where the F/M ratio was much lower than the first experiment. The second peak indicates the existence of a second substrate. Since glucose was the only substrate initially added to the medium, it can be said that either a storage material, or a primary metabolite is being synthesized and consumed, following the completion of the readily biodegradable substrate, glucose. Since the production and consumption of acetate is in agreement with our model, it can be concluded that the second peak is the result of the growth on acetate. Besides, it was found that no glycogen was stored during the growth.

Accourding to our model, during the catabolism of glucose, glucose is converted to acetate very quickly. But the rest of the Krebs Cycle is slower and excess acetate was removed from the cytoplasm and taken out of the cell. When glucose was totally consumed, the excess acetate was used as the new substrate and the growth was continued on acetate. Since the amount of acetate was much less than glucose, the second peak in the OUR profile was significantly smaller than the first one. Further investigation is needed in order to model the complete growth of the culture.

Another finding is that, when the first respirometric experiment was compared with the last two, the second peak doesn't occur under very high F/M ratios. This may indicate that *E. coli* stores some of its substrate under well aerated conditions and high F/M ratio. When F/M ratio is extremely high, which is the case in the first experiment, there may be no storage or the storage does not affect the OUR profile. Also the comparison made between the kinetic parameters of these two cultures indicated that high F/M ratios caused substrate inhibition on the culture growth.

The question of which of the kinetic models and pathways are applicable to this growth profile could be answered by making further model simulations on the OUR profiles of the *E. coli* culture.

In light of our results, the future studies necessary would be to conduct: inhibition experiments with other metals such as zinc, lead, and cadmium, etc. can be carried out for E. coli culture and a detailed analysis of inhibition kinetics under different conditions.

Respirometric experiments could also be performed on the other two cultures. Especially *M. phosphovorus* shows an interesting PHA storage behaviour (AkarA.personal communication), thus it needs to be investigated under different F/M ratios and and inhibition conditions.

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BIOGRAPHY

Gamze Çelikyılmaz was born in October 1979 in İstanbul. She graduated from Kadıköy Anatolian High School. She has been as a visiting student to Puerto Rico where she graduated from Escuella Jose Collazo Colon High school. Then she had her Bachelors Degree in Chemical Engineering in Istanbul Technical University. She has continued to her Masters Degree in Molecular Biology and Genetics-Biotechnology Program which is a part of Advanced Technologies in engineering Graduate Program in Istanbul Technical University. She has been working as an assistant in the same program since January 2003.