INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

UMI®

RECOVERY OF COPPER FROM LOW-GRADE ORES BY *ASPERGILLUS NIGER*

MAHTAB KAMALI

A THESIS
IN
THE DEPARTMENT
OF
CIVIL BUILDING AND ENVIRONMENTAL ENGINEERING

PRESENTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF MASTER OF APPLIED SCIENCE
CONCORDIA UNIVERSITY
MONTRÉAL, QUÉBEC, CANADA

JULY 2001

0-612-64055-8

Abstract

RECOVERY OF COPPER FROM LOW-GRADE ORES BY *ASPERGILLUS NIGER*

Mahtab Kamali

The main concern of this study is to find a feasible and economical technique to recover metals from oxide low-grade ore microbially. Owing to the large quantities of metals that are embodied in low - grade ores and mining residues. these are considered new sources of metals. On the other hand they potentially imperil the environment. as the metals they contain may be released to the environment in a hazardous form. Hence. mining industries are seeking an efficient technique to handle these ores.

Pyrometallurgical and hydrometallurgical techniques are either very expensive or have a negative impact on the environment. That is why. a new environmental strategy called "sustainable development" is coming into perspective. This study employs heterotrophic leaching that falls into this new environmental strategy.

In this study. by employing *Aspergillus niger*. the feasibility of recovery of metals from a mining residue is shown. *A. niger* exhibits good potential in generating varieties of organic acids effective for metal solubilization.

Organic acid effectiveness was enhanced when sulphuric acid was added to the medium. Different agricultural wastes were evaluated and the maximum solubilization of 68%. 46% and 34% was achieved for copper, zinc and nickel. respectively. Also iron codissolution of 7% was gained.

Acknowledgments

I would like to thank my supervisor Dr. Catherine Mulligan for her guidance, enthusiasm, encouragement and free donation of her time. This thesis owes much to her; however, any mistakes are my own.

Grateful acknowledgment is made for the valuable suggestions and help given by Juliet Dunphy and Lee Harris.

My deepest thanks go to my husband, Ali, for his support and help.

To ALI

Contents

List of Figures

List of Tables

Chapter 1

Introduction

1.1 Introduction

In the late 1960s, industrial waste caused hundreds of people to be affected by paralysis and sensory loss along Minamata Bay in Japan. In the late 1970s, chemically induced problems of many children in the neighborhood of an elementary school were found to be the effect of the waste disposal of Hooker Chemical Co. Such news heightened public awareness of the disasters which could be caused by industrial waste. Industrial waste not only caused many health problems but also imposed expenses. In the United States alone, $70 million were spent on waste disposal in 1980 (LaGrega, 1994). Due to the considerable cost it caused waste management became an important concern for industrial companies. Driven by cost incentives, good engineering economics finally came to be characterized by the need to produce salable products rather than waste. This impetus was the first inkling of a very important environmental movement towards waste minimization (LaGrega, 1994).

In the early 1980s, negative environmental consequences which were caused by industrial waste mishandling became the main environmental issue for industrial societies. While waste disposal imposed expenses were rising, the adverse health and environment consequences of industrial wastes were being revealed. These factors encouraged environmental organizations to think of new criteria for the problem of waste treatment. This motivation finally led to the concept of waste minimization, a concept which was introduced in Congress in the 1984 Hazardous and Solid Wastes Amendments to the Resource Conservation and Recovery Act (RCRA) to legitimize a policy called waste minimization. As it is mentioned in Hazardous Waste Management by LaGrega(1994) waste minimization came into environmental perspective formally when in October 1986 the U.S. Environmental Protection Agency (EPA) in a report to Congress defined waste minimization as:

> "...any source reduction or recycling activity undertaken by a generator that results in either (1) the reduction in total volume or quantity of hazardous waste, or (2) the reduction of toxicity of hazardous waste, or both, so long as the reduction is consistent with the goal of minimizing present and future threats to human health

and environment ."

Afterwards, many efforts were concentrated on techniques which had the potential of producing less waste, so that reclamation and recovery methods caught the attention of industrial companies since these techniques not only met the goal of waste reduction but also had economic profits.

The waste minimization concept was modified in order to lead to a more environmentally sustainable solution. Hence, sustainable development was the next step towards cleaner technology. In the last decade, the idea of environmentally sustainable development has arisen as a possible way to combine economic growth with environmental protection. Sustainable development as a concept to be reached by all nations was introduced during the 1992 Earth Summit-UN Conference on Environment and Development (UNCED) - at Rio de Janeiro. Preparation of a 400-page document blueprint in this conference endorsed the importance of this framework (UNCED,1992). Since then, this strategy has become a guide to many communities, which are seeking economic development approaches that also benefit the environment. Even though no industry can claim that it is already achieved this, many efforts are focused on approaching this goal. Among these industries are metal producing industries. Metals are of concern particularly as important elements which are necessary for technology on one hand, and dangerous for the environment on the other hand. They are comprised of around seventy of the ninety- six naturally occurring elements and are supposed to be one of the major hazardous wastes sources. By EPA (1985) definition hazardous wastes are wastes which by reason of 'their chemical activity or toxic or explosive or corrosive or other characteristics cause danger or likely will cause danger to health or the environment, whether alone or when coming into contact with other waste...'.

Metal is not a discovery of the modern world. The first human experience of metals was in about 6000 BC when native copper was first hammered. Humans need metals for their ever widening purposes. Metals are added to fertilizers in agriculture. The existence of aircraft, automobiles, computers or electrical appliances without metals is inconceivable. Life without these naturally occurring, ubiquitous elements is unimaginable. While mines are the primary source of metals, the availability of metals for future generations is uncertain.

Besides metals' significant role in technology advancement, their natural presence in the environment sometimes imposes risks to the environment. For instance, lead has a carcinogenic effect on the kidneys, cadmium has potential carcinogenic effects on the lungs, and other heavy metals have other toxic effects (LaGrega, 1994). Furthermore, great amounts of metals which are disposed of by industrial companies annually, are sources of many health and environmental problems. The importance of recycling wasted metals was understood better when it was realized that the availability of metals for future generations is uncertain as long as mines are the only sources of metals. With respect to the importance of metals in our lives and the limited sources that exist, the sustainable development approach seems a feasible solution.

In order to understand how to apply a sustainable development framework to the metal production process, a brief background of metal resources is required.

Generally metal resources can be categorized into two main groups:

a) environment resources which embrace the majority of metals available for industries

b) metals present in industrial waste and used material which can be seen as second hand resources.

Mining is the main activity to obtain metals. A metal's origin is mostly ore. Ore is usually a mineral, a naturally occurring inorganic substance, or an aggregate of minerals in which a valuable constituent, i.e. a metal, exists, of which its concentration is high enough to make extracting process profitable. Ore is often classified as containing ore minerals, valuable constituents, and gangue minerals where gangue is undesired minerals which associate with ore and are mostly nonmetalic. Metal concentration in ores varies and, based on the amount of metal concentration, ores are divided into low-grade ores which are ores with little concentration of metal, and high-grade ores, the ones in which metal concentration is relatively high. Metals are extracted from ore by a variety of techniques.

1.2 Extraction metallurgy

The history of extraction metallurgy can be divided into two main periods, the ancient period and the recent one. The ancient period, namely the time when very small numbers of metals were known, is usually referred to by the term "early extraction metallurgy".

Early extraction metallurgy history can be traced back to an early time when humans melted certain rocks and minerals to gain new materials known as metal. Probably the first readily available resource of metals for humans were the rocks on the surface of the earth with a character like colour. It is reported by Walsh (1927) that most probably the first ancient mining was done by Greek naturalists and physicians around 166 A.D. on several copper mines of Cyprus. The extracting process was passing surface water through a permeable copper-bearing rock and obtaining a copper- sulfate containing solution.

Generally, at that time operators selected their raw material entirely by appearance and products succeeded or failed according to how they behaved in use. There was no knowledge of chemical analysis and mechanical testing.

The recent period of development of extraction metallurgy probably commenced in about 1700 A.D. when zinc was recognized as a metal. This new era began with the development of chemistry. Chemists started to isolate and classify all elements that could be found on earth. New methods of metal extraction developed, many of which in fact evolved from the old methods.

At present, extractive metallurgy or mining, in a broad sense is the study of metals and their properties in bulk and at the atomic level, involving winning metals from a raw material by physical and chemical processes.

Generally, the first step is concentration of the ore to liberate it from undesired minerals, gangue. These processes are collectively called "ore dressing" which involves fragmentation to small sizes to permit easy separation of the different kinds of mineral. This process is followed by sorting operations in which, based on some physical properties such as density, magnetism or surface energy valuable mineral particles will be separated from the rest. These steps will assist the chemical process to be performed with higher efficiency.

Afterwards, in the extraction process, the metal is produced. The extraction process may be carried out by the following techniques:

- Pyrometallurgy techniques involve heating operation which basically are processes that are performed at high temperatures (Alcock, 1976).
- Hydrometallurgy techniques which are characterized by the use of aqueous solutions and inorganic solvents to achieve the desired reactions (Arsdale, 1953).
- Electrometallurgical techniques in which various electrical processes are used for the working of metals, eg., electrowinning, electrorefining and operations in electrical furnaces (Alcock, 1976).
- Biohydrometallurgy techniques are the ones that employ microorganisms. It should be noticed that all these techniques may be utilized in the extraction of a particular metal. The purpose of each method is to prepare material for the next procedure as cheaply and as suitably as possible .

1.2.1 Pyrometallurgy Techniques

These processes are mainly applied to ores which are already in oxide form such as iron and tin, or ores that have been converted to an oxide, such as through roasting lead sulfide.

In this process the ore is heated with a reducing agent under high temperature in a furnace, which results in the production of molten metal and slag, a usually fusible and easy to handle fluid, which is separated from the metal at the working temperature. The main function of the slag is to dissolve all impurities and ideally it should not have any power to dissolve metal. The molten metal will be separated from slag in the high temperature since slag specific gravity is lower than metal specific gravity.

Apart from these, the smelting flux in creation of the slag is very important. The function of this flux is to combine and neutralize the gangue and products of decomposition.

The pyrometallurgy process may be easily explained as the reaction of a reducing agent with the nonmetal part of a metallic compound which will yield the metal as follows:

$$MX \text{ (the metallic compound)} + R \text{ (reducing agent)} \longrightarrow M \text{ (free metal)} + RX$$

Generally, a proper reducing agent would have a greater affinity for oxygen under the condition of treatment. Since this process is performed at very high temperature the energy consumed is very high and the ease of reducibility of a compound under lower temperatures would make the process more cost effective. The reducing agent could be another metal, or C, H_2, or CO. The selection of the reduction compound depends on technical feasibility and the economics of the process. For instance very stable metallic oxides require high temperatures and a powerful reducing agents (Arsdale,1953).

Overall, pyrometallurgy techniques are performed in various stages as follows:

- Preliminary processes: physical or chemical processes which will change the properties of the ore and will make it more suitable for the main metal extracting processes, such as roasting and calcination which are performed at temperatures below the melting point. In brief, roasting involves chemical changes other than decomposition like; burning out sulphur from sulphides and replacing them with oxides, volatilizing to eliminate other unwanted volatile oxides, such as ZnO. Calcination is another ore treatment which thermally effects its decomposition and the elimination of volatile products such as carbon dioxide or water (Alcock, 1976).
- Reduction to liquid metal: These processes embrace two main operations, liquidation and smelting: liquidation is the process in which the metal melts and separates from other materials present; Smelting is the process in which the ore is all melted and separated out into two or more liquid layers, one of which contains the metal (either as metal or sulphide).

There are more pyrometallurgy operations which may be necessary, such as the conversion of molten sulphide to metal or of pig iron to steel by blowing air through it to oxidize out sulphur and carbon respectively.

1.2.2 Hydrometallurgy Techniques

Development of these methods began when pyrometallurgical methods were not cost effective for low-grade ore. These methods are also viable for the case where the metal to be extracted is more reactive than the impurities. These methods are favorable when high purity metallic products are needed, or for ores with a complex nature such as ceramics, composites, and nanostructure materials. They are also viable when air pollution concerns and water pollution can be controlled (Alcock,1976).

These techniques are performed in an aqueous environment. The three main steps of these methods are as follows:

- Leaching. The purpose of leaching is to dissolve the desired metal in a selective aqueous solvent in order to separate it from the gangue material. The ore is soaked and leached for sufficient time in a dilute aqueous mineral solvent, of which the solvent power should be as selective as possible, and it should be inexpensive or easily regenerated .
- Purification or removal of the impurities and/or concentration of the solution. Usually this process involves many steps, which may be physical processes such

as washing, clarification, thickening, filtering, drying, evaporation etc (Suri *et al*, 2001).

- Recovery of metal from solution. The last step is to recover metal from the solution. In this process, which is mostly referred to as reduction, metal is released from the chemically combined state in which it exists, into an elemental or metallic state (Suri *et al*, 2001). There are several techniques to accomplish recovery. Some of them are as follows:
  - Precipitation, it may occur by either diluting the pregnant liquor and reducing the pH or lowering the temperature.
  - Cementation, the removal of the desired metal by displacing it with a more reactive metal.
  - Ion exchange, where the desired metal can be removed from the aqueous solution by ion exchange at the surface of an organic resin.

1.2.3 Electrometallurgical techniques

In this process electrical energy supplies power for the system, which causes a reaction to proceed against its chemical affinity and in the direction of its electrochemical affinity.

Employing electrical energy will make the extraction process very efficient on the one hand, but very expensive on the other hand.

There are many different electrometallurgy processes and some of them are explained as following:

- Electrowinning: In this process metal is recovered by electrochemical reduction of one of its compounds which is dissolved in a appropriate electrolyte. Among the various types of solutions, sulphuric acid and sulphate solutions are preferred as an electrolyte. Being less expensive and less corrosive are the most significant advantages of these types of solutions.

  Electrowinning is also one of the recovery methods. It is a very selective process and usually yields a pure metal. However, it is the most expensive process in comparison to other recovery methods (Felsher *et al*,2000).

  In principle, this process consists of three main steps:

  1. A metal is placed in a solution either by leaching of a calcine or by direct leaching of low grade ores containing oxidized minerals or weathered sulphides.
  2. The pregnant solution is purified in order to remove metallic impurities other than the metal to be electrowon, and impurities that are capable of reducing current efficiency. The pregnant solution can be treated by

solvent extraction to produce a more concentrated electrolyte.

3. The purified solution is fed to the electrolysis tanks in which the metal is plated on a cathode, and oxygen is developed at an inert anode, which is usually made of lead or one of its alloys.

Metals more active than zinc and manganese are not suitable for this process.

- Electrolysis: Electrolysis is a process in which a liquid (or electrolyte) is split into its chemical parts by passing an electric current through it. The liquid, in most cases, is molten salts. In this operation a mixture of salts with a low melting point, suitable viscosity, density and a high conductivity will be achieved by dissolving a compound of the desired in the salts of more active metals (Felsher *et al*, 2000).

1.2.4 Biohydrometallurgy techniques

Biohydrometallurgy or biological metal extraction, is a microorganism assisted ore - leaching process which involves microorganisms and/or their metabolic products. In these techniques, microbes fulfill the major role. Biohydrometallurgy is a branch of classical metallurgy which is not as widely publicized as other fields of metallurgy such as pyrometallurgy.

Biohydrometallurgy processes are less expensive, almost waste-free and less energy intensive compared to other conventional processes (Karavaiko *et al*,1988), so that they have the advantages of compatibility with sustainable development. However, compared to large numbers of patents obtained in the Patent Cooperation Treaty concerning pyro- and hydro- metallurgical techniques, very few - approximately 15 international patent applications - have been claimed in biohydrometallurgy since 1990.

In this research we are mostly concerned with biohydrometallurgy techniques. Due to their importance in this study, they will be explained in detail later, in Chapter 2. In addition to that a short explanation about different commercially used techniques offers. Chapter 3 explains the purpose of this particular research and the experimental works and analytical methods will be covered in Chapter 4. Discussion about results achieved in this study will be in Chapter 5. The conclusion of the work done is coming in Chapter 6.

Chapter 2

Biohydrometallurgy

2.1 Introduction

The history of biohydrometallurgy traces back to around 2000 years ago, centuries before the discovery of microorganisms, when the Chinese in 200 BC and Europeans in 200 AD extracted metals by biological methods (Rossi, 1990). Hallberg and Rickard, claimed that more than 2 million tons of copper have been bioleached from the copper deposit of the Falun Mine in central Sweden since 1687 (Hallberg and Rickard, 1973). Rossi (1990) in his book "Biohydrometallurgy" cited that the first commercially leached copper which involved bacteria to some extent, was performed at the Rio Tinto in Spain in 1752 (Gerbella, 1940). Then, the dissolved copper present in pregnant solution was precipitated on contact with metallic iron, a process first explained by the Chinese. Even though, in 1922, Rudolfs and Helbronner reported that an unidentified sulphur-oxidizing microorganism had oxidized zinc sulphide and, based on that, they suggested a biological method for utilization of low-grade zinc sulphide ores economically (Rudolfs and Helbronner, 1922), it was believed that metal-solubilization process is a chemical reaction mediated by water and oxygen. It was only in 1947 that , bacterial catalysis of iron oxidation and sulphuric acid formation was illustrated by Colmer and Hinkle in mine waters (Colmer and Hinkle,1947).

The first reports of involvement of identified bacteria, were in the 1950's in the formation of acid mine drainage from pyrite inclusions in bituminous coal deposits (Colmer *et al.*, 1950; Temple and Colmer, 1951 ; Leathen *et al.*, 1953). Kennecott Copper Corporation in the late 1950s finally reported copper bioleaching by which method they patented the process of microbial leaching for the first time (Zimmerley *et al*, 1958).

Today, biohydrometallurgy is an interdisciplinary subject affiliating geomicrobiology, microbial ecology, microbial biogeochemistry and hydrometallurgy (Rossi, 1990). It is a promising technology which may be used for obtaining valuable metal compounds from ores, e.g., recovery of low-grade metal, or for detoxifying industrial waste products (Brombacher *et al.*, 1997). Generally, it has many apparent advantages, particularly in processing of low-grade mineral resources: low energy costs, a cost-effective recovery of valuable metal, and an industrial process similar to the global biogeochemical cycles in the biosphere.

On the other hand, the same microbial activities can impose severe environmental hazards. For instance the natural weathering process and uncontrolled microbial activity in mine waste deposits result in 60% of the metals released to water in Sweden (Notter, 1993). Even though different techniques are present to reduce the environmental impact of mine water, they are associated with high costs (Ladin and Pedesen, 1995). Moreover, most studies on reclamation methods are concentrated on physico-chemical techniques, and biological treatment methods are rarely taken into account. Agate (1996), Bosecker(1994), Rossi(1990)and Torma (1987) have provided information about companies applying bioleaching techniques for metal recovery in addition to sites where these techniques are currently in operation.

Besides commercial application of biohydrometallurgy in bioleaching of metals, some of the recent applications are : the biotic metal extraction of industrial waste (Bosecker, 1986; Francis and Dodge, 1990; Strasser *et al.*, 1993;Müller *et al.* 1995; Bousshard *et al.*, 1996); sewage sludge (Blais et al.,1993; Benmussa *et al.*, 1994; Strasser *et al.*, 1995), and soil (Bartlett, 1993). Microorganisms are also used to desulphurize or depyritize coal (Dugan, 1986; Andrews *et al.*,1993) or fossil fuels (Finnerty and Robinson, 1986). So far, few biohydrometallurgical patents for pilot- or commercial-scale plants have been published. However, modifying laboratory scale techniques could lead to establishing patents in pilot- or commercial-scale plants.

This chapter offers a brief explanation on different leaching techniques, followed by some patent applications published since 1990. After that, different types of metal and microorganism interactions and microbial leaching will be propounded.

2.2 Leaching techniques

Based on working volume, leaching techniques can be divided into three main areas:

- The laboratory-scale ($0 - 10dm^3$), Laboratory-scale leaching techniques can be divided in two main groups; The first type involves a qualitative or semi-quantitative estimate of the agreeableness of an ore type to bioextraction by a well-specified microorganism. This class includes manometric, stationary flask and air sparging techniques. The second type, on the other hand, favors the assessment of quantitative parameters in an analytical approach. This class, which involves the preliminary step of pilot plant testing, includes air-lift percolator and shake flask, tank, and pressure bioleaching (Rossi,1990). Some of the techniques are as follows:
  - Manometric technique : In the manometric technique the objective is to assess the amount of evolved gas in a well-specified physicochemical or

biochemical process at constant temperature (Rossi,1990).

- Stationary Flask technique : This technique is one of the simplest methods of microbial culture for its modest equipment costs and experimental simplicity. In this procedure a flask with a special shape is kept at a constant temperature. The opening is plugged by absorbent cotton to allow only filtered air to enter. The flask is placed under ultraviolet lamps, ambient atmosphere sterilizing light . The culture medium, substrate and inoculum are then introduced into the flask, which is then plugged again. The area of air-liquid contact should be maximized to favor the diffusion of air into the liquid mass.

  This technique is useful when, the physiology of microorganisms, the amenability of minerals to bioleaching and the physicochemical parameters in the process are of concern; however, the information it provides is very limited (Rossi,1990).

- Shake Flask technique : This technique is very similar to the stationary flask technique; however, it has two advantages over it: problems of time-dependent heterogeneity of the suspension and the slowness of gas diffusion have been overcome in this method. This system consists of several Pyrex glass Erlenmeyer flasks which are fixed by clamps to a reciprocating or rotary action shaker. Shaking the suspension, therefore, assuring mixing, homogenization and stirring the surface and thereafter, enhancing the dissolution of atmospheric gases essential for microorganisms such as oxygen and carbon dioxide. Usually each test is conducted in three identical prepared flasks to guarantee a minimum statistical reliability of the results. To have an accurate measurement of kinetic parameters, bioleaching tests should be conducted under controlled temperature and environmental conditions. Shake flask techniques have many applications and a great volume of published work on this subject is available (Rossi,1990).

- The pilot-plant ($< 10m^3$), Many problems could arise in using the data obtained from laboratory- scale tests for developing trustworthy models for the design of commercial-scale plants. Most laboratory-scale techniques can supply the desired information in a relatively short time; however, it is difficult to apply these data to commercial-scale since they are carried out under different conditions. For instance, particle size is a very important factor; as particle size increases, its exposed surface decreases so that the accessibility of solution and microorganisms to valuable metals decreases, and therefore metal solubilization decreases. In addition to this, differences in length of solution path, permeability, oxygenation and temperature distribution within the rock mass are different in laboratory-scale and commercial cases (Rossi, 1990).

  Furthermore, the controlled environmental condition in laboratory-scale tests may not be applied to commercial-scale plants. By pilot-plant techniques which model the real environmental condition, it is possible to have a more liable assessment of a commercial size plant. There are different types of pilot-plant techniques, such as column leaching and agitated tanks and reactors, which are not the focus of this study.

- The commercial-scale ($> 10m^3$) . Commercial - scale bioleaching techniques, main applications are in the mining industry. As mentioned above, they were first patented by the copper industry in the early 1950's and thereafter they were introduced into the gold (Olson and Kelly, 1986; Olson, 1994), uranium (Khalid *et al.* ,1993) and zinc (Agate, 1996) industries for production of metal from low-grade ores . Commercial - scale leaching has been employed in various forms; for instance:
    - In situ leaching . Without removing rocks from the orebody, metals are solubilized and recovered during in situ leaching.
    - Dump leaching . Material obtained from research or preparation works in underground mines or from stripping of open-cast mines form the dump. Generally, dump leaching can be applied to very lean ore mixed with rocks in order to recover metal/metals.
    - Heap leaching . It has application when the recovery of metal from run-of-mine ore on an appropriate area, is desirable. The run-of-mine, which

frequently has undergone pre- treatments such as crushing, screening or even partial roasting, forms heaps in the so- called prepared area.

- Vat leaching : In vat leaching the ore, after crushing to a suitable size, is dumped on the concrete vats which are lined with acid-proof material . Each vat is fed through a bottom opening and then the leach solution percolates upwards through the ore mass and the overflow is pumped into the next vat (Rossi,1990).
- Reactor leaching : This method is more expensive than all above - mentioned leaching methods, due to the elevated installation and operating costs. Thus, it is limited to the products of conventional concentration processes, such as flotation concentrates. This process is carried out in reactors consisting of adequately stirred tanks in which the pulp to be leached resides for a definite time interval Rossi,1990). This process, therefore, appears to offer the best potential for leaching operations, since it is possible to achieve higher reaction rates compared to other process systems.

All the above-mentioned processes are termed bioleaching processes. In situ, dump and heap leaching operations are often conducted on submarginal run-of-mine ores. Vat leaching and heap leaching can also be applied to high grade run-of-mine ores. However, reactor leaching is mainly applied in metal extraction using flotation concentrates.

As yet, biohydrometallurgy's techniques have been mostly applied to mine industries in the leaching process. In 1980, approximately 10% of the world's copper was extracted by microbiological leaching (Ingledew, 1990). Today this percentage has increased and it accounts for 15%-30% of the world production of copper, which is roughly 2-3 Mt (Brombacher *et al.*, 1997). Moreover, Brierley (1995) proposed that in 1992, 2.1 kt gold, which is about 20% of the world production of gold, was obtained by biological leaching. Agate(1996) , Bosecker(1994), Rossi (1990) and Torma (1987) have reviewed commercial information on bioleaching techniques for metal recovery in addition to sites where these techniques are currently in operation.

2.3 Biohydrometallurgy patents

As mentioned before, since 1990, approximately 15 patents with world- wide importance are known to have been issued, not a considerable number when compared to large numbers of issued patents in other fields of metallurgy, such as hydrometallurgy. Some of the applications of biohydrometallurgy which have been published as patents are reviewed here:

- Processing of low-grade ore: since high-grade ore deposits are becoming rare, the importance of processing of low-grade ore has been recognized. Many studies have been published but few have had patents issued as a result. The followings are the most important ones. Among reactor leaching patents is the Hoffmann et al. (1989) patent in the California Institute of Technology. They elaborated on a process in which ferric iron was biologically reduced to ferrous iron. In this process a *Pseudomonas sp.* strain with domestic wastewater as substrate under anoxic conditions was employed to reduce ferric iron existing in an aqueous mixture of iron ore. This method is applicable to steel production, where coke is introduced to provide reducing conditions to produce elemental iron. This microbial action would result in a significant saving of coke. Portier (1991), as well, described a reactor leaching process for the recovery of gold from carbonaceous or carbonaceous pyritic ores. Usually, in ordinary gold recovery processes it is extracted by cyanidation processes; however, it is impossible to treat this kind of ore with standard cyanidation processes. This is because, the graphitic carbon absorbs the gold cyanide complex from the cyanide leaching solution. Moreover, long chain organic compounds, similar to humic acid, form stable complexes with the gold. Portier employed fungal and bacterial strains such as *Aspergillus brunneio-uniseriatus, Penicillium citrinum* and *Citrobacter freudii*, to pre-treat these ores. This pre-treatment mainly reduces the carbon content of ore by consumption of the carbon by microorganisms. Thereafter, the pre-treated ore is exposed to autotrophic leaching by *Thiobacillus* strains.

Rusin (1992), patented a process for recovery of metals, particularly silver, from refractory manganiferous ores. In this process a manganese-reducing *Bacillus* strain is utilized, in which its metal-resistant genes could be transferable to other microorganisms. The transformed host can be used as the parent strain. In this process more than 90% of silver and manganese can be recovered. The bacteria he suggested was *Bacillus polymyxa.* Without bacteria, up to 2% of manganese was solubilized and silver recovery by cyanidation was only 8%-15%. In the recommended process, manganiferous silver ore is mixed with a culture of *Bacillus sp.* and incubated for 2-7 days, in order to solubilize metal contaminants in the ore. The culture liquid is separated from the ore and manganese and silver are then recovered from the solution. The residual ore, yet embodying some silver, is recovered using metallurgical techniques. This process is carried out under anoxic conditions at room temperature. Rusin (1993) has also patented a remediation method for soil with the same bacterium in a batch or continuous bioreactor in the presence of a chelator.

In addition to reactor leaching processes, several heap leaching processes have been patented also, which mainly focus on an economical recovery of gold. Kleid et al. (1991) illustrated the use of cyanide-producing microorganisms, such as *Chromobacterium violaceum, Chlorella vulgaris,* or *Anacystis nidulans,* to form a cyanide-ion-containing solution, which is introduced into the refractory gold ore. Hence, gold-cyanide complexes are formed and absorbed into the microorganisms through a biosorption process. Thereafter, gold is recovered from the cultures. This process is less harmful to the environment because, in contrast to chemical cyanidation which causes a concentrated cyanide solution, the cyanide concentration is much lower; the organisms metabolize the generated cyanide in a very rapid and efficient way.

Steemson et al.(1994) patented a continuous bioleaching solvent extraction process to recover metallic zinc from a zinc concentrate. Then, they scaled it up from a bench-scale operation to a fully continuous pilot-plant. They developed an efficient and cost effective method to separate zinc metal from lower-grade zinc sulphide concentrate. In this method, by using *T. ferrooxidans, L. ferrooxidans, T. thiooxidans, Sulfobacillus* strains and heterotrophic organisms as inocula, a recovery yield of up to 97% was achieved.

- Processing of soil : as bioremediation of heavy-metal- contaminated soil by microorganisms is a new process, only a few patents have been published in this area. However, removal of toxic metal substances from soil is possible in a similar manner to ore. Rusin (1993) patented a biological process for plutonium recovery from contaminated soils by iron-reducing microbes. Although the recovery of plutonium from contaminated soil is very difficult, in this process a recovery of 89% was achieved. This process was very efficient compared to current ways of plutonium extraction. Current processes include:

    1. Transport and storage of wastes in depositories, which requires high cost ;
    2. Solubilization of plutonium by strong acids such as nitric or nitric/hydrofluoric

acid. This process will result in destruction of the soil matrix in addition to the corrosion problems, which oblige companies to provide acid-resistance equipment;

3. Volatilization of PuO_2 as PuF_6 ,by using fluorine which is a highly reactive and corrosive gas. This process is a rather uneconomical process, through which after 6 days only 20% solubilization is obtained. In the Rusin biological process iron-reducers isolated from ore, soil or water were added to the contaminated soil and the growth medium. The mixture was then incubated and kept under anoxic conditions for 2-7 days. Subsequently, plutonium was recovered from the leach solution by standard procedures such as ion exchange, solvent extraction, precipitation, volatilization etc.

- Processing of coal fly ash : since coal fly ash, a waste product of coal-fired power plants, contains a variety of metals such as Al, Ti, Zn, Cu, Co and others, it is supposed to be a source of toxic metals. In many countries great amounts of fly ash are produced annually. For instance, in Russia approximately 100 Mt coal fly ash is created per annum (Pavlenko *et al.*, 1997). The most common way of discarding fly ash is disposal in landfills or in the sea (Brombacher *et al.*, 1997) but since there is a possibility of leaching in this method, it may threaten the environment. Fass et al.(1994) patented a biological method in which *Thiobacillus thiooxidans* was used. This bacterium was first adapted to an acidic sea -water-based medium and then it was inoculated with a suspension of 50% coal fly ash. This culture was kept in this medium for 4 months, in order to select a viable strain capable of growing under extreme conditions, which was a saline environment with toxic metals. This process resulted in a strain of *T. thiooxidans* that grew in the presence of at least 4000 ppm aluminum. The bioleaching process was carried out in 50-l plastic containers with a circulation and aeration system. The coal ash concentration was 20% and the incubation period was 2-3 weeks. Then, the metals were recovered by precipitation steps by increasing the pH.

- Processing of industrial waste : industrial wastes such as galvanic sludge, filter-press residue and filter dust carry great amounts of valuable metals, e.g., copper or zinc, but in a very low concentration. Therefore, generally, extracting these metals by techniques other than biological methods are not effective. Using microorganisms not only makes the recovery process cost-effective, but also minimizes the pollution level of the environment through detoxification actions. In 1994, Hahn (1994) described a biological method for recovering copper from solid wastes containing 5% of copper. He utilized a microbial culture from *Bacillus sp.*. The culture was fed continuously with saccharose. After 18 days 320 g/L of saccharose had been utilized and 99% copper had been extracted.

2.4 Microorganisms and metal interactions

In the last decade, numerous literature and review papers concerning metals and microorganisms interference have been published. Overall, all the alluded to mechanisms which can remove or concentrate metals may be categorized as follows (Gaylard and Videla, 1995):

- Extracellular interactions. These interactions mainly involve extracellular polymers, proteins, acid metabolites and local environment changes because of biochemical processes.

  In this kind of interaction microorganisms do not have to be in direct contact with metals. Microbes will contact with metals indirectly by production of metabolites or by some biochemical reactions. These processes can benefit the environment in several ways:

  a) Leaching of metals from alloys or natural ores by acid production. Leaching of different metals from solid substrate by utilizing organic and inorganic acids generated by microorganisms, has been studied by Schinner and Burgstaller (1989).

  b) Release of metals bound to iron and manganese oxides by microbial reduction. Since iron and manganese oxide formation causes serious problems such as corrosion, many studies have been conducted on the mechanisms of iron and manganese oxidation and reduction (Ghiorse, 1984; Jones, 1986; Nealson *et al.*, 1989, Abdelouas *et al.*, 2000).

c) Immobilization of metals by formation of insoluble salts. Some metal salts are insoluble, mostly in the hydroxide, carbonate, phosphate and sulphide forms. Their formation results in separation of these metals from the containing solution. The most significant microbial immobilization process is sulphide production by sulfate-reducing bacteria (Gaylard and Videla, 1995).

- Cell- surface interactions. Some metals tend to bind to microbial cell surfaces as a result of functional groups. One of the most important cell-surface interactions is biosorption. Biosorption is one of the significant mechanisms by which microorganisms remove or concentrate metals from solutions. This process may be cited as a passive process, in the sense that it may occur even if the microorganism is dead. In other words it is not associated with any physiological function of the living microorganism (Gadd *et al.*, 1998).

  Biosorption is essentially an adsorption-type phenomenon. Electrostatic attraction causes metal cations to be drawn to the negatively charged cell-surface of the microbe. Moreover, metal binding capacity for each microorganism depends on the chemical composition of its cell wall. It is noteworthy that the amount of metal bound to the cell wall is always more than what can be accounted for in terms of stoichiometric interaction between metal ion and active sites on the cell wall. It occurs because of a two-stage mechanism proven by electron microscopic observation as follows: in the first stage all the metal ions trapped by the cell wall are those which by stoichiometric bound have been absorbed. In the second stage, the metal ions bound to the cell wall act as nucleation sites for the deposition of more metal ions from solution with development of aggregates, called crystaloids (Beveridge *et al.*, 1980). In conclusion, it may be stated that any microorganism which has negatively charged groups on its cell surface should have an affinity for cations. The goal would be, therefore, to recognize the microorganisms displaying the highest metal binding effectiveness and, in addition, selectivity. Furthermore, the cost-effectiveness of an organism as biosorbent, should be regarded on the basis of its overall metal adsorption capacity, weight of biomass multiplied by concentration of metal adsorbed on biomass, rather than its absolute metal uptake. Metal adsorption capacity is a more accurate tool as long as the presence of toxic metals may slow down the growth of metal-tolerant organisms.

- Intracellular interactions. Some particular transport processes may effect metals accumulation in microbial cells. This can cause environmental detoxification through metals conversion to insoluble or more volatile forms, or incorporation of specific metals into enzymes. Among these interactions is bioaccumulation, which will be covered here (Rossi, 1990).

Bioaccumulation relies on the metabolic energy ATPase activity and trans-membrane proton gradient, which is referred to as active absorption (White and Gadd, 1987). It is considered the second phase of the process of metal uptake by living microorganisms. Despite passive adsorption which is rapid and independent of the presence of specific nutrients, active absorption is slow and nutrient dependent. In general, bioaccumulation of metal into a cell needs particular transport systems and depends on the toleration of the microorganism to relatively high concentrations of toxic elements in the intracellular cytoplasm and other subcellular components (Gadd *et al*, 1998). Metal cations can reach the cytoplasm through the highly specific micronutrient transport systems developed by microorganisms. In this regard, metals can be divided into two categories: metabolically essential metals which utilize their specific transport systems, and non-metabolic metals. The former group includes K, Fe, Mg and traces of Cu and Ni. The non metabolic metals such as Co, Ni, Cu and Ag primarily utilize the pathway existing for the uptake of the first group (Rossi, 1990).

It should be noted, that the bioaccumulation capacity of microorganisms Is generally much lower than the biosorption. Its range is approximately 0.5% to 2.0% of the organism's dry weight (Kelly *et al.*, 1979). This number is slightly lower with bacteria than with yeasts and fungi when the organisms are compared on an equal dry weight basis.

The concentration of this research is on extracellular interactions, especially metal interactions with organic acids, which eventually led to leaching out of the metal.

2.5 Microbial leaching

Microbial leaching is a special hydrometallurgical process. In this process either metabolic activities or products of microorganisms are involved. Up to now, two types of microbial leaching are known: Chemolithoautotrophic leaching and heterotrophic leaching. Chemolithoautotrophic (a term, which encompasses the autotrophic creatures that oxidize sulphur as a source of energy), mostly *Thiobacillus* type, leaching processes, which have been surveyed intensively and have been subject of many studies. It is a suitable type of leaching for minerals rich in sulphur or sulphides. This type of leaching will be explained in brief later on. Heterotrophic (a term, which encompasses the organisms that need organic carbon sources to survive) leaching processes, in which metabolites excreted by microorganisms are the results of chemical reactions, occur between ore and microorganisms. This type of leaching is well suited for minerals low in sulphur and sulphides. This sort of leaching can happen under various pH regimes: under low pH Due to organic acid production by microorganisms; at pH values of 6-9 when appropriate leaching by-products or non-acidic complexing are excreted; or where pH is high due to ammonia production from catabolism of proteins. In this study, heterotrophic leaching, specially the first mentioned mechanism is mostly of concern.

2.5.1 Chemoautotrophic leaching process

Colmer et al. (1950) for the first time reported the role of bacteria in the formation of acid mine drainage from pyrite in 1950. Following that Bryner and Beck and their students at Brigham Young University in Provo Utah reported that *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans* have been found in acid mine drainage in two different types of copper sulfide mineral mines. Moreover, involvement of *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans* has been reported by Malouf et al. (1963) and Silver et al. (1974) in bioleaching, in many metal sulfides such as ZnS, NiS and PbS as well.

Today the most common type of bioleaching, which is now in commercial scale, is extraction of different metals by the aid of *Thiobacillus* genus, mostly *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans*. These bacteria are mesophilic and acidophilic and grow optimally at the temperature between 25 to 30°C and a pH between 1.5 to 2.0. They are obligate autotrophs as well. *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans* participate in different chemical reactions.

Thiobacillus ferrooxidans gains energy from oxidation of either ferrous iron or reduced sulfur compounds, whereas *Thiobacillus thiooxidans* can obtain energy only by oxidation of reduced sulfur compounds while both of them decompose mineral sulfides in pure culture.

Metal constituents mobilization is the main process in bioleaching of sulfide ores. This is accomplished through microbial oxidation of metal sulfides, which is basically the conversion of an insoluble form of metal to a soluble form. Silverman et al. (1964) first reported that bacterial attack takes place in two different modes, direct and indirect. In direct mode the cell is attached to the sulfide minerals and oxidizes metal sulphide enzymatically to sulphate and metal cations. The sulphur moiety of the mineral is oxidized to sulphate without any detectable process (Ehrlich, 1999). The direct process can be summarized as follows:

$$FeS_2 + 3\frac{1}{2}O_2 + H_2O \longrightarrow Fe^{2+} + 2SO_4^{2-} + 2H^+$$

$$2Fe^{2+} + \frac{1}{2}O_2 + 2H^+ \longrightarrow Fe^{3+} + H_2O$$

In the indirect mechanism the cell doesn't attach to the mineral. In this mechanism the oxidizing potential of iron (III) causes mineral sulfide to be solved. During this chemical reaction iron (II) and elemental sulfur (S^8) might be produced and then are biologically oxidized to iron(III) and sulfate (Sand *et al.*, 1999). This mode can be summarized as following:

$$FeS_2 + 14Fe^{3+} + 8H_2O \longrightarrow 15Fe^{2+} + 2SO_4^{2-} + 16H^+$$

$$MS + 2Fe^{3+} \longrightarrow M^{2+} + S^\circ + 2Fe^{2+}$$

$$S^\circ + 1\tfrac{1}{2}O_2 + H_2O \longrightarrow 2H^+ + 2SO_4^{2-}$$

Although these definitions are the most common ones they are some how imprecise and uncertain. The hypothesis of direct mechanisms has not been proven yet and there are reports either supporting or rejecting that hypothesis (Rossi,1990; Ehrlich, 1996) . Even recently it is suggested that there is no difference between these two mechanisms, since Fe(III) oxidizes metal sulfide in both modes(Sand *et al.*, 1995).

However up to now results of recent research have shown that the bioleaching of sulfide minerals occurs mostly through an indirect mechanism, in which the rates of both the ferric leaching and ferrous-iron oxidation are governed by the ferric/ferrous-iron ratio (Breed *et al.*, 1999).

There is another strain which is capable of decomposing sulfide ore and is called *Leptospirillum ferrooxidans.* This strain is mesophilic, acidophilic and its optimal environment of grow is the same as the two above-mentioned strains. It consumes only ferrous iron as a source of energy (Harrison, 1984).

Among so called bacteria *Thiobacillus ferrooxidans*, plays the principal role in most bioleaching operations. Since this bacterium is autotrophic, the carbon source it consumes is carbon dioxide from the atmosphere. By mainly oxidation-reduction reactions, it obtains its energy needs for the carbon dioxide fixation process. In this oxidation- reduction reaction, ferrous iron (FeS_2) and reduced sulfur compounds serve as the electron donor and oxygen is the electron acceptor.

Although bacterial leaching has gained great promise in extracting of metals from sulfide-ore, knowledge about biomining of oxide and silicate ore is quite limited (Tzeferis, 1994a).

2.5.2 Heterotrophic leaching process

Although this kind of leaching deals with all types of ores low in sulphur, studies have been focused on metal extraction from oxide ores, which are the most prevalent type of ores. In this respect, this research has mostly been concentrated on oxide ores.

Two mechanisms are thought to be the main mechanisms during the process of heterotrophic microbial metal extraction from oxide ores;

a) The first one occurs when an inorganic mineral acid such as sulphuric acid with a strong acid pH displaces divalent metal cations by hydrogen ions from the ore matrix.

b) The second one happens when compounds produced by the microorganisms can seclude metals into soluble complexes by chelation.

A combination of these two mechanisms may be observed when a microorganism generates organic acids.

2.5.2.1 Fungal leaching

Fungi are of importance in the heterotrophic leaching process. Although their ability in leaching metal has been proven (Bull *et al.*, 1976; Kristiansen *et al.*, 1983; Brown, 1988; Burgstaller *et al.*, 1992) the feasibility of the process is under question.

Advantages and disadvantages of fungi associated leaching have been suggested. Burgstaller et al. (1992) have alluded to some points one may need to consider in order to disregard fungi as a possible means for leaching:

1. Generally, fungi require large amounts of organic carbon for growth and generation of a leaching agent.
2. Biohydrometallurgists are not well acquainted with fungi treatment.
3. The leaching process led by fungi is slower than leaching by bacteria in most cases; for instance, *Thiobacillus*.

However, those points are less significant after the following facts are considered:

1. Some materials embodying metals will augment the pH of the medium during the leaching process, which makes the environment unsuitable for acidophilic *Thiobacillus* species.
2. The formation of complexes with metal ions, which are either more soluble in neutral environments or less toxic, is another superiority of the presence of fungal leaching agents.
3. As mentioned before, conventional leaching processes are only applicable for materials containing sulphur compounds.
4. Tightened environmental standards will force industry to move towards less-polluting metal winning processes, mainly less corrosive. Compared to chemolithoautotrophic leaching processes, fungal leaching's effective pH is not a very low pH.
5. Fungal microorganisms are capable of consuming organic carbon sources, which may be supplied by inexpensive organic wastes.

Thus, fungi can be taken into account as microorganisms which have the potential for metal extraction.

The metabolites' action in fungal leaching has not been totally investigated and there is controversy about different metabolites and their solubilization action on metals. However, Berthelin (1993) suggested three mechanisms by which solid metal compound can be leached:

a) Acidolysis. Oxygen atoms around the surface of metal compound can be protonated. In this mechanism the protons and oxygen associate with water and displace the metal from the surface.

b) Complexolysis. Complexing capacity of a molecule will result in the solubilization of the metal ion (Sigg and Stumm, 1989). This mechanism takes place slower than acidolysis. Some examples of stable complexes are oxalic acid and iron, magnesium as well as citric acid and calcium and magnesium. The complexolysis of the metal ions which have been solubilized via acidolysis would be augmented. That is why this mechanism is very important in solubilization action. Besides, this process often reduces the toxicity of heavy metals for the fungi.

c) Redoxolysis. Reduction of metal ions in an acidic environment, such as reduction of ferric iron and manganese under the influence of oxalic acid.

Chapter 3

Research objectives

The large amount of mine wastes in Canada has made mining industries one of the most debated environmental issues. According to The State of Canada's Environment in 1994, 6811 million tons of tailings were produced in Canada annually of which about one third was produced in Quebec. The great amount of wastes produced annually not only has side effects on the environment but also embodies a huge amount of valuable metals. For instance, generation of acid mine drainage (AMD) which is one of the great threatening environmental issues, is among the mining industries' concerns. AMD not only would affect water streams, but also it may cause metals such as arsenic and cadmium to leach from mine wastes. The latter matter which is the metal load in the environment will cause greater damage than the acidity in environmental terms. The main reason for this problem is the mishandling and misplacing of tailings. Besides that, in Canadian mines huge amounts of rocks embodying trivial amount of precious metal exist, which are exposed to the atmosphere. These rocks may undergo different environmental changes, such as weathering which make huge amounts of metal leach out to natural bodies of water.

Currently, in order to diminish these side effects, Canadian mining management is pursuing the following policies:

1. reduction of the volume of surface waste streams;
2. securing the confinement of mining residues and
3. recovery of valuable matter from the residues.

There are different methods focusing on each of the mentioned strategies; however, the third one is the only one that can be categorized in the sustainable development framework.

This research deals with biological leaching of mine residue that falls into the third category. A heterotrophic fungus called *Aspergillus niger* which has been shown to produce different organic acids was selected and preliminary studies were conducted to find an inexpensive process to recover metals.

Overall, the objectives of this research can be outlined as follows:

1. To evaluate the effectiveness of citric acid and oxalic acid in metal leaching;
2. To evaluate the efficiency of different substrates in organic acid production and metal removal;
3. To evaluate the effects of different types of pre-treatment on metal removal.
4. To investigate whether some organic wastes could be used as substrates.

Chapter 4

Materials and Methods

4.1 Introduction

Great volumes of literature have been published concerning the ability of fungi to extract metals from different types of sources. Wenberg et al.(1971) introduced the idea of winning metal from mining wastes and carbonaceous low-grade ores by using fungi. In addition to that, Silverman and Munoz(1971) investigated the process of titanium leaching from rock by fungi in 1971.

A large research study about metal leaching of non-sulphide nickel using fungi was done by Tzeferis (1994a; 1994b; 1994c). In his studies a variety of fungal screening, adaptation and leaching techniques were tested in order to determine the optimum conditions for nickel recovery. In his research with *Aspergillus niger* up to 72% nickel recovery was achieved. In his studies, molasses was shown to be a suitable substrate for fungi.

Golab and Orlowska (1988) reported that tartaric acid and citric acid produced by *Aspergillus niger* are able to leach out zinc oxide, with up to 90% efficiency.

Müller et al.(1995) studied the leaching of zinc from an industrial filter dust with a fungus (*Penicillium simplicissimum*) and two bacteria (*Corynebacterium glutamicum* and *Pseudomonas putida*). They compared the effects of amino acids and citric acid in the leaching process. Based on their experience, the produced citric acid solubilized considerable amounts of zinc, whereas amino acids did not perform well in the leaching process.

Burgstaller et al. (1992) studied the solubilization of zinc oxide from filter dust with *Penicillium*. Their reports shows 30 g/L of zinc solubilization in a period of 9 days. They indicated that zinc oxide stimulates the excretion of citric acid, which may not happen with the oxides of other metals.

Cameselle et al. (1995) investigated the ability of *Aspergillus niger* in the solubilization of iron existing in kaolin. It was then concluded that iron oxalic acid is the main leaching agent and not citric acid which has a negative effect.

Bioleaching of fly ash by fungi (*Aspergillus niger*) for the first time was reported in 1996 (Bousshard *et al.*, 1996). The experiments were conducted in shake flasks and the leaching of different metals such as Al, Cd, Cr, Cu, Zn and Mn present in fly ash was recorded. Gadd et al. (1998) endorsed employing *Aspergillus niger* in bioremediation of radionuclide pollution. They also mentioned that, the process commercially has not yet feasible. Mulligan et al. (1999) conducted various experiments with *A. niger* in the presence of different concentrations of low-grade ore.

With respect to these facts, *Aspergillus niger* was selected as a microorganism capable of leaching metal.

4.2 Fungal strain and growth conditions

Aspergillus niger strain ATCC 6275 was obtained from American Type Culture Collection (ATCC), in a freezed dried state. The medium utilized for growth of culture was based on the method of Bousshard et al.(1996), in which a culture was inoculated on a Potato Dextrose Agar (3.9% w/v). In order to obtain sufficient numbers of spores, the culture was kept for 7 days at $26°C$ in this medium.

Afterwards, sodium dodecyl sulfate (0.2% w/v) was used to recover the spores. The spores were counted using a Petroff-Hausser counting chamber where 1 mL of spores (approximately 3×10^7 spores) was obtained. The spores were then added to a 500 mL flask containing 150 mL of prepared medium.

4.3 Characterization of the mining residues

An oxidized sample of mining residues was obtained from a mine in the Gaspé region. Because of the large size of these residues, they were crushed. The crushed ore residue (obtained by passing crushed ore through sieve no. 4 and remaining on sieve no. 8 (grain size varied from 2.38 mm to 4.75 mm) was selected. The quantity of metals was determined after nitric acid/hydrogen peroxide digestion of the residues as described by Environment Canada (1990). The analysis of the metals was performed by PERKIN ELMER atomic absorption spectrophotometer model AAnalyst 100. The results of the analysis are shown in Table 1.

Table 1: Concentration of metals in the mining residue

Heavy metal	Concentration (mg/ kg residue)
Copper	7245
Iron	26470
Zinc	201
Nickel	27

4.4 Chemical leaching experiments

Preliminary chemical leaching tests were carried out in order to evaluate effectiveness of oxalic and citric acids with various concentrations in nickel, zinc, iron and copper leaching. Four different concentrations (w/v) 0.01%, 0.05%, 0.1%, 0.5% of citric and oxalic acids with 10% (w/v) of residue in duplicate were shaken for 48 hours. Liquid samples were taken at the end and they were filtered, then the analysis of the metals dissolved in each sample was performed by atomic absorption.

4.5 Leaching of the mining residue by *A. niger*

All assays were carried out in a 150 mL volume. Medium, ore samples (100 g/L) and salts(1.6 g/L yeast extract, 1.5 g/L $NaNO_3$, 0.5 g/L KH_2PO_4, 0.025 g/L $MgSO_4$ * $7H_2O$, 0.025 g/L KCl)were sterilized by autoclaving for 20 minutes at 121°C before spores were added. Afterwards, all flasks were sealed with removable cotton. Temperature was kept at 20°C during the experiment. Each flask was shaken by a BURRELL WRIST ACTION shaker during the period of the experiment in order to keep everything in a homogenous slurry form. Samples were collected by disposable sterilized pipettes. Then they were centrifuged and filtered as mentioned in section 4.6.1. Metals and organic acid quantities were then measured in the supernatant as mentioned in section 4.6.1 and 4.6.2. Each experiment was done in triplicate and a control flask, one without the microorganisms, was handled maintaining the same conditions. All media compositions are summarized in the following table and supplementary details appear subsequently.

Table 2: Summary of media content

	Substrate	sampling day (day)	pre-treatment
medium no.1	100 g/L sucrose	5;7;9;11;13	Autoclaved
medium no.2	100 mL/L Molasses (80% diluted)	5;7;9;11;13	Autoclaved
medium no.3	40 g/L sawdust passing through sieve no.16	5;7;9;11;13	Autoclaved
medium no.4	40 g/L leaves passing through sieve no. 10	7;9;12;14;23	Autoclaved
medium no.5	40 g/L potato peels	7;9;12;14;23	Autoclaved
medium no.6	27 g/L sawdust and 13 g/L sucrose	7;9;12;14;23	Autoclaved
medium no.7	40 g/L potato peels and 1 g/L sucrose	9;11;14;15 17;25	Autoclaved no yeast extract
medium no.8	40 g/L potato peels and 1 g/L sucrose	9;11;14;15 17;25	Not autoclaved no yeast extract
medium no.9	40 g/L sawdust passing through sieve no.16	9;11;14;15 17;25	Sulphuric acid not autoclaved no yeast extract
medium no.10	40 g/L leaves passing through sieve no. 10	9;11;14;15 17	Sulphuric acid not autoclaved no yeast extract
medium no.11	1 L/L potato chips waste	9;11;14;15 17	Sulphuric acid not autoclaved no yeast extract
medium no.12	40 g/L potato peels	9;11;14;15 17;25	Sulphuric acid not autoclaved no yeast extract
medium no.13	20 g/L corn kernel 20 g/L corn kernel	9;11;14;15 17;25	Sulphuric acid not autoclaved no yeast extract

First set: In the first assay 100 g/L sucrose as the standard medium was tried. This set of experiments was carried out for 13 days.

Second set: Another medium chosen was molasses, since many studies (Clark *et al.*,1963; Hamissa *et al.*, 1977; Szczodrak, 1981; Ilczuk, 1983; Roukas and Alichanidis, 1988) recommended its suitability as an alternative source of sucrose for citric acid production. In the second set 100 mL/L molasses (diluted, 20 mL/(20 mL molasses + 80 mL/L distilled water))was employed. This set was carried out for 13 days. Results confirmed that assumption and, besides, it appeared to be very effective especially for nickel extraction which was reported by Tzeferis (1994b) also.

Third set: Tzeferis (1994a) has pointed out that the main obstacle of heterotrophic leaching is its being more expensive than autotrophic leaching, which can be overcome by providing cheap organic carbon and energy sources. In this line, organic waste products containing hydrocarbons or by-products from the food, paper and agriculture industries seem to be alternatives. Sawdust, leaves and potato peels as three examples of agricultural wastes, which are cheap and abundant, were selected as substrates. The third assay was made of $40g/L$ sawdust passing through sieve no.16.

This set was conducted in a 13 day period.

Fourth set: The fourth was composed of 40 g/L of air dried leaves passing through sieve no.10.

The fourth set of experiments ran for 23 days to evaluate whether the amount of leached metal would have considerably increased.

Fifth set: The composition of fifth medium was $40 g/L$ of air dried potato peels.

This set of experiments ran for 23 days to determine whether the amount of leached metal would have considerably increased.

Sixth set: Among the three substrates, sawdust and leaves and potato peels, potato peels seemed to work well. Sawdust and leaves without any pre-treatment did not appear to be a good substrate, since most of the carbohydrates present in them are not consumable for these fungi. Consequently, to make the fungi production thrive, a certain percentage of sucrose (30% (weight of sucrose / weight of sucrose + weight of sawdust)) was added to the sawdust medium to see whether larger numbers of fungi could perform more effectively. So, the next medium was 27 g/L of saw dust passing through sieve no.16 and 13 g/L sucrose.

This set of experiments was conducted for 23 days and samples were collected on the seventh, ninth, twelfth, fourteenth and twenty-third day of the experiment. This medium worked well and it performed almost as well as pure sucrose medium. However, since the sucrose concentration was still high enough to make the process economically expensive, a low concentration of sucrose was added to the next medium.

Seventh sets: In order to see whether it is possible to find a less expensive substrate, in the next set of experiment, yeast extract as an expensive matter was eliminated. In this experiment potato peels as a substrate which is a waste was tried. In the seventh set a small amount of sucrose was added and it was autoclaved. The medium was composed of 40 g/L of potato peels and 1 g/L sucrose.

This set ran for 25 days.

Eighth set : In the eighth set potato peels as a substrate was chosen and in order to make the process more cost effective, the assay was not autoclaved. This medium was composed of 40 g/L of potato peels and 1 g/L sucrose.

This set ran for 25 days.

Ninth set: In this set of experiment, in addition to elimination of yeast extract, sawdust was soaked in a very dilute sulphuric acid (pH 2) for 24 hours. Sulphuric acid could work out in several ways: It helps breaking of the cellulose chains, which may make them easier to be consumed by *A. niger*; In addition, it could will make the leaching process more effective. McKenzie et al. (1987) pointed out two reasons for this event. Firstly, since hydrogen ions are consumed by ores through reactions, the efficiency of the attack mechanism of hydrogen ions will be reduced. In that case, sulphuric acid would lower the pH and therefore increase hydrogen ions. The second reason is, it is possible that the organic acids act as chelators in low pH; however hydrogen ions will not function as chelators.

The medium was composed of 40 g/L of saw dust passing through sieve no.16.

This experiment ran for seventeen days.

Tenth set: By the same account, dried leaves were soaked in dilute sulphuric acid under the same condition and the medium composition was 40 g/L of air dried leaves passing through sieve no.10 .

This set was carried out for seventeen days.

Eleventh set: Since the potato peels appeared to be a suitable substrate, in this set another type of potato waste, which was basically liquid waste from potato chips, was utilized. The assays were autoclaved but yeast extract was eliminated. This set medium was 1 L/L of potato chips waste.

This set was carried out for seventeen days.

Twelfth set: As mentioned before, potato peels showed good potential as a substrate. To investigate whether it could be more economical yeast extract was eliminated. In addition, the medium was soaked in dilute sulphuric acid with pH 2 for 24 hours. Then, the experiment was conducted in non- sterilized assay. The media composition was 40 g/L of potato peels waste.

These sets of experiments were conducted for 25 days.

Thirteenth set: In this set another type of agricultural waste was tested as substrate. The medium was composed of ground kernels, husks and leaves of corn. They were air-dried for more 10 days. The medium was then immersed in dilute sulphuric acid of pH (2) for 24 hours. Yeast extract was absent and the medium was not autoclaved as well. The medium composition was 20 g/L of corn kernels and 20 g/L of corn husks and leaves.

This experiment were carried out for 25 days.

4.6 Analytical Methods

4.6.1 Determination of organic acids produced by *A. niger.*

Samples were analyzed for organic acid after centrifugation with ICE HN-S II centrifuge at 3500 rpm for 10 min. Then the samples were filtered using a 0.5 micron microfilter. The method of Bousshard et al. (1996) was employed, in which the concentration of different organic acids such as citric acid, oxalic acid, gluconic acid, malic acid and tartaric acid were determined by a Beckman Coulter System GOLD Model HPLC (High Pressure Liquid Chromatography). In this method an A-312 YMC-Pack column (6.0 mm x 150 mm in length) at a flow rate of 5 ml/min (room temperature), with a mobile phase of 50 mM monoammonium phosphate adjusted to pH 2.4 with H_3PO_4 was utilized.

Concentrations of different organic acids were measured based on a standard solution made with 0.1% citric acid, 0.1% malic acid, 0.1% tartaric acid, 0.01% oxalic and 0.1% phytic acid. For these standard solutions the following retention times were achieved:(Table 3)

Table 3: Retention times

	Concentration (%)	Retention times (min)
Phytic acid	0.1	7.93
Oxalic acid	0.01	8.47
Citric acid	0.1	10.94
Tartaric acid	0.1	11.50
Malic acid	0.1	12.90

4.6.2 Determination of metals

At the end of each set of experiments, residue samples were washed with distilled water three times. Thereafter, the residues were air- dried for 24 hours. 5 g of residue from each assay was digested by nitric acid/hydrogen peroxide method, illustrated by Environment Canada (1990). All samples were filtered as mentioned in the previous section and the metal concentrations in all samples were determined by PERKIN ELMER Atomic Absorption model AAnalyst 100. Concentration of copper in all samples and concentration of zinc, nickel and iron in some samples were measured. The maximum difference in copper concentrations in each medium was about 15%, which with respect to residue heterogeneity is an acceptable difference.

Copper concentrations via time period were plotted for all media and in each graph copper concentrations in control media were indicated as well. For zinc, nickel and iron concentrations in some media, the same graphs were plotted. Organic acid concentrations were measured and they were plotted versus time. In addition to that, the pH of all samples was measured by Accumet AR 25 pH meter and plotted with respect to time.

Chapter 5

Results and Discussion

5.1 Chemical leaching

5.1.1 Copper solubilization

Citric acid showed a better potential than oxalic acid to solubilize copper. As illustrated in Figure 1 and Figure 2, in lower concentrations, both acids worked equivalently, whereas in higher acid concentrations, citric acid was more effective than oxalic acid. In 0.5% concentration citric acid dissolved copper 4 times more than oxalic acid. Lower pH of oxalic acid (1.9-2.8) assays indicate that oxalic acid is a stronger acid than citric acid (pH: 2.7-4.5). With respect to the chelating capacity of both acids, it can be concluded that citric acid chelates copper more effectively than oxalic acid, as it is proved that citric acid is a stronger chelater than oxalic acid. Besides, stability constants for copper indicate the tendency of this metal to be chelated by citric acid. Thus, it could be postulated that copper citrate complex may generate in the solution as copper and citric acid interacts.

5.1.2 Zinc solubilization

Oxalic acid solubilized zinc 3 fold more than citric acid. This phenomenon could be explained since citric acid and zinc oxide are capable of producing Zn_3Cit_2 which precipitates. Besides that, as mentioned before oxalic acid is a stronger acid than citric acid. Burgstaller et al. (1992) have illustrated that in acid and zinc interaction H^+ ions governs the dissolution process and the maximum dissolved amount of zinc corresponds to the maximum amount of H^+ ions available. Hence, the pH of the acid is the factor by which the solubility of this metal oxide is distinguished. Consequently, since oxalic acid assays had lower pH compared to their citric acid co- assays, zinc solubility is higher.

5.1.3 Nickel solubilization

As was expected, oxalic acid appeared to be a very inefficient agent in nickel solubilization. This observation could be explained as oxalic acid precipitates the leached nickel as nickel oxalate which has a very low solubility (Linke, 1965). This was illustrated by Tzeferic (1994b) as well. In his research a 0.5 M oxalic acid (pH: 1.1-1.33) solubilized 2%-3% of the nickel. Thus, it should be considered that oxalic acid is preventing the nickel solubilization in the leaching process and is the least desirable organic acid in nickel recovery process.

Citric acid on the other hand solubilized up to 60% of nickel and it is regarded as an effective agent in nickel bioleaching.

5.1.4 Iron solubilization

Oxalic acid solubilized iron 5 times more than citric acid. As Tzeferic (1994b) suggested, oxalic acid could be very effective in the iron leaching process, due to both its complexing capacity and reducing capacity.

A reason for low recovery ability of citric acid could be the formation of iron (II) citrate and iron (III) citrate which dissolve very slowly. In addition to that iron (III) and iron (II) citrates are very stable compounds.

5.2 Organic acid production

As was mentioned before, during each experiment different types of organic acids and amino acids have been produced, however, according to Golab and Orlowska (1988) organic acids are more effective than amino acids. We dealt with some significant organic acids produced such as citric acid, oxalic acid, tartaric acid, malic acid and phytic acid. Some of these organic acids are chelators which can form complexes with metals. An organic acid is called a chelator if it has two or more electron donor groups. Hence, one or more rings are formed, then the organic acid can be termed a chelating agent and the resulting complexes termed metal chelates (Martell and Calvin, 1952). Such complexation is dependent on the relative concentrations of anions and metals in solution, the pH and the stability constant of the various complexes (Denevre *et al.*, 1996).

Organic acids present in several media were measured, in order to investigate the effectiveness of different organic acids in the leaching process.

As indicated in Figure 3 in medium no.1, 0.6% phytic acid was produced. This acid is defined as a chelator. Citric acid and oxalic acid were produced as well, however the concentration of oxalic acid was much lower. The amount of all acids reached their maximum in the last day of the experiment. That could be the reason for the higher metal solubilization at the last day of experiment.

In the second medium as it is shown in Figure 4, malic acid and phytic acid were produced as the major products besides citric and oxalic acids. The concentration of acids kept increasing in this medium as well, which supports the evidence of higher metal concentrations in the medium on the last day of experiment.

In the third medium, no phytic acid was found and oxalic acid was the essential organic acid generated (see Figure 5). This medium did not solubilize a considerable amount of metal, as will be discussed in the next section, which could be due to the generation of oxalic acid.

In the medium no. 5 (see Figure 6) and medium no. 6 (see Figure 7) a large quantity of phytic acid was produced, and the amount of acid was augmented slightly during experiment periods. High concentrations of phytic acid in the fifth and sixth media could be due to the existence of phosphorus in the potato peels and sawdust.

The high concentration of phytic acid observed in medium no. 10 (see Figure 8) and medium no. 12 (see Figure 9) could be as a result of phosphorus existing in potato peels and leaves. These media effectively dissolved considerable amounts of copper, as is shown in the next chapters.

5.3 Biological leaching

5.3.1 pH

In order to observe the variation of pH during the experiments and the effect of it on the metal solubilization process, the pH of some media were measured. As is illustrated in Figure 10 and Figure 11 and Figure 12, pH decreased several days after experiments started. In some media pH did not alter much, however in some it did. Since most of the media were composed of agricultural wastes, the pH was not adjusted at the beginning, in order to be closer to real conditions.

5.3.2 Copper solubilization

Copper concentrations in all assays and controls were measured. The first set, as mentioned before, was sucrose medium as a standard medium. The results showed good correlation with experiments presented in literature(Mulligan *et al.*, 1999).

In this experiment up to 60% of copper (see Figure 13) was solubilized. Media pH at the beginning of the experiment was about 6, which after 5 days of experiment reached to 2.9, that implies production of acid, and after day 5^{th} increased slightly, which can be the result of consuming H^+ by metals. The high copper recovery may be an outcome of the acidic affect of the environment and the chelating capacity of organic acids such as citric acid. Comparing copper solubilized in this medium and in the citric acid shows that combinations of citric acid with other organic acids are more effective than citric acid alone (see Figure 3).

Molasses, which consists of 30 to 40% sucrose, showed good potential for solubilization of copper, it is noteworthy that molasses is a cheap byproduct of sugar. Although by day 14, only 48% of copper was solubilized in the molasses medium, the graph has an increasing slope. As is shown in Figure 14 the positive slope of the curve indicates that *A. niger* is adapting with the environment, therefore, this medium has the potential to solubilize a higher percentage of copper in a longer period of time. In this medium the pH did not fall as much as it did in the sucrose medium and the final pH was about 4.5 (see Figure 10). High amounts of organic acids generated, especially tartaric acid and citric acid, as shown in Figure 4. These acids are chelators which are capable of chelating copper. Even though the organic acids concentration produced in medium no.2 was higher, a higher copper concentration in medium no.1 was achieved. This could be due to the production of other metabolites which would lower the pH. The only obvious difference was in phytic acid concentration in both media.

Sawdust and leaves which were chosen as agricultural wastes did not work well. The maximum copper solubilized in sawdust medium was about 8% (see Figure 15) and in leaves medium was about 3.5% (Figure 16). It is noteworthy that leaves medium initial pH was very low (2.89)(Figure 11) whereas, sawdust initial pH was about 5.7 (Figure 10). The culture did not grow well in either medium which indicates the poor potential of these media as being substrates, it can be explained, as they are made of lignin and cellulose and *Aspergillus niger* cannot consume these materials.

In medium no.6 sucrose was added to sawdust. Even though the sucrose concentration in this medium was one third of the sucrose concentration in medium no.1, copper recovery in both media was almost the same (Figure 17).

However, as presented in Figure 7, the generated organic acid types and concentrations were different. Phytic acid was the main generated acid in this medium. Probably phytic acid has worked as a chelator in copper dissolution. The medium efficiency started to decline after 15 days; indicating The deficiency of the substrate for the culture.

Potato peels, as another type of agricultural waste showed a better potential than sawdust and leaves. These media solubilized 35% of copper(Figure 18). Assay pH fell from 5 to 2 after 7 days but within the next 16 days it reached 3.8. Some citric acid, oxalic acid and phytic acid were generated during the experiment. Supplementary amounts of sucrose did not alter the results much, where the maximum leached copper in that stage reached to 32% (Figure 19). To observe the potential of potato peels with less pre-treatment, potato peels were not sterilized in the next step. The auxiliary amount of sucrose added, however, very little amount of copper solubilized. The maximum leached copper was 14% (Figure 20) in this stage and in control medium 8% copper was found in soluble form. This peak started to fall off after 17 days.

Cellulose and lignin chains available in these agricultural wastes are very hard to break down. The complexity of lignin chemical bonds has been proven to be difficult to biodegrade by microorganisms. Therefore, the elevation of the hydrolysis process which essentially is cleavage by means of water may help in breaking down cellulose chains and lignin. In this line, diluted sulphuric acid was utilized. Sulphuric acid can promote hydrolysis processes and so it will make these substrates an easier substrate to consume for *A. niger*. In addition to that, it can make produced organic acids more effective. The results endorsed this hypothesis.

Leaves and sawdust were soaked in sulphuric acid for 24 hours and then autoclaved. Although the leaves medium was not effective solely and the maximum solubilized copper was less than 4%, after immersing leaves in sulphuric acid the potential of the medium was augmented 8 fold (see Figure 21). It is noteworthy that the copper solubilized in control medium was about 14% which is very high and it could be due to 1- copper concentration present in mine residue; 2- solubilization of copper present in leaves. The former assumption is more likely, since the copper concentration in leaves is very low (about 10-15 ppm) compared to the copper concentration in residue. On the other hand suphuric acid did not alter the leached copper concentration in sawdust and sulphuric acid medium. The copper concentration reached to 8% at maximum (see Figure 22), which showed the poor ability of pre-treatments on sawdust in modifying it as a consumable substrate for *A. niger*. Comparing leaves medium to sawdust medium results illustrates that leaves have a better potential and pre-treatment can be effective for this medium. In addition to that, the slope trend of leaves medium shows that it kept its capability to leach metal after 17 days and in a longer period of time its performance could be better, whereas in sawdust medium the copper concentration seemed not to increase. The higher copper concentration in leaves medium could be due to the lower pH as well. Furthermore, copper concentration in the control medium of sawdust did not exceed 5%, whereas solubilized copper in leaves medium was 15%. This 10% difference could be the result of different factors. Since the control media were almost the same, the lower pH of medium no. 10 could be on account of the leaves. The lower initial pH of leaves medium (medium no. 4) in comparison to sawdust (medium no. 5) justified this evidence. It may be concluded that leaves are making the environment more efficient for this kind of leaching.

Another medium tested was potato peels that were soaked in sulphuric acid. In this medium about 68% of copper was recovered (see Figure 23). This medium's effectiveness diminished after 14 days, where the solubilized copper started to decline.

This evidence can be explained regarding a deficiency of available substrate for *A. niger*. Probably at the time that usable substrate started to terminate, the culture began to consume more available organic carbon sources, which in this case are organic acids. The sudden fall in peak of copper solubilization graph could be due to this matter, which was confirmed regarding the descending concentration of organic acids, specifically after day 15 (see Figure 9). In this case, it is suggested to terminate the experiment before approaching the inefficient point. It is also possible to supply continuous amounts of substrate in order to avoid the undesirable effect caused by the substrate depletion.

The other medium tested was potato starch, which was waste from a potato chips company. To investigate the potential of this waste as being a consumable substrate for *A. niger*, a sterilized assay was conducted, in addition to a control. The initial pH of this liquid was 3.5 and it increased during the experiments (see Figure 12). The *A. niger* did not grow well in this medium. The copper solubilized in sterilized and control assays did not vary much. The maximum solubilized copper in the sterilized sample was less than 25% which was almost the same as copper solubilized in the control (Figure 24). Consequently, it is postulated that this starch with no pre-treatment is not a good medium for *A. niger*. It is noteworthy as well that the low initial pH of this medium could be the main reason for 14% copper concentration in the control.

The last assay was ground corn cobs immersed in dilute sulphuric acid. This assay was done only in one repeat to find out if it could be a proper substrate or not. Even though it shows a very good potential it lost its effectiveness after 15 days (see Figure 25). This preliminary experiment confirmed that corn waste with little pretreatment could be a potential substrate for *A. niger.*

5.3.3 Zinc solubilization

Research on the effect of different organic acids on zinc oxide leaching by Golab et al.(1988) shows that among citric, gluconic, oxalic and tartaric acids, tartaric acid is the most effective in zinc solubilization. Tartaric acid is among the chelating agents produced by *A. niger.* However, zinc solubilization was not considerable in all assays. One reason for this could be that tartaric acid effectiveness has been overlaid by other factors.

In many of the assays conducted in this research, no significant amounts of zinc were dissolved. Therefore only the significant ones are discussed here. In medium no.1 (sucrose medium) 28% (see Figure 26)of zinc was solubilized and in medium no.2 20% (see Figure 27)of zinc was solubilized, which compared to previous literature (Műller *et al.*,1995) is considered a good result. This is because, in the zinc leaching process the maximum will be achieved later than the copper leaching maximum.

By comparing the pH of these media, it can be seen that pH is an important factor. For instance, since the pH in medium no. 1 is much lower than the pH in medium no. 2 (see Figure no. 10)the dissolved zinc in medium no.2 was 70% of what dissolved in medium no.1. However, the slopes of both figures have an increasing trend which shows that in a longer period of time, better results may be achieved.

Zinc in medium no.6, which was composed of sawdust and sucrose, reached its maximum after 23 days and the curve has a very slight slope on this day(see Figure 28). The constitution of organic acid existing in this medium shows that only oxalic acid and phytic acid were generated, which caused a low pH. Even though this medium pH is lower than medium no.1 and medium no.2, the copper dissolved was lower. Hence, one can conclude that the lower zinc recovered in this medium could be due to the lower efficacy of metabolites, especially organic acids generated during this experiment. However, a point that should be kept in mind is that ore structure is heterogeneous and this may cause up to 15% difference in similar samples.

As was expected, sulphuric acid had a high ability to solubilize zinc. For instance, as is illustrated in Figure 29, medium no.9 with diluted sulphuric acid up to 10% zinc was solubilized in control medium and in medium no.12, 24% of zinc was solubilized (see Figure 30) in the control sample. In medium no.12, 45% of zinc was solubilized, which is a good result considering the little pre-treatment performed on the potato peels.

5.3.4 Nickel solubilization

As mentioned previously, oxalic acid is the least desired acid in nickel extraction, as nickel oxalate has a very low solubility and makes the leached nickel precipitate. Hence, oxalic acid generation is not an appreciated incident. Whereas, according to Tzeferis (1994a) oxalic acid combined with other organic acids gives a better potential. Sulphuric acid can promote its potential in nickel solubilization as well. Regarding these facts, the media that contained sulphuric acid might have a better potential.

Leached nickel in the sucrose medium was about 20% in a 13 day period (Figure 31). With respect to the graph slope, one could suppose that the solubilized nickel would have been enhanced if the time period had been extended. This assumption has been supported by the Tzeferis experiments (1994b). In his experiments, maximal nickel recovery was achieved between 25 to 30 days; moreover, his experiments showed that in the first 15 days of experiments only 15% of nickel had been recovered, but after 40 days it reached 60%. Thus, it is concluded that in a longer period of time nickel recovery will be enhanced.

Nickel solubilized in molasses medium was about 15.5% after 13 days (see Figure 32). Nevertheless, it is considered a good result with respect to the long period of time required to recover nickel. Leaves, sawdust and potato peel media did not leach a considerable amount of nickel, since oxalic acid concentration was substantial in these media.

As was expected, sulphuric acid augments the capability of medium no.9 in the recovery of nickel. It is noteworthy that sulphuric acid is an effective acid in nickel leaching. This hypothesis was proven in our experiments, when the amounts of leached nickel in the control medium was about 20%. Nickel recovery up to 34% (see Figure 33) in this medium could be explained as sulphuric acid's positive affect on organic acid which has made the medium 60% more effective compared to the control medium in the absence of organic acids.

5.3.5 Iron solubilization

The main purpose of this recovery process is to recover copper, and secondly zinc and nickel. The iron existing in this ore is unwanted, for it makes recovery of above-mentioned metals more difficult. Iron dissolution is undesirable, therefore, it is preferred to solubilize less iron. As mentioned previously, oxalic acid is an undesirable acid in nickel solubilization, and regarding the results achieved in our chemical experiments, it solubilized considerable amounts of iron. Consequently, it is an unwelcome acid in this recovery experiment.

Overall, in most of the media, a low concentration of iron was leached and the iron concentration in all of these media was less than 10%.

Among all media, the ones with higher iron concentration will be discussed. After 13 days of experiment, iron concentration in medium no.1 reached to 7% (see Figure 34), but it seems possible that in a longer period of time, more iron would solubilize, as its slope has an increasing tendency. In medium no.2 the amount of leached iron reached 7% (see Figure 35); the slope has an increasing tendency so it suggests that iron concentration would increase in a longer period of time. The concentration of iron in the rest of the media was less, especially in the media with sulphuric acid a little amount of iron was solubilized. However, iron concentration in autoclaved media, regardless of the media composition, was higher;therefore the autoclave process seems to promote iron extraction significantly. Regarding this point, one may infer that the elimination of the autoclave process may help to make the recovery process more efficient. In addition to that, diluted sulphuric acid used to elevate recovery process effectiveness did not have a significant influence on iron solubilization.

5.4 Mass balances

Finally analysis of the leftover solids was done in order to close the mass balance. Six of the samples chosen for detailed mass balance. Table 4 illustrates the results. In this table it is illustrated that 10 to 20% of copper was missing. Furthermore, in most cases more than 50% of the copper was removed from the solid residues, which indicates the capability of this method in soil remediation as well.

Some of the missing copper could be adsorbed in the biomass, since the biomass has not been digested besides that residue heterogeneity could be another reason.

Table 4: Mass balance results

	Cu leached (%)	Cu left in residue (%)	Cu missing (%)
medium no.1	59	30	11
medium no.2	48	37	15
medium no.5	34	50	16
medium no.6	54	36	10
medium no.10	25	60	15
medium no.11	30	50	20

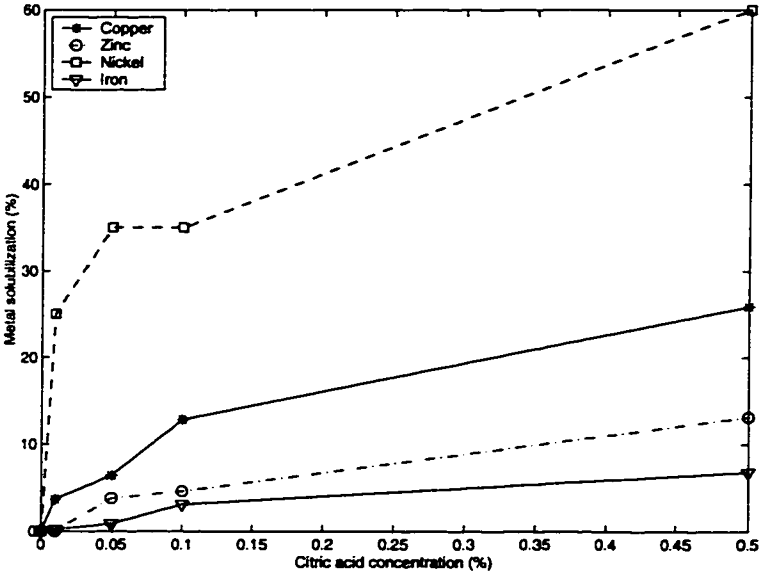

Figure 1: Metal solubilization by citric acid.

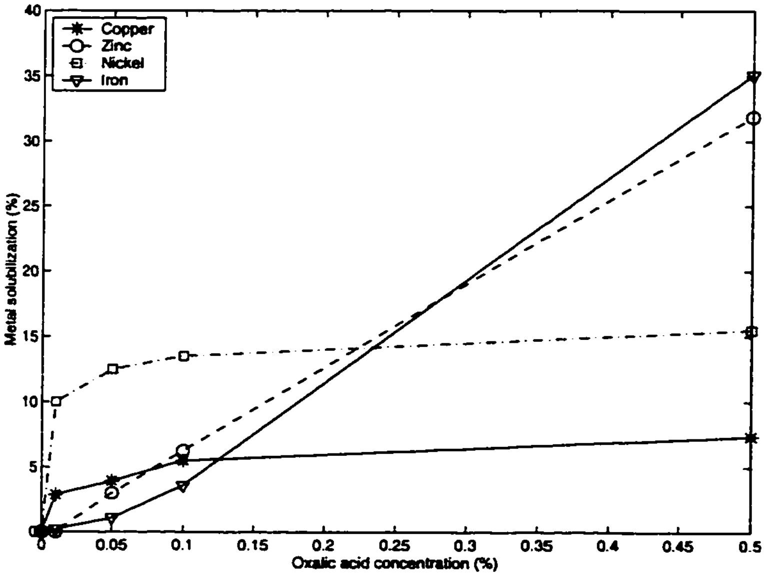

Figure 2: Metal solubilization by oxalic acid.

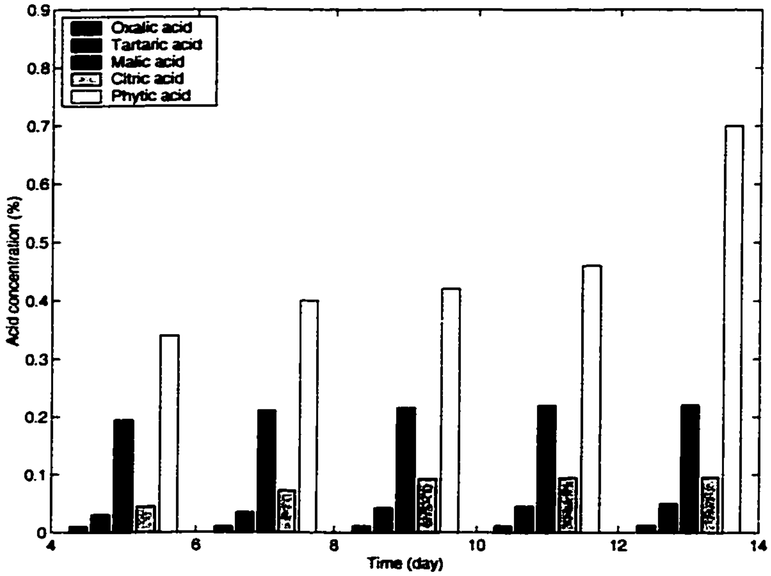

Figure 3: Organic acid concentration in medium no.1 (organic acid concentration at the beginning of experiment was zero.)

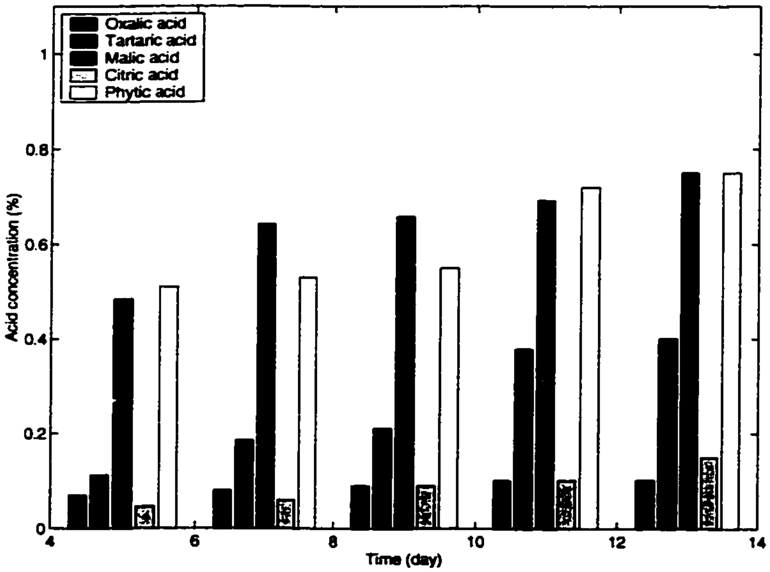

Figure 4: Organic acid concentration in medium no.2 (organic acid concentration at the beginning of experiment was zero.)

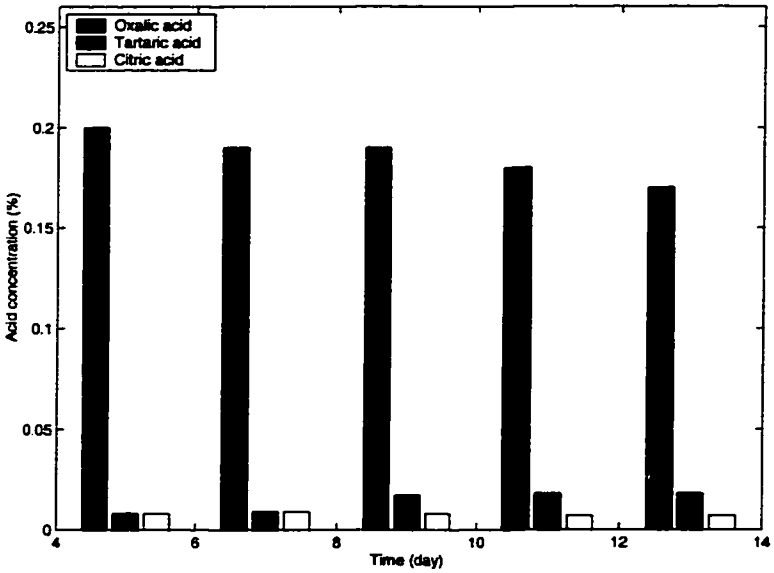

Figure 5: Organic acid concentration in medium no.3 (organic acid concentration at the beginning of experiment was zero.)

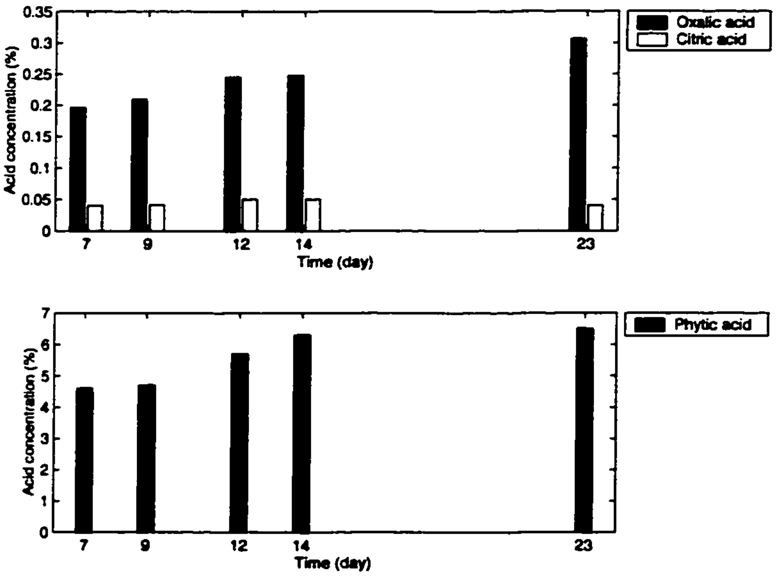

Figure 6: Organic acid concentration in medium no.5 (organic acid concentration at the beginning of experiment was zero.)

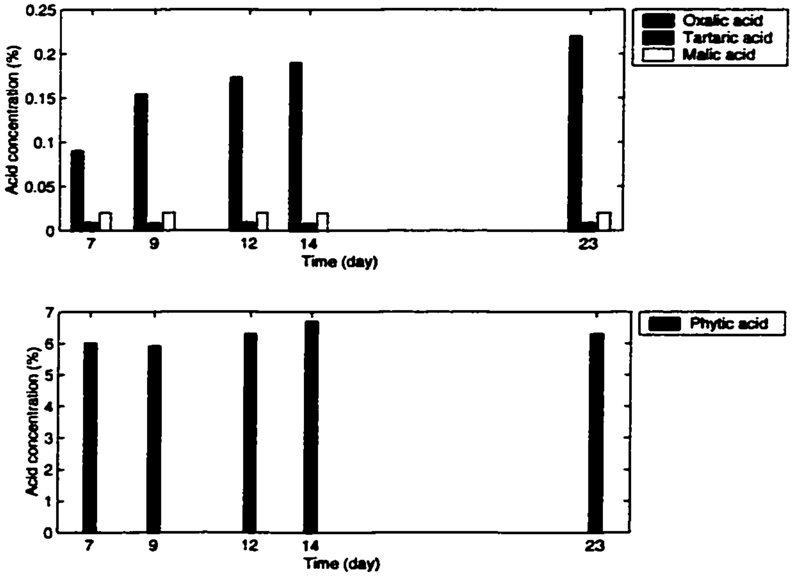

Figure 7: Organic acid concentration in medium no.6 (organic acid concentration at the beginning of experiment was zero.)

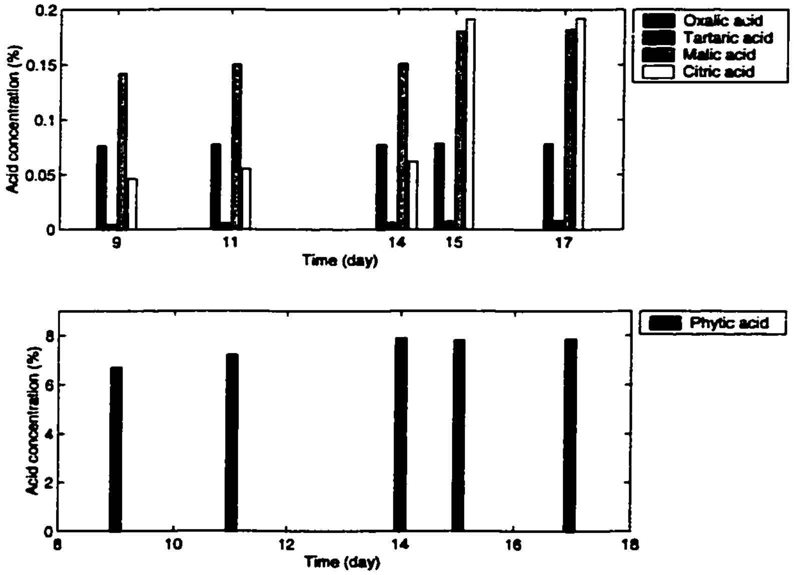

Figure 8: Organic acid concentration in medium no.10 (organic acid concentration at the beginning of experiment was zero.)

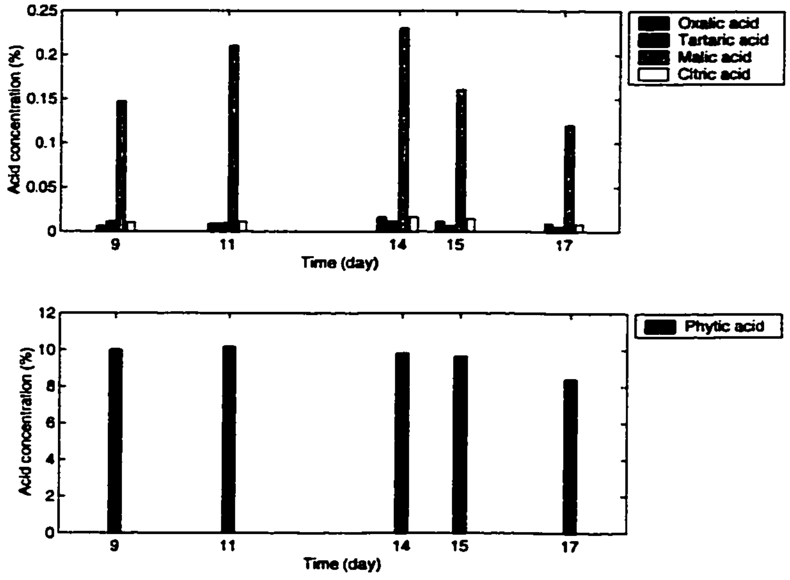

Figure 9: Organic acid concentration in medium no.12 (organic acid concentration at the beginning of experiment was zero.)

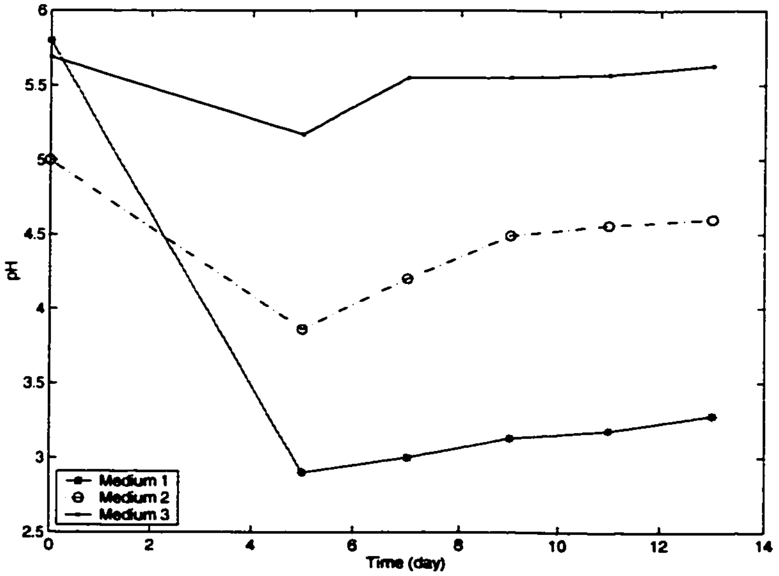

Figure 10: Variation of pH in medium no.1, medium no. 2 and medium no. 3

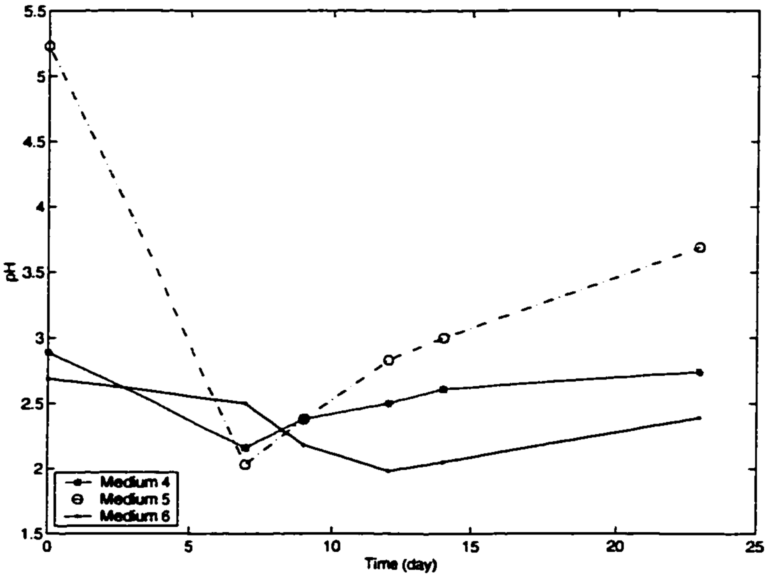

Figure 11: Variation of pH in medium no.4, medium no. 5 and medium no. 6

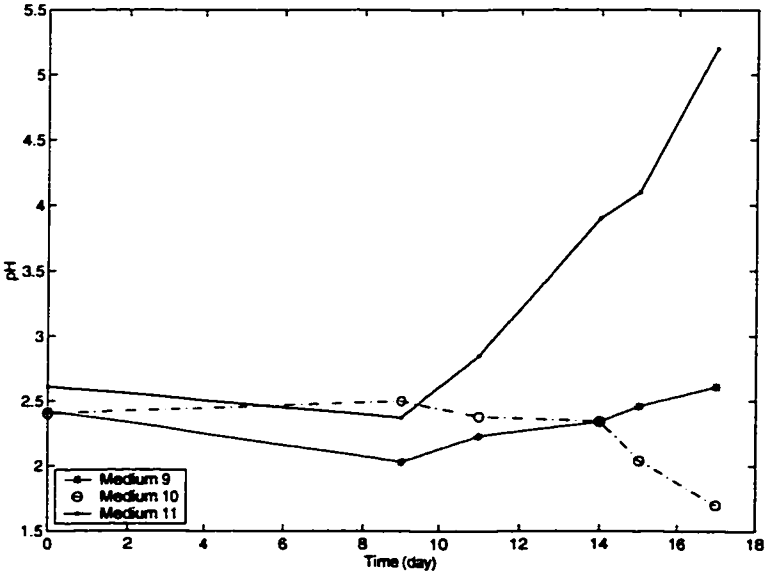

Figure 12: Variation of pH in medium no. 9, medium no. 10 and medium no. 11

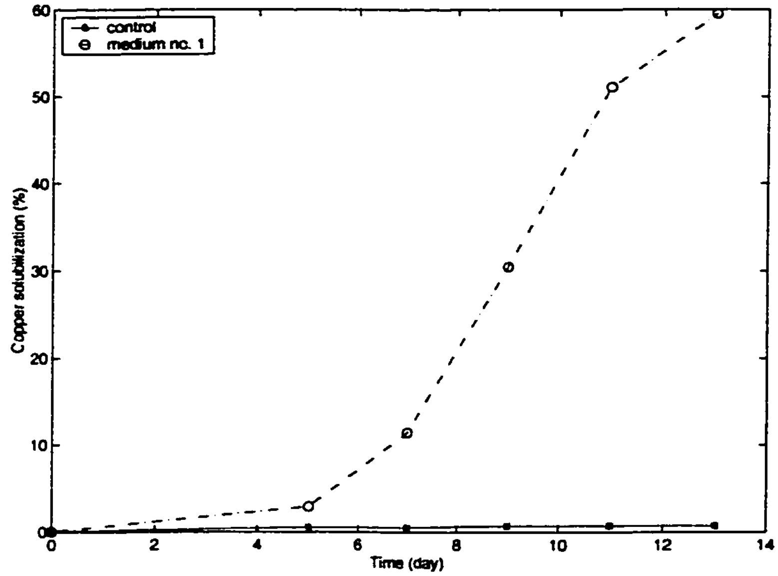

Figure 13: Copper solubilization in medium no.1

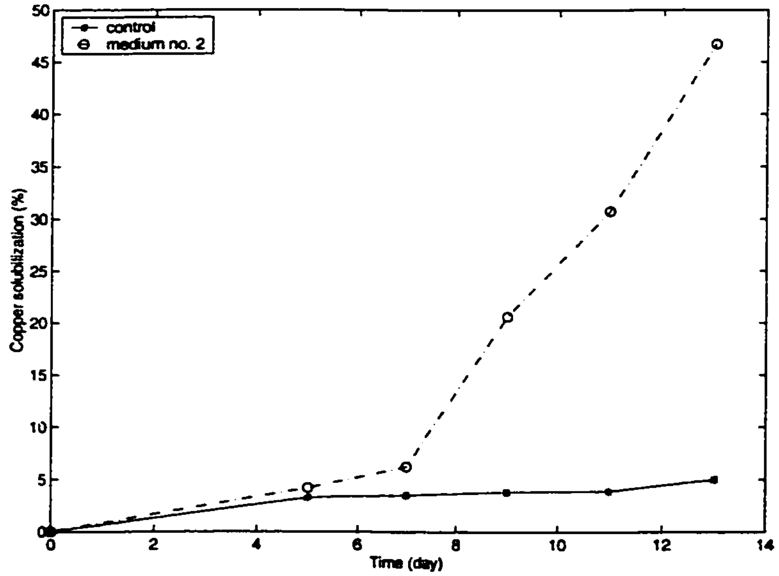

Figure 14: Copper solubilization in medium no.2

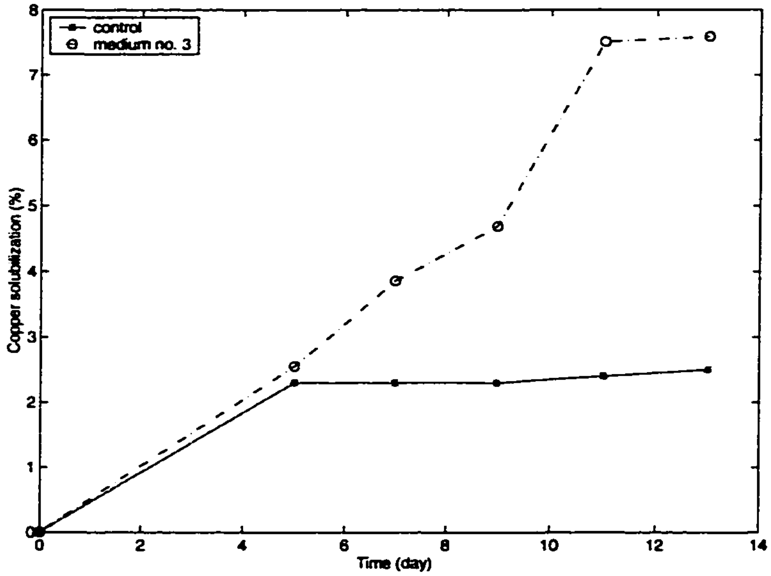

Figure 15: Copper solubilization in medium no.3

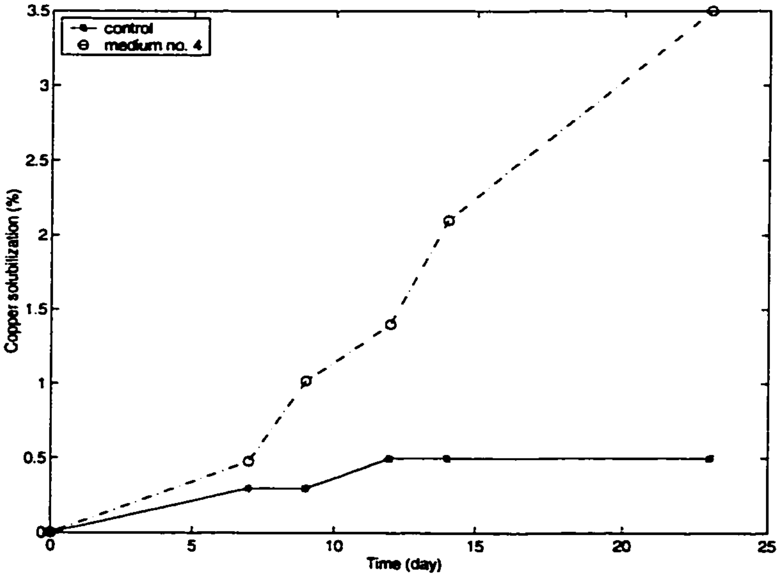

Figure 16: Copper solubilization in medium no.4

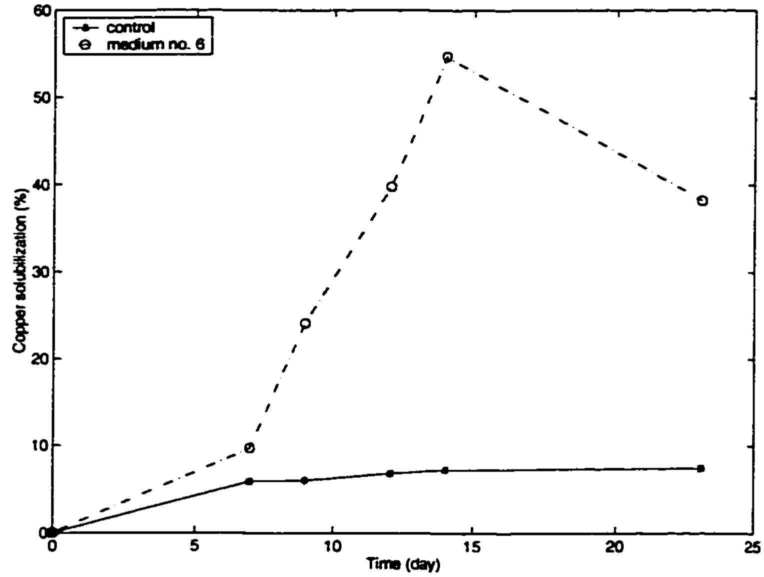

Figure 17: Copper solubilization in medium no.6

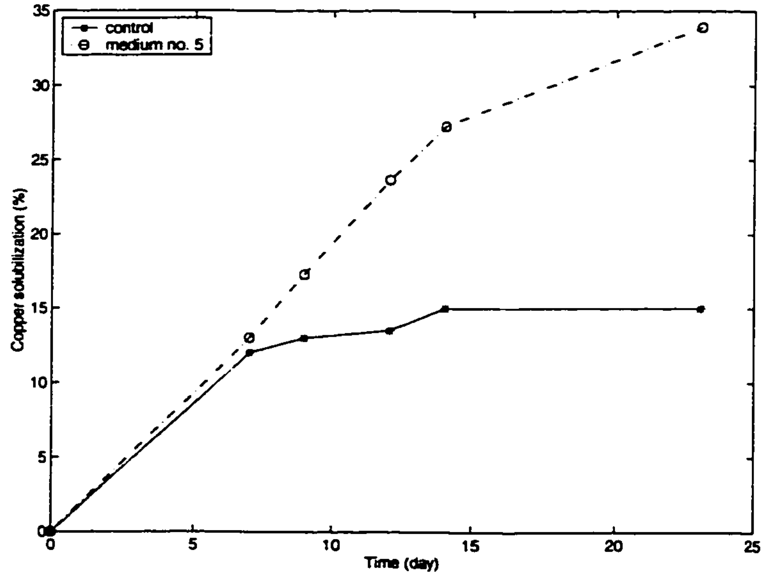

Figure 18: Copper solubilization in medium no.5

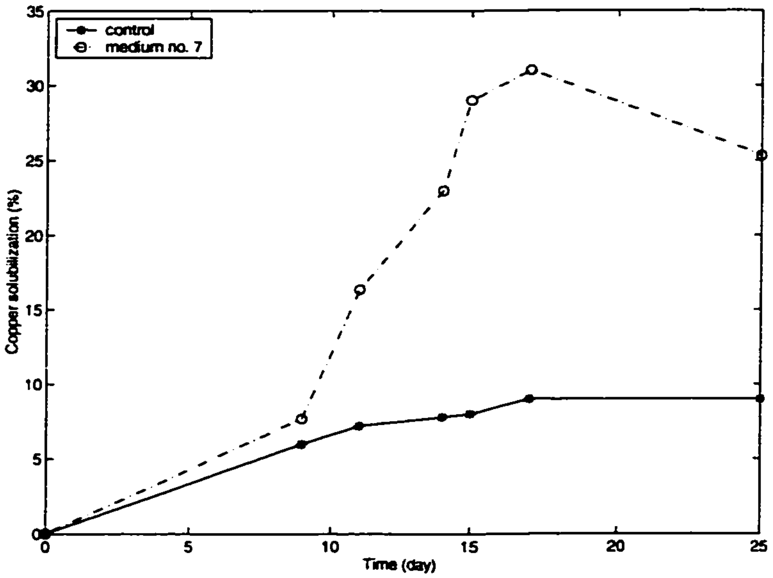

Figure 19: Copper solubilization in medium no.7

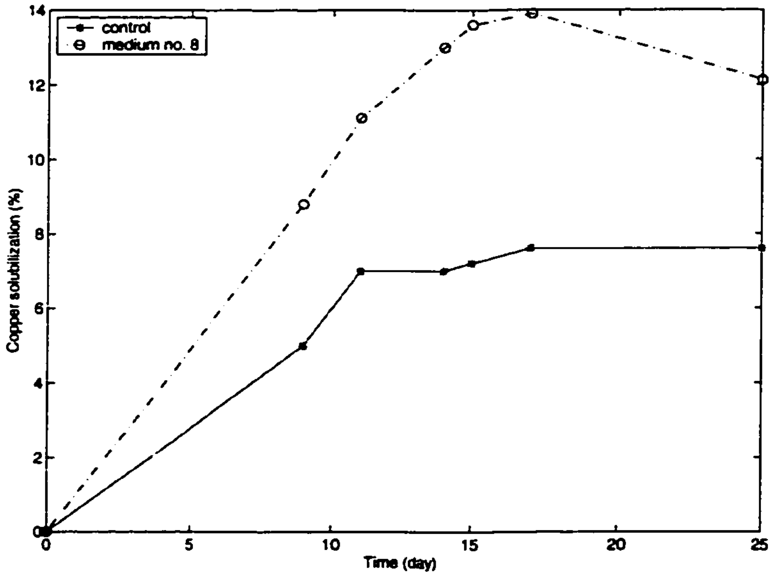

Figure 20: Copper solubilization in medium no.8

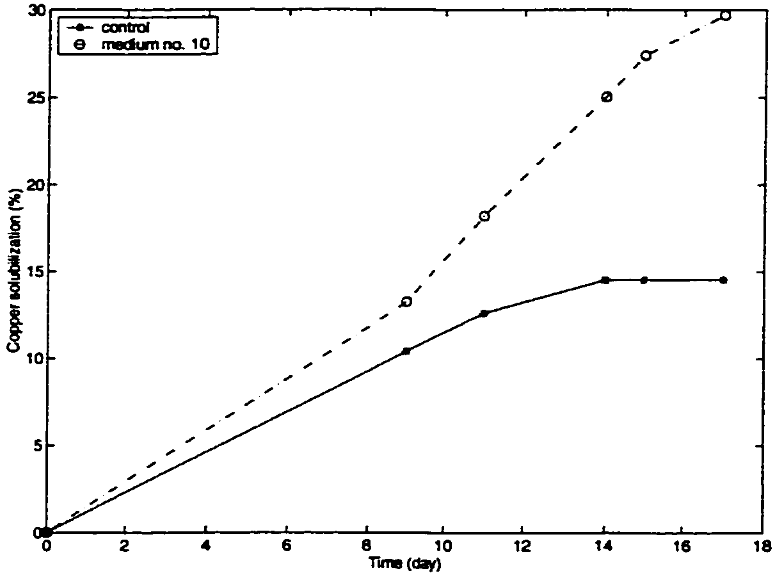

Figure 21: Copper solubilization in medium no.10

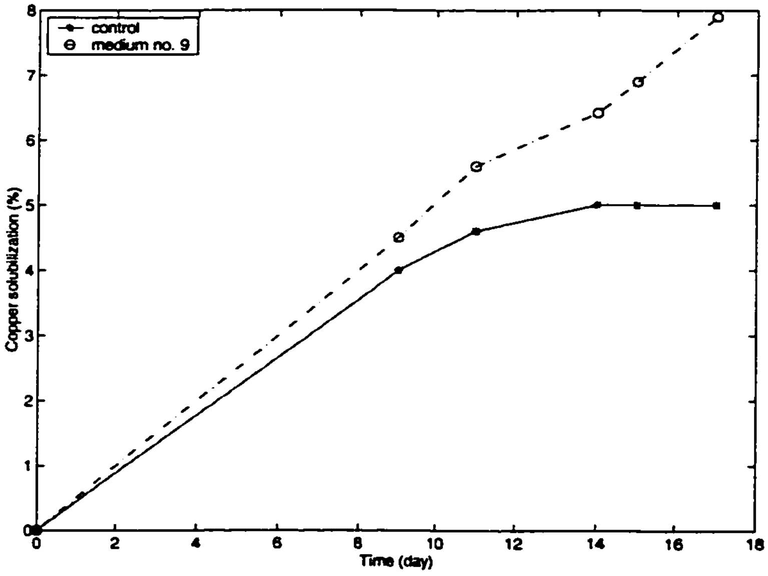

Figure 22: Copper solubilization in medium no.9

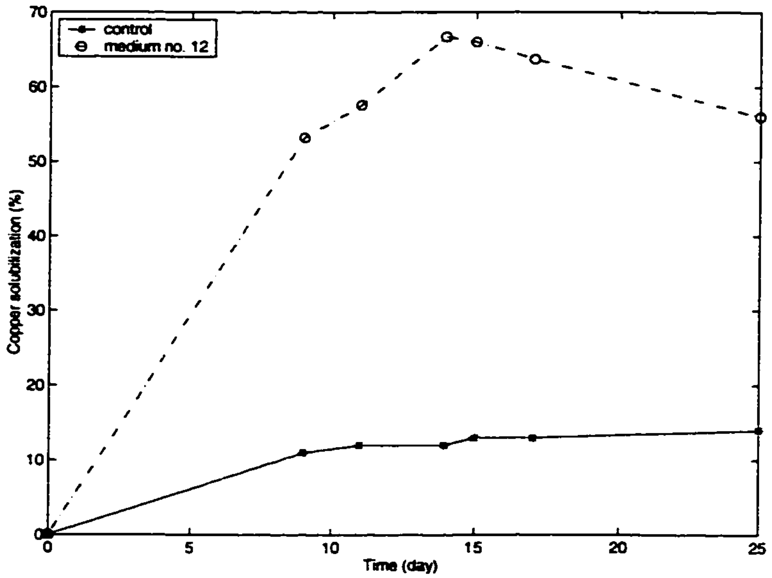

Figure 23: Copper solubilization in medium no.12

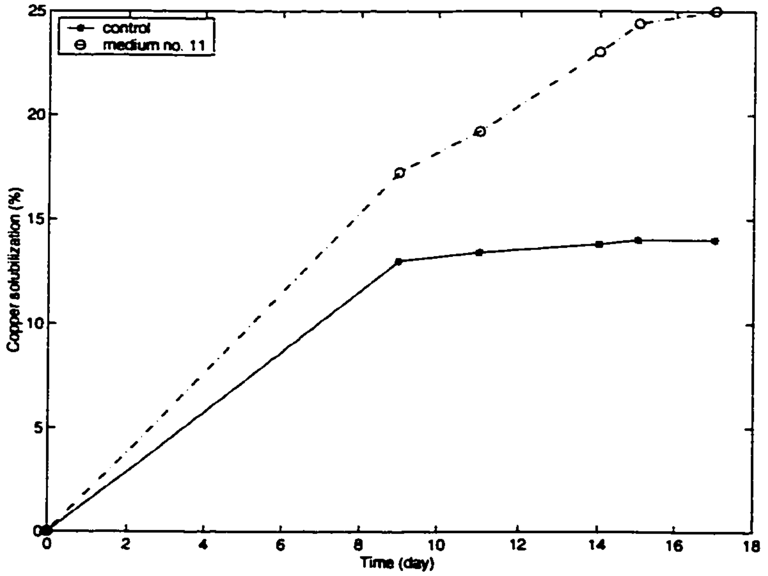

Figure 24: Copper solubilization in medium no.11

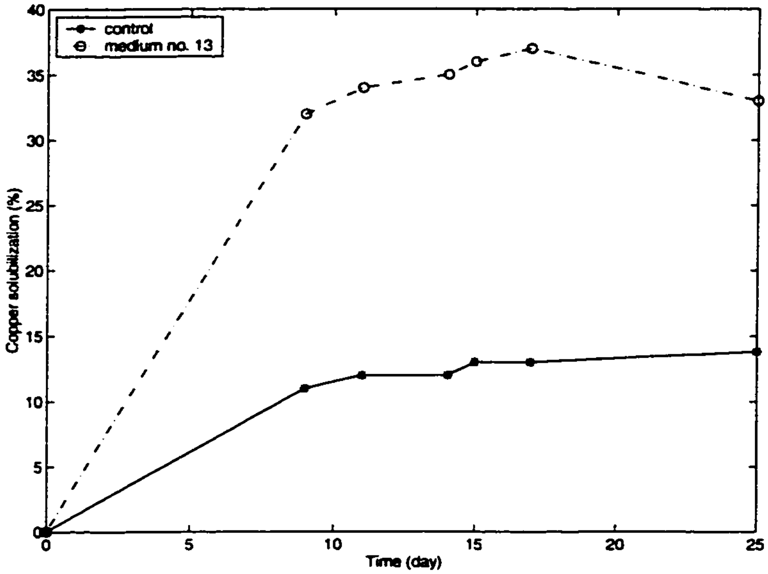

Figure 25: Copper solubilization in medium no.13

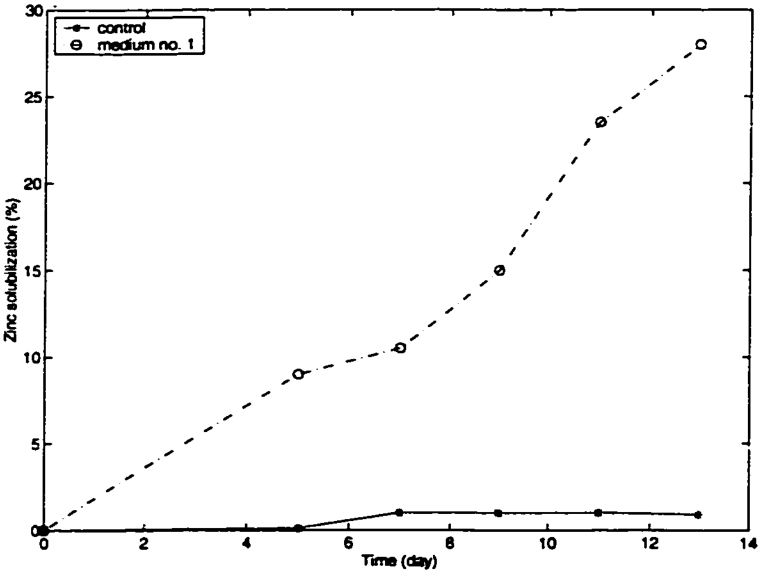

Figure 26: Zinc solubilization in medium no.1

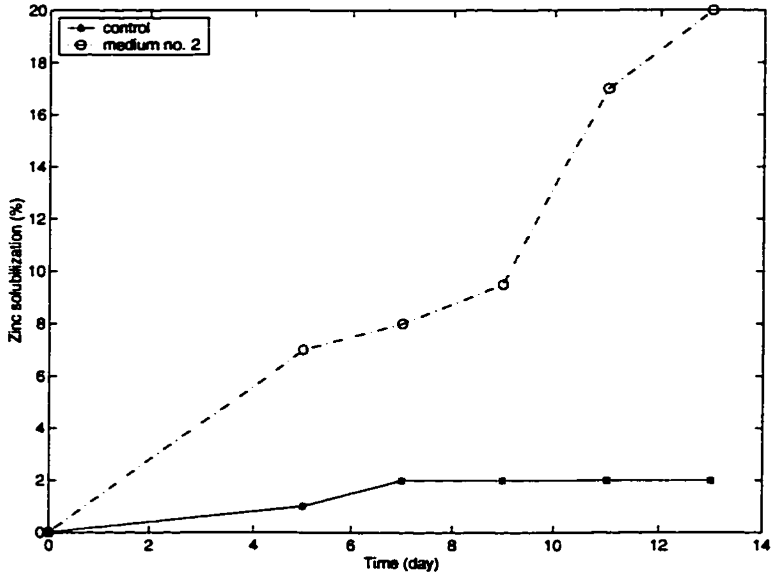

Figure 27: Zinc solubilization in medium no.2

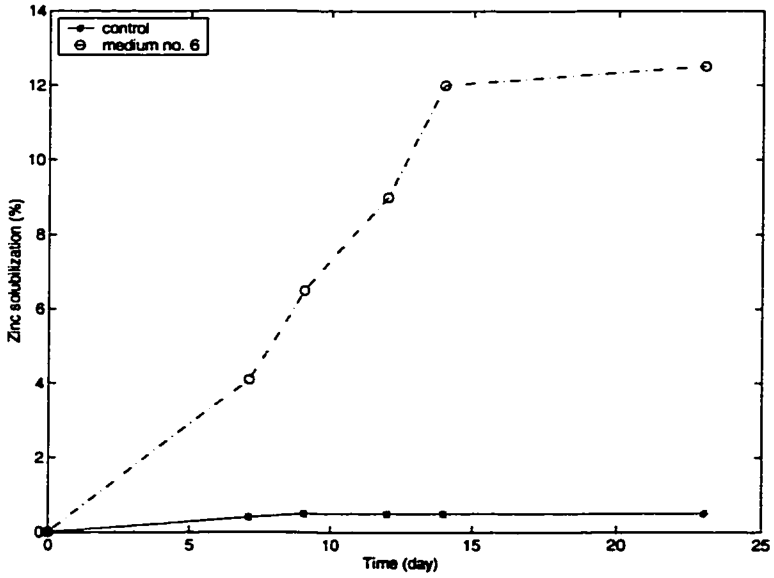

Figure 28: Zinc solubilization in medium no.6

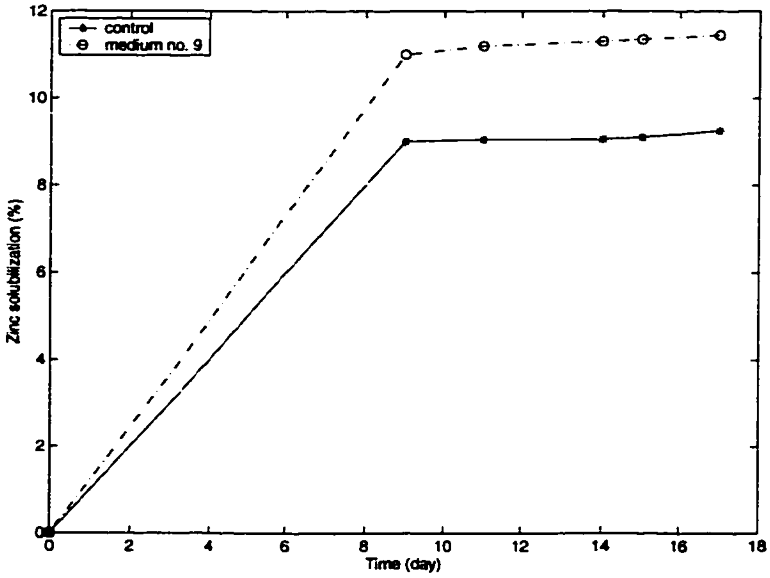

Figure 29: Zinc solubilization in medium no.9

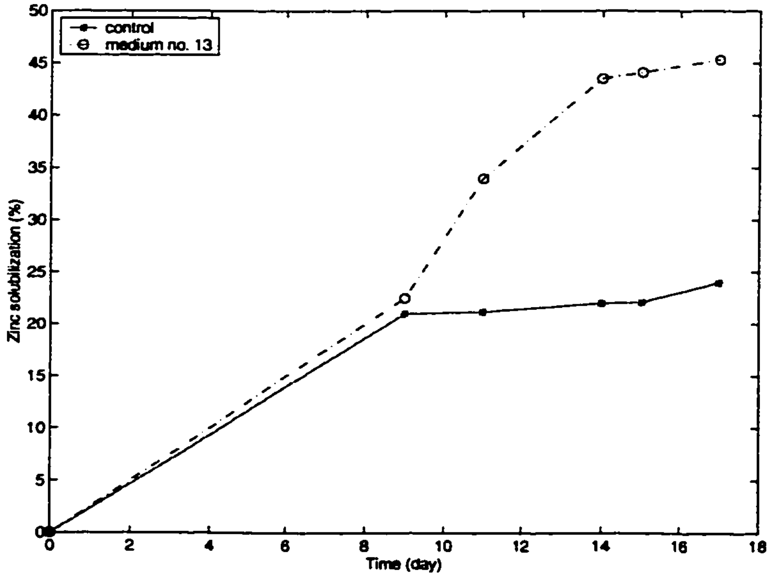

Figure 30: Zinc solubilization in medium no.12

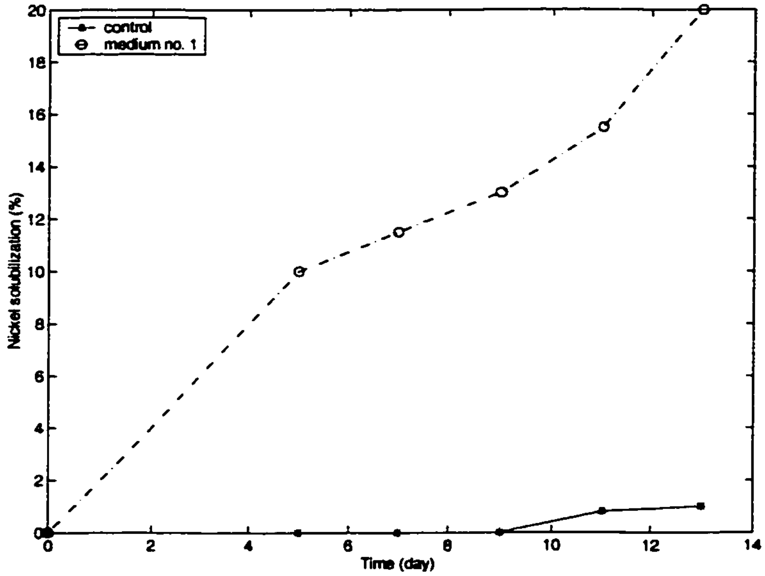

Figure 31: Nickel solubilization in medium no.1

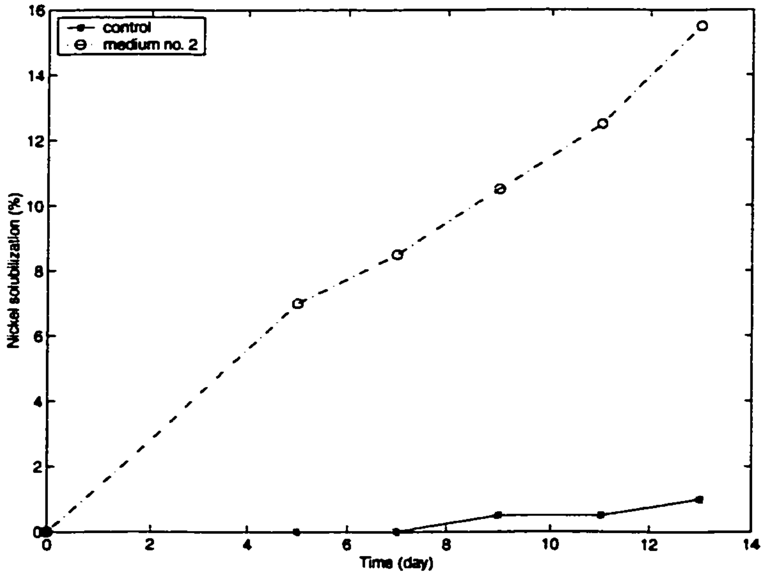

Figure 32: Nickel solubilization in medium no.2

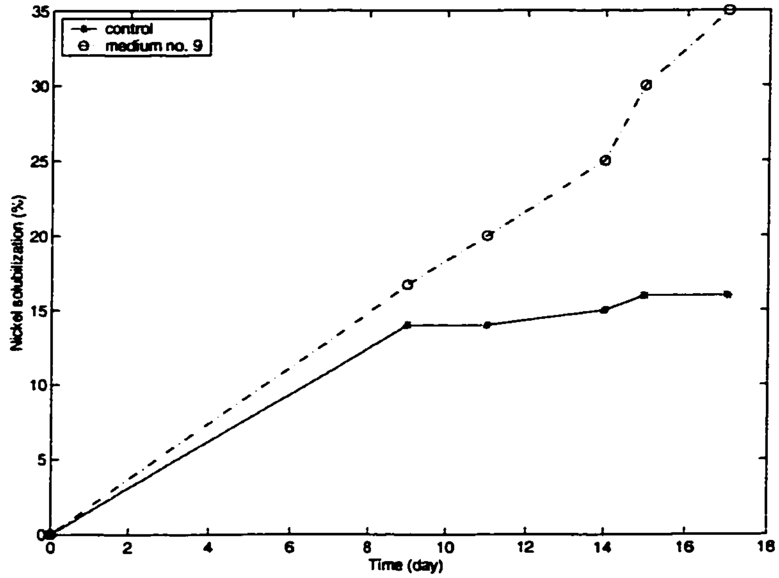

Figure 33: Nickel solubilization in medium no.9

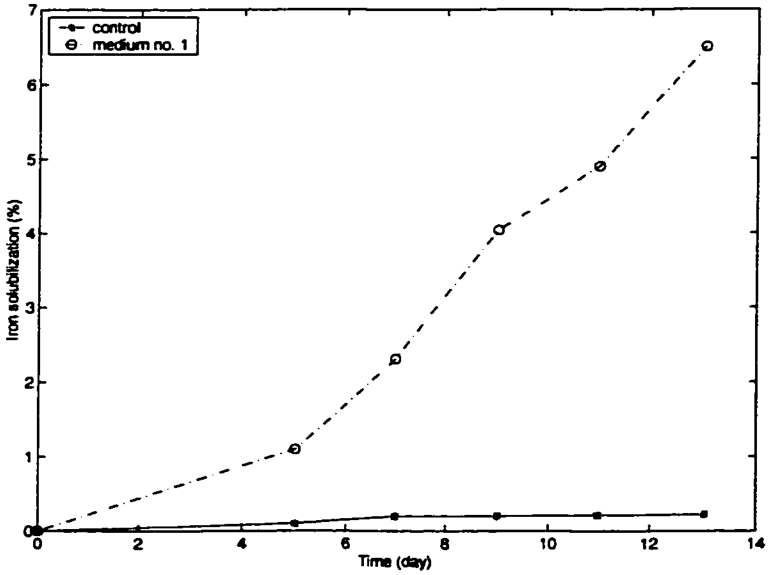

Figure 34: Iron solubilization in medium no.1

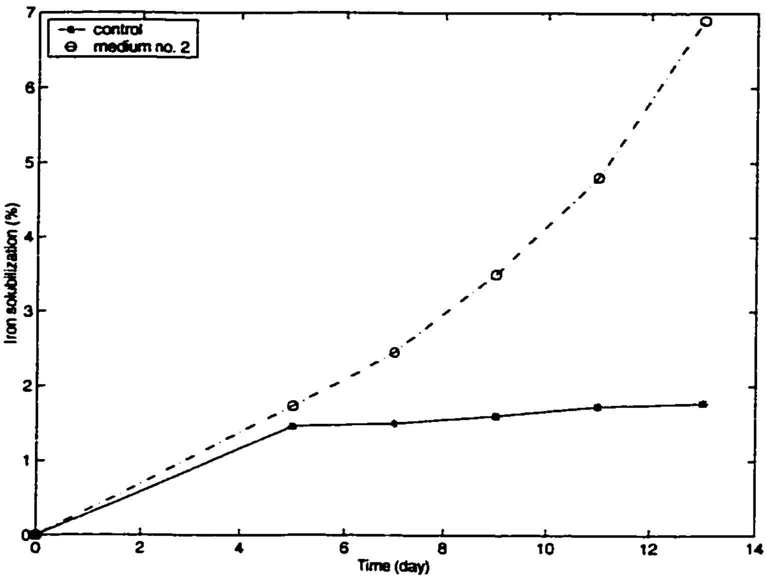

Figure 35: Iron solubilization in medium no.2

Chapter 6

Conclusions

The results of this study show that recovery of metal constituents of mine residue is feasible. In this study *A. niger* exhibits a good potential for generating varieties of organic acids effective in metal solubilization. Organic acid effectiveness was enhanced when sulphuric acid was added to the medium. Different food and agricultural wastes were evaluated with the results of a maximum of 68% for copper dissolution, 46% for zinc, 34% for nickel and just up to 7% of iron codissolution. In this work the sterilizing process, which is one obstacle in making the process cost effective, was overcome; however, it makes the process slower. Among these wastes, potato peels showed good potential and acceptable results were achieved. In summary some of the results achieved are as follows:

Table 5: Summary of copper removal results

	max. Cu solubilization (%)	max. rate of Cu solubilization (mg/day)	time interval (day)	type of pre-treatment
medium no.1 (sucrose)	59	7.24	7-11	Autoclaved
medium no.2 (molasses)	48	5.79	7-9	Autoclaved
medium no.3 (sawdust)	7.5	1.08	7-9	Autoclaved
medium no.4 (leaves)	3.5	0.18	7-9	Autoclaved
medium no.5 (potato peel)	34	1.45	0-12	Autoclaved
medium no.6 (sawdust and sucrose)	55	5.43	7-9	Autoclaved
medium no.7 (potato peel and sucrose)	32	4.13	14-15	Autoclaved; no yeast extract
medium no.8 (potato peel and sucrose)	14	0.73	0-11	Not autoclaved; no yeast extract
medium no.9 (sawdust)	8	0.365	0-9	Sulphuric acid; no yeast extract; not autoclaved
medium no.10 (leaves)	29.5	1.70	9-14	Sulphuric acid; no yeast extract; not autoclaved
medium no.11 (starch)	25	1.12	0-9	Sulphuric acid; no yeast extract; not autoclaved
medium no.12 (potato peel)	68	4.06	0-9	Sulphuric acid; no yeast extract; not autoclaved

As indicated in this table, in medium no. 1 which is sucrose medium, the rate of copper removal was maximum, whereas the overall maximum copper removal occurred in medium no.12 (Potato peel). Rate of removal was high in the sixth medium (sawdust and sucrose) as well, which shows the role of sucrose in accelerating the copper removal process. Maximum rate of copper removal was low in the fifth medium (Potato peel), however, the overall copper removal was 50% which seems promising. In most of the media, the rate of removal had its maximum between day 9 and 11.

6.1 Future recommendations

Yet, a number of technical and economical problems have remained, concerning the practicalities of applying heterotrophic leaching techniques applicable on an industrial scale. Reaction kinetics were too slow to be applicable to stirred tank technology. For heap leaching and dump leaching, in which the ore is already seeded with the culture and nutrient feed is applied by spraying onto the ore, the problem of contamination by other microorganisms exists.

Other problems that probably will be confronted are firstly plugging problems, as microorganisms congregate together and prevent solution flow; and secondly, environmental polluting problems because of severe biomass presence. However, these problems may be overcome by a two-step procedure in which the production of organic acids and metal leaching are performed as separate processes. Even though this type of bioleaching does not suffer from the technical problems mentioned previously, it is very much slower than the one-step bioleaching method. So the profitable application is a matter of cost-analysis. Finally, it is hoped that this study will stimulate further investigation in this field.

Bibliography

Abdelouas A, Lutze W, Gong W, Nuttall EH, Strietelmeier BA and Travis BJ (2000) “Bilogical reduction of uranium in groundwater and subsyrface soil”. *Science of the Total Environment 250 no. 1-3* 21-35.

Agate AD (1996) “Recent advances in microbial mining ”. *World J Microbiol Biotechnol. 12* 487-495.

Alcock CB (1976), *Principle of Pyrometallurgy* ,Academic Press.

Andrews GF, Stevens CJ, Glenn A, Noah KS (1993) “Microbial coal depyritization :why and how ? ” *Torma AE, Wey JE, Lakshmanan VI (eds) Biohydrometallurgical technologies, 1* . The Minerals, Metals & Materials Society, Warrendale, Pa,381-391.

Arsdale GD (1953), *Hydrometallurgy of Base metals,*McGraw-Hill Press.

Bartlett RW (1993)“ Soil decontamination by percolation leaching” *Hager J, Hansen B,Imrie W, Pusatori J, Ramachandran V (eds). Extraction and processing for the treatment and minimization of wastes* . The Minerals, Metals & Materials Society, Warrendale, Pa, 411-424.

Benmoussa H, Tyagi RD, Campbell PGC (1994) “Heavy metal bioleaching and stabilization of municipal sludges: effect of the form of elemental sulphur used as substrate”. *Rev Sci Eau 7* 235-250

Berthelin J (1993)" Microbial weathering processes ". *Krumbein, W.E.(Ed.), Microbial Geochemistry* . Blackwell, Oxford, 223-262.

Beveridge TJ, Murray RGE (1980)" Sites of Metal Deposition in the Cell Wall of textit Bacillus subtilis ". *J. Bacteriol 141* . 876.

Blais JF, Tyagi RD, Auclair JC (1993)" Bioleaching of metals from sewage sludge microorganisms and growth kinetics". *Water Res. 27.* 101-110.

Bosecker K (1986) "Bacterial metal recovery and detoxification of industrial waste ". *Ehrlich KL, Holmes DS (eds) Workshop on biotechnology for the mining, metal-refining and fossil fuel processing industries. Biotechnology and Bioengineering Symposium 16* .Wiley, New York, 105-120.

Bosecker K (1994) "Mikrobielle Laugung (Leaching)." *Prave P,Faust U, Sittig W, Sukatsch DA (eds) Handbuch der Biotechnologie. Oldenbourg, München* .835-858.

Bousshard PB, Bachofen R, Brandl H (1996) "Metal leaching of fly ash from municipal waste incineration by Aspergillus niger". *Environ. Sci. Technol. 30* , 3066-3070.

Breed AW, Hansford GS (1999) *Biotechnology and Bioengineering 64 no.6* , 671-677.

Brierley CL (1995) "Bacterial oxidation ".*Eng Mining Journal 196* 42-44.

Brombacher C.,Bachofen R., Brandl H. (1997) "Biohydrometallurgical processing of solids;

a patent review". *Appl Microbiol Biotechnol 48* 577-587

Burgstaller W, Strasser H, W"bking H and Schinner F (1992) "Solubilization of zinc oxide from filter dust with Penicillium simplicissimum : Bioreactor leaching and stochiometry ". *Environ. Sci. Technol. 26* 340- 346.

Cameselle C., Nonez MJ, Lema JM and Pais J (1995) "Leaching of iron from kaolins by a spent fermentation liquor: influence of temperature, pH, agitation and citric acid concentration". *Journal of Industrial Microbiology 14* , 288-292.

Clark DS, Lentz CP(1963) "Submerged citric acid fermentation of beet molasses in Tank-Type fermentation". *Biotechnol. Bioeng. 5* 193.

Colmer AR,Hinkle HE (1947)."The role of microorganisms in acid mine drainage :a preliminary report". *Science 106*253- 256

Colmer AR ,K.L. Temple and HE. Hinkle (1950). *J.Bacteriol. 59* 317.

Denevre, O., Garbaye, J., and Botton, B. (1996)" Release of complexing organic acids by rhizosphere fungi as a factor in Norway Spruce yellowing in acidic soils". *Mycol. Res. 100* 1367-1374

Dugan PR (1986) "Microbiological desulfurization of coal and its increased monetary value". In : Ehrlich KL, Holmes DS (eds) Workshop on biotechnology for the mining, metal-refining and fossil fuel processing industries.*Biotechnology and Bioengineering Symposium 16* .Wiley, New York 185-203.

Ehrlich H.L. (1996), *Geomicrobiology* , Marcel Dekker, Inc.,New York.

Ehrlich H.L.(1999) "Past,present and future of biohydrometallurgy ". *Proceedings of the International Biohydrometallurgy Symposium IBS'99,Part A* 3-12.

Environnement Canada, Service de conservation et protection Region Quebec Methode d'analyse des metaux dans sediments, les boues et les sols, 1990.

Fass R, Geva J, Shalita ZP, White MD, Fleming JC (1994) "Bioleaching method for the extraction of metals from coal fly ash using Thiobacillus ". US patent 5,278,069.

Felsher D, Finch JA, and Distin PA (2000), *Electrowinning from copper complexes.* Canadian Metallurgical Quarterly Vol. 39 No. 3

Finnerty WR, Robinson M (1986) "Microbial desulfurization of fossil fuels :a review. : Ehrlich KL, Holmes DS (eds) Workshop on biotechnology for the mining, metal-refining and fossil fuel processing industries ".*Biotechnology and Bioengineering Symposium 16* .Wiley, New York, 205- 221.

Francis AJ, Dodge CJ (1990) " Anaerobic microbial remobilization of toxic metals coprecipitated with iron oxide. *Environ Sci Technol. 24* 373-378.

Gadd GM, Gharieb MM, Ramsay LM, Sayer JA, Whatley AR and White C (1998) "Fungal proceses for Bioremediation of Toxic Metal and Radionuclide Pollution". *Journal of Chemical Technology, Bio, 71* , x.4 364-366.

Gaylard CC and Videla HA (1995) *Bioextraction and biodeterioration of metals* Cambridge University Press.

Gerbella L.(1940) ."II problema del rame in Italia".*Ingegnere 14* 281-288

Ghiorse W C (1984) "Biology of iron- and manganese-depositing bacteria". *Annual Review of Microbiology, 38* 37-40.

Golab Z, Orlowska B (1988) "The effect of amino and organic acids produced by the selected microorganisms on metal leaching". *ACTA MICROBIOLOGICA POLONICA. 37* no. 1, 83-93.

Hahn M (1994) "Verfahren zum L"sen von Metallen aus Feststoffen". DE patent 43 12 906.

Hallberg R.O ,and D.T. Rickard . De (1973). sma "gruvarbetarna" i Falun. *Swedish Natural Science Research Council* Stockholm,102.

Hamissa FA, Radwan AJ (1977) "Production of citric acid from cane molasses on a semi-pilot scale". *Gen. Appl. Microbiol. 23*, 325.

Harrison, A.P. (1984)."The acidophilic Thiobacilli and other acidophilic bacteria that share their habitat ". *Annu. Rev. Microbiol. 38*265-292.

Hoffmann MR, Arnold RG, Stephanopoulos G(1989)" Microbial reduction of iron ore". US patent 4880740.

Ilczuk Z (1983) "Attempts at improving citric acid fermentation on molasses solutions". *Eur. J. Appl. Microbiol. Biotechnol.17*, 69.

Ingledew WJ (1990)" Acidophiles ". *In: Edwards CA (ed) Microbiology of extreme environments.* Open University Press, Milton Keynes, 33-54

Jones JG (1986) "Iron transformations by freshwater bacteria ". *Advances in Microbial Ecology, 9* 149-185.

Karavaiko GI, Golovacheva RS, Pivovarova TA, Tzaplina LA, Vartanjan NS, (1988) "Thermophilic bacteria of genus sulfobacillus". *BIOHYDROMETALLURGY : PROCEEDINGS OF THE INTERNATIONAL SYMPOSIUM; Warwick, 1987. Norris PR and Kelly DP (eds)* , 29-42

Kelly DP, Norris PR and Brierley CL (1979) "Microbiological Methods for the Extraction and Recovery of Metals ". *Microbial Technology, Current State , Furure Prospects , Bull ,A.T., Ellwood, D.C and Ratledge C (eds)* , Cambridge Univ. Press . 263 Advances in Microbial Ecology, 9 149-185.

Khalid AM, Anwar MA, Shemsi AM, Niazi G and Akhtar K (1993) "Biohydrometallurgy of low-grade, carbonate bearing sandstone uranium ore. In: *Torma AE, Wey JE, Lakshmanan VI (eds) Biohydrometallurgical technologies, 1* . The Minerals, Metals & Materials Society, Warrendale, Pa,285-292.

Kleid DG, Kohr WJ, Thibodeau FR (1991) "Processes to recover and reconcentrate gold from its ores". European patent 0 432 935.

Ladin M.and Pedersen K. (1995) "The environmental impact of mine waste-role of microorganisms and their significance in treatment of mine waste.". *Swedish Waste Research Council (AFR report 83).*

LaGrega MD, Buckingham PL, Evans JC (1994) *Hazardous Waste Management.* McGraw-Hill Series in Water Resources and Environmental Engineering.

Lakshamanan VL, Christison J, Knapp R, Scharer J, and Sanmugasunderam V (1985) "A review of bioadsorption techniques of recover heavy metals from mineral-processing streams, In : Proc. 2nd Anuual General Meeting of BIOMINET, McCready,R.G.L.(ed.)". *Canmet Special Publication SP85-6* Ottawa, 76.

Leathen W. W., Braley Sr. S. A., and McIntyre L.D.. Appl. Microbiol. 1 (1953) 65.

Linke W (1965). *Editor of Seidell's Solubilities of Inorganic and Metal-Organic Compounds, Fourth Edition, 2 Vols* American Chemical Society, Washington, DC.

Malouf EE and Prater J (1963) *J. Metals 13* .353

Martell, A. E. and Calvin, M. (1952) *Chemistry of the metal chelate compounds.* Prentice-Hall Inc., New York.

Müller B, Burgstaller W, Strasser H, Zanella A and Schinner F (1995) "Leaching of zinc from an industrial filter dust with Penicillium, Corynebacterium Psudomonas : citric acid is the leaching agent rather than amino acids". *Journal of Industrial Microbiology 14* 208-212.

Mulligan C N, Galvez-Cloutier R, and Renaud N. (1999) "Biological leaching of copper mine residues by *Aspergillus niger.* In: Procc of the International Biohydrometallurgy Symposium IBS'99" . *Biohydrometallurgy and the environment toward the mining of the 21st century, part A.* R. Amils and Ballester (ed.) pp 453-461.

Nealson KH, Rosson RA, Myers CA (1989) "Mechanisms of oxidation and reduction of manganese". *Metal Ions and Bacteria* .TJ Beveridge and RJ Doyle(ed.)Wiley, New York, 383-411.

Notter M (1993) "Metals and the environment". *Swedish Environmental Protection Agency* (report 4245).

Olson GJ (1994) "Microbial oxidation of gold ores and gold bioleaching." *FEMS Microbiol Lett 119* 1-6

Olson GJ, Kelly RM (1986) " Microbial metal transformations : biotechnological applications and potential." *Biotechnol Prog 2* 1-15

Pavlenko SI, Permvakov AA, Tsymbal VP, Tsai TN (1997) "Development of technology for producing iron, aluminium oxids and organisation of production of structural materials from ash of thermal power plant". *Proceedings of R'97 - Recovery, recycling, re-inegration* . Geneva, February 4-7, vol V. EMPA, St. Gallen, 162-167.

Portier RJ (1991) "Biohydrometallurgical processing of ores and microorganisms therefor". US patent 5,021,088.

Rawlings Douglas E. (Ed.)(1997). "Biomining:Theory ,Microbes and Industrial Processes", *Springer-verlag Berlin Heidelberg and Landes Bioscience Georgetown* , TX,U.S.A.,Printed in Germany)

Rossi G. (1990)*Biohyrometallurgy* .McGraw- Hill,Hamburg,Germany.

Roukas T, Alichanidis E (1988) "The effect of pH on the production of citric acid from beet molasses by surface fermentation". *Abst. 8th Inter. Biotechnol. Symp.Paris.* 218,

Rudolfs W.and Helbronner A. (1922) "Oxidation of zinc sulfide by microorganisms". *Soil Sci. 14* 459-464.

Rusin P. and Ehrlich H.L. (1995). Adv. Biochemical Engineering/Biotechnology 52 1.

Rusin PA (1992)." Biological processes for recovering heavy metals". PCT patent WO 92/14848.

Rusin PA (1993). "Biological processes for recovery of plutonium from contaminated soils". PCT patent WO 93/19184.

Sand W. , Gerke T., Hallmann R. and Schippers A.(1995). *Appl. Microbiol. Biotechnol. 43* 961.

Sand W. , Gerke T., Jozsa PG and Schippers A (1999). "Direct vesus indirect bioleaching ". *Procc of the International Biohydrometallurgy Symposium IBS'99 .Biohydrometallurgy and the environment toward the mining of the 21st century, part A.* R. Amils and Ballester (ed.) 27-49.

Schinner F ,Burgstaller W (1989) "Extraction of zinc from industrial waste by a Penicillium sp. ". *Applied and Environmental Microbiology, 55* 1153-1156

Scott T.R.(1976) "Recent advances in hydrometallurgy with implications for the developing countries" . *Economics of Mineral Engineering ,Seminar Organized by the United Nations in Cooperation with the Government of Turkey ,Ankara* 56-89.

Sigg L, and Stumm W (1989) "Aquatische chemie". *Verlag der Fachvereine, Zurich.*

Silver Mand Torma AE (1974). *Can. J. Microbiol 20* 141.

Silverman MP, Ehrlich (1964) *Adv. Appl. Microbiol 6* 153.

Silverman MP, Munoz EF (1971) "Fungal leaching of titanium from rock". *Appl. Microbiol. 22* 923-924.

Steemson ML, Sheehan GJ, Winborne DA, Wong FS (1994) "An integrated bioleach/solvent extraction process for zinc metal production from zinc concentrates". PCT patent WO 94/28184.

Strasser H, Brunner H, Schinner F (1993) "The feasibility of nickel and vanadium recovery from a filter dust of a combustion furnace. *Torma AE, Wey JE, Lakshmanan VI (eds) Biohydrometallurgical technologies, 1* . The Minerals, Metals & Materials Society, Warrendale, Pa,157-162.

Strasser H, Brunner H, Schinner F (1995) "Leaching of iron and toxic heavy metal from anaerobically-digested sewage sludge ". *J Inc Microbiol 14* 281-287.

Suri AK, Gupta CK, and Tekin A (2001). *Purification of refractory metals.* Mineral Processing and Extractive Metallurgy Review Vol. 22, Nos. 1-3.

Szczodrak J (1981) "Biosynthesis of citric acid in relation to the activity of selected enzymes of the Kerbs cycle in Aspergillus niger mycelium". *Eur. J. Appl. Microbiol. Biotechnol. 13* 107.

Temple KL and Colmer AR (1951). *J. Bacteriol. 62* 605. 605.

Torma AE (1987)" Impact of biotechnology on metal extractions". *Miner Process Extract Metal Rev 2* 289-330.

Tzeferis P.G (1994a)" Mineral leaching of low-grade laterite ores using bioacids by molasses fungal metabolism". *Minerals and Metallurgical Processing* . pp 125-129.

Tzeferis P.G (1994b) "Leaching of nickel and iron from Greek non- sulphide nickeliferous ores by organic acids ". *Hydrometallurgy 36* 345-360

Tzeferis P.G (1994c) "Leaching of low-grade hematitic laterite ore using fungi and biologically produced acid metabolites". *Int. J. Miner. Process. 42* 267-283.

United Nations Conference on Environment and Development (1992).

Walsh J(1927) "Galen visits the dead sea and the copper mines of cyprus (166 A.D.)". *Bull Geogr Soc Philadelphia. 25* 93-110.

Wenberg G M, Erbisch FH and Volin ME (1971) "Leaching of copper by fungi". *Min.*

Eng. 23 207-212.

White C and Gadd GM (1987)) " The uptake and cellular distribution of zinc in saccharomyces cerevisiae " .*J. Gen. Microbiol. 133* 727.

Zimmerley SR,Wilson DG and Prater JD. (1958) " Cyclic leaching process employing iron oxidizing bacteria. ". U.S. Patent No. 2,829,964 .